The assessment and characterisation of obstructive sleep apnoea in severe obesity

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by

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CONTENTS

Abbreviation List

ACR: Albumin-Creatinine Ratio

AHI: Apnoea-Hypopnoea Index

Aix: Augmentation Index at Heart Rate 75

AP:Augmentation Pressure

BMI: Body Mass Index

BP: Blood Pressure

CE-MS: Capillary Electrophoresis-Mass Spectrometry

95%CI: 95% confidence interval

CPAP: Continuous Positive Airway Pressure

ESI: Electron Spray Ionisation

ESS: Epworth Sleepiness Scale

FEV1: Forced Expiratory Volume in 1 sec

FVC: Forced Vital Capacity

FEV1 % pred: Percentage predicted forced expiratory volume in 1 sec

FVC % pred: Percentage predicted forced vital capacity

HbA1c: Glycated haemoglobin

HDL: High Density Lipoprotein

HOMA: Homeostatic model assessment

HR: Heart Rate

hsCRP: highly sensitive C-Reactive Protein

IDF: International Diabetes Federation

IQR: Interquartile range

LDL: Low Density Lipoprotein

MAP: Mean Arterial Pressure

MDRD-GFR: Glomerular Filtration Rate using Modification of Diet in Renal Disease Equation

MS: Mass Spectrometer

ODI: Oxygen Desaturation Index

OSA: Obstructive Sleep Apnoea

PCO2: partial pressure of carbon dioxide

PWA: Pulse Wave Analysis

RCT: Randomised controlled trial

SD: Standard Deviation

SDB: Sleep-disordered breathing

SEVR: Subendocardial Viability Ratio

TSH: Thyroid Stimulating Hormone

Abstract

Background Obstructive Sleep Apnoea (OSA) is associated with cardiovascular disease. The current evidence regarding the effects of OSA in individuals with severe obesity is limited and has hitherto been largely unexplored. With the growing population of diabetes and obesity globally, the identification of severely obese individuals who have OSA is important given the risk of adverse outcomes associated with OSA. In this regard, the use of urinary proteomic (urinary peptide) profiling as a potential tool to characterise adult severely obese patients for the diagnostic assessment of OSA remains to be investigated.

Aims The aims of the studies described in this thesis were to (1) investigate the effects of OSA in severe obesity in relation to measures related to cardiovascular risk, specifically, arterial stiffness and serum urate; (2) assess current clinical practice of OSA assessment; and (3) explore the use of urinary proteomics in characterising subjects with severe obesity and OSA.

Methods In the arterial stiffness, urate and urinary proteomic studies, patients with severe obesity, were assessed at baseline and at follow-up. Anthropometric, respiratory and cardio-metabolic parameters were measured. All subjects had overnight polysomnography. Subjects with OSA were initially naive to OSA treatment at baseline were subsequently offered CPAP.

For the arterial stiffness studies, pulse wave analysis (PWA) was performed. In the urate studies, serum urate measurements were taken to identify associations between OSA and urate at baseline; and CPAP with change in urate at follow-up. OSA assessment in diabetes clinical practice was explored by a national survey in relation to the International Diabetes Federation (IDF) guidance. In the urinary proteomics studies, urine samples were analysed by capillary electrophoresis-mass spectrometry (CE-MS) at baseline and at follow-up.

Results Severely obese patients with OSA had increased arterial stiffness. Although sleepiness and blood pressure improved with CPAP in severe obesity, CPAP alone was not sufficient to modify PWA measures to levels comparable with non-OSA patients. In the urate study, serum urate was associated with OSA in severely obese females and there was a trend for reduced urate levels in CPAP-treated patients. The questionnaire study revealed a disappointing low awareness of the IDF statements and of the perceived importance of assessing for OSA in type 2 diabetes. The urinary proteomic studies identified trends in the urinary peptide profiles suggesting that there may be inherent differences between OSA and non-OSA patients, even following a period of effective CPAP treatment. The identified peptide panel included collagens, cadherin and fibrinogen subtypes.

Conclusions The theme that links the studies in this thesis has been the importance of OSA in relation to cardiovascular risk. Severely obese patients with OSA have increased arterial stiffness that may increase cardiovascular risk. Likewise, in severely obese individuals with OSA who have hyperuricaemia or recurrent gout, there may be a need to consider OSA assessment as elevated urate levels are associated with increased cardiovascular risk. From the questionnaire study, it is clear that more work needs to be done to raise awareness about OSA assessment in diabetes teams as obesity is a risk factor for type 2 diabetes. Urinary proteomic CE-MS profiling has provided novel insights into the urinary proteome in OSA, with and without CPAP. Although there is insufficient evidence to support its use for OSA diagnosis, the urinary peptides identified may be linked with mechanisms underlying cardiovascular disease in OSA and may be associated with treatment effects of CPAP on OSA progression that influences expression of urinary peptides.

Declaration

This thesis is the result of work performed whilst registered as a candidate for the degree of Doctor of Philosophy at the University of Liverpool. I declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

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Prof John Wilding
Prof Kevin Hardy
Dr Jatin Burniston

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Chapter 1

Introduction

This chapter begins with a discussion of the epidemiology of obesity and diabetes, and the association with sleep-disordered breathing, in particular obstructive sleep apnoea. Aspects of sleep physiology are then described, followed by a review of the literature concerning the pathophysiological aspects, associations and complications of OSA in relation to impaired metabolism in obesity and type 2 diabetes. Assessment and treatment of OSA are then discussed. Finally, the concept of screening for OSA in obesity and type 2 diabetes, the rationale for the work presented in this thesis and the use of urinary proteomics are described.

Background

There has been a substantial increase in the prevalence of diabetes mellitus and obesity worldwide that is of concern to health care providers and continues to be a significant public health issue. In terms of obesity, global estimates of prevalence have nearly doubled since 1980. At present, nearly 500 million individuals are obese (World Health Organisation, 2014), and by 2030, this is projected to be about 1.12 billion individuals after adjusting for secular trends (Kelly et al., 2008).

In England, there was a marked rise in the proportion of adults with obesity between 1993 and 2012 from 13.2% to 24.4% among males and from 16.4% to 25.1% among females (Health and Social Care Information Centre, 2014). By 2050, 60% of males, 50% of females will be obese if current trends continue; with NHS costs attributable to obesity expected to double to £10 billion and wider costs to society estimated to reach £49.9 billion per year (Foresight, 2007).

Obesity is defined as an excess accumulation of body fat and is classified according to the body mass index (BMI), that is calculated as weight(kg)/ height(m)^2. According to World Health Organisation guidance, adults with a body mass index (BMI) of 25 to 30kg/m^2 are classed as 'overweight', and those with a BMI of 30kg/m^2 or more may be referred to as having obesity. Those with a BMI with a body mass index (BMI) of 35kg/m^2 or more may be referred to as having severe (previously termed 'morbid') obesity. It should be noted that the threshold values may differ amongst populations (WHO Expert Consultation, 2004), with Asian populations in particular having lower BMI cut-offs. It is recognised that the regional distribution of body fat, such as abdominal and visceral adiposity may also be important in determining metabolic and cardiovascular health risks in obesity (Tchernof and Despres, 2013).

The rapid increase in obesity is closely linked to the rising prevalence of type 2 diabetes. The world prevalence of diabetes mellitus among adults is 6.4%, affecting an estimated 285 million adults, and estimates indicate that the prevalence is projected to reach 7.7% with patient numbers expected to reach 439 million worldwide by 2030 (Shaw et al., 2010). Although there is considerable heterogeneity in body weight in different populations, the majority of individuals with type 2 diabetes are overweight or obese (Inzucchi et al., 2012). Obesity, in particular abdominal obesity, is central to the development of type 2 diabetes and is therefore a focus for attention in the reduction of risk of diabetes (Alberti et al., 2007). Hence the term 'diabesity' has been used to describe the association between the two conditions (Sims et al., 1973). Most patients with type 2 diabetes are also overweight or obese, and this is a major contributor to the insulin resistance and progressive β-cell dysfunction that characterises the condition (Kahn et al., 2006).

Individuals with obesity and/or diabetes may be at risk of complications such as cardiovascular disease and other obesity-related complications, such as endocrine dysregulation, arthritis and increased cancer risk that may increase the complexity of management for these patients (Haslam and James, 2005) (Tchernof and Despres, 2013). It is recognised that sleep-disordered breathing (SDB), in particular, obstructive sleep apnoea (OSA) is associated with obesity and diabetes.

Regulation of Sleep

Sleep is a state of reversible unconsciousness in which the brain is relatively more responsive to internal than external stimuli, and is characterised by sleep cycles with specific electroencephalogram (EEG) patterns and physiological changes (Schupp and Hanning, 2003). Non-rapid eye movement (NREM) sleep transitions into rapid eye movement (REM) sleep that follows a regular cycle which lasts about 90 min in adults. The cycles may be separated by a period of wakefulness and are repeated 3–6 times each night. The wakeful state is characterised by low-voltage high frequency electroencephalogram (EEG) activity and high muscle tone. In NREM sleep, high amplitude low-frequency EEG activity and decreased muscle tone occurs; whereas REM sleep has low-voltage fast electroencephalogram (EEG) activity coupled with muscle atonia and characteristic rapid eye movements (Brown et al., 2012).

Wakefulness is mediated by the ascending arousal systems in the brainstem and posterior hypothalamus that project to the forebrain. Cholinergic neurones in the peduculopontine and laterodorsal tegmental areas (PPT/LDT) activate the thalamus and other forebrain targets (Espana and Scammell, 2004). Activation of the cerebral cortex to facilitate processing of thalamic inputs

arises from monoaminergic neurones in the tuberomammillary nucleus (TMN) containing histamine, the dorsal and median raphe nuclei containing serotonin, and the locus coeruleus (LC) containing noradrenaline. Inputs are also received from peptidergic neurones in the lateral hypothalamus (LH) containing orexin and melanin-concentrating hormone, and from basal forebrain (BF) neurones that contain γ-aminobutyric acid (GABA) or acetylcholine (Saper et al., 2005). Cholinergic activation of the thalamus is necessary for thalamocortical activation, which results in the low-amplitude, high-frequency EEG characteristic of wakefulness. Other neurotransmitters that are active in wakefulness include histamine, dopamine, serotonin, noradrenaline and orexins (Saper et al., 2005). These are summarised in **Figure 1.1.**

Figure 1.1

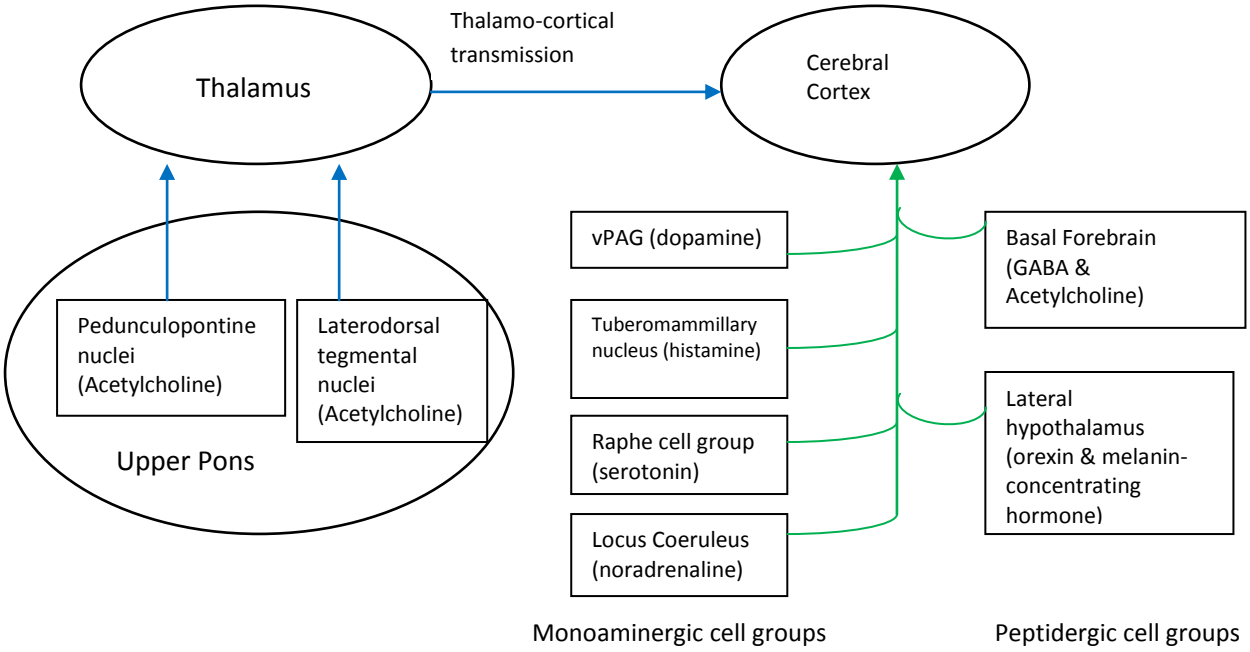


Figure 1.1 Schematic Representation of the ascending arousal (wakefulness) pathways that traverse the junction between brainstem and forebrain. Neurotransmitters are in brackets. Inputs in blue facilitate thalamocortical transmission. Pathways in green activate the cerebral cortex to allow processing of thalamic inputs. Key: vPAG, ventral periaqueductal grey matter; GABA: γ-aminobutyric acid. Adapted from Saper et al (2005).

The preoptic area in the anterior hypothalamus contains neurons that help produce NREM and REM sleep. These cells are located within the ventrolateral preoptic area (VLPO), and within adjacent regions of the preoptic area and basal forebrain, and are active during sleep. VLPO neurons contain inhibitory neurotransmitters γ-aminobutyric acid (GABA) and galanin that produce sleep by inhibiting wake-promoting brain regions such as the tuberomamillary nucleus (TMN), lateral hypothalamus, locus coeruleus (LC), dorsal raphe, laterodorsal tegmental nucleus (LDT), and pedunculopontine tegmental nucleus (PPT) (Espana and Scammell, 2004).

The interaction of cholinergic and aminergic brainstem neurons controls REM sleep (Espana and Scammell, 2004). Cholinergic neurones in the LDT and PPT are active during REM sleep and depolarise thalamic neurones, thereby activating thalamocortical signalling to produce high frequency cortical EEG activity. During wakefulness and NREM sleep, these REM-active cholinergic cells are inhibited by noradrenaline, serotonin and histamine. However, the REM sleep-generating neurons are disinhibited during REM sleep (Espana and Scammell, 2004). Sleep promoting pathways are presented in **Figure 1.2.**

Figure 1.2

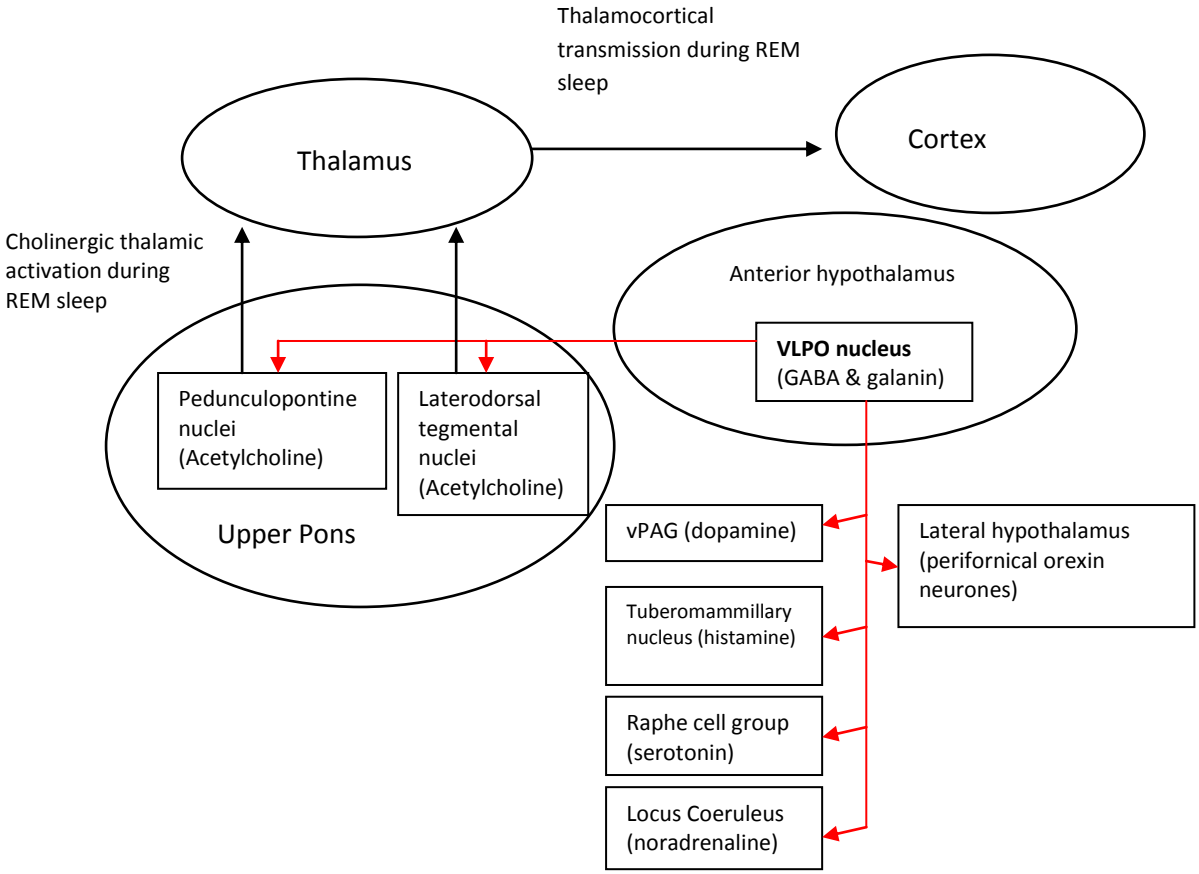


Figure 1.2 Schematic Representation of the sleep-promoting ventrolateral preoptic (VLPO) pathways inhibiting the components of the wake promoting regions. Projections in red represent inhibitory pathways. Pathways in black represent thalamic stimulation during REM sleep by cholinergic neurones, producing thamalocortical stimulation. Neurotransmitters are in brackets. Key: VLPO, ventrolateral preoptic nucleus; vPAG, ventral periaqueductal grey matter; GABA: γ-aminobutyric acid. Adapted from Saper et al (2005).

The timing, quality and duration of sleep are controlled by the duration of prior wakefulness (homeostatic control, process S) and by the interaction of time of day (circadian control, process C) (Borbely, 1982). The suprachiasmatic nucleus regulates circadian rhythms through local hypothalamic circuits via signals that conveyed to the supraventricular zone and dorsomedial nucleus of the hypothalamus (Chou et al., 2003) **(Figure 1.3)**. The output from the sub-paraventricular zone and dorsomedial nucleus of the hypothalamus are integrated with behavioural and endocrine information to drive circadian cycles of sleep, feeding, activity and corticosteroid secretion (Saper et al., 2005). Excitatory wake signals are relayed to arousal regions such as the lateral hypothalamus and locus coeruleus, and arousal inhibitory projections extend to the sleep-promoting VLPO (Saper et al., 2005).

Figure 1.3

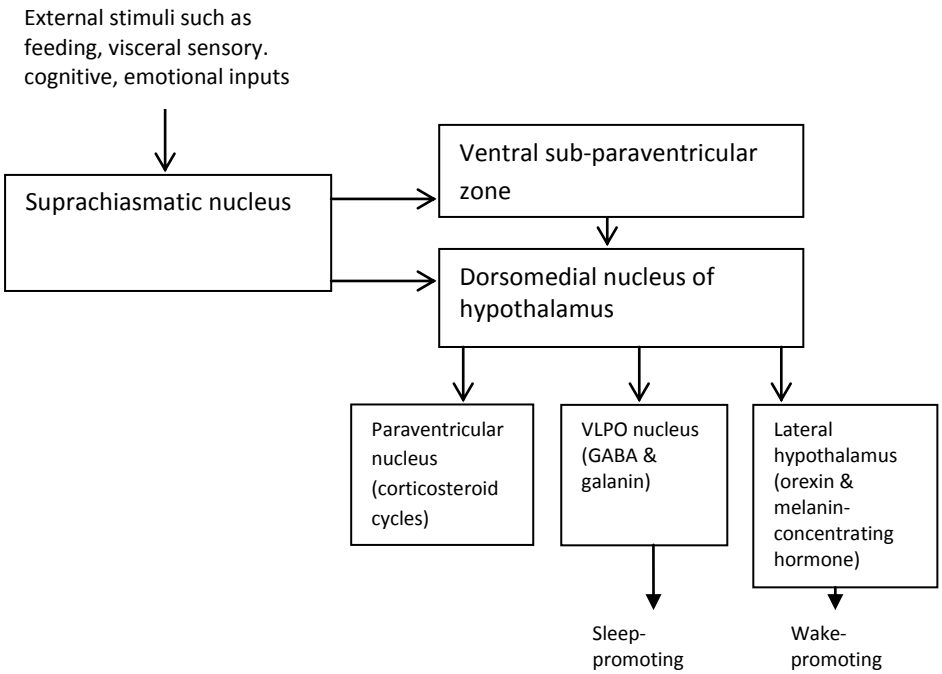


Figure 1.3 Schematic diagram of circadian regulation of sleep and wakefulness. VLPO, ventrolateral preoptic nucleus; GABA: γ-aminobutyric acid. Adapted from Saper et al (2005).

Adequate sleep is essential for optimal cognitive function as sleep loss or disrupted sleep alters behaviour and performance in terms of attention, vigilance, executive function, emotional reactivity, memory formation, decision making and judgment (Brown et al., 2012). Sleep deficiency may arise from insufficient sleep schedules, shift work and from conditions with sleep-disordered breathing such as OSA (Depner et al., 2014).

Sleep-Disordered Breathing

Sleep-disordered breathing (SDB) is characterised by a spectrum of altered sleep homeostasis that ranges from simple snoring to obstructive sleep apnoea with excessive daytime somnolence. SDB is associated with hypertension, cardiovascular disease, type 2 diabetes and metabolic impairment (Nieto et al., 2009). There is also evidence linking disrupted sleep patterns to other adverse health consequences such as neurocognitive deficits, impaired quality of life and an increased risk of accidents (Beebe et al., 2003). Whilst there are different forms of SDB, the majority of research studies have focused on OSA and its complications and the potential interactions with obesity, diabetes, metabolic syndrome and its components. The focus of this thesis will be on obstructive sleep apnoea (OSA) which is the most common form of SDB.

Obstructive Sleep Apnoea

OSA is a common condition with an estimated prevalence of 13% in men and 6% in women between 30 and 70 years having moderate to severe OSA (AHI>15 events per hour of sleep) (Peppard et al., 2013). The major risk factors of OSA are obesity, gender and increasing age (Malhotra and White, 2002) Cross-sectional estimates that up to 40% of OSA patients will have type 2 diabetes (Meslier et al., 2003), and the prevalence of OSA may be up to 23% in patients who are known to have type 2 diabetes (West et al., 2006). Prevalence estimates of OSA in severe obesity have been reported to be between 40-90% (Schwartz et al., 2008).

In OSA, repeated apnoeas or hypopnoeas occur during sleep with subsequent daytime hypersomnolence (Young et al., 1993). These episodes are characterised by recurrent episodes of upper airway obstruction and changes in intra-thoracic pressure that result in recurrent periodic oxygen desaturations, with frequent sleep arousals and fragmented sleep (Young et al., 1993, Tasali and Ip, 2008). An apnoea is defined as the complete cessation of airflow for at least 10 seconds. A hypopnoea is defined as a reduction in airflow that is followed by an arousal from sleep or a decrease in oxyhaemoglobin saturation (Punjabi, 2008).

Excessive somnolence
Loud snoring
Concerned partner who has witnessed apnoeic episodes
Choking symptoms whilst waking up
Loss of concentration
Unrefreshed sleep
Other symptoms that may be reported
Nocturia
Reduced libido
Nocturnal sweating

Table 1.1 Clinical Features of OSA

The presence of OSA may be assessed by formal polysomnography studies with the number of apnoeas and hypopnoeas per hour during sleep, termed the apnoea-hypopnoea index (AHI) classifying the severity of OSA. The AHI measures the frequency of reduction in airflow associated with collapse or narrowing of the airways (Caples et al., 2005). This index classifies the severity of OSA based on the number of obstructive breathing episodes per hour during sleep; mild AHI: 5 to 15 events per hour; moderate OSA: 15 to 30 events per hour and severe OSA>30 events per hour (Flemons et al., 1999).

Diagnosis	**Events per hour**
Normal	<5
Mild OSA	5 - 15
Moderate OSA	15 - 30
Severe OSA	>30

Table 1.2 AHI for diagnosis and classification of OSA (Flemons et al., 1999)
AHI = apnoea-hypopnoea index; OSA = obstructive sleep apnoea

Besides the AHI, the frequency of oxygen desaturation episodes and severity of somnolence symptoms are also used (Flemons et al., 2003). Although the diagnosis of OSA can be made when the AHI is >5 events/hr in a patient with excessive daytime sleepiness, it is also important to distinguish the severity of daytime somnolence symptoms as patients with mild OSA (AHI 5-15 events/hr) may not always require treatment if there are no sleepiness symptoms with only minor impairment of daily functioning. Patients with moderate and severe OSA (AHI > 15 events/hr) who have a history of daytime sleepiness with impaired social and occupational function should be offered treatment (Flemons et al., 1999).

Associations and complications of OSA

OSA is associated with a clustering of clinical cardio-metabolic manifestations. There is evidence linking OSA to the metabolic syndrome that comprises obesity, insulin resistance, hypertension and dyslipidaemia (Coughlin et al., 2004). It is also well documented that OSA affects cardiovascular risk. In the Wisconsin Sleep Cohort Study and the Sleep Heart Health Study, it was found that there was a relation between OSA and hypertension (Nieto et al., 2000) (Young et al., 1997). OSA patients have an increased risk of developing coronary artery disease (Peker et al., 2006), cerebrovascular disease (Yaggi et al., 2005), atherosclerosis (Kylintireas et al., 2012), arterial stiffness (Doonan et al., 2011), cardiac arrhythmias (Hoffstein and Mateika, 1994, Mehra et al., 2006) and heart failure (Gottlieb et al., 2010). OSA poses important perioperative risks for obese individuals who are particularly vulnerable during anaesthesia and sedation and are at an increased risk of developing postoperative respiratory and cardiopulmonary complications (Chung and Elsaid, 2009) (Liao et al., 2009).

The reduction in time spent sleeping that is typical of OSA may have profound effects on glucose regulation, insulin resistance, appetite and energy balance (Knutson et al., 2007). OSA may induce fatigue and with increasing daytime somnolence, weight reducing activities are pursued less often with a decrease in energy expenditure that may lead to weight gain, insulin resistance, and further worsening of OSA.

Understanding the potential influence of OSA on the components of the metabolic syndrome and the mechanisms by which such interactions may contribute to metabolic dysregulation is important because such knowledge may define appropriate targets and lead to more-precisely administered preventive actions in addressing health outcomes such as cardiovascular disease, obesity, and type 2 diabetes.

Compromised metabolism

OSA, Insulin resistance and type 2 diabetes

OSA severity is correlated with the degree of perturbation in glucose homeostasis, an effect that is independent of obesity (Tasali et al., 2008) (Shaw et al., 2008). The prevalence of OSA in type 2 diabetes has been reported with different rates in several population-based studies that are not directly comparable due to differences in populations studied and how OSA was assessed. This has been found to range from 18% of patients in primary care based on an assessment of electronic records (Heffner et al., 2012), 23% in a mixed primary and secondary care population of patients

with type 2 diabetes who had overnight oximetry testing (West et al., 2006) to as high as 86% in the Sleep AHEAD (Action for Health in Diabetes) study cohort comprising obese subjects with type 2 diabetes who had polysomnography (Foster et al., 2009).

There is substantial evidence for the association between OSA, insulin resistance and type 2 diabetes. OSA is independently associated with alterations in glucose metabolism and an increased risk of type 2 diabetes (Tasali et al., 2008, Shaw et al., 2008). Sleep-disordered breathing as assessed by AHI was observed to be independently correlated with insulin resistance; this association was seen in both obese and non-obese subjects (Ip et al., 2002). Punjabi et al (2002) found that sleep-disordered breathing, based on the AHI was associated with glucose intolerance and insulin resistance in mildly obese men without diabetes or cardiopulmonary disease and that increasing AHI was associated with worsening insulin resistance independent of obesity (Punjabi et al., 2002). The Sleep Heart Health Study showed that OSA was associated with impaired glucose tolerance and insulin resistance (Punjabi et al., 2004) and in the Wisconsin Sleep Study cohort, there was an association between OSA and type 2 diabetes (Reichmuth et al., 2005). Botros et al (2009) found that OSA increased the risk of type 2 diabetes in a cohort of 544 patients referred for evaluation of SDB after adjusting for age, gender, ethnicity, blood glucose, body mass index, and weight change (Botros et al., 2009). Tamura et al (2012) observed that OSA-induced hypoxia was associated with increases in glycated haemoglobin regardless of glucose tolerance status (Tamura et al., 2012). In a longitudinal study, men without diabetes were followed up for a mean of 11 years, and it was demonstrated that OSA was independently related to the development of insulin resistance (Lindberg et al., 2012). In a cross-sectional analysis in the European Sleep Apnoea Cohort (ESADA) study, the prevalence of type 2 diabetes and poorer glycaemic control was observed to be associated with increasing severity of OSA assessed by the ODI (Kent et al., 2014). Another study that retrospectively analysed health data in a cohort of OSA patients found that the initial severity of OSA was associated with subsequent risk of diabetes (Kendzerska et al., 2014) It should be noted that these studies have been based on observational data that can only suggest an association and not causality. Vgontzas et al. (2000) evaluated obese males with symptomatic sleep apnoea with age and BMI-matched controls and found mean fasting blood glucose and insulin levels were higher in OSA than in obese controls suggesting that sleep-disordered breathing is an independent risk factor for hyperinsulinaemia (Vgontzas et al., 2000). Conversely, two studies did not find an independent association between OSA and insulin resistance (Onat et al., 2007) (Gruber et al., 2006). In summary, despite mixed findings in relation to the link between OSA and insulin resistance, there is

nevertheless evidence that suggests a role for increased insulin resistance as a potential intermediary mechanism that may influence cardio-metabolic risk.

Several potential mechanisms could explain the pathophysiological links between OSA and impaired glucose metabolism **(Figure 1.4)**. These mechanisms are interacting and potentially synergistic and therefore all pathways shown in **Figure 1.4** are equally important. These include intermittent hypoxia and sleep fragmentation that may alter sympathetic activity (Somers et al., 1995), effects on endocrine (Meston et al., 2003) and hypothalamic–pituitary–adrenal axes (Vgontzas et al., 2007); oxidative stress and inflammatory responses (Lavie, 2009); and changes in adipokines that may alter glucose metabolism (Drager et al., 2010c, Punjabi and Polotsky, 2005).

Figure 1.4

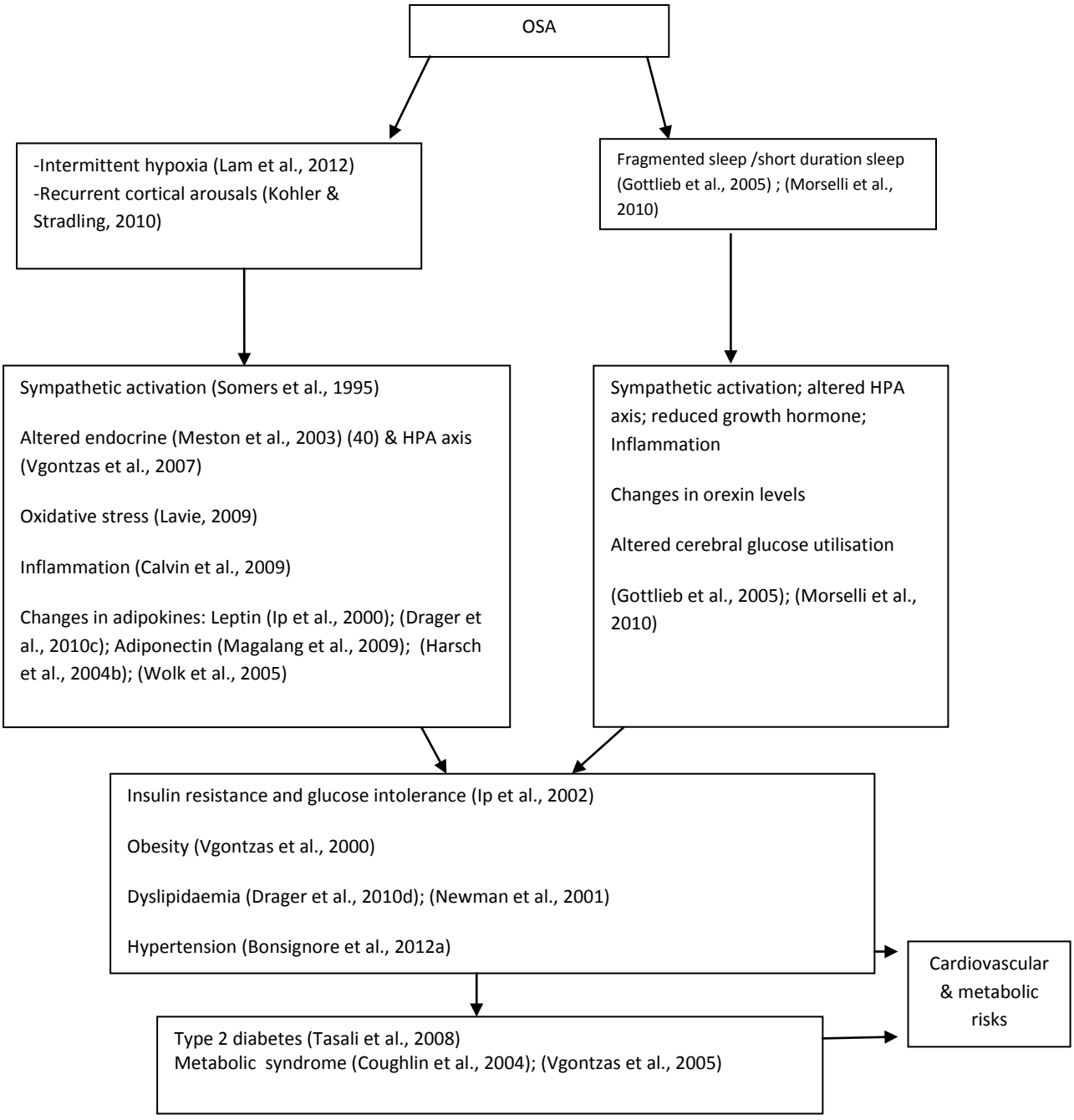


Figure 1.4 Interacting pathophysiological pathways in OSA and impaired metabolism including type 2 diabetes and metabolic syndrome. These pathways are interacting and potentially synergistic and therefore all the mechanisms shown are equally important. Selected references are in parentheses.

Animal studies of intermittent hypoxia have demonstrated glucose intolerance and insulin resistance in lean mice with acute intermittent hypoxia (Iiyori et al., 2007), and in obese mice exposed to chronic intermittent hypoxia for 12 weeks (Polotsky et al., 2003). Human volunteers exposed to acute sustained hypoxia for 30 minutes (Oltmanns et al., 2004) and acute intermittent hypoxia simulating moderate OSA for 5 hours (Louis and Punjabi, 2009) also demonstrated impaired glucose tolerance.

Autonomic function

Several studies have found autonomic abnormalities in OSA that are attenuated with CPAP therapy (Somers et al., 1995, Imadojemu et al., 2007). During sleep in OSA, intermittent hypoxia and recurrent arousals stimulate sympathetic responses by mechanisms that include chemoreflex and baroreflex changes, vasoconstrictor effects of nocturnal endothelin release and endothelial dysfunction (Narkiewicz and Somers, 2003, Kohler and Stradling, 2010). Sympathetic activation increases hepatic glycogenolysis and gluconeogenesis (Punjabi and Polotsky, 2005). The stimulation of lipolysis increases circulating free fatty acid metabolites that inhibit insulin signalling and reduce insulin-mediated glucose uptake, contributing to insulin resistance (Delarue and Magnan, 2007).

Hypothalamic-pituitary-adrenal axis

Activation of the HPA axis may provide a further mechanistic link between OSA and diabetes. There is evidence for potential OSA effects on the HPA axis as several studies have reported enhanced cortisol secretion in OSA (Lanfranco et al., 2010). Increased cortisol levels may contribute to hyperglycaemia by reducing insulin secretion and glycogen synthesis, and increasing gluconeogenesis (Rosmond, 2003). It should be noted that an increased cortisol secretion in OSA has not been consistently reported in the literature (Lanfranco et al., 2010). For example, Dadoun et al (2007) found no association in cortisol profiles and OSA in obese men (Dadoun et al., 2007). This may reflect an interaction between OSA and the HPA axis that may involve different mechanisms. Alterations in HPA axis activity may potentially be induced by intermittent hypoxia or by altered neural control of corticotroph function (Drager et al., 2010c, Lanfranco et al., 2010). Obese patients with OSA were found to have an increased adrenocorticotrophic hormone (ACTH) response to Corticotrophin Releasing Hormone (CRH) compared with obese controls (Lanfranco et al., 2004). Additionally, sleep deprivation has been associated with increased HPA axis activity that can potentially affect insulin resistance and may also predispose to obesity and metabolic syndrome (Spiegel et al., 1999) (Buckley and Schatzberg, 2005).

Several studies have examined the effects of OSA treatment on the HPA axis. The results have been mixed in terms of effects of CPAP treatment on cortisol production. Meston et al (2003) found no relation between cortisol and OSA severity, and no measurable response to nasal CPAP treatment (Meston et al., 2003). Vgontzas et al (2007) demonstrated that OSA in obese men is associated with increased nocturnal cortisol levels, compared with controls, that were corrected after the use of CPAP for 3 months (Vgontzas et al., 2007). Consistent with this, another study found that 3 months of CPAP therapy decreased evening salivary cortisol concentrations in severe OSA patients (Schmoller et al., 2009).

Oxidative stress & Activation of Inflammatory pathways

Repeated episodes of intermittent hypoxia and reoxygenation in OSA may cause oxidative stress with increased production of reactive oxygen species that may contribute to altered glucose homeostasis. The formation of reactive oxygen species may impair pancreatic beta cell function and insulin secretion (Punjabi and Polotsky, 2005). This may be due to increased pancreatic beta cell proliferation and apoptosis with mitochondrial oxidative stress (Xu et al., 2009). Hypoxia-Inducible Factor-1 is up-regulated in many tissues during hypoxia and modulates the expression of proteins that mediate adaptive responses to hypoxia some of which may influence glucose metabolism (Lavie, 2003, Punjabi and Polotsky, 2005, Lavie, 2009).

Additionally, reactive oxygen species formation may have an important role in activating inflammatory responses and have been implicated in the up-regulation of transcription factors nuclear factor-κB (NF-κB), and activator protein 1 with increased expression of proinflammatory cytokines, including tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-8 (Calvin et al., 2009). TNF-α and IL-6 are increased in patients with OSA (Vgontzas et al., 2000) and there is evidence that these cytokines may have a role in insulin resistance (Alam et al., 2007). Expression of TNF-α and plasma IL-6 are higher in subjects with insulin resistance (Kern et al., 2001). TNF-α may downregulate genes required for normal insulin action and the peroxisomal proliferator-activated receptor-γ (an insulin-sensitising nuclear receptor), and may have direct effects on insulin signalling and induction of elevated free fatty acids via stimulation of lipolysis (Moller, 2000). IL-6 levels have been found to be correlated with insulin resistance in adipose tissue (Bastard et al., 2002).

Changes in Adipokine Profiles

There is evidence that OSA may be associated with altered adipokine levels. The adipokines leptin and adiponectin are hormones produced mainly by adipocytes that have a range of effects on physiological processes. The main function of leptin is its role in regulation of appetite and energy regulation but it also influences glucose regulation (Ceddia et al., 2002). Adiponectin has roles in fat distribution, inflammation and insulin sensitivity (Trujillo and Scherer, 2005). It acts as an insulin-sensitising hormone and low levels are found in type 2 diabetes (Harsch et al., 2004b). One study found elevated serum leptin levels in OSA that are reduced following CPAP treatment (Ip et al., 2000), consistent with the observation that intermittent hypoxia increases leptin expression and inhibits insulin secretion (Drager et al., 2010c). However other studies on leptin levels and OSA have shown no effect independent of adiposity (Lui and Ip, 2010). Insulin resistance is associated with low levels of adiponectin (Magalang et al., 2009), although the mechanism remains unclear; intermittent hypoxia has been proposed as a putative mechanism so adiponectin concentrations have been studied in OSA, with equivocal findings (Harsch et al., 2004b) (Wolk et al., 2005).

Sleep duration/Fragmented Sleep

Shortened sleep duration from fragmented sleep may be associated with altered glucose regulation and insulin resistance (Morselli et al., 2010) (Spiegel et al., 2009). Short sleep duration is associated impaired glucose regulation and an increased prevalence of diabetes (Gottlieb et al., 2005). Potential mechanisms underlying this effect may include sympathetic activity which may impair glucose regulation via lipolysis; alterations in the hypothalamic-pituitary-adrenal axis, reduced growth hormone secretion, appetite changes, and inflammatory responses that may influence glucose and insulin homeostasis. Other mechanisms include; up-regulation of orexin neurons and altered cerebral glucose metabolism (Gottlieb et al., 2005) (Morselli et al., 2010).

Diabetes complications

A number of studies have shown that OSA may be associated with increased risk of the complications of diabetes, independently of glucose control. These studies were not randomised controlled trials and were based on observational findings at a point in time from the populations examined. It may be possible that control group comparisons were difficult given the nature of the complications necessitating treatment. West et al (2010) showed that in males with type 2 diabetes, the presence of OSA is associated with diabetic retinopathy (West et al., 2010). Mason et al (2012), found a high prevalence of SDB in patients with type 2 diabetes and diabetic macular oedema, although no relationship was observed between the severity of SDB as defined by ODI and macular

oedema (Mason et al., 2012). Proposed mechanisms that may influence retinal damage include increases in blood pressure and sympathetic activation; oxygen desaturation causing retinal hypoxia with production of vascular growth factors and autonomic dysregulation (Mason et al., 2012). Tahrani et al (2012) reported an association between OSA and peripheral neuropathy in patients with type 2 diabetes and proposed increased oxidative stress and microvascular changes as potential mechanisms (Tahrani et al., 2012).

In relation to diabetes nephropathy, a prospective study to evaluate the evaluate the OSA and microalbuminuria found no correlation between urinary albumin excretion and OSA in a cohort with type 2 diabetes although this may have been due to the small study sample size (Buyukaydin et al., 2012). In another study of Japanese subjects, nocturnal intermittent hypoxia was found to be associated with microalbuminuria in females with type 2 diabetes (Furukawa et al., 2013).

In summary, there is evidence to suggest that OSA may be associated with an increased risk of complications in diabetes. The evidence has been from observational studies as it is conceivable that randomised controlled trials would potentially be difficult to perform as patients would need urgent treatment for their complications. For example, proliferative retinopathy would require ophthalmological review and potential laser treatment.

OSA and metabolic syndrome

One of the major implications of the global rise in obesity has been an associated rise in the prevalence of the metabolic syndrome (Zimmet et al., 2001). The metabolic syndrome, also known as the insulin resistance syndrome, or 'syndrome X' (Reaven, 1988) has been variably defined based on clustering of abnormalities of factors including insulin resistance, dyslipidaemia (low HDL cholesterol and raised triglycerides), hyperglycaemia and blood pressure (Eckel et al., 2005), with the most recent definition of the syndrome requiring central adiposity as a key feature (Alberti et al., 2009) **(Table 1.3)**. These definitions have been proposed by different organisations to unify the diagnostic criteria. In this thesis, the NCEP ATP III criteria (Cleeman et al., 2001) were used as it is established and applied in clinical practice. Moreover, this allowed comparisons with other studies within the group that have applied similar criteria for defining metabolic syndrome (Coughlin et al., 2004) (Coughlin et al., 2007).

Table 1.3

World Health Organisation (1999)	**European Group for the Study of Insulin Resistance (1999)**	**US National Cholesterol Education Program: Adult Treatment Panel III (2001)**	**International Diabetes Federation (2005)**	**International Diabetes Federation (2009)**
(Alberti et al., 1998)	(Balkau and Charles, 1999)	(Cleeman et al., 2001)	(Alberti et al., 2005)	(Alberti et al., 2009)
Impaired glucose regulation or diabetes mellitus and/or insulin resistance together with two or more of: ***Raised arterial pressure*** ≥ 160/90 mmHg ***Raised plasma triglycerides*** (≥ 1.7 mmol/l; 150 mg/dl) and/or ***low HDL-cholesterol*** (<0.9mmol/l, 35 mg/dl males; < 1.0 mmol/l, 39 mg/dl females) ***Central obesity*** (males: waist to hip ratio >0.90; females: waist to hip ratio >0.85) and/or BMI >30 kg/m^2 ***Microalbuminuri***a (urinary albumin excretion rate ≥20 µg/min or ***albumin:creatinine ratio*** ≥20 mg/g)	Excluding people with diabetes, presence of insulin resistance or fasting hyperinsulinaemia and two of: ***Hyperglycaemia*** (fasting plasma glucose & 6.1 mmol/l, but without diabetes); ***Hypertension*** (systolic/diastolic blood pressures ≥ 140/ 90 mmHg or treated for hypertension); ***Dyslipidaemia*** (Triglycerides > 2.0 mmol/l or HDL-cholesterol < 1.0 mmol/l or treated for dyslipidaemia); ***Central obesity*** (waist circumference≥94cm in males and ≥80cm in females). Hyperglycaemia was defined as fasting plasma glucose ≥6.1 mmol/l or impaired fasting glucose in individuals without diabetes.	Presence of any three of: ***Central obesity*** (waist circumference) males>102cm, females>88cm; ***Raised blood pressure***≥130/≥85); ***Raised triglycerides*** (≥1.7 mmol/l; 150 mg/dl); ***Low HDL-cholesterol*** (males<1.03mmol/l; 40mg/dl, females< 1.29mmol/l; 50mg/dl); ***Fasting hyperglycaemia*** (≥6.1mmol/l; 110mg/dl).	**Central obesity** ***Waist circumference*** according to ethnicity and any two: ***Raised triglycerides*** >1.7 mmol/L; 150 mg/dL or on treatment for this ***Reduced HDL-cholesterol*** Males <1.03 mmol/L; 40 mg/dL Females <1·29 mmol/L; 50 mg/dL or on treatment for this ***Raised blood pressure*** Systolic≥ 130 mmHg and/or Diastolic ≥85 mmHg Or on treatment for previously diagnosed hypertension ***Raised fasting plasma glucose*** Fasting plasma glucose≥ 5·6 mmol/L; 100mg/dl Or known type 2 diabetes	Presence of any three of: ***Waist circumference*** According to specific population and country definitions ***Elevated triglycerides*** ≥1.7 mmol/L; 150 mg/dL or on treatment for elevated triglycerides ***Reduced HDL-C*** <1.0 mmol/L; 40 mg/dL in males; <1.3 mmol/L; 50 mg/dL in Females or on treatment for reduced HDL-cholesterol ***Elevated blood pressure*** Systolic ≥130 mmHg and/or Diastolic ≥85 mmHg or on antihypertensive drug treatment ***Elevated fasting glucose*** ≥5.6mmol/l; 100 mg/dL or on treatment for elevated glucose

Table 1.3 Definitions of metabolic syndrome

There is also evidence that OSA is associated with the metabolic syndrome, independently of adiposity (Coughlin et al., 2004)'(Nieto et al., 2009). The term 'syndrome Z' reflects the close interaction between OSA and cardiovascular disease risk (Wilcox et al., 1998). In a study of 529 newly diagnosed OSA patients who had polysomnography, metabolic syndrome based on the National Cholesterol Education Program Adult Treatment Panel (NCEP-ATP III) definition occurred in about half of the patients; prevalence rates of metabolic syndrome in OSA have previously been reported to be between 23-87% using this definition (Bonsignore et al., 2012a).

The association between OSA and the metabolic syndrome may influence cardio-metabolic dysregulation (Lam et al., 2012). OSA patients are more likely to have abnormalities in components characterising the metabolic syndrome and conversely, there may be a higher prevalence of OSA in patients with metabolic syndrome (Basoglu et al., 2011).

Many studies have demonstrated relationships between OSA and metabolic syndrome. In patients well matched for total adiposity, it was found that OSA was independently associated with metabolic syndrome components including increased blood pressure, higher fasting insulin and dyslipidaemia and increased insulin resistance (higher HOMA values) (Coughlin et al., 2004). In another study, this association was found to be independent of obesity mainly from increased triglyceride and glucose but not insulin resistance (Gruber et al., 2006). In Chinese OSA subjects, there was a five-fold risk of having metabolic syndrome that was associated with waist circumference, diastolic blood pressure and fasting glucose (Lam et al., 2006). Such effects may be independent of obesity: the severity of OSA as measured by the AHI was found to be a strong predictor of metabolic syndrome parameters that included hypertension, dyslipidaemia and hyperglycaemia (Kono et al., 2007). Another study demonstrated that non-obese OSA subjects had metabolic abnormalities associated with dyslipidaemia and hypertension (Lin et al., 2012). Thus, the coexistence of metabolic syndrome and OSA may have detrimental effects on cardiovascular risk and glycaemia. The extent to which OSA has direct effects on components of the metabolic syndrome was found to be dependent on the severity of OSA (Kono et al., 2007) and was correlated with insulin resistance and inflammatory markers (Peled et al., 2007). It is conceivable that OSA itself may represent a complex marker of adverse metabolic and cardiovascular factors. OSA may simply be a marker for upper body obesity, as most of the studies did not measure this directly, however those studies that controlled for total body fat and fat distribution, for example using bioimpedance (Coughlin et al., 2004), computed tomography (Kono et al., 2007) and waist-hip ratio (Basoglu et al., 2011), still show a striking excess of metabolic

syndrome in OSA patients. It seems unlikely that small residual differences in body composition explain the entire clinical picture, furthermore there are a number of plausible biological mechanisms that link OSA itself to components of the metabolic syndrome.

There have been advances in our understanding of the important contributions of OSA to metabolism with findings from studies on the components of the metabolic syndrome and the influence of OSA, with potential interactions that may influence predisposition to cardio-metabolic dysregulation. The parameters defining metabolic syndrome may be important in determining susceptibility to cardiovascular mortality. In an observational study a significant trend was noted between all-cause or cardiovascular mortality and the number of risk factors; and that risk was increased with the number of metabolic syndrome components (Ho et al., 2008). In another study, it was found that metabolic syndrome did not predict cardiovascular mortality independently of the individual variables (Sundstrom et al., 2006). Further longitudinal studies are needed to elucidate the prognostic implications of metabolic syndrome parameters in OSA (Bonsignore et al., 2012a).

Obesity

In OSA patients, many factors may contribute to the effects on metabolism. OSA patients were found to have greater visceral adiposity compared with BMI-matched obese controls and there was a strong association between visceral obesity and OSA (Vgontzas et al., 2000). Intra-abdominal and visceral adiposity has been closely associated with insulin resistance with increased lipolysis and fatty acid availability (Bjorntorp, 1991); as well as dyslipidaemia, hypertension, and hyperglycaemia (Kono et al., 2007).

OSA may perpetuate the cycle of obesity by inducing fatigue and tiredness; the symptoms of somnolence from sleep fragmentation might have the effect of discouraging weight reducing activities in these patients. Conversely, obesity is associated with mechanical effects that may predispose to upper airway obstruction and OSA. The factors causing upper airway obstruction in obesity are only partly understood (Schwartz et al., 2008). Suggested mechanisms include increased collapsibility of pharyngeal structures during air movements, altered chest wall dynamics, respiratory muscle compliance and function (Schwartz et al., 2008). The adiposity in the neck leading to increased neck circumference, with pharyngeal airway narrowing and enlargement of tissue in the upper airways such as the lateral pharyngeal wall and posterior tongue. Lung volumes are also reduced due to the effects of central obesity in recumbency that may decrease tracheal traction forces and pharyngeal wall tension (Isono, 2012). Upper airway collapsibility is increased and lung

volumes may be decreased (Schwartz et al., 2008). Other reported mechanisms include neuromechanical changes such as adipokines and inflammatory cytokines that may modulate upper airway patency (Punjabi, 2008) and ventilatory stability (Fogel et al., 2004) (Schwartz et al., 2008).

Hypertension

It is known that OSA has an adverse effect on blood pressure and OSA patients have an increased risk of hypertension, independent of obesity and age (Peppard et al., 2000b) (Lavie et al., 2000). Furthermore, untreated patients with proven OSA have been found to have an increased risk of hypertension.(Marin et al., 2012) This may be attributable to increased sympathetic activity, with chemoreflex activation, oxidative stress, systemic inflammation and endothelial dysfunction from repeated arousals and intermittent hypoxia (Kapa et al., 2008) (Pedrosa et al., 2011). There may be a role for vasoactive hormones such as renin–angiotensin–aldosterone system activation in OSA-related hypertension with evidence of increased angiotensin II and aldosterone levels in OSA although this remains to be clarified (Moller et al., 2003) (Pedrosa et al., 2011). Additionally, the effects of reduced slow wave sleep on vascular function in OSA may affect cardiorespiratory function by increasing sympathetic activation and pressor responses (Monahan and Redline, 2011). There is evidence that suggests that CPAP treatment may have BP-lowering effects in patients with severe OSA by reducing renin-angiotensin system activity (Fava et al., 2014) (Nicholl et al., 2014).

Dyslipidaemia

In patients with metabolic syndrome, it was observed that patients with OSA had higher triglycerides, cholesterol, LDL, cholesterol:HDL and triglycerides:HDL ratios, furthermore OSA severity (AHI) was independently associated with increased triglycerides and cholesterol/HDL ratio (Drager et al., 2010d). In the Sleep Heart Health Study, alterations in lipid profiles were found in relation to the respiratory disturbance index (RDI) in different subject groups. For example, high-density lipoprotein (HDL) cholesterol levels were inversely related to the RDI in women and in men less than age 65 years; triglyceride levels were associated with the level of RDI only in younger men and women. Total cholesterol level did not vary across quartiles of RDI, although there was a trend towards higher cholesterol in those with higher RDI in the men less than age 65 years (Newman et al., 2001). Another study of OSA patients observed a significant association between the AHI and HDL cholesterol that was independent of age, gender, BMI, diabetes and lipid lowering medication; at 6 months, there were improvements in lipid levels with CPAP therapy (Borgel et al., 2006).

The link between severity of OSA and lipid metabolism is not fully understood and may involve activation of the inflammatory cascade (Alam et al., 2007) and intermittent hypoxia (Drager et al., 2010b). Several murine studies have provided evidence for potential mechanisms. Chronic intermittent hypoxia may induce dyslipidaemia through increased triglyceride and phospholipid synthesis (Li et al., 2005b), and may lead to upregulation of expression of pathways involved in lipid synthesis (Li et al., 2005a). Impaired clearance of triglyceride-rich lipoproteins and inactivation of lipoprotein lipase, and upregulation of lipoprotein lipase inhibition may contribute to hyperlipidaemia fasting levels of plasma triglycerides and very low density lipoprotein cholesterol (Drager et al., 2012) (Drager et al., 2013). With animal models of intermittent hypoxia, there may be more control over variables such as diet, genotypes, oxygen profile, obesity and sleep fragmentation (Jun and Polotsky, 2009). The protocols may however vary in frequency, intermittent hypoxia cycle length and severity of the hypoxic stimulus (Drager et al., 2010c). Levels of intermittent hypoxia may be more severe than that seen in OSA. Furthermore, intermittent hypoxia causes hypoxaemia with hyperventilation and hypocapnia rather than hypercapnia that occurs with airway obstruction (Jun and Polotsky, 2009). These factors may potentially affect gene expression and may limit generalisability of these findings.

OSA and serum urate

Uric acid is a by-product of purine metabolism. The enzyme xanthine oxidase may have a role in cardiovascular dysfunction (Dopp et al., 2007). Studies have reported an association between serum urate and cardiovascular risk including ischaemic heart disease, hypertension and a more adverse cardiovascular risk profile (Feig et al., 2008). Putative mechanisms for the increased cardiovascular risk that have been proposed include an up-regulation of renin release and altered endothelial function (El Solh et al., 2006) (Dopp et al., 2011). Furthermore, xanthine oxidase inhibition (for example with allopurinol), which leads to reduced serum urate, has been shown to improve endothelial dysfunction (El Sohl et al., 2006). Epidemiological studies have suggested that there may be an association between gout and OSA (Roddy et al., 2013). Elevated levels of serum urate have been associated with OSA and may influence cardiovascular risk (Feig et al., 2008). Intermittent hypoxia may lead to increased adenosine triphosphate degradation and purine catabolism that may increase urate levels (Marinchev, 2013). Superoxide radical production by xanthine oxidase in the vascular endothelium may impair vasodilation (Price et al., 2000). Thus there may be a possible role for serum urate in the link between cardiovascular risk and OSA.

OSA and cardiovascular disease

With disruption of the normal sleep architecture, the association of OSA with metabolic syndrome, obesity and type 2 diabetes may increase the risk of cardiovascular disease. Indeed there is evidence suggesting that OSA may contribute or potentially exacerbate cardiovascular disease (Peker et al., 2006) (Marin et al., 2005). Studies have shown an association between OSA with hypertension, coronary disease, stroke, atrial fibrillation and heart failure (Monahan and Redline, 2011). Potential mechanisms include the effects of intermittent hypoxia, recurrent arousals and intrathoracic pressure changes, leading to sympathetic activation, inflammation and oxidative stress that may increase the risk of hypertension (Kohler and Stradling, 2010). Impaired cardiac contractility and negative intrathoracic pressure may increase left ventricular afterload, decrease preload and stroke volume (Bradley and Floras, 2009). Additionally, OSA is also linked with atherosclerosis (Drager et al., 2010a). Therefore, OSA may have potentially serious consequences if discovered later on in the course of the disease. Several studies have suggested that continuous positive airway pressure (CPAP) treatment can attenuate the cardiovascular effects of OSA although large scale randomised controlled trials are still needed (Bradley and Floras, 2009). Furthermore, long-term adverse cardiovascular events are lower in patients with severe OSA who are treated with CPAP (Marin et al., 2012).

Diagnosis and Assessment tools

When approaching patients, the need for diagnostic investigation is a clinical decision that should take account all available clinical information such as symptoms, quality of life and comorbidities. The reason it is difficult to diagnose OSA may be because many of its symptoms are common in the general population. Sleepiness is a non-specific symptom that can arise from many conditions. Subjective sleepiness is common and is not sufficiently discriminating to diagnose OSA (Strohl and Redline, 1996). Furthermore, using the Epworth Sleepiness scale alone may not distinguish snorers from OSA (Osman et al., 1999). Dixon et al. found that symptoms were poor predictors of OSA, while observed sleep apnoea, male gender, age, fasting insulin, glycated haemoglobin, neck circumference and BMI independently predicted severe apnoea with a sensitivity of 96% and specificity 71% (Dixon et al., 2003).

Regardless of sleepiness reported, a combination of patient history that suggests sleep-disordered breathing: including witnessed apnoeas or hypopnoeic events during sleep, snoring, coupled with clinical factors that increase predisposition to OSA such as obesity, may trigger further assessment for OSA. Different approaches have been developed to facilitate the assessment process. In addition,

there is increasing information of the possible role of biomarkers of OSA and other potential screening tools that may yield a sustainable strategy in which sleep-disordered breathing can be screened for **(Table 1.4)**.

Anthropometric measurements

Although clinical features such as obesity (body mass index> 30 kg/m^2) and other anthropometric measurements such as neck and waist circumference, oral pharyngeal attributes (narrow mandible, narrow maxilla, retrognathia, dental malocclusion, overbite, reduced nasal patency, high and narrow hard palate, elongated and low-lying uvula, enlarged tonsils and adenoids, macroglossia) may be useful to consider when screening patients, there are limitations in terms of the predictive values of these variables in making the diagnosis (Riha, 2010) (Ward Flemons and McNicholas, 1997). Weight per se is not useful due to differences in body height and size. The body mass index has predictive power but cannot distinguish between differences in body composition. Neck circumference may be an important factor. Central obesity as determined by waist and hip measurements may be associated with impaired diaphragmatic function and reduced pharyngeal area. Kushida et al. (1997) developed a model combining morphometric measurements of the oral cavity, body mass index and neck circumference to predict OSA (Kushida et al., 1997). In another study, differences in cephalometric measurements in terms of soft palate and lingual and oropharyngeal areas were reported in OSA patients compared with simple snorers (Battagel et al., 2000). However, these methods may not identify some patients with OSA (Ramachandran and Josephs, 2009).

Questionnaires

Many studies have evaluated sleep questionnaires as well as physical and demographic data as tools to screen for possible OSA. A meta-analysis of screening tools by Ramachandran and Josephs (2009) concluded that the Berlin questionnaire and the Sleep Disorders Questionnaire were the two most accurate questionnaires, whereas morphometry and combined clinical– cephalometry were the most accurate clinical models. However, false negatives may still occur by the use of these screening methods (Ramachandran and Josephs, 2009). In a review by Abrishami et al. (2010), eight screening questionnaires were evaluated based on the findings from ten studies that met the inclusion criteria. It was noted that the validation studies of these questionnaires had been performed in different populations with variations in study design. Although the questionnaire approach may be useful to identify patients at risk for OSA, due to inconsistent evidence from the evaluated studies, it was not possible to conclude which questionnaire was the most accurate (Abrishami et al., 2010).

Clinical prediction models

Clinical prediction models and artificial neural nets are methods that are capable of recognising complex patterns of biological data. Although clinical prediction rules may have reasonably high sensitivities, the presence of intermediate specificities means that at least some type of additional testing is required to confirm the diagnosis (Ward Flemons and McNicholas, 1997).

Portable screening devices

Portable devices may be divided into four types: Type 1: full attended polysomnography (≥ 7 channels) in a laboratory setting; Type 2: full unattended polysomnography (≥ 7 channels); Type 3: limited channel devices (usually using 4–7 channels) and Type 4: 1 or 2 channels usually using oximetry as 1 of the parameters (Collop et al., 2007). There have been several portable OSA screening devices reported in the literature (**Table 1.4**) Devices such as the pulse oximeter, QUISI ambulatory electroencephalogram, NovaSom QSG, WatchPat, Somnocheck, Sleep Strip have been reviewed prevously (Pang and Terris, 2006). The RUSleeping RTS is a screening device that provides real-time screening by measuring airflow nasal pressure and detects apnoea events, supplementing other screening tools and provides an assessment for the need for more formal testing (Phillips Respironics). ApneaLink is a portable screening device that uses nasal pressure cannulae and pressure sensors (Clark et al., 2009). ApneaLink was shown to be useful in screening high-risk patients (Clark et al., 2009) (Ragette et al., 2010). Nasal pressure recordings in 30 subjects using SleepCheck, an ambulatory device, overscored disordered breathing events in subjects with OSA, but correlation was reported with the apnoea–hypopnoea index and respiratory disturbance index (de Almeida et al., 2006). The Embletta is a portable device that has been shown to produce AHI results that are correlated with AHI results from sleep laboratory polysomnography (Ng et al., 2010). Other portable devices include the MediByte, SOMNIE, ApnoeaScreen, Morpheus, ARES Unicorder, Stardust II, the SIESTA sleep system, North-East Combined Holter-Oximetry monitoring, NovaSom QSG, the Remmers Sleep Recorder, SD-101 pressure sensors, APV2 Remote analysis and automated plethysmography (**Table 1.4**). These will not be discussed further in this thesis. An evaluation of many of these portable devices has been described previously (Collop et al., 2011).

Potential for biomarkers

More recently, analysis of exhaled air provides a non-invasive approach to investigate airway inflammation. Increased inflammatory markers such as IL-8, ICAM, nitric oxide (NO), nitrates, 8-isoprostane, leukotriene B4, hydrogen peroxide have been found in exhaled air, breath condensate and induced sputum of OSA patients (Petrosyan et al., 2008). The increase of markers has been

found to be related to the apnoea–hypopnoea index, suggesting that the assessment of inflammation may help in predicting OSA severity (Carpagnano et al., 2010) (Bucca et al., 2011). There have been studies investigating the relationship between biomarkers such as C-reactive protein, IL-6 and TNF-alpha that have yielded mixed results. There has also been interest in the link between hypertension and OSA, given the beneficial effects of CPAP on lowering blood pressure. Hypertension and OSA frequently coexist, but the high prevalence in the general population reduces its predictive value (Montesi et al., 2012a). Other potential markers include carotid intima-media thickness, glycated haemoglobin A1c (HbA1c), cysteine and homocysteine although further studies are necessary to define the potential of these variables (Montesi et al., 2012a). Reduced leptin and raised ghrelin levels have been found to be associated with short sleep duration. These perturbations in appetite-regulating hormones may contribute to the association between obesity and OSA (Taheri et al., 2004).

In summary, the major features for OSA such as snoring, observed apnoeas, anthropometric measurements such as abnormal upper airway examination, and obesity indices and clinical biomarkers such as blood pressure may be of value for clinical assessment but are at present not sufficiently specific to be applied as screening tools.

Table 1.4. Tools to facilitate the assessment process (Seetho & Wilding 2013)

Screening Method		Reference	Sample Size	Threshold	Outcome
Questionnaire	Epworth Score	(Pouliot et al., 1997)	354	AHI <20	Sensitivity 42%, Specificity 68%
		(Osman et al., 1999)	46	AHI 0-26	Low correlation with AHI (r=0.12)
	Berlin	(Netzer et al., 1999)	100	RDI>5	Sensitivity 86%, Specificity 77%, PPV 89%
		(Sharma et al., 2006)	104	AHI>5	Sensitivity 86%, Specificity 95%, PPV 96%, NPV 82%
		(Ahmadi et al., 2008)	130	RDI>5	Sensitivity 68%, Specificity 49%, PPV 50%, NPV 49%
	STOP Questionnaire	(Silva et al., 2011)	4770	RDI 15-30	Sensitivity 62%, Specificity 56.3%, LR+ 1.4, LR- 0.67
				RDI>30	Sensitivity 68.8%, Specificity 59.5%, LR+ 1.5, LR- 0.69
		(Chung et al., 2008a)a	177	AHI>5	Sensitivity 65.6%, Specificity 60%, PPV 78.4%, NPV 44%
				AHI>15	Sensitivity 74.3%, Specificity 53.3%, PPV 51%, NPV 76%
				AHI>30	Sensitivity 79.5%, Specificity 48.6%, PPV 30.4%, NPV 89.3%
	STOP-BANG questionnaire	(Chung et al., 2008a)a	177	AHI >5	Sensitivity 83.6%, Specificity 56.4%, PPV 81%, NPV 60.8%
				AHI>15	Sensitivity 92.9%, Specificity 43%, PPV 51.6%, NPV 90.2%
				AHI>30	Sensitivity 100%, Specificity 37%, PPV 31%, NPV 100%
		(Silva et al., 2011)	4770	Moderate-severe SDB (RDIs of ≥ 15 – < 30)	Sensitivity 87%, Specificity 43.3%, LR+ 1.5, LR- 0.3
				Severe SDB (RDI≥ 30)	Sensitivity 70.4%, 59.5%, LR+ 1.7, LR- 0.49
	American Society of Anaesthesiologists (ASA)	(Chung et al., 2008b)b	177	AHI >5	Sensitivity 72.1%, Specificity 38.2% PPV 72.1, NPV 38.2%
				AHI>15	Sensitivity 78.6%, Specificity 37.4%, PPV 45.1%, NPV 72.7%
				AHI>30	Sensitivity 87.2%, Specificity 36.2%, PPV 27.9%, NPV 90.9%
	Sleep Disorders Questionnaire	(Weatherwax et al., 2003)	125	AHI>5	For men, an SA-SDQ score of 29 provided a sensitivity of 75% and a specificity of 65%. For women, an SA-SDQ score of 26 provided a sensitivity of 80% and a specificity of 67%

Screening Method		Reference	Sample Size	Threshold	Outcome
Clinical models	Artificial Neural Network with 45 variables	(Kirby et al., 1999)	150		Sensitivity 98.9%, Specificity 80%, PPV 88.1%, NPV 98%
	Neural network with 12 variables	(El-Solh et al., 1999)	189	AHI 10 AHI 15 AHI 20	Sensitivity 94.9%, Specificity 64.7, PPV 87.9%, 85.2% Sensitivity 95.3%, Specificity 60%, PPV 83.7%, NPV 85.7% Sensitivity 95.5%, Specificity 73.4%, PPV 83.3%, NPV 92.1%
	Decision rule based on crico-omental space, pharyngeal grade, overbite	(Tsai et al., 2003)	75	RDI>10	Sensitivity 100%, Specificity 46%, PPV 76%, NPV 100%* (*based on crico-omental space>1.5cm) Sensitivity 40%, Specificity 96%, PPV 95%, NPV 49%** (**based on 3 variables model)
	Clinical decision support system (CDSS) in veterans with ischaemic heart disease	(Laporta et al., 2012)	91	AHI>5	Sensitivity 98.5%, Specificity 86.9%
Screening Method		Reference	Sample Size	Threshold	Outcome
Screening Devices	ApneaLink®	(Clark et al., 2009)	67	AHI>15	Sensitivity 92%, Specificity 96.7%, NPV 94%
		(Chen et al., 2009)	50	AHI 5 AHI 10 AHI15 AHI20 AHI 30	Sensitivity 97.7%, Specificity 66.7% Sensitivity 95%, Specificity 90% Sensitivity 87.5%, Specificity 88.9% Sensitivity 88%, Specificity 88% Sensitivity 88.2%, Specificity 93.9%
	RUSleeping RTS®	(Grover and Pittman, 2008)	25	AHI>5	Sensitivity 89%, Specificity 86%, LR+ 6.2, LR- 0.13
		(Watkins et al., 2009)	34	AHI>15	Sensitivity 70%, Specificity 83%, PPV 64%, NPV 87%
	MediByte®	(Driver et al., 2011)	73	AHI>5 AHI>15 AHI>30	Sensitivity 97%, Specificity 67%, PPV 94%, NPV 80% Sensitivity 80%, Specificity 97%, PPV 97%, NPV 76% Sensitivity 70%, Specificity 100%, PPV 100%, NPV 88%
	SleepCheck®	(de Almeida et al., 2006)	30	AHI 10 AHI 20	Sensitivity 86.4%, Specificity 75% Sensitivity 88.9%, Specificity 81%
	Embletta® portable diagnostic system	(Ng et al., 2010)	80	AHI>5 AHI>10 AHI>15 AHI>20	Sensitivity 92.4%, Specificity 85.7%, PPV 96.8%, NPV 70.6%, LR+ 6.462, LR- 0.089 Sensitivity 90%, Specificity 86.7%, PPV 91.8%, NPV 83.9%, LR+ 6.767, LR- 0.115 Sensitivity 87.8%, Specificity 94.9%, PPV 94.7%, NPV 88.1%, LR+17.216, LR- 0.129 Sensitivity 85.3%, Specificity 95.7% PPV 93.5%, NPV 89.8%, LR+ 19.837, LR- 0.154

Screening Method		Reference	Sample Size	Threshold	Outcome
Screening Devices	SOMNIE® -Single Channel Airflow monitor for screening	(Nakano et al., 2008)	100	AHI>5 AHI>15 AHI>30	Sensitivity 96%, Specificity 82%, LR+ 5.3, LR- 0.05 Sensitivity 91%, Specificity 82%, LR+ 5.2, LR- 0.11 Sensitivity 89%, Specificity 96%, LR+ 21.2, LR- 0.12
	Apnoeascreen I® A five channel recording device recording variations in oronasal airflow, body position, arterial oxygen saturation, wrist actimetry	(Golpe et al., 2002)	44	AHI>10	Findings from device agreed with hospital polysomnography agreed in 75% cases, 77% of clinical decisions based on the device agreed with polysomnography.
	Apnoeascreen II® Respiratory polygraphy device with actigraphy	(Garcia-Diaz et al., 2007)	62	AHI>10 AHI>10	Sensitivity 91.6-96.9%, Specificity 92-96.7% (measured in sleep laboratory) Sensitivity 83.8-87.5%, Specificity 94.7-100% (measured at home)
	Morpheus® Portable Device	(Takama and Kurabayashi, 2010)	83*	AHI>20	*patients with cardiovascular disease Sensitivity 81%, Specificity 86%
	Apnea Risk Evaluation System (ARES)® Unicorder	(Ayappa et al., 2008)	97	RDI>15	Sensitivity 95%, Specificity 94%, LR+ 17.04, LR- 0.06 (measured in laboratory) Sensitivity 85%, Specificity 91%, LR+9.34. LR- 0.17 (measured in home)
		(Westbrook et al., 2005)	284 (in-lab ARES testing) 187 (home ARES testing)	AHI>10 AHI>10	Sensitivity 97.4%, Specificity 85.6%, PPV 93.6%, NPV 93.9% Sensitivity 91.5%, Specificity 85.7%, PPV 91.5%, NPV 85.7%
	Stardust II®	(Santos-Silva et al., 2009)	80 (70 suspected OSA & 10 without suspicion of OSA	AHI>5 AHI>15 AHI>30	Sensitivity 93%, Specificity 59%, PPV 85%, NPV 76% Sensitivity 85%, Specificity 80%, PPV 80%, NPV 84% Sensitivity 77%, Specificity 93%, PPV 81%, NPV 91%
	Siesta Sleep System®	(Campbell and Neill, 2011)	30	AHI>5 AHI>10 AHI>15	Sensitivity 88%, Specificity 50%, LR+ 1.76, LR- 0.24 Sensitivity 90.5%, Specificity 88.9%, LR+ 8.14, LR- 0.11 Sensitivity 93.7%, Specificity 76.9%, LR+ 4.06, LR- 0.081
	WatchPat 100®	(Choi et al., 2010)	25	AHI>5 AHI>15 AHI>30	Sensitivity 100%, Specificity 83%, PPV 95%, NPV 100% Sensitivity 81%, Specificity 77%, PPV 87%, NPV 70% Sensitivity 92%, Specificity 92%, PPV 92%, NPV 92%

Screening Method		Reference	Sample Size	Threshold	Outcome
Screening Devices	NorthEast Monitoring Combined Holter-oximeter	(Heneghan et al., 2008)	59	AHI 5 AHI10 AHI15	Sensitivity 93.8%, Specificity 100%, LR+ infinity, LR- 0.06 Sensitivity 81.6%, Specificity 90.5%, LR+ 8.6, LR- 0.2 Sensitivity 74.2%, Specificity 96.4%, LR+ 20.8, LR- 0.27
	NovaSom QSG®	(Reichert et al., 2003)	51	AHI15	Sensitivity 95%, Specificity 91%, PPV 91%, NPV 96% (NovaSom in lab) Sensitivity 91%, Specificity 83%, PPV 83%, NPV 91% (NovaSom at home)
	SNAP®	(Su et al., 2004)	60	RDI>5 RDI>10 RDI>15	Sensitivity 98%, Specificity 40%, PPV 89.1%, NPV 80% Sensitivity 87.8%, Specificity 73.7%, PPV 87.8%, NPV 73.7% Sensitivity 83.9%, Specificity 75.9%, PPV 78.8%, NPV 81.5%
	Remmers Sleep Recorder (RSR)	(Jobin et al., 2007)	94	RDI>5 RDI>10 RDI>15	Sensitivity 75.3%, Specificity 81%, LR+ 3.95, LR- 0.3 Sensitivity 67.9% , Specificity 87.8%, LR+ 5.57, LR- 0.37 Sensitivity 62.8%, Specificity 96.1%, LR+ 16, LR- 0.39
	SD-101 Pressure sensors sense gravitational alterations in body respiratory movements	(Agatsuma et al., 2009)	n=201 suspected OSA & n=165 screening group	RDI 14	Sensitivity 89.5%, Specificity 85.8%
	Automated plethysmography	(Amir et al., 2012)	64	AHI>15	Sensitivity 98%, Specificity 96%
	APV2 Remote Analysis	(Tiihonen et al., 2009)	20 (10 for APV2, 10 for Embla)	Records breathing movements, nasal & oral airflow, position, snore, oxygen saturations, Heart Rate	Similar diagnostic capability compared with Embla reference instrumentation. Close correlation between AHI and ODI recorded with both APV2 and Embla (r=0.996-0.997)
Screening Method		Reference	Sample Size	Threshold	Outcome
Biomarkers	Analysis of exhaled air, breath condensate and sputum	(Depalo et al., 2008) (Carpagnano et al., 2008)	43 60		Exhaled Nitric oxide (NO) significantly increased in obese OSA subjects, with increase in neutrophils, decrease in macrophages in induced sputum compared with healthy controls Concentration of exhaled NO and percentage of neutrophils in sputum was greater in obese subjects compared to controls. The pH of exhaled air was also lower in the obese subjects
	Changes in Leptin and Ghrelin	(Taheri et al., 2004)	856		Short sleep was associated with low leptin and high ghrelin
	Plasma adhesion molecules –selectins	(Cofta et al., 2013)	80		Progressive increase in the concentrations of E, L and P-selectins with the severity of OSA

Screening Method		Reference	Sample Size	Threshold	Outcome
Biomarkers	Inflammatory markers, carotid intimal-media thickness, HbA1c, IL-6, Cysteine	(Steiropoulos et al., 2010)	61	AHI>15	Obese OSA patients had significantly higher TNF alpha levels
		(Drager et al., 2005)	42	AHI>5	Increased pulse wave velocity, Increased atherosclerosis (carotid intimal-media thickness and diameter) in OSA (AHI>5)
		(Carpagnano et al., 2002)	43	AHI>20	Increased 8 isoprostane and IL-6 in OSA
		(Cintra et al., 2011)	150	AHI>20	Significant increase in cysteine levels in OSA patients compared with controls. CPAP treatment significantly reduced plasma cysteine levels after 6 months
		(Aronsohn et al., 2010)	60	AHI>5	OSA severity was positively correlated with increasing HbA1c levels
	Heart rate variability using Electrocardiogram algorithm	(Babaeizadeh et al., 2011)	25 (development) 1907 (test)		Sensitivity 74%, Specificity 62%, PPV 64%, NPV 72%
	Heart rate changes measured by pulse oximetry	(Adachi et al., 2003)	33		Sensitivity 88%, Specificity 86%
	Electrocardiogram (ECG) based cyclic variation of heart rate (CVHR)	(Hayano et al., 2011a)a	862	AHI>15	Sensitivity 83%, Specificity 88%
		(Hayano et al., 2011b)b	862	AHI>15	PPV 96.1%, NPV 95.1%, LR+ 50.6, LR- 0.11
				AHI>30	PPV 95.6%, NPV 95.1%, LR+ 97.3, LR- 0.23
	ECG derived respiration	(Babaeizadeh et al., 2011)	25 (development) 1907 (test)	Measures respiratory waveforms using an ECG algorithm	Sensitivity 45%, Specificity 76%, PPV 64, NPV 60
	Snore based studies	(Abeyratne et al., 2005)	45 (16 training subjects & 29 testing subjects)	Intrasnore Pitch Jump	Sensitivity 86-100%, Specificity 50-80%
	Multiparametric snore sound analysis	(Karunajeewa et al., 2010)	41	Logistic regression model with snore parameters	Sensitivity 89.3%, Specificity 92.3%
	24 hour BP monitoring	(Gresova et al., 2009)	116		Increased nocturnal average systolic & diastolic blood pressure with sleep apnoea according to disease severity. 24 hour average systolic and diastolic blood pressure increases in sleep apnoea
	Cardiac & Respiratory Monitor (CPAM) (recorder placed on upper chest & attached with 2 ECG electrodes)	(Dillier et al., 2012)	85	AHI >5	Sensitivity 57.9%, Specificity 89.3%, PPV 91.7%, NPV 51%
				AHI 15-30	Sensitivity 74.3%, Specificity 86%
				AHI>30	Sensitivity 50%, Specificity 88.1%

Table 1.4 Different methods to facilitate the assessment process (Seetho & Wilding 2013) AHI: Apnoea-Hypopnoea Index, RDI: Respiratory Depression Index, PPV: Positive predictive value, NPV: Negative predictive value, LR+: Positive likelihood ratio, LR-: Negative likelihood ratio, HbA1c: glycated haemoglobin, ODI: oxygen desaturation index

Treatment of OSA

The different modalities for the treatment of OSA are presented in the table below.

CPAP
Weight loss (Lifestyle & Bariatric Surgery Interventions)
Oral devices
Surgery (Upper Airway)
Possible others (pharmacotherapy; positional therapy; novel methods such as upper airway muscle training & stimulation; transnasal insufflation; oral pressure therapy)

Table 1.5 Different modalities for treating OSA

Treatment - CPAP

CPAP treatment abolishes repetitive upper airway obstruction during sleep by splinting the airway open to facilitate airflow to reduce daytime sleepiness and improve health status and quality of life (Ballester et al., 1999, Jenkinson et al., 1999). It is the treatment of choice in patients with moderate-severe OSA. It is also recommended in mild OSA if symptoms are severe enough to affect quality of life or daily activities and if lifestyle or other treatments have been unsuccessful (National Institute for Health and Care Excellence, 2008). CPAP intervention studies have allowed the study of the potential effects of OSA treatment and whether specific effects in patients can be modified and provided important insights on the relation between SDB and altered metabolism. OSA therapy may be a potential avenue for addressing cardio-metabolic risk given the role of OSA in glycaemic and metabolic dysregulation.

Diabetes

Studies have explored the influence of continuous positive airway pressure (CPAP) therapy on glucose homeostasis have thus far yielded mixed findings (Surani and Subramanian, 2012) (Hecht et al., 2011) (Iftikhar and Blankfield, 2012). Harsch et al (2004) reported that there was improved insulin sensitivity in patients without diabetes at 2 days and at 3 months of CPAP treatment (Harsch et al., 2004a), but these subjects were non-obese, limiting the generalisability of this finding. Although Dawson et al. (2008) found no significant change in HbA1c levels after an average of 41 days of CPAP treatment, it was noted that nocturnal glucose levels were decreased with CPAP therapy and therefore a potential effect on glycaemia (Dawson et al., 2008). In an observational study by Babu et al (2005), there were lower fasting and post-prandial glucose levels following three months of CPAP, with improved glycaemic control in patients with glycated haemoglobin (HBA1c)>7% (Babu et al., 2005). Nevertheless, these studies were limited by an absence of a placebo treatment group.

Several randomised controlled trials have been performed **(Table 1.6)**. It was demonstrated that nasal CPAP treatment of OSA for 1 week improved insulin sensitivity in males without diabetes and this improvement appeared to be maintained after 12 weeks of treatment in those with moderate obesity (Lam et al., 2010). Another study by Weinstock et al (2012) found that CPAP did not aid a reversion to normal glucose tolerance in subjects with impaired glucose tolerance. However, there was suggestion of improved insulin sensitivity in those patients with severe OSA (AHI>30) (Weinstock et al., 2012). However, West et al (2007) reported that CPAP treatment compared with sham-CPAP treatment in men with OSA and type 2 diabetes improved Epworth Sleepiness Scale (ESS) scores, but did not improve glycaemic control or insulin resistance (West et al., 2007).

Table 1.6

Study	Population	Parameters	CPAP regimen	findings
West et al (2007)	42 men with type 2 diabetes and newly diagnosed OSA (>10 oxygen desaturation of >4% per hour) randomised	Glycaemic control and insulin resistance (Insulin resistance was assessed by both HOMA and euglycaemic hyperinsulinaemic clamp); lipid profile, adiponectin, hsCRP	Randomised to therapeutic (n = 20) or placebo CPAP (n = 22) for 3 months	CPAP did not affect glycaemic control or insulin resistance.
Comondore et al (2009)	Subjects with AHI>15 (13 patients completed protocol)	Cardiovascular measures (urinary microalbumin, catecholamines, blood pressure, homeostasis model for insulin resistance score and endothelial function resistance score (decreased by 1.11);	Randomised to either CPAP or no therapy for 4 weeks followed by washout for 4 weeks, and then a crossover to the other intervention	Although insulin resistance (HOMA) decreased with CPAP, the difference was not significant. Likewise, the other cardiovascular measures did not show significant improvements.
Lam et al (2010)	61 men randomised with moderate-severe OSA (AHI>15)	Effects of nasal CPAP treatment of OSA on insulin sensitivity in male subjects without diabetes mellitus	Randomised to therapeutic nasal CPAP (n=31) or sham CPAP (n=30). (n=29 completed 12 weeks therapeutic CPAP, n=30 completed 1 week of sham CPAP)	Therapeutic nasal CPAP treatment for 1 week improved insulin sensitivity in obese men (BMI>25) without diabetes, and the improvement was maintained after 12 weeks of treatment
Weinstock et al (2012)	50 subjects with OSA (AHI > 15) and impaired glucose tolerance	normalisation of the mean 2 hour OGTT; a secondary outcome was improvement in the Insulin Sensitivity Index	Randomised to 8 weeks of CPAP or sham CPAP; patients crossed over to other therapy after a one-month washout	CPAP did not normalise impaired glucose regulation. insulin sensitivity improved in subjects with severe OSA (AHI ≥ 30)
Hoyos et al (2012)	65 men randomised with AHI>20 & ODI >15 (46 completed protocol)	Visceral abdominal & liver fat, insulin sensitivity	Real or sham CPAP for 12 weeks; At the end of the 12-week blinded period all participants had real CPAP for an additional 12 weeks	No group differences at 12 weeks. At 24 weeks, insulin sensitivity was improved but not visceral abdominal or liver fat.

Table 1.6 Therapeutic vs sham CPAP studies in glucose control and insulin resistance

Individual heterogeneity in terms of glycaemic responses to CPAP suggests most of these studies were probably underpowered to show significant effects on glycaemic control, and that the encouraging results seen in uncontrolled observational studies are in general not confirmed in randomised controlled trials. It is possible that for individuals with impaired glucose tolerance, other approaches such as intensive lifestyle intervention may be more effective in preventing progression to type 2 diabetes (Pepin et al., 2012) (Gillies et al., 2007) (Tuomilehto, 2009).

Nevertheless, a recent observational study of OSA patients with type 2 diabetes assessed clinical outcomes and cost-effectiveness of CPAP treatment compared with non-treatment. It was found that CPAP use was associated with significantly lower blood pressure, improved glycaemic control, and was more cost-effective compared with patients who were not treated with CPAP (Guest et al., 2014). However, this study had limitations because patients were not randomised to the treatments received, by the use of observational data and reliance on clinical outcome findings that were based on clinical entries in patient records. Therefore, no cause-and-effect inferences regarding CPAP treatment and these outcomes can be made.

Metabolic Syndrome

In order to understand CPAP treatment effects on cardio-metabolic profiles, studies have examined the effect of CPAP on the metabolic syndrome components as endpoints. Randomised controlled trials have evaluated the metabolic syndrome by comparing therapeutic and sham-CPAP **(Table 1.7)**.

Study	Population	Parameters	CPAP regimen	findings
Coughlin et al (2007)	Randomised controlled trial. 35 OSA subjects randomised (34 completed protocol)	Metabolic syndrome (using NCEP ATIII); blood pressure, glucose, lipids, insulin resistance,	Each group received 6 weeks of therapeutic or sham CPAP, then treatment crossover for further 6 weeks	Significant decreases in systolic and diastolic blood pressure. No change in glucose, lipids, insulin resistance or the proportion of patients with metabolic syndrome.
Hoyos et al (2013)	Analysis of results from a randomised controlled trial. 65 men with OSA randomised (46 completed protocol)	Effect on metabolic syndrome (using international consensus guidelines & NCEP ATPIII criteria) (analysis of results from Hoyos et al 2012)	Real or sham CPAP for 12 weeks	12 weeks of CPAP therapy had did not affect numbers of patients with metabolic syndrome.

Table 1.7 Therapeutic vs sham CPAP studies in metabolic syndrome

A meta-analysis of randomised trials found that there were favourable effects of CPAP treatment in terms of improving blood pressure responses (Haentjens et al., 2007). In terms of the effects on lipid profiles, there have been mixed results from different studies although the current evidence suggests that CPAP treatment may decrease total and LDL cholesterol (Bonsignore et al., 2012b). Previous work by Coughlin et al (2007), a randomised crossover trial with sham CPAP as control did not show any differences in lipids (Coughlin et al., 2007). In other controlled trials, Comondore et al (2009) randomised subjects to 4 weeks of CPAP or no therapy, with crossover after washout for 4 weeks; although no significant difference in lipid levels were found, there appeared to be reduced triglyceride levels (Comondore et al., 2009). Robinson et al (2004) randomised patients to either 1 month therapeutic or sub-therapeutic CPAP and found a trend towards a decrease in total cholesterol after CPAP (Robinson et al., 2004). One randomised cross-over trial with two months of therapeutic and sham-CPAP showed that treatment with CPAP improved postprandial triglyceride and total cholesterol levels (Phillips et al., 2011).

An interventional controlled study in males without diabetes did not find a significant change in weight, body fat, insulin resistance, or lipid profiles with 6 weeks of CPAP treatment as opposed to sham-CPAP therapy (Coughlin et al., 2007). Nevertheless, there were improvements in blood pressure control following CPAP intervention (Coughlin et al., 2007). A controlled study by Hoyos et al (2012) evaluated 12 weeks of therapeutic versus sham-CPAP on visceral adiposity and insulin sensitivity in males without diabetes and found that there was no significant effect of CPAP on either parameter (Hoyos et al., 2012).

In a further analysis, it was noted that the 12 weeks of CPAP therapy did not have a significant effect on the number of subjects with metabolic syndrome (Hoyos et al., 2013). In another placebo-controlled study by Kritikou et al (2014) compared 2 months of therapeutic and sham-CPAP in non-obese subjects and found no significant difference in metabolic markers including IL6, tumour necrosis factor, leptin, adiponectin and highly-sensitive C-reactive protein. CPAP treatment was not sufficient to alter these factors in this study although it was noted that the short duration of therapy may have limited metabolic alterations (Kritikou et al., 2014). Taken together, the results of studies suggest that CPAP in isolation may not be sufficient for OSA patients with the metabolic syndrome, although there is reasonably consistent evidence for a beneficial effect on blood pressure. It has been proposed that there may be a role for a multifaceted approach for these individuals in order to manage their cardio-metabolic risk (Pepin et al., 2012). Thus it is envisaged that measures to promote proper sleep hygiene and weight loss may be important.

Treatment – weight loss

It is clear that the promotion of weight loss activities and lifestyle changes has the potential to improve glucose regulation. There is also evidence that supports the role of weight loss in the treatment of OSA (Veasey et al., 2006). **(Table 1.8)**.

In the Wisconsin Sleep Cohort Study, subjects were prospectively observed for change in AHI and the development of SDB with weight change; weight control was associated with decreased AHI and a reduced likelihood of OSA (Peppard et al., 2000a). These findings are supported by evidence from randomised controlled trials. In the Sleep AHEAD study, obese subjects with type 2 diabetes and OSA were either randomised to a programme of intensive lifestyle intervention comprising behavioural weight loss and physical activity or to diabetes support and education. It was found that the intensive lifestyle intervention reduced weight and AHI over 4 years more than diabetes support and education alone (Kuna et al., 2013). Other randomised trials investigating various lifestyle modifications such as very low calorie diets combined with active lifestyle counselling (Tuomilehto et al., 2009), intensive exercise training (Kline et al., 2011), cognitive-behavioural weight loss and very low calorie diet (Kajaste et al., 2004) showed improved OSA severity with these interventions. These effects of lifestyle measures in OSA may potentially be sustained over time (Tuomilehto et al., 2013) (Kuna et al., 2013). In a randomised parallel group trial that compared the effects of CPAP, weight loss or both treatments for 24 weeks in adults with obesity, combination therapy with weight loss and CPAP had a beneficial effect in reducing insulin resistance, serum triglyceride levels and blood pressure (Chirinos et al., 2014).

Table 1.8 Selected studies relating to weight loss with lifestyle and bariatric surgery in OSA

Lifestyle interventions

Study	Population	Study protocol	Key findings
Peppard et al (2000)	690 randomly selected employed Wisconsin residents	Prospective study of change in AHI and odds of developing moderate-severe SDB with respect to change in weight	Relation between weight gain and increased SDB severity. Weight loss was associated with reduced SDB severity and likelihood of developing SDB.
Kuna et al (2013)	264 obese adults with type 2 diabetes and OSA	Randomised to either intensive lifestyle intervention with a behavioural weight loss program (controlled diet, physical activity) OR diabetes support and education (3 group sessions annually) over 4 years	Intensive lifestyle intervention produced greater reductions in weight and AHI over 4 years than diabetes support and education. Effects on AHI persisted at 4 years, despite an almost 50% weight regain.
Tuomilehto et al (2009)	72 patients (BMI 28-40) with mild OSA completed the protocol	Randomised to either very low calorie diet (VLCD) program with supervised lifestyle modification OR routine lifestyle counselling for 1 year	VLCD combined with active lifestyle counselling effectively reduced body weight and a significant reduction in AHI compared with the control group.
Tuomilehto et al (2013)	Follow-up study to Tuomilehto et al (2009). 57 patients completed the follow-up over 4 years	Assessment of long-term efficacy of lifestyle intervention during 4-year follow-up in OSA subjects who participated in the initial 1 year randomised intervention trial –(See Tuomilehto 2009)	Intervention achieved reduction in the incidence of progression of the OSA compared with the control group. The improvement in OSA that was sustained even 4 years after the active intervention.
Kline et al (2011)	43 sedentary and overweight/obese adults with OSA AHI≥15	Randomised to intensive exercise training OR low intensity stretching regimen	Compared with stretching, exercise resulted in a significant reduction in AHI and ODI despite non-significant changes in body weight
Kajaste et al (2004)	31 obese male symptomatic OSA patients (ODI>10)	All subjects had active weight reduction based on the cognitive-behavioural approach (CBT) (24 months) and a very-low-calorie diet (6 weeks) & were randomly selected to have either nasal CPAP OR without CPAP for the initial 6 months	Weight loss and improvement of OSA was achieved by the CBT weight loss program. CPAP in the initial phase of the weight reduction program did not result in significantly greater weight loss.
Thomasouli et al (2013)	Systematic review and meta-analysis of 12 randomised controlled trials with lifestyle interventions in adults with OSA. Diet and diet plus CPAP therapy were compared in three studies (n=261), and intensive lifestyle programmes and routine care were compared in six studies (n=483).	To evaluate the impact of diet, exercise and lifestyle modification programmes on indices of obesity and OSA parameters	Intensive lifestyle management can significantly reduce obesity and OSA severity. Lifestyle modification combined with CPAP may confer additional benefits in OSA.
Chirinos et al (2014)	181 obese patients, moderate-severe OSA and CRP>1mg/l. 136 completed the study. Intention to treat analysis for 146 subjects.	Randomised parallel group 24-week trial that compared the effects of CPAP, weight loss or both in adults with obesity. (weight loss interventions included weekly counselling, unsupervised exercise and cognitive behavioural therapy).	Weight-loss with CPAP therapy had an incremental effect in reducing insulin resistance and serum triglyceride levels, compared with CPAP alone. Additionally, combined treatment may result in a larger reduction in blood pressure than either treatment alone.

Metabolic-bariatric surgery

Study	Population	Parameters studied	Key findings
Dixon et al (2012)	60 obese patients (BMI 35-55), recently diagnosed OSA AHI≥20	Randomised to a conventional weight loss program (regular consultations with dietitian and physician, with use of very low-calorie diets) OR to laparoscopic adjustable gastric banding	Bariatric surgery produced greater weight loss but did not result in a statistically greater reduction in AHI compared with conventional weight loss therapy
Buchwald et al (2004)	Systematic review and meta-analysis of 136 fully extracted studies (total 22094 patients)	Systematic review and meta-analysis to determine the impact of bariatric surgery on weight loss, diabetes, hyperlipidaemia, hypertension, and OSA	Bariatric surgery produced effective weight loss in morbidly obese individuals with beneficial effects in the majority in terms of diabetes, hyperlipidaemia, hypertension and OSA

For patients who are unable to lose sufficient weight by lifestyle interventions alone, pharmacotherapy may be useful adjunct therapy. The influence of weight loss with the use of medications such as Glucagon-Like Peptide-1 (GLP-1) agonists in type 2 diabetes may potentially influence OSA. GLP-1 is an incretin that is released by intestinal L-cells in response to nutrient ingestion, which enhances glucose-stimulated insulin release by pancreatic beta cells and acts on satiety pathways to reduce food intake. The SCALE sleep apnoea trial was a randomised double blind placebo-controlled trial that investigated the effects of liraglutide (a GLP-1 agonist) in obese and overweight subjects compared with placebo. It was shown that there were significant and sustained improvements in body weight, with improved OSA severity (AHI scores) at 32 weeks in individuals with moderate to severe OSA (Blackman et al., 2014).Thus the results indicated that weight loss improved OSA.

Concerning the effects of surgical weight loss, in a meta-analysis of bariatric surgery outcomes, Buchwald et al (2004) reported improvements in glycaemic control, dyslipidaemia, hypertension, blood pressure and OSA following bariatric surgery, but the included studies were often of poor quality with a high proportion of patients lost to follow up (Buchwald et al., 2004). However, a randomised controlled trial investigated whether weight loss by bariatric surgery (laparoscopic gastric banding) was more effective than conventional weight loss therapy (medical consultations and low calorie diets) in the management of OSA. Despite more significant weight loss, it was found that surgical weight loss did not lead to significantly greater improvements in OSA, and therefore most patients would still require CPAP treatment post bariatric surgery (Dixon et al., 2012). Hence bariatric surgery alone may not be sufficient to obviate the need for CPAP therapy. In a review that sought to compare surgical and non-surgical weight loss studies in relation to BMI and AHI, no definitive statement could be made regarding the relative benefits of surgical therapy in OSA mainly because of inherent differences between trials and the need for more comparative studies between surgical and non-surgical methods of treating OSA (Ashrafian et al., 2012). Nevertheless, in relation to metabolic effects, improved glycaemic control is seen in type 2 diabetes with surgical weight loss approaches (Dixon et al., 2008) (Schauer et al., 2012).

It is possible that the different pathophysiological mechanisms operating in OSA may not be completely reversed by surgical weight loss alone despite our knowledge of the potential for amelioration of cardio-metabolic risks with weight loss in obesity and type 2 diabetes by lifestyle modifications. Conceivably, it would be expected that the extent of patient responses to different

approaches may differ according to individual circumstance. Thus a combination of weight loss interventions and CPAP may be complementary in influencing health outcomes for OSA patients (Thomasouli et al., 2013).

Treatment – Oral appliances

Dental or mandibular advancement devices (MADs) have a role in the treatment of OSA and are worn during sleep, holding the lower jaw forward in order to prevent retroglossal collapse (Malhotra and White, 2002). The American College of Physicians has recommended MADs for patients who are unable to tolerate CPAP but who prefer the MAD (Qaseem et al., 2013). In studies that compared MAD with no treatment and sham oral devices, the MAD was found to improve OSA in terms of signs and symptoms, AHI score, arousal index score, and minimum oxygen saturation (Qaseem et al., 2013). In studies that compared MAD with CPAP, CPAP was found to be more effective for improving OSA including AHI and arousal index scores and minimum oxygen saturation (Qaseem et al., 2013). The potential side effects of MAD use includes teeth pain, temporal mandibular junction discomfort, dry mouth, and excessive salivation, and in the long term, potentially altered bite with teeth movement (Young and Collop, 2014).

Treatment – Upper Airway Surgery

Various upper airway surgical procedures may be performed for OSA including uvulopalatopharyngoplasty (UPPP) in which redundant soft palate tissue is resected. Other procedures include laser-assisted palatal procedures and radiofrequency ablation techniques (Malhotra and White, 2002). However, at present, there is insufficient evidence to conclusively demonstrate that upper airway surgical interventions are more effective than CPAP or MADs because of the heterogeneity in surgical procedures and outcomes that have been evaluated (Young and Collop, 2014). Furthermore, surgical procedures may have attendant risks; with insufficient evidence to show benefits of this approach to OSA, it is not recommended as initial treatment (Qaseem et al., 2013). It has been suggested that these procedures may potentially be beneficial for alleviating snoring symptoms for patients whose main complaint is snoring, but with minimal or no apnoea on formal testing (Malhotra and White, 2002).

Other possible treatments

The use of pharmacological agents has been explored as a possible treatment modality for OSA (Qaseem et al., 2013). Positional therapy involves the use of assistive devices to avoid the supine position during sleep, for example a vibrating device when the supine position is assumed, in order

to reduce the frequency and severity of obstructive events when supine(Young and Collop, 2014). At present there is insufficient evidence in the literature to support the use of pharmacological or positional therapy as treatment for OSA (Qaseem et al., 2013). Other novel methods that have been described that require further research include upper airway muscle training to increase upper airway muscle tone and other methods such as transnasal insufflation to deliver continuous high flow humidified air through an open nasal cannula and oral pressure therapy which involves the application of a vacuum to the mouth, pulling the soft palate forward to stabilise the tongue for improved airway patency (Young and Collop, 2014).

Screening for OSA

With the growing population of diabetes and obesity globally, it is becoming apparent that identifying OSA in patients at high risk of OSA is becoming increasingly crucial in order to reduce the risk of adverse outcomes associated with OSA. Despite awareness of the association between OSA, obesity, diabetes, cardiovascular disease and the metabolic syndrome, at present, it may not be practically and economically viable at present to test entire populations for OSA with formal sleep studies, in the absence of sufficient evidence exploring the costs and benefits of such a large undertaking. Polysomnography is both expensive and time-consuming, with many sleep centres having long waiting lists. It would therefore be prudent to focus screening efforts primarily at symptomatic 'at-risk' patients, who are likely to benefit from intervention. Those patients who are deemed at higher risk for OSA should be assessed based on the clinical picture. These high-risk groups would include those patients who provide a clinical history that is consistent with OSA (snoring, apnoeic events during sleep and excessive somnolence), with obesity, type 2 diabetes, metabolic syndrome, cardiovascular disease (Shaw et al., 2008). Obese patients should be screened prior to any surgical procedures because of the perioperative risks associated with undiagnosed OSA.

There is a growing demand for diagnostic studies and treatments with an increased appreciation and recognition of OSA by patients and healthcare providers. Conversely, the symptoms and signs of OSA may still not be perceived as important to many and patients may remain unaware of the potential diagnosis and continue to be undiagnosed for a long time. Additionally, the high prevalence of OSA poses a demanding challenge to healthcare providers in order to provide sufficient resources and facilities for patient diagnosis and treatment.

Rationale behind research in this thesis

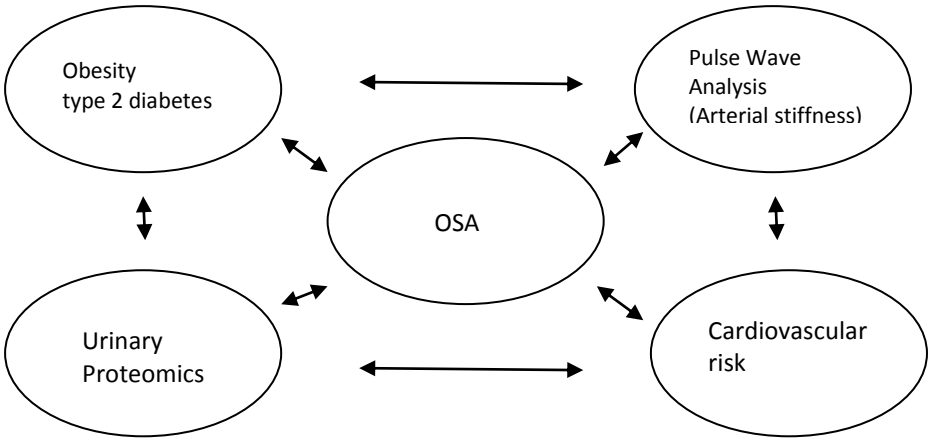


Figure 1.5 The central themes for this thesis are indicated. It should be noted that all are interlinked with each other indicating the complex nature of OSA.

The main work in this thesis relates to the study of subjects with severe obesity and OSA and it is important to explain the rationale behind this research. With cogent arguments for the need to screen patients with obesity and diabetes for OSA, there was a need to support these arguments with evidence of the effects of OSA in severe obesity. With this in mind, pulse wave analysis (PWA) studies on arterial stiffness in obesity were performed as this may reflect potential heightened cardiovascular risk. Furthermore, as elevated levels of serum uric acid have been associated with obstructive sleep apnoea and increased cardiovascular risk, urate levels were also studied as part of the studies performed. Next, a national assessment of current clinical practice for OSA assessment in diabetes services was performed. Finally, the urinary proteome in obesity with and without OSA was characterised using capillary electrophoresis (a tool in urinary proteomics) in order to assess if this method of analysis could distinguish and characterise OSA in severe obesity.

A potential role for urinary proteomics

To date, there is emerging evidence that molecular profiling using proteomics may be a powerful tool in the study of OSA (Arnardottir et al., 2009). The use of urinary proteomic analysis has been applied in studies in coronary artery disease and renal disease. The kidney is particularly sensitive to the effects of hypoxia and plays a key role in blood pressure regulation which is often altered in patients with OSA. There have been several studies involving urinary protein profiles that have been carried out in the paediatric OSA setting (Table 2). In a two-dimensional gel-based analysis, Gozal et al. (2009) studied n=30 OSA subjects and n=30 controls and identified concentrations of uromodulin

(P<0.0001), urocortin-3 (P<0.001), orosomucoid-1 (P<0.0001) and kallikrein-1 (P<0.001) as having favourable predictive properties (sensitivity 95% and specificity 100%) that were specific for OSA (Gozal et al., 2009a). Krishna et al. (2006) examined urine samples in children (n=11) with OSA and n=11 controls using gel electrophoresis coupled with matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI TOF MS), identifying two differentially expressed proteins in OSA: Gelsolin (P=0.00008), heparan sulphate proteoglycans (P=0.002) (Krishna et al., 2006). Shah et al. (2006) used surface-enhanced laser desorption ionisation time-of-flight mass spectrometry (SELDI-TOF MS) in n=20 children with OSA and n=20 habitual snoring with no OSA. Three proteins with masses 5896KDa, 3306KDa and 6068KDa were identified as capable of diagnosing paediatric OSA with 93% sensitivity and 90% specificity (Shah et al., 2006). In another study, Snow et al. (2010) used surface-enhanced laser desorption ionisation MS (SELDI-TOF MS) to discover a specific increase in urocortins in OSA in the paediatric setting n=30 OSA compared with n=25 controls [sensitivity 93% and specificity 97%] (Snow et al., 2010b).

There has been encouraging evidence from a study on adults by Jurado-Gamez et al. (2012), using serum proteomics and subsequent MALDI TOF mass spectrometry analysis (n=30 OSA, n=10 controls) found different expressions of 103 proteins. The proteins were expressed differently according to the severity of OSA and were associated with lipid and metabolic pathways suggesting that differential protein expression occurs in adults with OSA (Jurado-Gamez et al., 2012).

These hypotheses will need testing using emerging scientific techniques that allow detailed investigation of urinary protein profiles. Crucially, the current literature in paediatric OSA justifies such further investigations in OSA in adults that may have the potential to serve as an assessment tool for obesity related breathing problems and as a driver for more targeted focused therapy and a means for disease monitoring. Clearly, with advances in scientific research on the proteomic profile of OSA, there are challenges to apply this knowledge to aid the diagnosis and management of those individuals who are at risk of OSA. Notwithstanding the recent efforts to approach OSA screening with novel applications such as biomarkers and proteomics, it would be prudent to consider the possible limitations arising from these techniques. There is great heterogeneity in terms of the course of disease and patients' exposure to treatment that may hinder the validation of identified markers in cohorts with OSA. Additionally, various technical and methodological issues, including costs and necessary expertise required for the validation and qualification process (as opposed to biomarker discovery per se), may be factors that limit the implementation of such markers in clinical practice (Mischak, 2012) (Mischak et al., 2012). In addition, further work is needed as different

biomarkers may apply according to the different manifestations and complications of the disease process (Montesi et al., 2012a).

Despite the potential limitations of these future tools, the identification of ubiquitous protein profiles and biomarkers remains a promising strategy in terms of defining individuals who should be considered for further investigations as potential targets for treatment and monitoring progression.

Table 1.9

Reference	Method of analysis	Sample source	Patients recruited	Findings	
(Gozal et al., 2009a)	2-Dimensional gel-based analysis	urine	n=30 OSA n=30 controls	uromodulin urocortin-3 orosomucoid-1 and kallikrein-1	(P<0.0001), (P<0.001), (P<0.0001), (P<0.001)
(Krishna et al., 2006)	gel electrophoresis coupled with matrix-assisted laser desorption ionisation-time-of flight mass spectrometry (MALDI-TOF MS)	urine	n=11 OSA n=11 controls	Gelsolin, heparan sulphate proteoglycans	(P=0.00008) (P=0.002)
(Shah et al., 2006)	surface-enhanced laser desorption/ionisation time of flight mass spectrometry (SELDI-TOF MS)	serum	n=20 OSA n=20 controls	Three proteins with masses 5896KDa, 3306KDa and 6068KDa were identified as capable of diagnosing paediatric OSA with 93% sensitivity and 90% specificity	
(Snow et al., 2010a)	surface enhanced laser desorption ionisation MS (SELDI-TOF MS)	urine	n=30 OSA n=25 controls	increase in urocortins	sensitivity 93% and specificity 97%
(Kim et al., 2009)	Western blotting, ELISA	serum	n=40 OSA n=34 controls	Haptoglobin and apolipoprotein M levels are independently related to AHI	P<0.01
(Jurado-Gamez et al., 2012)	MALDI TOF mass spectrometry	serum	n=30 OSA n=10 controls	Different expressions of 103 proteins. The proteins were expressed differently according to the severity of OSA	

Table 1.9 Proteomics studies in OSA (Seetho & Wilding 2013)

In summary, there are cogent arguments in favour of screening for SDB, especially OSA in the growing diabetes and obesity population. While there are current and potential future approaches that may enable screening, it must be acknowledged that novel tools such as biomarkers and proteomics methods have limitations, are at present experimental and will require validation against established physiologically based tests. Clearly, there are opportunities for the application of the knowledge gained from current research findings on such tools in future studies with the expectation that these advances may potentially aid improved screening and yield new insights into disease mechanisms and complications, thereby translating into relevant clinical benefit for these patients. The identification of screening methods is a promising strategy that would enable early recognition and treatment of OSA, thereby yielding improved cardiovascular and metabolic outcomes. Likewise, such a screening process may have a crucial role in potentially ameliorating the adverse consequences of obesity and diabetes.

Aims of the thesis

The principal aims of work described in this thesis was to further our understanding of the effects of OSA in severe obesity, assess current clinical practice, and to explore the use of urinary proteomics, using capillary electrophoresis-mass spectrometry, in the identification and characterisation of individuals with severe obesity and OSA. This was initially studied in subjects with and without OSA. Subsequently, follow-up studies were performed to investigate the effects of CPAP in this cohort.
The hypotheses and aims of these studies were:

Arterial stiffness studies (Chapter 3)
It was hypothesised that the presence of OSA in severe obesity would affect arterial stiffness measurements derived from PWA.
1. To evaluate arterial stiffness in severe obesity with and without OSA at baseline using pulse wave analysis.
2. To investigate the changes in arterial stiffness and other indices of pulse wave analysis following CPAP in the severely obese cohort at follow-up.

Serum Urate study (Chapter 4)
It was hypothesised that OSA was associated with increased serum urate, and that CPAP treatment would influence serum urate levels.
1. To explore whether the presence of OSA is associated with serum urate.
2. To identify whether use of CPAP treatment is associated with fall in serum urate.

Assessing for obstructive sleep apnoea in clinical practice (Chapter 5)
In 2008, the International Diabetes Federation (IDF) released a consensus statement emphasising the need for OSA assessment in type 2 diabetes.
This study aimed to obtain an understanding of current practice in relation to the IDF recommendations with regards to the assessment of OSA in patients in diabetes clinics.

Urinary Proteomics in OSA and severe obesity (Chapter 6)
It was hypothesised that urinary profile patterns were different in severely obese subjects with OSA compared with subjects without OSA
1. To undertake discovery profiling of urinary peptides using capillary electrophoresis-mass spectrometry (CE-MS) in severely obese subjects with and without OSA.

2. To characterise the urinary proteome in severely obese adult subjects with OSA receiving CPAP compared with severely obese subjects without OSA.

Chapter 2 Methods

Introduction

In this chapter, the study populations are introduced and the materials and methodology involved in the arterial studies (chapter 3), urate study (chapter 4) & proteomics studies (chapter 6) are described. In addition, an account of the main statistical methods used in this thesis is given.

Participants

Subjects were recruited from the weight management and sleep clinics at University Hospital Aintree.

Weight management service at Aintree

The multi-disciplinary weight management service for severely obese patients at University Hospital Aintree was established in 1998 and comprises the specialist medical team, dieticians, physiotherapists and psychologists, that has close links with bariatric surgical services. The service provides care across the North West Region including Cumbria, Lancashire, Merseyside. It has formed successful partnerships for shared care with primary care teams across the region. The service delivers an expert-led patient focused programme, providing education, guidance and support for patients to help them achieve and maintain their goals in relation to healthy weight management by appropriate diet, physical activity and psychological support. The programme is individually tailored to each patient's needs. At each visit, patients are assessed and treatment plans are agreed. Regular follow-up allows problems to be identified and treatment optimised. Suitable patients may subsequently be referred on for bariatric surgery.

Aintree Sleep Service

The Aintree Regional Sleep Centre serves Southport, Ormskirk, Merseyside for OSA and Cheshire West and North Wales for complex sleep disorders, and also receives referrals from the Isle of Man and neighbouring Welsh boroughs. It sees approximately 40 new patients per week and about 1800 new patients per year. The sleep laboratory performs 4400 diagnostic sleep studies per year, with 1300 CPAP trials per year and has a total of 5500 patients on CPAP. Approximately 2200 CPAP reviews and 2400 CPAP maintenance services are performed each year (Personal Communication Dr Stephen Embego, Sleep Laboratory, University Hospital Aintree).

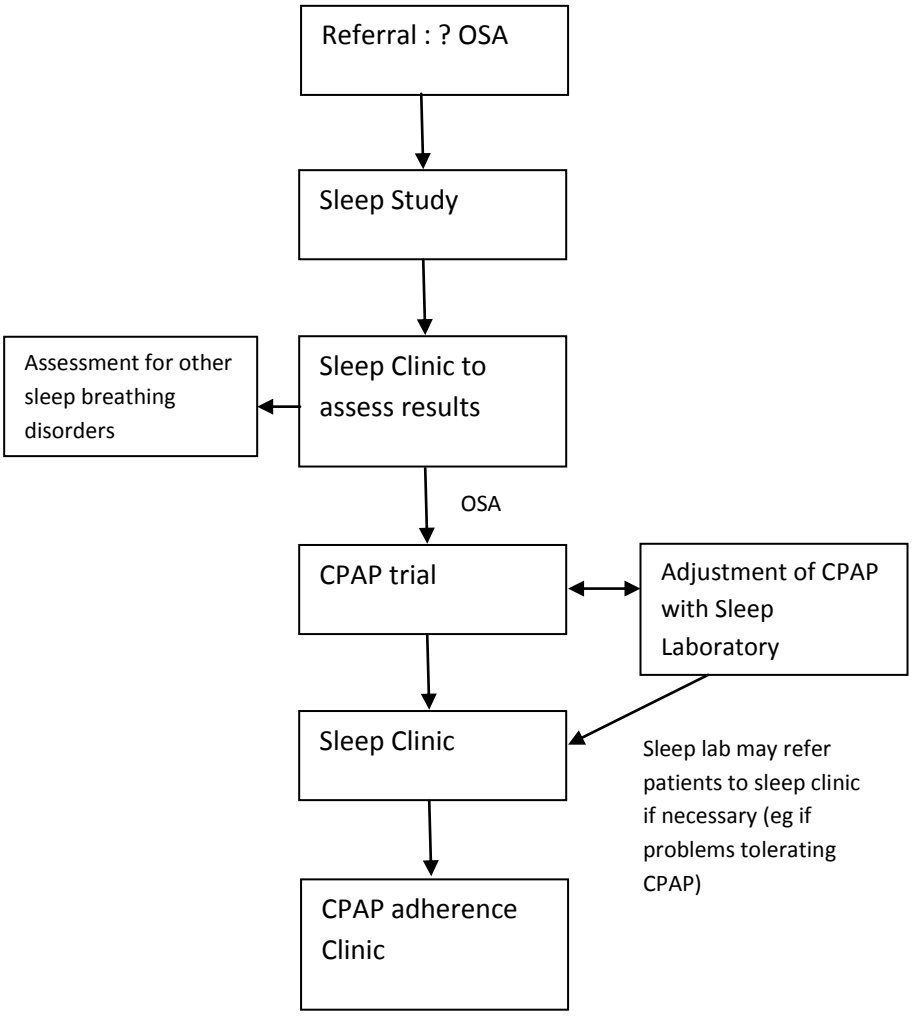


Figure 2.1 Sleep assessment pathway for patients. (For baseline visits, subjects with OSA were assessed and sampled prior to commencement of CPAP)

Recruitment

Subjects were recruited from weight management and sleep clinics at University Hospital Aintree. The NRES North West Ethics Committees at Haydock and Preston granted ethical approval for the studies in this thesis. All studies were performed in accordance with the Declaration of Helsinki. All subjects received the patient information sheet in advance before giving written informed consent.

Participants

All study participants attended a study visit morning at baseline. These subjects were then subsequently followed-up for assessment.

Baseline studies

Severely obese patients from multidisciplinary weight management and sleep clinics at University Hospital Aintree were recruited from March 2012 to January 2013. Inclusion and exclusion criteria were assessed according to the clinical history, physical examination and analysis of the medical notes.

Patients were eligible if they were ≥21 years old and had a BMI ≥35 kg/m². Exclusion criteria were patients who were being treated or had prior treatment for OSA and those with known cardio-respiratory disease; hypertension [defined as BP>140/90 or on blood pressure-lowering medications (Chobanian *et al*., 2003)]; current smokers or those with more than 10 pack years smoking history; kidney and liver disease; acute illnesses and pregnancy.

Follow-up studies

All subjects were invited to return for a clinical assessment, sampling and measurements after at least 12 months. Follow-up took place from September 2013-February 2014. Inclusion and exclusion criteria were assessed according to clinical history, physical examination and analysis of medical notes. Patients were eligible if they were ≥21 years, with BMI≥35kg/m² and had measurements of cardiovascular and metabolic parameters, and arterial stiffness taken at baseline. Exclusion criteria included cardio-respiratory disease; hypertension [BP>140/90 or on BP-lowering medications (Chobanian et al., 2003)]; current smokers or those with more than 10 pack years smoking history; kidney and liver disease; acute illnesses and pregnancy. Patients with OSA who were not on CPAP treatment or not compliant with CPAP (usage<4hrs/night) were subsequently excluded from the analyses in PWA (Chapter 3) and proteomics (Chapter 6) follow-up studies. In the urate study in Chapter 4, subjects on CPAP (<4hrs/night) were included in the statistical analysis at follow-up.

Protocol

Baseline visit

All patients attended a study visit day between 0800-1000hrs (March 2012 – January 2013) and underwent a detailed history and physical examination. Body composition measurements, anthropometry, fasting blood and urine biochemical tests, pulse wave analysis (PWA), venous blood gases (to assess for hypercarbia), and spirometry testing were performed. Daytime somnolence was assessed using an Epworth Sleepiness Scale Questionnaire (ESS); a score >10 indicated increased sleepiness. An overnight sleep study was performed 2-3 weeks later and patients then grouped according to their sleep status. This ensured that the recruiter was blinded to the OSA status of the patient, especially in relation to the PWA measurements taken on the study visit day.

Follow-up visit

All subjects attended a second study visit between 0800-1000hrs (September 2013 – February 2014) and had a detailed history and physical examination. Body composition measurements, anthropometry, fasting blood and urine sampling, PWA, venous blood gases were performed. Daytime somnolence was assessed using an Epworth Sleepiness Scale Questionnaire (ESS); a score >10 indicated increased sleepiness.

Blood Pressure

Blood pressure was measured at the arm in a sitting position after a rest for at least 5 minutes at 1 minute intervals between each measurement according to Hypertension Society guidelines (O'Brien et al., 2003, Pickering et al., 2005), using an oscillometric digital blood pressure monitor (HEM-705CP, Omron, Japan). The mean of three measurements was calculated.

Body measurements

All measurements were done in triplicate. Weight and height were measured without shoes and with light clothing. Body composition measurements used bioimpedance scales (TBF-521,TANITA, Japan). This method has been previously validated (Jebb *et al.*, 2000). 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.

Biochemical and blood gas measurements

Serum samples were collected using standard phlebotomy vials and immediately sent to the local pathology laboratory for analysis in accordance with local protocol and standards. Serum biochemistry was measured using standard laboratory assays (Roche, West Sussex, UK). Blood gases were analysed with a Cobas Blood Gas Analyser (Roche, UK). A raised PCO_2>6kPa would indicate CO_2 retention. Furthermore, bicarbonate measurements were performed as chronic CO_2 retention would lead to abnormally high bicarbonate levels which would indicate metabolic compensation for chronic CO_2 retention.

Metabolic syndrome

Subjects were assessed for metabolic syndrome according to the National Cholesterol Education Program (NCEP) guidelines (Cleeman *et al.*, 2001). Patients had metabolic syndrome if three or more risk factors were present: waist circumference (males>102cm; females>88cm), triglycerides≥1.7mmol/l, HDL cholesterol (males<1.04 mmol/l; females<1.3mmol/l), blood pressure ≥130/≥85 mmHg, and fasting glucose ≥6.1mmol/l.

Spirometry Assessment

Figure 2.2 The Spiro Air system

Spirometry was performed at baseline with a Spiro Air LT system (Medisoft, Sorinnes, Belgium), supervised by an experienced technician. The forced expiratory volume (FEV1) is the volume of air that can be blown out in 1 second. The forced vital capacity (FVC) is the maximum amount of air that can be blown out completely. The ratio provides information on the degree of airflow obstruction. A FEV1:FVC ratio less than 70% was deemed to indicate the presence of obstructive airways disease.

Sleep Diagnostic Assessment

All subjects underwent sleep studies according to the standard hospital protocols as provided for in clinical practice. Diagnosis at baseline was confirmed by overnight multichannel respiratory limited polysomnography (Somnoscreen Digital PSG & EEG acquisition system, Version 2.0, SomnoMedics, Randersacker, Germany), using a montage of pulse oximetry, chest and abdominal excursion, airflow by oronasal thermistry, single bipolar electrocardiogram and body position. Studies were examined by respiratory physiologists with software (Domino PSG analysis software (version 2.5.0), SomnoMedics, Germany). 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. Sleep apnoea was diagnosed if the apnoea-hypopnoea index (AHI) was ≥5 (Flemons et al., 1999). The oxygen desaturation index (ODI) is another measure that may be used to assess the severity of OSA. It is a measure of the hourly average number of desaturation episodes during sleep. An ODI≥5 recorded during the sleep study was the diagnostic threshold for OSA.

Body composition measurements

Bioelectrical impedance (TANITA) scales

Body weight and body fat percentage were measured using the Tanita TBF-521 bioimpedance scale (TBF-521, Tanita, Tokyo, Japan). During this test, subjects were asked to stand on the metal sole plates of the instrument. The scales passed a safe electrical signal between each foot via the lower half of the body. Age, gender, height and weight are used to calculate fat percentage.

The prediction is derived from body density calculated as follows (Jebb et al., 2000):

Body Density= 1.100696-0.107903 X weight X impedance/(height)^2+ 0.00017 X impedance

where impedance is measured in ohms (Ω), weight (kg), height (m)

Body Fat percentage is then calculated as:

Fat percentage (%) = (4.57/Body density -4.142) x 100

This method was previously validated against the four compartment model for measuring body composition (Jebb et al., 2000). The values for fat measured by Tanita analyser were slightly higher than those determined by the four-compartment model (body fat percentage bias +0.9 (SD10.2) and fat mass +0.8 (SD7.9). However, correlation between the two methods was significant for percentage body fat (r=0.89) and fat mass (r=0.93) P<0.05 (Jebb et al., 2000).

Figure 2.3 TANITA scales (left) and demonstration of use of the scales (right). Subjects were asked to wear minimal clothing or a hospital gown during measurements. (Permission granted by Sister Birch for this and subsequent photos of her demonstrating use).

Air displacement plethysmography (BODPOD)

Body fat composition was measured by air displacement plethysmography using BodPod (Life Measurement Inc, Concord, CA) whole body air-displacement plethysmography. The BodPod is a dual-chambered, fibreglass plethysmograph that determines body volume by measuring changes in pressure within a closed chamber. The anterior test chamber provides a seat for the subject to sit on. A partition separates it from the rear reference chamber. The BodPod is connected to a dedicated weighing scale and computer that provides operating controls and data storage. The door to the front chamber is secured by a series of magnets during data collection. The BodPod also has a large acrylic window that allows subjects to look out and also serves to alleviate claustrophobia concerns for some subjects.

Figure 2.4 Picture of BodPod (left) and demonstration of use of the BodPod. Subjects would be asked to wear minimal skin-tight clothing for this test.

During this test, subjects were asked to wear minimal skin-tight clothing whilst seated within the BodPod plethysmography chamber (Life Measurement Inc, Concord, CA) for 2-4 minutes. When in a sealed chamber of a known volume, any change in pressure and volume would be attributable to subject's volume. Body volume was determined by subtraction of the chamber volume when empty and the corresponding pressure change was measured. Poisson's law describes the pressure volume relationship:

$$\mathbf{P_1/P_2 = (V_2/V_1)^{\gamma}}$$ (Fields and Hunter, 2004)

(where γ is the ratio of the specific heat of the gas at a constant pressure to that of the constant volume and equals ~1.3 for water and carbon dioxide).

Each subject's thoracic gas volume (V_{TG}) was either measured during normal tidal breathing using a tube connected to the breathing circuit or was based on a predicted estimate based on age, sex, and height where an accurate measurement was not possible (Nunez et al., 1999). It has been previously shown that there is no significant difference between measured and predicted V_{TG} in adults (McCrory et al., 1998). The measured body volume was used in estimating body density and percentage body fat was then computed by the software (Life Measurement Inc, Concord, CA) based on a standard algorithm (Fields et al., 2002).

Corrected body volume (L) = raw body volume (L) -SAA+40% (V_{TG}) (Fields and Hunter, 2004)

Surface area artefact (SAA) was calculated by the software:
SAA (L) = κ (L/cm^2) x body surface area (cm^2) (Shuter and Aslani, 2000)
Where k is a constant derived by the manufacturer; body surface area using the DuBois formula (Shuter and Aslani, 2000); Body Surface Area=C(Weight)^A x (Height)^B where the units of BSA are cm^2, weight(kg) and height(cm) respectively, with constants C=71.84, A=0.425 and B=0.725.

Once body mass (Mass) and corrected body volume are known, body density is calculated as:

Body Density=Mass/corrected body volume

Body Density is then inserted into a standard formula for estimating body fat based on the 2-compartment model of Siri :

% fat= [(495/Body density)-450]x100 (Siri, 1961)

The BodPod has previously been validated against other methods. Variations in body fat measurements by the BodPod are within 1% body fat for adults when compared with hydrostatic weighing and Dual Energy X-Ray absorptiometry (DEXA). Compared with multicompartment models, body fat composition assessed by BodPod varied by 2–3% (Fields et al., 2002).

Pulse Wave Analysis measurements

Pulse Wave Analysis (PWA) was used for the studies in chapter 3.

Background

Pulse wave analysis (PWA) uses peripheral arterial waveforms to provide estimates of central systolic pressure and PWA indices. This is achieved by using direct carotid artery measurements as a surrogate for the aorta or by application of a mathematical transfer function to measurements at the radial artery. This non-invasive measurement technique allows the study of peripheral pulse waveforms with measurements using a micromanometer-tipped probe that is attached to an integrated system for PWA. The SphygmoCor (AtCor Medical, Sydney, Australia) is an integrated system for PWA that derives central aortic pressure waveforms non-invasively from pulse pressures recorded at the radial artery by applanation tonometry. The elastic and geometric properties of arteries cause arterial pressure to change its shape as it travels along the arterial tree. Intra-arterial pulse pressure is transmitted through the arterial wall to the sensor and peripheral pressure waveforms are calibrated using peripheral blood pressure (BP) readings, with subsequent derivation of central aortic pressures based on a transfer function (O'Rourke et al., 2001). Previous work has

shown that PWA demonstrates high levels of reproducibility (Wilkinson et al., 1998). The use of PWA can provide information concerning haemodynamic status; enabling derivation of arterial stiffness, augmentation pressure and subendocardial viability ratio measurements that may allow assessment of cardiovascular risk.

Figure 2.5 The SphygmoCor (left) and demonstration of use of the applanation tonometry at the radial artery.

PWA indices

The augmentation index (Aix) is a composite measure of central arterial stiffness and peripheral wave reflection. The reflected waves cause changes in pulse wave morphology that affects haemodynamic performance and vascular compliance. Aix is the difference between the pulse height of the primary systolic and reflected peak pressure waves divided by the central pulse pressure expressed as a percentage (Siebenhofer et al., 1999). **Figure 2.6** shows an example of a central pressure waveform.

Aix(%) $=$ $\frac{\text{Difference between early (inflexion) and late (peak) systolic pressures}}{\text{Pulse Pressure}}$

The augmentation pressure (AP) is defined as the difference between the primary and reflected systolic peak pressures. It is a measure of central wave reflection in the vasculature and is a marker of cardiovascular risk in OSA (Noda et al., 2008).

AP (mmHg)= Difference between early (inflexion) and late (peak) systolic pressures

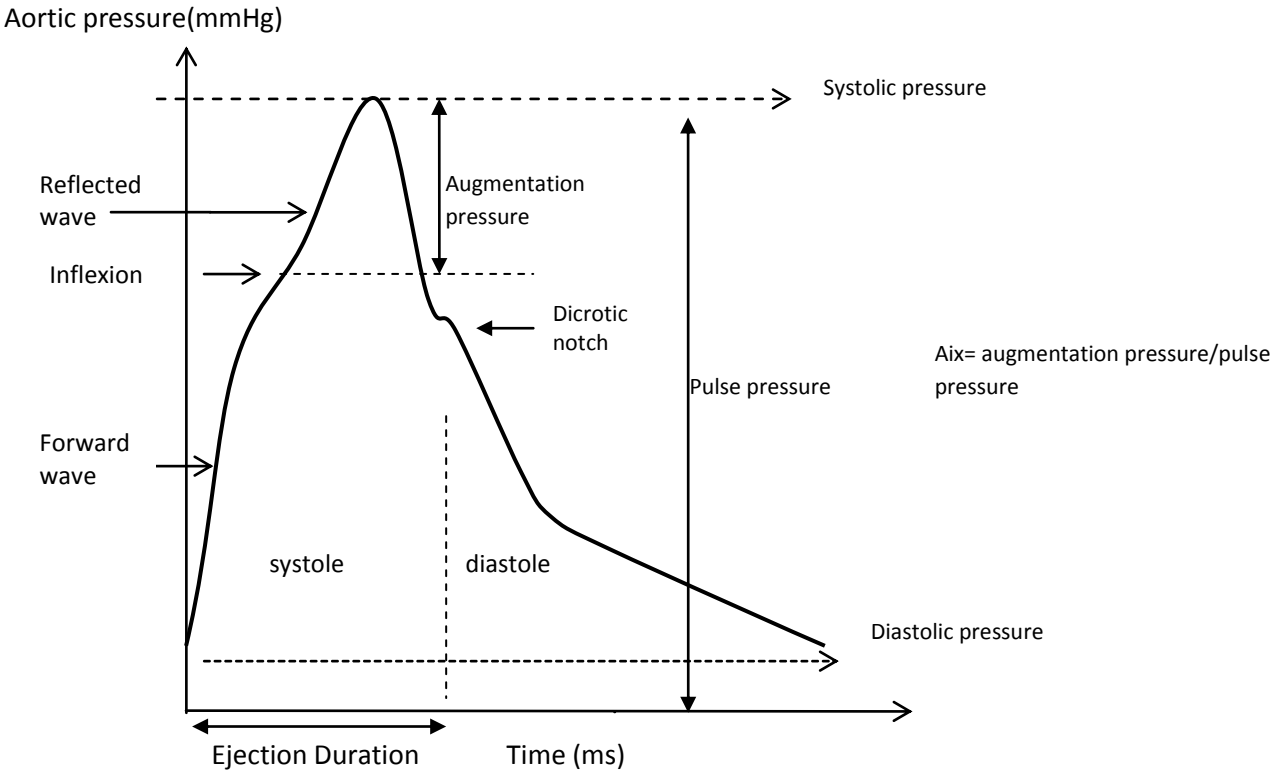


Figure 2.6 Central pressure waveform. The peak and troughs of the waveform are systolic and diastolic pressures respectively. The inflexion point represents time to wave reflection and is the point of onset of the reflected wave. The reflected wave augments systolic flow. Augmentation pressure is the additional pressure to the forward wave due to the reflected wave. Augmentation index is the augmentation pressure as a percentage of the pulse pressure. The dicrotic notch (incisura) is the closure of the aortic valve. Adapted from (Nelson et al., 2010).

Subendocardial Viability Ratio (SEVR) is expressed as a ratio of diastolic pressure time integral to systolic pressure time integral. It is a marker of myocardial oxygen supply and demand, and subendocardial ischaemia (Siebenhofer et al., 1999) (Chemla et al., 2008). A higher SEVR is better in terms of cardiovascular health as lower values (at ~50%) indicate decreased diastolic perfusion times and reduced coronary perfusion (Hoffman and Buckberg, 1978, Ferro et al., 1995). Low values are found in patients with coronary artery disease, with subendocardial ischaemia due to reduced diastolic perfusion times (Ferro et al., 1995).

$$\textbf{SEVR (\%)} = \frac{\textbf{Diastolic pressure time integral}}{\textbf{Systolic pressure time integral}} = \frac{\textbf{Area under diastolic curve}}{\textbf{Area under systolic curve}}$$

$$= \frac{\textbf{(Diastolic time x mean aortic diastolic pressure)}}{\textbf{(Systolic time x mean aortic systolic pressure)}}$$ (Chemla et al., 2008)

PWA protocol

PWA was performed at the same time each day (~0930hrs) for all patients in order to ensure uniformity in measurements. All measurements were made in the fasting state, with abstinence from alcohol and caffeine for at least 8 hours, in the seated upright position, in a temperature-controlled room (24^{o}C). PWA measurements were taken by applying a hand-held tonometer attached to the SphygmoCor system (AtCor Medical, Australia). The tonometer comprised a high fidelity micromanometer-tipped probe that was gently applied to the skin surface at the radial artery of the non-dominant arm in a non-occlusive manner, with the wrist slightly flexed and palm facing upwards. By this means, radial artery waveforms were acquired and digitized through the SphygmoCor software linked to a computer. Radial artery waveforms were calibrated from brachial pressures that were measured using an oscillometric digital blood pressure monitor (HEM-705CP, Omron, Japan). A minimum of 10 radial waveforms for each patient was required to generate a corresponding ascending aortic pressure waveform by a validated mathematical transfer function within the SphygmoCor software. To ensure quality control, only measurements with a Quality Index ≥80% and a signal strength ≥500 units were accepted. As heart rate influences the Augmentation Index (Aix), all values were normalised to a heart rate of 75 bpm. The tonometry data acquired were utilised by the SphygmoCor software to derive values for Aix, AP and SEVR.

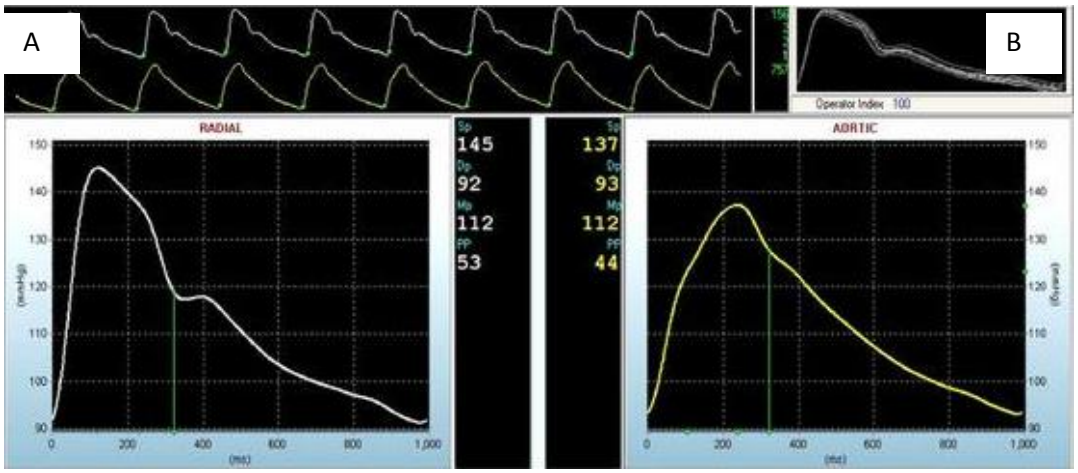


Figure 2.7 Showing an example of a radial artery applanation tonometry recording from the SphygmoCor. (A) The long panel shows the radial pressure waveform above the derived central pressure waveform. (B) The upper right panel shows operator Index, quality control indices and overlaid radial waveforms. The central panel shows systolic and diastolic pressures (white) and central pressures (yellow). The bottom left and right panels demonstrate the magnified radial arterial (white graph) and derived central pressure waveform (yellow graph) respectively [x axis- (time) msecs, y-axis- mmHg].

Capillary Electrophoresis-Mass Spectrometry (CE-MS)

In Chapter 6, studies to investigate and characterise the urinary proteome in the subjects were performed.

Background

Proteomics is the study of the proteome which is the protein complement expressed by the genome (Wilkins et al., 1996). Proteomics provides information on peptide interactions and post translational changes that may not be seen at the level of the genome (Arnardottir et al., 2009). Urine is ideal for proteomic analysis because it can be obtained non-invasively and usually in large quantities and the dynamic range of analytes is lower compared to plasma, with proteins and peptides that are stable (Albalat et al., 2014). Urine contains water, glucose, salts, metabolites and peptides of the kidney, urogenital tract and from the glomerular filtration of plasma (Siwy et al., 2011). Approximately 70% of urinary proteins originate from the kidney, and 30% are derived from plasma (Thongboonkerd, 2007).

Capillary electrophoresis coupled to mass spectrometry (CE-MS) is a sensitive proteomic analysis technique that is robust with high reproducibility of results in an acceptable time frame (Ahmed, 2009) (Mischak and Schanstra, 2011). CE-MS is capable of resolving up to 6000 different peptides per sample within approximately 45 minutes (Albalat et al., 2011). In CE-MS, capillary zone

electrophoresis is interfaced with high resolution mass spectrometry. In capillary electrophoresis (CE), molecules are separated within a narrow tube or capillary by application of an electrical field across the capillary. The electrophoretic separation is a function of charge and size (mass to charge ratio (*m/z*)) and occurs by electro-osmotic flow (Albalat et al., 2014). The electro-osmotic flow within the capillary is a consequence of the surface charge on the interior capillary wall and the effect of the applied electric field on the capillary (Heiger, 2000).

Coupling between the CE and MS instruments is by a closed electrical circuit with high voltage maintained across the capillary. Electrospray ionisation (ESI) couples the CE to the MS. Spray formation is facilitated by a conductive sheath-liquid interface together with an optional gas flow that is applied coaxially with the sheath liquid (Albalat et al., 2014). The separated peptides are detected and analysed in the mass spectrometer (Mischak and Schanstra, 2011). The accuracy, precision, selectivity, sensitivity, reproducibility, and stability of the CE–MS measurements have been previously demonstrated (Theodorescu et al., 2005).

The rationale for the use of CE-MS relative to other methods such as liquid chromatography-Mass spectrometry (LC-MS) is the high reproducibility, comparability of datasets (Albalat et al., 2011), and the ability to effectively recondition the CE without issues of 'carry-over' of residual analytes that may arise from the previous samples in subsequent analyses (Mischak et al., 2013).

CE-MS has been previously compared to LC-MS (Mullen et al., 2012) and Matrix Associated Laser Deionisation (MALDI)-MS (Molin et al., 2012). CE-MS showed better reproducibility of results when compared with the latter two proteomic methods. Furthermore, CE allowed the calibration of migration time as a function of mass and charge of the peptide that facilitated sample-sample comparisons by enabling peaks from consecutive runs to be aligned according to these parameters (Mullen et al., 2012).

CE-MS has been used to study biomarker panels of urinary peptides for conditions such as coronary artery disease (Delles et al., 2010) and chronic kidney disease (Rossing et al., 2008) (Good et al., 2010) that may indicate the presence of the relevant condition (Coon et al., 2008) (Siwy et al., 2011).

Figure 2.8 Showing the CE-MS instruments in the proteomics laboratory at Glasgow University where the proteomic experiments were performed. The CE is on the left, the MS is in the middle which is connected to the computer.

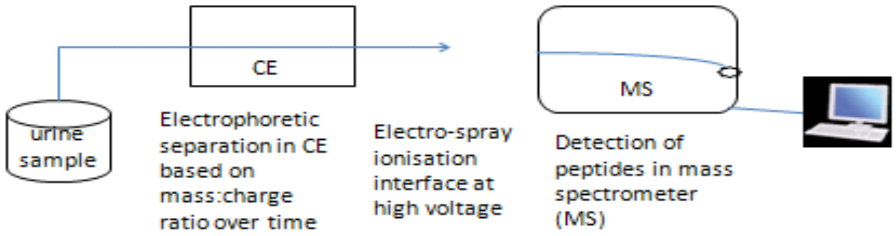


Figure 2.9 Schematic diagram of CE-MS set-up

CE-MS materials & methods

Urine sample preparation

Spontaneously voided urine samples for CE-MS analyses were collected at the study visit using urine Monovettes (Sarstedt AG & Co, Nümbrecht, Germany). Second void samples were collected at the same time each morning (~09:00 hours) after an overnight fast for 8 hours, at their study visit prior to the administration of any medication. The samples were stored at -80^{o}C in urine Monovettes until the sample preparation stage. Sample preparation for proteomic analysis was performed as previously described (Albalat et al., 2013). In this process, each 0.7 mL aliquot of urine was thawed immediately before use and diluted with 0.7 mL of 2M urea, 10mM NH_4OH containing 0.02% SDS. Each sample was ultrafiltered (2000g, 60 mins, 4^{o}C) to remove higher molecular mass proteins, such as albumin and immunoglobulin G using Centrisart ultracentrifugation filter devices (20kDa 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% NH_4OH in HPLC-grade H_2O to decrease matrix effects by removing urea, electrolytes, and salts, and to enrich polypeptides present. Finally, all samples were lyophilized, stored at 4^{o}C.

CE-MS Analysis

Samples were re-suspended in HPLC-grade H_2O shortly before CE–MS analyses, as described (Albalat et al., 2013). 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) (Albalat et al., 2013). 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 seconds, over a range of m/z 350 to 3000 over 60 minutes (Albalat et al., 2013). The accuracy, precision, selectivity, sensitivity, reproducibility, and stability of the CE-MS measurements have been previously demonstrated (Theodorescu et al., 2005).

CE-MS Data Processing

Mass spectra were processed using MosaiquesVisu software (Mosaiques Diagnostics, Hannover, Germany), including peak picking, deconvolution and deisotoping (v. Neuhoff et al., 2004). 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, normalized 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 (Jantos-Siwy et al., 2009). 'Housekeeping' peptides that consistently appear in urine samples and are unaffected by disease states provided ideal reference mapping points for CE migration times (Coon et al., 2008). 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 (Albalat et al., 2013). 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, allowing further statistical analysis. Non-OSA and OSA-specific polypeptide patterns were generated using support vector machine (SVM)-based MosaCluster software (Mosaiques Diagnostics, Hannover, Germany) for comparison.

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 (Coon et al., 2008) (Zurbig et al., 2006). These human urinary peptide sequences can be accessed at (http://mosaiques-diagnostics.de/diapatpcms/mosaiquescms/front_content.php?idcat=257).

The database is used for the identification of peptides detected in clinical samples and currently holds >20,000 individual datasets. A total of 5616 different peptides characterized by molecular

mass [Da], normalized CE-migration time [min], and sequence (if known). Currently, the sequences of >1400 peptides are known and are included in the human urinary proteome database (Mischak et al., 2013). Therefore the database provides information that allows comparisons of results between experiments (Mischak et al., 2013).

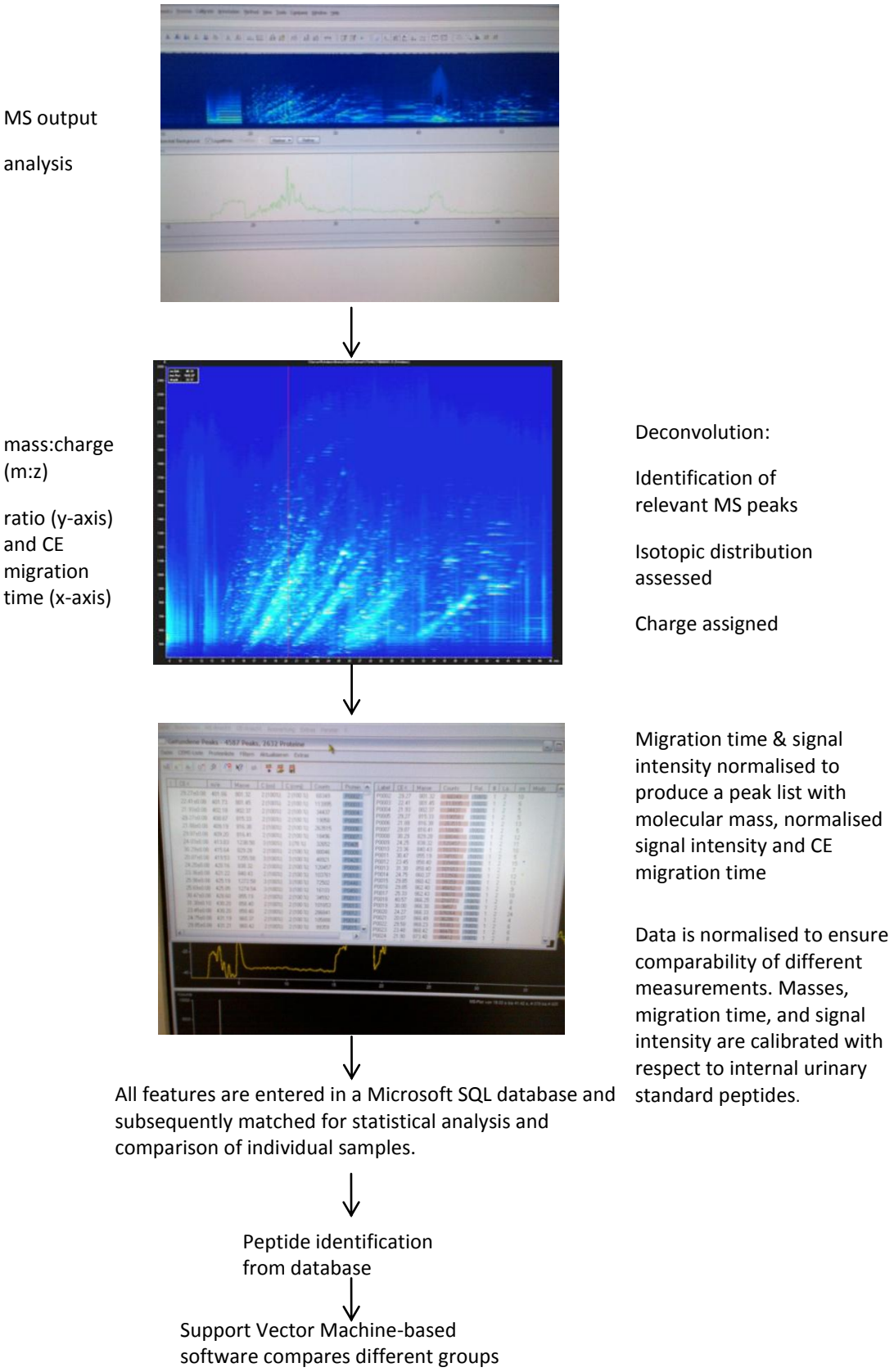


Figure 2.10 Showing the steps in CE-MS data processing

The Support Vector Machine (SVM) algorithm classifies subjects by constructing an n-dimensional hyperplane optimally separating subjects into case and controls. Features that describe each subject are called vectors. The aim of SVM classification is to find the optimal hyperplane that separates clusters of vectors in such a way that the case subjects are on one side of the plane and the controls are on the other side of the plane and the most suitable features that are case-specific markers are identified using statistical testing (Mischak et al., 2013).

CPAP therapy

Subjects with OSA were offered CPAP treatment and were assessed at follow-up studies described in Chapters 3, 4 and 6.

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 (hrs/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 hrs/night on >70% of nights in the treatment group (Engleman and Wild, 2003).

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 (Dakna et al., 2010), and was used to determine significance in peptide abundance between the groups. Statistical significance was assigned at $P\leq0.01$. 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. This was necessary to correct for peptides that may

have been found to be significant by chance. Multiple testing correction adjusts the individual p-value for each peptide to keep the overall error rate to less than or equal to the user-specified p-cutoff value, which was P≤0.01 for the proteomics data sets. This test provides a good balance between discovery of statistically significant peptides and the limitation of false positives.In this test, P-values relating to each peptide were ranked in order from the smallest to the largest P-value. The largest P-value is multiplied by the number of peptides being tested. Next the second largest P-value is multiplied by the total number of peptides divided by its rank. Hence the corrected P-value for each peptide= P-value X (n/rank of peptide), where n is the total number of peptides (Benjamini and Hochberg, 1995).

Chapter 3

Arterial stiffness in obstructive sleep apnoea and severe obesity

This chapter comprises two studies and it describes pulse wave analysis studies in severely obese subjects with and without OSA. In the first part, arterial stiffness was measured using PWA at baseline. Subjects with OSA were subsequently offered CPAP. In the second study, the subjects were invited to return for follow-up assessment of their arterial stiffness and other PWA measures.

Abstract

Background Obstructive sleep apnoea (OSA) is associated with obesity and metabolic syndrome, leading to greater cardiovascular risk. Severely obese patients with OSA may still be at risk of adverse health outcomes, even without previous cardiovascular disease. Pulse Wave Analysis (PWA) non-invasively measures peripheral pulse waveforms and derives measures of haemodynamic status. It was hypothesized that the presence of OSA in severe obesity, even in the absence of an antecedent history of cardiovascular disease would affect measurements derived from PWA.

Methods Severely obese adult subjects were characterised using anthropometric, respiratory and cardio-metabolic parameters, as well as sleep studies. Pulse wave analysis, using applanation tonometry at the radial artery, measured arterial stiffness, augmentation pressure and the subendocardial viability ratio. Patients diagnosed with OSA were studied prior to CPAP.

Results Seventy-two severely obese adult subjects [OSA 47 (BMI 42±7kg/m^2), non-OSA 25 (BMI 40±5kg/m^2)] were recruited. Groups were similar in age, BMI and gender. More OSA subjects had metabolic syndrome (OSA 60%, non-OSA 12%). Those with OSA had greater arterial stiffness, augmentation pressure and decreased subendocardial viability ratio (all $P<0.001$); with significantly higher systolic ($P=0.003$), diastolic ($P=0.04$) and mean arterial pressures ($P=0.004$) than non-OSA patients. Arterial stiffness correlated with mean arterial blood pressure ($P=0.003$) and OSA severity [Apnoea-Hypopnoea Index (AHI)] ($P<0.001$). AHI significantly predicted arterial stiffness in multiple regression analysis, but components of the metabolic syndrome did not.

Conclusions OSA patients with severe obesity have increased arterial stiffness that may potentially influence cardiovascular risk independently of metabolic abnormalities. The presence of obstructive sleep apnoea in severe obesity identifies a group at high cardiovascular risk; clinicians should ensure that risk factors are managed appropriately in this group whether or not treatment of OSA is offered or accepted by patients.

Introduction

OSA is characterised by repetitive intermittent hypoxia, periodic arousals and sleep fragmentation, with cardiovascular and metabolic sequelae. Randomised trials have shown that OSA treatment with continuous positive airway pressure (CPAP) can improve hypertension and atherosclerosis (Pepperell et al., 2002) (Drager et al., 2007). Furthermore, fewer long-term adverse cardiovascular events have been observed when severe OSA is treated with CPAP (Marin et al., 2005).

The proposed mechanisms underlying the cardiovascular associations of OSA include increased sympathetic drive, hypothalamic-pituitary-adrenal axis activation, insulin resistance and associated metabolic disturbances, increased systemic inflammation, oxidative stress and a prothrombotic state (Kohler and Stradling, 2010).

OSA is associated with increased arterial stiffness that may contribute to the associated cardiovascular risk (Doonan et al., 2011). It has been reported that increased arterial stiffness (measured as augmentation index) derived from the radial artery is predictive of cardiovascular events (Weber et al., 2005). Arterial stiffness is associated with OSA severity (Doonan et al., 2011), and is elevated in OSA (Kohler et al., 2008). This is attenuated following continuous positive airway pressure (CPAP) therapy (Drager et al., 2007). Such changes in arterial stiffness indicate that it may be linked with an increased cardiovascular risk in OSA patients (Buchner et al., 2012).

Pulse Wave Analysis (PWA) is a non-invasive measurement technique that allows the study of peripheral pulse waveforms with measurements using a micromanometer-tipped probe. Intra-arterial pulse pressure is transmitted through the arterial wall to the sensor and peripheral pressure waveforms are calibrated using peripheral blood pressure (BP) readings, with subsequent derivation of aortic pressures waveforms based on a transfer function (O'Rourke et al., 2001). Previous work has shown that PWA demonstrates high levels of reproducibility (Wilkinson et al., 1998). The use of PWA can provide information concerning haemodynamic status; enabling derivation of arterial stiffness, augmentation pressure and subendocardial viability ratio measurements that may allow assessment of cardiovascular risk. The augmentation index (Aix) is a composite measure of central arterial stiffness and peripheral wave reflection. The reflected waves cause changes in pulse wave morphology that affects haemodynamic performance and vascular compliance. Aix is the difference between the pulse height of the primary systolic and reflected peak pressure waves divided by the central pulse pressure expressed as a percentage (Siebenhofer et al., 1999).

Augmentation pressure (AP) is defined as the difference between the primary and reflected systolic peak pressures. It is a measure of central wave reflection in the vasculature and is a marker of cardiovascular risk in OSA (Noda et al., 2008). Subendocardial Viability Ratio (SEVR) is expressed as a ratio of diastolic pressure time interval to systolic pressure time interval. It is a marker of myocardial oxygen supply and demand, and subendocardial ischaemia (Siebenhofer et al., 1999). A higher SEVR is better in terms of cardiovascular health as lower values (at ~50%) indicate decreased diastolic perfusion times and reduced coronary perfusion (Hoffman and Buckberg, 1978, Ferro et al., 1995).

Although PWA has been used to investigate the effects of obstructive sleep apnoea on arterial stiffness (Phillips et al., 2013), to date, few studies have investigated these effects in severely obese cohorts (defined as a body mass index (BMI)≥35kg/m^2). Jelic et al (2002) studied changes in arterial stiffness during sleep cycles with OSA in an obese cohort with a mean BMI 35kg/m^2 (Jelic et al., 2002). In another study, the differential effects of OSA severity on arterial properties in subjects with mean BMI 38kg/m^2 was investigated but a control group was not used (Protogerou et al., 2008). Yim-Yeh *et al*. (2010) investigated the effects of age and OSA on vascular function in a cohort with a mean BMI 37kg/m^2 (Yim-Yeh et al., 2010). Bakker *et al.* (2011) investigated the effects of continuous and auto-adjusted positive airway pressure on 12 severely obese patients but did not study patients of similar BMI without OSA (Bakker et al., 2011).

Severely obese patients with OSA may still be at risk of adverse health outcomes, even without a previous history of cardiovascular disease. Therefore, this knowledge will be useful when patients are assessed in clinical practice, especially in the presence of OSA, enabling risk factors to be modified, and potential to improve health outcomes with early treatment.

In this study, it was hypothesized that the presence of OSA in severe obesity, even in the absence of an antecedent history of cardiovascular disease would affect measurements derived from PWA. In order to test this, we measured the augmentation index, augmentation pressure and subendocardial viability ratio derived from PWA in patients recruited from weight management clinics. In order to determine the independence of the observed associations relating to the augmentation index, a regression analysis adjusted for obesity and other variables such as age, blood pressure, lipid levels, fasting glucose, body measurements (neck and waist to hip ratio) and body fat percentage and the apnoea-hypopnoea index (AHI) was performed.

Research Design and Methods

Although this was broadly discussed in the methods chapter (chapter 2), the methods specifically pertaining to the arterial studies presented in this chapter are described below.

Ethics Statement

The study was approved by the local research ethics committee (NRES 12/NW/123). The study was performed in accordance with the Declaration of Helsinki. All patients gave written informed consent.

Participants

Severely obese patients from multidisciplinary weight management and sleep clinics at University Hospital Aintree were recruited from March 2012 to January 2013. Inclusion and exclusion criteria were assessed according to the clinical history, physical examination and analysis of the medical notes.

Patients were eligible if they were ≥21 years old and had a BMI ≥35 kg/m². Exclusion criteria were patients who were being treated or had prior treatment for OSA and those with known cardio-respiratory disease; hypertension (defined as BP>140/90 or on blood pressure-lowering medications (Chobanian et al., 2003); current smokers or those with more than 10 pack years smoking history; kidney and liver disease; acute illnesses and pregnancy.

Protocol

All patients attended a study visit day between 0800-1000hrs and underwent a detailed history and physical examination. Body composition measurements, anthropometry, fasting blood and urine biochemical tests, pulse wave analysis, venous blood gases (to assess for hypercarbia), and spirometry testing were performed. An overnight sleep study was performed 2-3 weeks later and patients then grouped according to their sleep status. This ensured that the recruiter was blinded to the OSA status of the patient, especially in relation to the PWA measurements taken on the study visit day. Given the high prevalence of OSA in obesity, it was anticipated that there would be more OSA than non-OSA patients. It was estimated that it would be necessary to screen approximately 90 patients to ensure adequate recruitment.

Blood Pressure

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

Body measurements

All measurements were done in triplicate. Weight and height were measured without shoes and with light clothing. Body composition measurements used bioimpedance scales (TBF-521,TANITA, Japan). This method has been previously validated (Jebb et al., 2000). 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.

PWA

PWA was performed at the same time each day (~0930hrs) for all patients in order to ensure uniformity in measurements. All measurements were made in the fasting state, with abstinence from alcohol and caffeine for at least 8 hours, in the upright position, in a temperature-controlled room (24^{o}C). PWA measurements were taken by applying a hand-held tonometer attached to the SphygmoCor system (AtCor Medical, Australia) (Kelly et al., 1989). The tonometer comprised a high fidelity micromanometer-tipped probe that was gently applied to the skin surface at the radial artery of the non-dominant arm in a non-occlusive manner, with the wrist slightly flexed and palm facing upwards. By this means, radial artery waveforms were acquired and digitized through the SphygmoCor software linked to a computer. Radial artery waveforms were calibrated from brachial pressures that were measured using an oscillometric digital blood pressure monitor (HEM-705CP, Omron, Japan). A minimum of 10 radial waveforms for each patient was required to generate a corresponding ascending aortic pressure waveform by a validated mathematical transfer function within the SphygmoCor software (Pauca et al., 2001). To ensure quality control, only measurements with a Quality Index ≥80% and a signal strength ≥500 units were accepted. As heart rate influences the Augmentation Index (Aix), all values were normalised to a heart rate of 75 bpm. The tonometry data acquired were utilised by the SphygmoCor software to derive values for Aix, AP and SEVR.

Spirometry Assessment

Spirometry was performed with a Spiro Air LT system (Medisoft, Belgium), supervised by an experienced technician.

Sleep Diagnostic Assessment

Daytime somnolence was assessed using an Epworth Sleepiness Scale Questionnaire (ESS) where a score >10 indicated increased sleepiness (Johns, 1993). Diagnosis was confirmed by overnight

multichannel respiratory limited polysomnography (Somnoscreen Digital PSG & EEG acquisition system, Version 2.0, SomnoMedics, Germany). Sleep studies were independently assessed by experienced sleep physiologists using software (Domino PSG analysis software (version 2.5.0), SomnoMedics, Germany). Apnoea was defined as a cessation of airflow for >10secs. Hypopnoea was defined as a 50% reduction in airflow accompanied by a >4% desaturation and a reduction in chest wall movement. Sleep apnoea was diagnosed if the apnoea-hypopnoea index (AHI) was ≥5 (Flemons et al., 1999).

Biochemical Measurements

Serum biochemistry was measured using standard laboratory assays (Roche, UK). Blood gases were analysed with a Cobas Blood Gas Analyser (Roche, UK).

Metabolic syndrome

Subjects were assessed for metabolic syndrome according to the National Cholesterol Education Program (NCEP) guidelines (Cleeman et al., 2001). Patients had metabolic syndrome if three or more risk factors were present: waist circumference (males>102cm; females>88cm), triglycerides≥1.7mmol/l, HDL cholesterol (males<1.04 mmol/l; females<1.3mmol/l), blood pressure ≥130/≥85 mmHg, and fasting glucose ≥6.1mmol/l.

Statistical Analysis

Published results of arterial stiffness with Aix as the measure in subjects with OSA have reported a standard deviation of ~1-9% (Doonan et al., 2011). With this information, it was calculated, that a minimum sample size of 17 subjects per group, would show a 2% difference in arterial stiffness, with a power of 80% at 0.05 significance level.

Statistical analyses were carried out using SPSS version 20 (IBM SPSS Inc.). Normal distribution of data was confirmed using the Kolmogorov–Smirnov test. Normally distributed data are presented as mean ± standard deviation while non-normally distributed data are presented as median (25th–75th percentile). Independent t test and Mann–Whitney test were used to compare parametric and nonparametric numerical variables between groups respectively, while the chi-squared test was used for categorical variables. Pearson's or Spearman's coefficient regression was used for univariate correlation analysis as appropriate. Statistical significance for all analyses was assigned at $P<0.05$. Multiple linear regression models were constructed with potential confounders as additional independent variables as needed.

Results

Ninety-seven consecutive patients were screened. 72 completed the protocol (47 obese OSA, 25 obese non-OSA). 20 were excluded because of hypertension. 5 subjects withdrew before their sleep studies and thus were excluded from the analyses. All subjects were of white European ethnicity.

Clinical and biochemical parameters of patients according to OSA status are presented in **Table 3.1**. The groups did not significantly differ in age, BMI and gender. Although OSA subjects had higher waist circumference, the small numerical differences in neck circumference, waist: hip ratio and body fat composition between the groups were not statistically significant. Smoking, diabetes and alcohol consumption were similar between groups.

Table 3.1 Patient Characteristics

Body measurements

Characteristics	**OSA n=47**	**Non-OSA patients n=25**	**P value**
Gender - male [c]	24 (51%)	13 (52%)	0.94
Age (years)[a]	47±9	49±10	0.13
BMI (kg/m^2)[a]	42±7	40±5	0.15
Neck circumference (cm)[a]	44.3±4.4	41.9±3.7	0.06
Waist: Hip Ratio [a]	0.98±0.12	0.96±0.11	0.42
Body Fat % (Tanita) [a]	44±8	41±9	0.16
Ever smoked[c]	13 (27%)	5 (20%)	0.58
Diabetes [c]	7 (15%)	3 (12%)	0.74
Alcohol (0-8 units/week)[c]	29 (62%)	17 (68%)	0.62

Respiratory parameters

AHI (events/hr)[a]	23.8±10.6	2.6±1.0	**P<0.0001**
ESS[a]	10.4±5.5	7.6 ±5.4	**0.04**
FEV_1 % predicted[b]	92.1 (84.4-105.9)	93.5 (87.0-104.1)	0.93
FVC % predicted[b]	101.8 (92.2-112.7)	101.6 (93.7-117.7)	0.64
FEV1:FVC ratio[b]	77.1 (73.1-81.6)	75.6 (73.7-79.3)	0.52
PCO_2 (kPa)[b]	5.4 (5.2-5.6)	5.41 (5.1-5.59)	0.58
Bicarbonate (mmol/l)[b]	25.0 (24.0-25.6)	24.5 (24.0-25.0)	0.08
O_2 Saturation (%)[a]	96.5±1.5	96.6±1.5	0.66

Cardio-metabolic parameters

MAP (mmHg)[a]	105±10	98±9	**0.004**
Heart Rate (bpm)[a]	74±10	70±12	0.13
ACR (mg/mmol)[b]	0.6 (0.3-1.5)	0.3 (0.1-0.8)	0.13
Creatinine (micromol/l)[b]	72 (63-85)	79 (68-88)	0.44
MDRD-GFR (mL/min/1.73m^2)[b]	94 (79-106)	88 (74-102)	0.31
LDL (mmol/l)[a]	2.9±0.8	2.9±0.9	0.46
Total cholesterol (mmol/l)[a]	5.1±0.9	4.9±1.1	0.14
HbA1c (mmol/mol)[a]	42±12	39±10	0.62
TSH (mU/l)[a]	2.1±1.0	2.7±1.5	0.24

Components of the metabolic syndrome

Characteristics	**OSA n=47**	**Non-OSA patients n=25**	**P value**
Metabolic syndrome[c]	28 (60%)	3 (12%)	**P<0.001**
Systolic BP (mmHg)[a]	138±12	128±12	**0.003**
Diastolic BP (mmHg)[a]	88±11	83±10	**0.04**
Waist circumference (cm)[a]	126.9±2.6	115.2±2.4	**0.01**
Triglycerides (mmol/l)[a]	1.9±1.0	1.5±0.6	0.18
HDL (mmol/l)[a]	1.2±0.3	1.3±0.4	0.62
Fasting glucose(mmol/l)[a]	5.6±1.4	5.6±2.0	0.89

Data are presented as: [a] mean±SD, [b] median (interquartile range), [c] n (%)

The AHI and ESS were significantly higher in those with OSA. No other significant differences in respiratory variables were observed. In terms of cardio-metabolic parameters, OSA subjects were found to have significantly higher systolic (P=0.003), diastolic (P=0.04) and mean arterial pressures (P=0.004) than non-OSA patients. More OSA subjects had metabolic syndrome, particularly meeting the waist circumference, blood pressure and triglycerides components of the criteria).

PWA measurements in OSA compared to control subjects

Severely obese OSA patients demonstrated significantly (all P<0.001) increased arterial stiffness (OSA Aix 23.2±5.2% (95%CI 21.1-25.1) vs non-OSA 11.3±5.2% (95%CI 8.9-13.5)) (Figure 1A), augmentation pressure (OSA AP 12.8±6.3mmHg (95%CI 10.9-14.7) vs non-OSA 6.5±4.1mmHg (95%CI 4.4-7.4)) (Figure 1B) and decreased subendocardial viability ratio (OSA SEVR 149±14% (95%CI 165.4-176) vs non-OSA 171±12% (95%CI 144.8-152.9)) (Figure 1C) compared with non-OSA patients.

Figure 3.1A

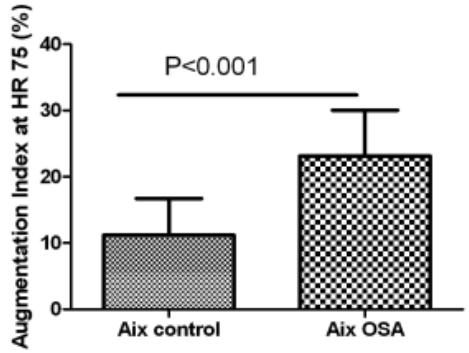


Figure 3.1B

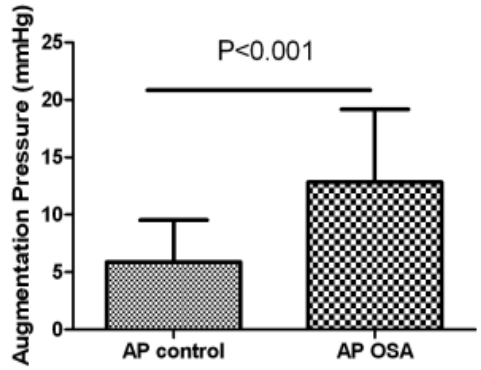


Figure 3.1C

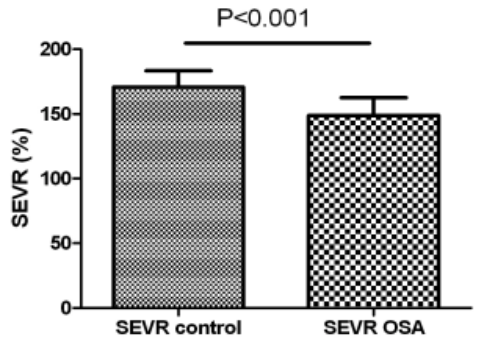


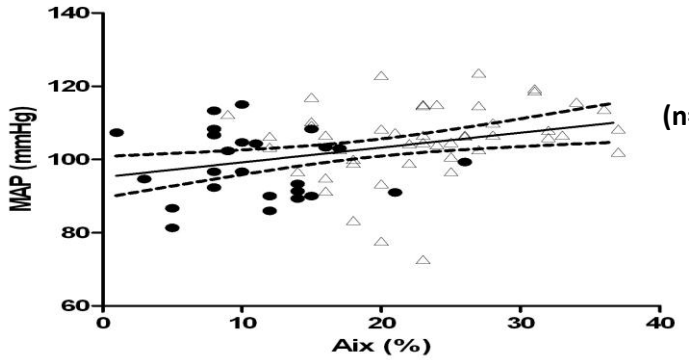
Figure 3.1(A-C) Differences in Aix (A), AP (B) and SEVR (C) between non-OSA and OSA groups.
Key: Aix (Augmentation index at HR 75), AP Augmentation pressure, SEVR Subendocardial viability ratio. Error bars expressed in SD.

Relationships between arterial Stiffness (Aix) & body measurements

Neck circumference was not correlated with arterial stiffness (n=72, r=-0.112, 95%CI(-0.3352 to 0.1228),P=0.348). The waist to hip ratio (n=72, r=-0.152, 95%CI(-0.3768 to 0.08938) P=0.201), and BMI (n=72, r=0.141, 95%CI(-0.1011 to 0.3666), P=0.239) were not significantly associated with arterial stiffness. Neck circumference was significantly correlated with OSA severity (AHI) (n=72, r=0.319, 95%CI(0.09433 to 0.5129), P=0.006).

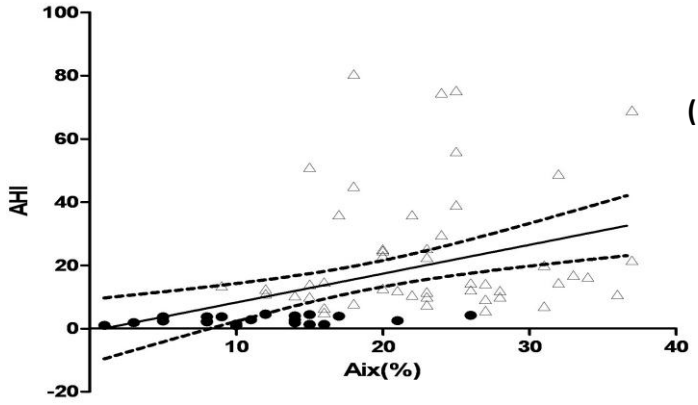
Relationships between arterial stiffness (Aix) and MAP & arterial stiffness (Aix) and AHI

Arterial stiffness (Aix) was associated with mean arterial blood pressure (MAP) (n=72, r=0.31, 95%CI(0.1086 to 0.5335), P=0.003)(**Figure 3.2A**) and with OSA severity as measured by the AHI (n=72, r=0.436, 95%CI(0.1974 to 0.5863),P<0.001)(**Figure 3.2B**). Age and augmentation index were not significantly correlated (n=72, r=0.117, 95%CI(-0.1245 to 0.3459), P=0.326).



(n=72, r=0.31, P=0.003)

Figure 3.2A Relationship between arterial stiffness (Aix) and MAP



(n=72, r=0.436, P<0.001).

Figure 3.2B Association between Apnoea-Hypopnoea Index and arterial stiffness
Key: Aix =arterial stiffness@HR75, MAP=mean arterial pressure, AHI: Apnoea-Hypopnoea Index.
(Triangles for OSA, dark circles for non-OSA patients)

Table 3.2

Model	Variables	Standardised beta coefficient	P value
1 (R^2=0.45)	Age	0.15	0.16
	Systolic BP	0.28	0.08
	Diastolic BP	0.09	0.46
	BMI	-0.20	0.26
	Waist:Hip Ratio	-0.25	0.13
	Neck	-0.08	0.63
	HDL	0.05	0.67
	TG	0.07	0.52
	Glucose	-0.06	0.60
	Fat Percentage	0.24	0.15
	AHI	0.50	**<0.0001**
2 (R^2=0.45) (Model 1 excluding HDL)	Age	0.17	0.09
	Systolic BP	0.28	0.08
	Diastolic BP	0.09	0.44
	BMI	-0.20	0.26
	Waist:Hip Ratio	-0.25	0.13
	Neck	-0.09	0.59
	TG	0.06	0.59
	Glucose	-0.06	0.55
	Fat Percentage	0.25	0.14
	AHI	0.50	**<0.0001**
3 (R^2=0.44) (Model 2 excluding TG)	Age	0.17	0.09
	Systolic BP	0.29	0.07
	Diastolic BP	0.09	0.45
	BMI	-0.21	0.23
	Waist:Hip Ratio	-0.23	0.14
	Neck	-0.08	0.60
	Glucose	-0.06	0.59
	Fat Percentage	0.25	0.13
	AHI	0.50	**<0.0001**
4 (R^2=0.44) (Model 3 excluding glucose)	Age	0.16	0.11
	Systolic BP	0.28	0.07
	Diastolic BP	0.09	0.45
	BMI	-0.21	0.22
	Waist:Hip Ratio	-0.24	0.12
	Neck	-0.09	0.55
	Fat Percentage	0.24	0.14
	AHI	0.51	**<0.0001**
5 (R^2=0.42) (Model 4 excluding fat percentage)	Age	0.15	0.12
	Systolic BP	0.27	0.08
	Diastolic BP	0.11	0.34
	BMI	-0.03	0.80
	Waist:Hip Ratio	-0.28	0.10
	Neck	-0.15	0.32
	AHI	0.52	**<0.0001**
6 (R^2=0.29) (Model 5 excluding Waist:Hip Ratio & Neck)	Age	0.15	0.16
	Systolic BP	0.21	0.13
	Diastolic BP	0.14	0.25
	BMI	0.04	0.70
	AHI	0.37	**0.001**
7 (R^2=0.20) (Model 6 excluding systolic and diastolic BP)	Age	0.18	0.11
	BMI	0.04	0.74
	AHI	0.41	**<0.0001**
8 (R^2=0.22) (Model 7 including ESS)	Age	0.20	0.07
	BMI	0.05	0.68
	ESS	0.15	0.18
	AHI	0.38	**0.001**
9 (R^2=0.20) (metabolic syndrome components with age & BMI)	Age	0.16	0.20
	Systolic BP	0.25	0.09
	Diastolic BP	0.14	0.30
	BMI	0.25	0.17
	Waist circumference	-0.14	0.41
	TG	0.07	0.61
	HDL	0.08	0.56
	glucose	-0.15	0.21

Model	Variables	Standardised beta coefficient	P value
10 (R^2=0.16) (metabolic syndrome components)	Systolic BP	0.27	0.08
	Diastolic BP	0.13	0.35
	Waist circumference	0.03	0.84
	TG	0.06	0.66
	HDL	0.16	0.20
	glucose	-0.10	0.39

Table 3.2 Results of multiple regression analysis using Aix as the dependent variable and independent variables (as indicated) in sequentially adjusted linear regression models.

Table 3.2 shows the results of multiple linear regression analyses exploring the association between Aix designated as the dependent variable and potential confounders as independent variables in sequentially adjusted models. The regression models showed that AHI remained a significant predictor of arterial stiffness. Regression analysis for the components of the metabolic syndrome (Table 3.2 Model 10) indicated that these variables did not predict arterial stiffness to a significant level.

Discussion

With the increasing global prevalence of severe obesity, the presence of associated complications such as OSA is now recognised as one of several factors leading to the development of cardiovascular disease. Previous studies have demonstrated associations between arterial stiffness (as measured by Aix) and cardiovascular disease (Weber et al., 2004), and a link between OSA and arterial stiffness (Doonan et al., 2011). However, to date, few studies have investigated these parameters in severely obese subjects. There were two main findings in this study. Firstly, severely obese OSA patients, without an antecedent history of cardiovascular disease have increased arterial stiffness. Secondly, OSA severity as measured by the AHI was significantly associated with arterial stiffness in a multivariate model adjusted for potential confounders. These findings suggest that OSA may increase cardiovascular risk in severely obese individuals. There are a number of potential reasons why arterial stiffness is increased in OSA. These include changes in vasculature with alterations in collagen and elastin, endothelial dysfunction and peripheral vascular resistance (Zieman et al., 2005); as well as inflammation and oxidative stress (Kohler and Stradling, 2010).

It was found that there were increased augmentation pressures (AP) and decreased subendocardial viability ratio (SEVR) in OSA relative to non-OSA subjects. AP has been previously shown to be increased in patients with OSA, but these subjects had a lower BMI (Noda et al., 2008). The increased AP would be expected given that increased arterial stiffness results in the amplification of ventricular pressure waves and enhanced peripheral wave reflection (Chirinos et al., 2005). It has been previously shown in a hypertensive population that decreased values of SEVR were an index of impaired coronary flow (Tsiachris et al., 2012). In the present study, it is possible that the observed differences in SEVR in OSA may be due to factors that increase predisposition to perfusion changes such as heart rate, left ventricular pressures and changes in vascular compliance (Algranati et al., 2011). Taken together, the findings of increased Aix and AP, and lower SEVR in the severely obese OSA group suggest that these patients may be at higher risk of vascular perturbations.

There was an association between arterial stiffness and OSA severity as measured by the AHI. In agreement with our study, Buchner *et al*. (2012) found that OSA was associated with increased arterial stiffness (Buchner et al., 2012). Phillips *et al.* (2005) studied 57 males without known cardiovascular disease and found that arterial stiffness was positively correlated with OSA severity (Phillips et al., 2005).

In this study, there was a significant difference in systolic/diastolic BP and MAP between the two groups, and there was also an association between arterial stiffness and the MAP. As previous studies have shown a well-established association between OSA and hypertension (Kohler and Stradling, 2010), I sought to control for this link from the outset by excluding subjects with known or treated hypertension. Differences in pressor responses between OSA and non-OSA subjects may have a potentially confounding influence on the arterial stiffness findings. Nevertheless, in relation to the independence of the Aix results from the differences in blood pressures, the regression analysis did not indicate that measured blood pressure was an independent predictor of arterial stiffness. There are a number of possible reasons for the differences in measured pressures. It is possible that there may be underlying mechanisms in OSA driving sympathetic activity such as repeated arousals from fragmented sleep leading to the release of excess catecholamines (Kohler and Stradling, 2010). The presence of obesity may predispose to alterations in vascular tone through mechanisms including hyperinsulinemia, oxidative stress, inflammation and activation of the renin-angiotensin system (Kotsis et al., 2010).

More patients in the OSA group met the criteria for metabolic syndrome according to NCEP guidelines (Cleeman et al., 2001). This is consistent with previous findings that showed an association between OSA and the metabolic syndrome (Coughlin et al., 2004). Given that cardiovascular risk is increased with a diagnosis of metabolic syndrome (Grundy et al., 2005), it may be argued that the increased arterial stiffness in the OSA group may be attributable to this and not to the presence of OSA. Indeed, there is evidence that arterial stiffness is increased in patients with metabolic syndrome (Czernichow et al., 2010). However, a separate regression analysis demonstrated that the components that comprise the metabolic syndrome did not significantly predict arterial stiffness (Table 3.2 Model 10).

Although waist circumference was increased in OSA subjects, differences in neck circumference, body fat percentage and waist-to-hip ratio were not significant. These factors may potentially influence the severity of OSA (Subramanian et al., 2012). Indeed, the neck circumference of the subjects was correlated with OSA severity. However, the correlation of these variables with arterial stiffness did not reach significance and regression modelling confirmed that these variables did not significantly predict arterial stiffness (Table 3.2).

Although the ESS scores were significantly higher in the OSA group, the severity of somnolence symptoms may not necessarily correlate with the severity of OSA as determined by sleep-studies

(Osman et al., 1999). Furthermore, it has been shown that OSA patients with minimal symptoms may still have increased arterial stiffness and cardiovascular risk (Kohler et al., 2008).

Clinical Implications

The changes observed in the severely obese OSA group suggest that early mechanisms may potentially influence cardio-metabolic risk, in patients without a history of cardiovascular problems. This has implications for the clinical assessment of severely obese patients. Early testing of symptomatic individuals for OSA is necessary so that treatment in the form of CPAP may be applied to relieve daytime sleepiness. However, identification of OSA in severely obese patients defines a subgroup with a predisposition to high cardiovascular risk; in the absence of RCT data supporting cardiovascular risk reduction with CPAP it would be clinically appropriate to ensure optimal management of cardiovascular risk factors in these patients whether or not CPAP treatment is given.

Direct matching of OSA and non-OSA patients per se was not performed as this would have restricted eligible patients who were willing to participate, potentially introducing a selection bias; and it was important to try to recruit as many patients as possible for both groups. Furthermore, the OSA status of patients could only be known after they had their sleep studies, thus making direct matching of patients less practicable.

It is also important to note that all subjects in this study were of white European ethnicity and this may impose limitations on the generalisability of the findings. Lastly, it was not possible to confirm a causal link between OSA and arterial stiffness as measured by PWA. However, it is a potentially useful early marker of cardiovascular disease risk in this patient group.

Conclusion

In summary, the results of this study indicate that severely obese patients with OSA may have an increased cardiovascular risk that is associated with increased arterial stiffness. This supports justification for earlier recognition and investigation of such patients where appropriate. This study improves our understanding of cardiovascular risk stratification in severely obese groups, and through the use of these measures, potentially improving outcomes.

Effect of CPAP on arterial stiffness in severely obese patients with obstructive sleep apnoea

Abstract

Background

Obstructive sleep apnoea (OSA) may independently increase cardiovascular risk in obesity. Although there is evidence that arterial stiffness is altered in OSA, knowledge of these effects with continuous positive airway pressure (CPAP) in severe obesity (BMI≥35kg/m^2) is limited. This study aimed to explore how arterial stiffness, as measured by the augmentation index (Aix), changed in severely obese patients with OSA who were treated with CPAP and in patients without OSA.

Methods

Forty-two patients - 22 with OSA; 20 without OSA and severe obesity were recruited at baseline and followed-up after a median of 13.5 months. Pulse wave analysis (PWA) was performed using applanation tonometry at the radial artery to measure augmentation index (Aix), augmentation pressure (AP) and subendocardial viability ratio (SEVR). Cardiovascular parameters and body composition were also measured.

Results

There were significant improvements in Aix, AP (both P<0.001) and SEVR (P=0.021) in OSA patients on CPAP compared with subjects without OSA. Epworth scores (P<0.001), systolic (P<0.001) and mean arterial pressures (P=0.002) improved with CPAP. Regression showed that CPAP was significantly associated with change in arterial stiffness from baseline. However patients with OSA on CPAP continued to have increased arterial stiffness (Aix) (P<0.001), AP (P=0.028) and reduced SEVR (P=0.002) relative to non-OSA patients.

Conclusion

Although sleepiness and blood pressure improve with CPAP in severe obesity, CPAP alone is not sufficient to modify PWA measures to levels comparable with non-OSA patients. This supports a need for a multifaceted approach when managing cardiovascular risk in patients with severe obesity and obstructive sleep apnoea receiving CPAP therapy.

Introduction

OSA is associated with cardiovascular disease (Monahan and Redline, 2011). Despite the lack of randomised controlled trial (RCT) data supporting cardiovascular risk reduction with continuous positive airway pressure (CPAP) therapy, RCT evidence does support that blood pressure is reduced with CPAP treatment (Montesi et al., 2012b).

Increased arterial stiffness in OSA may contribute to increased cardiovascular risk (Doonan et al., 2011). Although there is evidence that arterial stiffness is altered in OSA, the evidence for these effects with CPAP in severe obesity (BMI≥35kg/m^2) is limited. Indeed, in a meta-analysis of studies examining arterial stiffness with CPAP, the studies that were evaluated comprised of subjects with lesser degrees of obesity (BMI<35kg/m^2)(Vlachantoni et al., 2013). Only one study by Bakker *et al.* (2011) investigated the effects of continuous and auto-adjusted positive airway pressure on 12 severely obese patients (BMI 49.9±5kg/m^2) and did not study patients of similar BMI without OSA (Bakker et al., 2011). Thus more work is needed in increasing our understanding of the role of effective CPAP treatment in arterial stiffness in severe obesity.

In the study of arterial stiffness at baseline that was described in the first part of this chapter, it was shown that OSA in a severely obese cohort of subjects was associated with increased arterial stiffness when compared with a similarly obese group without OSA, in the absence of a previous history of cardiovascular disease and prior to the commencement of CPAP treatment (Seetho et al., 2014a). In the present study, the original cohort of patients was invited to return for a follow-up assessment. The aim was to determine changes in PWA indices (augmentation index, augmentation pressure and subendocardial viability ratio) and other metabolic parameters in the severely obese OSA group who were now on established CPAP treatment, compared with the group of severely obese patients without obstructive sleep apnoea.

Research Design and Methods

Ethics Statement

The study was approved by the local research ethics committee (NRES 13/NW/589), and performed in accordance with the Declaration of Helsinki. All patients gave written informed consent.

Participants

Severely obese patients with a confirmed diagnosis of OSA and severely obese subjects without OSA (non-OSA) were recruited from the multidisciplinary weight management and sleep clinics at

University Hospital Aintree. All patients were recruited from the cohort who had their cardiovascular, metabolic and PWA parameters (Aix, AP and SEVR) measured at baseline (Seetho et al., 2014a). All OSA subjects were naïve to OSA treatment when measurements were performed at baseline. Subsequently, they were offered CPAP treatment. All OSA and non-OSA patients were invited to return for a clinical assessment and measurement of their PWA indices after at least 12 months. PWA indices and other cardio-metabolic parameters were studied in order to assess for changes between baseline and at follow-up, comparing OSA patients who received CPAP and non-OSA patients.

Patients were recruited from March 2012-January 2013 and follow-up took place from September 2013-February 2014. Inclusion and exclusion criteria were assessed according to clinical history, physical examination and analysis of medical notes. Patients were eligible if they were ≥21 years, with BMI≥35kg/m² and had measurements of cardiovascular and metabolic parameters, and arterial stiffness taken at baseline. Exclusion criteria included cardio-respiratory disease (ischaemic heart disease or chest disease for example, chronic obstructive pulmonary disease and interstitial lung disease); hypertension (BP>140/90 or on BP-lowering medications) (Chobanian et al., 2003); current smokers or those with more than 10 pack years smoking history; kidney and liver disease; acute illnesses and pregnancy. Patients with OSA who were not on CPAP treatment or compliant with CPAP (usage<4hrs/night) were subsequently excluded from the analyses. Likewise, patients who had bariatric surgery were excluded from subsequent analysis as the study sought to investigate changes in those compliant with CPAP treatment over time (CPAP≥4hrs/night).

Protocol

All patients attended their follow-up study visit between 0800-1000hrs and had a detailed history and physical examination. Body composition measurements, anthropometry, fasting blood and urine sampling, pulse wave analysis, venous blood gases (to assess for hypercarbia) were performed. Daytime somnolence was assessed using an Epworth Sleepiness Scale Questionnaire (ESS); a score >10 indicated increased sleepiness.

Blood pressure, body measurements, biochemical measurements and spirometry

Blood pressure, body measurements and biochemical measurements were assessed as described in the first study in this chapter. Spirometry measurements were taken at baseline as previously described.

PWA

PWA measurements of Aix, AP and SEVR were performed as described in the baseline study earlier in this chapter.

CPAP therapy

Following their baseline study, patients with OSA received standard CPAP therapy with S8/S9 Escape machines (ResMed, UK). CPAP compliance was based upon usage in hours per night (hrs/night) at the prescribed pressure. Compliance data was recorded by the CPAP machines and was downloaded using ResScan software (Version 4.2, ResMed, UK). This was assessed at each patient's CPAP adherence clinic. Adequate compliance was defined as a usage time of >4hrs/night (Engleman and Wild, 2003).

Metabolic syndrome

Metabolic syndrome was assessed according to the National Cholesterol Education Program (NCEP) guidelines as described previously in this chapter (Cleeman et al., 2001). Patients with hypertension or patients on blood pressure-lowering medications were not included in this study. In relation to diabetes or lipid lowering treatment, if patients had diabetes or were treated with statins, then they were assessed as having that component of metabolic syndrome. There were few patients on metformin and statin medications and the doses taken did not change during the course of the study.

Statistical Analysis

Patient demographics were summarised 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.

Linear regression with robust standard errors which allowed for the within-person correlation was fitted to model the change in augmentation index (Aix). Univariate associations of the change in Aix and demographic/clinical variables were tested using a two-sample t-test for categorical variables and Pearson's correlation for continuous variables. Model selection was performed using a stepwise procedure; the significance level for a variable to be included in the model was set at 0.025, and to be removed was 0.05. Univariate associations with a p-value<0.25 were included as candidate

variables along with a time and group-by-time interaction term. Model fit was assessed using QQ and residual plots. P-values<0.05 were considered statistically significant. All statistical analyses were conducted using Stata 13 (StataCorp. 2013. *Stata Statistical Software: Release 13*. College Station, TX: StataCorp LP.)

Results

Ninety-seven consecutive patients were screened. 20 were excluded because of hypertension. 5 subjects withdrew before their sleep studies and thus were excluded from the analyses. Therefore, seventy-two were assessed at baseline (47 obese OSA, 25 obese non-OSA) **(Figure 3.3)**. Fourteen declined to participate at follow-up. Seven patients were not eligible for inclusion in the study; 5 patients with CPAP did not tolerate CPAP and 4 patients used their CPAP<4hrs/night. Forty-two patients were entered into the present study (**Figure 3.3**). Twenty-two had OSA (12 males, 10 females) and were on CPAP and 20 were non-OSA patients (11 males, 9 females). Patients attended their follow-up after median duration of 13.5 (IQR 13,15) months. OSA patients had a mean CPAP use of 14 months (SD 1.4). All subjects were of white European ethnicity. OSA subjects on CPAP required a median of 10cmH_2O (IQR 9,11) treatment pressures. Mean CPAP compliance was 4.5 (SD 0.5) hrs/night (median 4.5hrs/night). In terms of the efficacy of CPAP treatment, the mean CPAP use per night may still represent systematic under treatment given the potential for greater hours of CPAP use per night

Figure 3.3

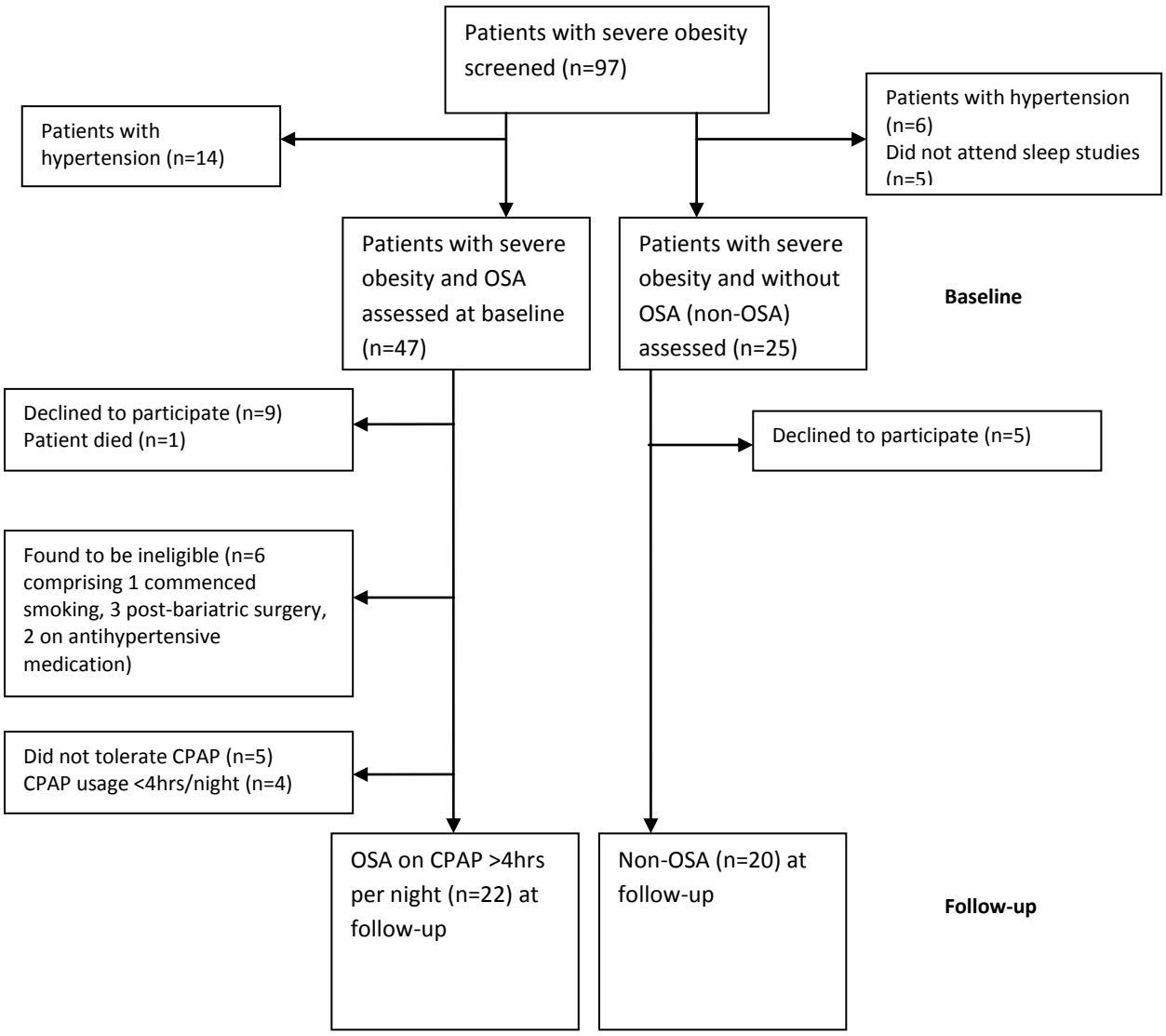


Figure 3.3 Study profile

Clinical and biochemical parameters of the patients according to non-OSA (control) and OSA groups are presented at baseline and at follow-up **(Table 3.3)**. At baseline, the OSA group (prior to CPAP) had significantly increased BMI, ESS, metabolic syndrome, systolic BP, Aix, AP, SEVR and mean arterial pressure. At follow-up, subjects with OSA on CPAP had a significantly higher neck circumference and a significant decrease in systolic blood pressure, mean arterial pressure and Epworth sleepiness scores was observed in OSA-on-CPAP compared with non-OSA patients.

	Baseline			Follow-up			Within-group difference		
	Control (non-OSA) (n = 20)	**OSA (prior to CPAP) (n = 22)**	**p-value**	**Control (non-OSA) (n=20)**	**OSA-on-CPAP (n=22)**	**p-value**	**$\Delta_{Control}$ (n=20)**	**Δ_{OSA} (n=22)**	**p-value**
Age (years)	52 (9)	47 (7)	0.059	53 (9)	48 (7)	0.056	-	-	-
BMI (kg/m^2)	38 (35, 41)	42 (35, 46)	**0.039**	38 (35, 42)	42 (35, 46)	0.092	0.6 (0.1, 2.3)	0.5 (-4.4, 1.2)	0.091
Neck Circumference (cm)	42 (39, 44)	44.5 (41, 48)	0.064	42 (38.5, 44)	43.5 (42, 48)	**0.045**	-0.2 (0.52)	0.23 (1.57)	0.254
Waist:Hip Ratio	0.95 (0.11)	1.00 (0.13)	0.236	0.94 (0.10)	0.99 (0.13)	0.248	0 (-0.1, 0)	0 (-0.04, 0)	0.865
Body Fat (%)	38 (33,51)	46 (38, 53)	0.064	41 (33, 51)	45 (39, 53)	0.195	0 (0, 2)	0 (-2, 1)	0.106
Gender:									
Female	9 (45%)	10 (45%)		9 (45%)	10 (45%)				
Male	11 (55%)	12 (55%)	0.976	11 (55%)	12 (55%)	0.976	-	-	-
Type 2 Diabetes:									
No	17 (85%)	19 (68%)		17 (85%)	15 (68%)				
Yes	3 (15%)	3 (32%)	0.284	3 (15%)	7 (32%)	0.284	-	-	-
Ever Smoked:									
No	14 (70%)	15 (68%)		14 (70%)	15 (68%)				
Yes	6 (30%)	7 (32%)	0.899	6 (30%)	7 (32%)	0.899	-	-	-
Alcohol Intake:									
<8 units per week	12 (60%)	13 (59%)		12 (60%)	13 (59%)				
≥8 units per week	8 (40%)	9 (41%)	0.952	8 (40%)	9 (41%)	0.952	-	-	-
Medications:									
Metformin	3 (15%)	3 (32%)	0.284	3 (15%)	7 (32%)	0.284	-	-	-
Statin treatment	3 (15%)	5 (23%)	0.524	5 (25%)	7 (32%)	0.625	-	-	-
Cardio-respiratory Parameters									
Systolic BP (mmHg)	126 (11)	139 (12)	**<0.001**	127 (11)	129 (10)	0.719	0 (-6, 5.5)	-8 (-13, 0)	**<0.001**
Diastolic BP (mmHg)	82 (10)	88 (13)	0.129	82 (9)	81 (8)	0.592	0 (-8, 1)	-6 (-11, 0)	0.070
Augmentation Index (%)	10.5 (8, 14.5)	24.5 (20, 31)	**<0.001**	10.0 (8,13)	20.5 (15, 27)	**<0.001**	-0.15 (2.62)	-4.18 (4.28)	**<0.001**
Augmentation Pressure (mmHg)	4.5 (3, 8.5)	12.5 (8, 15)	**<0.001**	4.5 (3,8.5)	8.5 (5, 12)	**0.028**	0.05 (2.16)	-3.82 (4.29)	**<0.001**
SEVR (%)	171 (167, 184)	146 (142, 157)	**<0.001**	177.5 (165, 182)	150 (146, 169)	**0.002**	0 (0, 3.5)	5 (0, 11)	**0.021**
Mean Arterial Pressure (mmHg)	98 (9)	106 (12)	**0.016**	99 (9)	97 (8)	0.459	-0.2 (-3.2, 3.5)	-8.3 (-12, -0.7)	**0.002**

Heart Rate (bpm)	69 (13)	76 (10)	0.079	71 (11)	76 (11)	0.129	1.3 (8.32)	-0.14 (7.77)	0.556
O2 Saturations (%)	96.8 (1.5)	96.8 (1.5)	0.884	97.1 (1.5)	96.9 (1.3)	0.583	0.35 (1.27)	0.05 (1.81)	0.536
PCO2 (kPa)	5.4 (5.0,5.5)	5.4 (5.1,5.5)	0.529	5.4 (5.2,5.5)	5.3 (5.1,5.4)	0.168	0(-0.1,0.1)	-0.1(-0.2,0)	0.186
FEV1 % predicted	95.5 (14.3)	99.3 (13.5)	0.369	-	-		-	-	-
FVC % predicted	105.8 (14.6)	105.9 (16.2)	0.977	-	-		-	-	-
FEV1:FVC ratio	76.2 (3.7)	78.3 (5.2)	0.134	-	-		-	-	-
AHI	2.5 (1.5,3.7)	21 (13.1,51)	**<0.001**	-	-				
Epworth Sleepiness Score	8 (3, 10)	11 (8, 16)	**0.013**	5 (3, 10)	3 (2, 10)	0.300	-0.5 (-2, 0)	-4.5 (-11, -2)	**<0.001**
CPAP treatment pressure (cmH_20)	-	-	-	-	10 (9,11)		-	-	-
Metabolic Parameters									
Metabolic Syndrome:									
No	19 (95%)	7 (32%)		15 (75%)	11 (50%)				
Yes	1 (5%)	15 (68%)	**<0.001**	5 (25%)	11 (50%)	0.096	-	-	-
Creatinine (micromol/l)	79 (17)	77 (15)	0.685	80 (13)	77 (13)	0.408	2 (0, 6)	1 (-4, 4)	0.211
MDRD-GFR (mL/min/1.73m^2)	87 (16)	90 (16)	0.476	84 (11)	90 (15)	0.176	-1 (-9, 0)	-2 (-6, 3)	0.377
LDL (mmol/l)	2.9 (1.0)	2.9 (0.9)	0.920	3.0 (1.0)	2.8 (0.9)	0.557	0 (-0.2, 0.3)	-0.1 (-0.4, 0.2)	0.342
HDL(mmol/l)	1.3 (0.4)	1.2 (0.4)	0.576	1.4 (1.2, 1.6)	1.2 (0.3)	0.058	0.13 (0.16)	-0.02 (0.22)	**0.016**
HbA1c (mmol/mol)	39 (35, 41)	41 (37, 46)	0.088	37 (34, 39)	39 (35, 50)	0.063	-1 (-4, 0)	-0.5 (-3, 2)	0.145
Total Cholesterol (mmol/l)	4.9 (1.2)	4.9 (1.0)	0.828	5.0 (1.1)	4.7 (1.0)	0.503	0 (-0.2, 0.3)	-0.2 (-0.6, 0.1)	0.093
Fasting Glucose (mmol/l)	5.2 (5.0, 5.6)	5.5 (4.5, 6.2)	0.390	5.4 (5.0, 5.7)	5.6 (4.7, 6)	0.512	0.1 (-0.3, 0.3)	-0.1 (-0.4, 0.5)	0.723
Triglycerides (mmol/l)	1.2 (1, 2.1)	1.7 (1.1, 2.5)	0.210	1.3 (0.8, 2.1)	1.6 (1.1, 2.6)	0.134	-0.1 (-0.4, 0.1)	0 (0, 0.1)	0.211
Bicarbonate (mmol/l)	24.6 (0.9)	24.6 (1.0)	0.923	23.9 (1.9)	24.2 (2.0)	0.580	-0.73 (1.95)	-0.43 (1.64)	0.587

Table 3.3 Patient demographics were summarised by OSA group as means (SD) (or median (IQR) if non-normal) for continuous variables and frequencies (%) for categorical variables. Significant P-values are highlighted in bold. $\Delta_{Control}$=Difference between baseline and follow-up for controls; Δ_{OSA}=difference between baseline and follow-up for OSA subjects. Medication doses did not change between baseline and follow-up.

Severely obese OSA patients on CPAP demonstrated significantly increased arterial stiffness (OSA-on-CPAP: 20.5%; IQR 15-17 vs. non-OSA: 10%; IQR 8-13; $p<0.001$), augmentation pressure (OSA-on-CPAP: 8.5mmHg; IQR 5-12 vs. non-OSA: 4.5mmHg; IQR 3-8.5; $p<0.001$) and decreased subendocardial viability ratio (OSA-on-CPAP: 150%; IQR 146-169 vs. non-OSA: 177.5%; IQR 165-182; $p=0.021$) compared with non-OSA patients at follow-up. Boxplots have been generated to graphically illustrate Aix, AP and SEVR at each time point, and change from baseline **(Figure 3.4).**

Figure 3.4

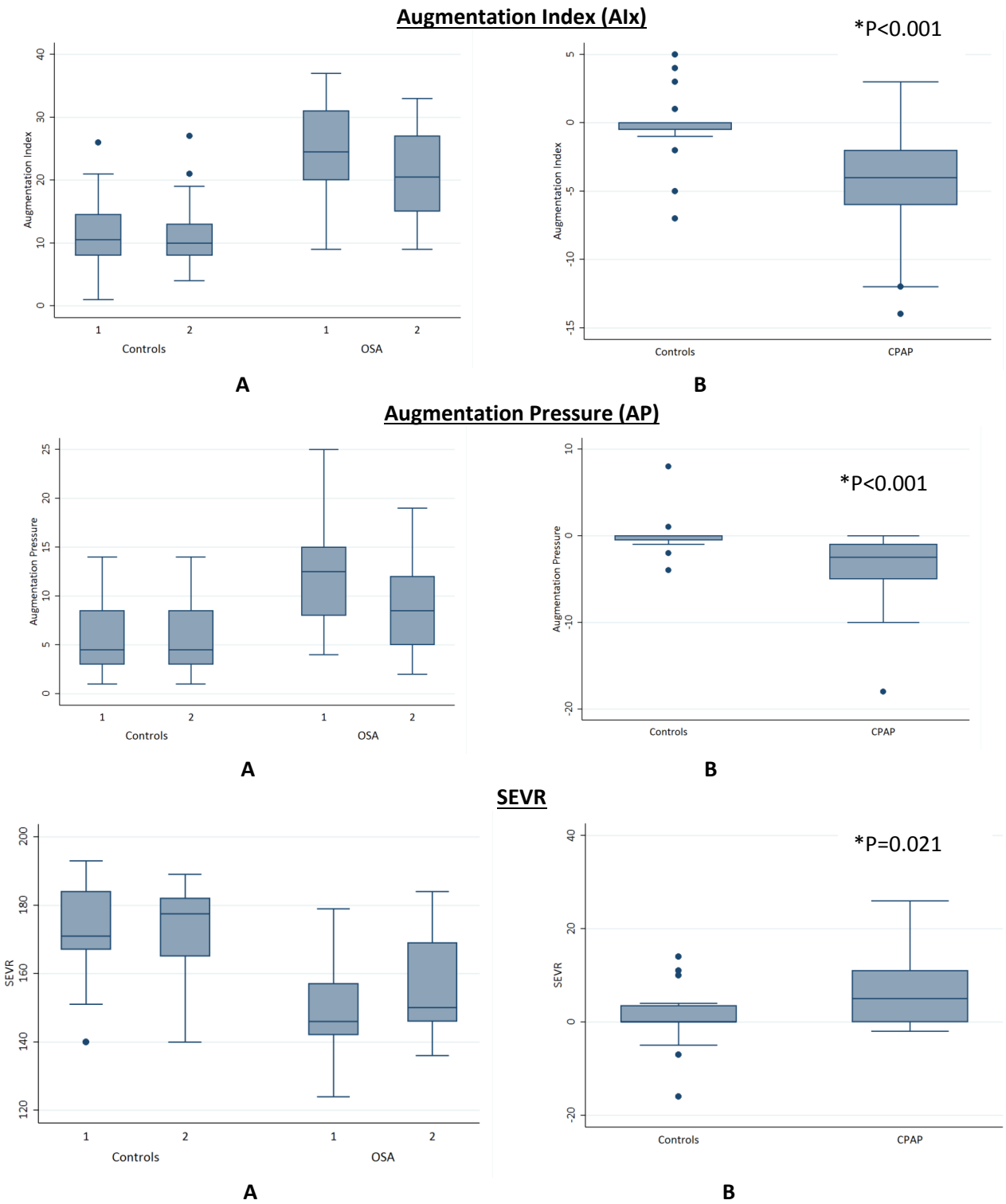


Figure 3.4 Boxplots graphically illustrate Aix, AP and SEVR at each time point (A) and the change from baseline (B) for each group. (1=baseline; 2=follow-up)

Regression analysis

In order to determine the independence of the observed associations relating to the augmentation index, a stepwise regression analysis was performed. The candidate variables for model selection were: group (CPAP or non-CPAP), time (baseline, follow-up), time/group interaction, metabolic syndrome, systolic BP, diastolic BP, neck circumference, triglycerides, mean arterial pressure and ESS (Epworth score). The final regression model included group, time (baseline, follow-up) and a group/time interaction.

Augmentation index was significantly higher in the OSA compared to the non-OSA group at baseline (13.3%; 95%CI 9.2-17.5; p<0.001) and follow-up (9.3%; 95%CI 5.3-13.3; p<0.001). There was a significant reduction from baseline in the OSA (CPAP-treated) group (4.2%; 95%CI 2.3-6; p<0.001), however this was not seen for the non-OSA group (0.15%; 95%CI 1-1.3; p=0.8), **(Table 3.4).**

	Estimate	95% CI	p-value
Difference between groups at baseline	13.3	(9.2, 17.5)	**<0.001**
Difference between groups at follow-up	9.3	(5.3, 13.3)	**<0.001**
Non-OSA group difference over time	-0.15	(-1.3, 1.0)	0.800
OSA group difference over time	-4.2	(-6.0, -2.3)	**<0.001**
Difference between groups (OSA and non-OSA) over time	-4.0	(-6.2, -1.8)	**0.001**

Table 3.4 Results of regression model

Discussion

In this study, there were significant improvements in arterial stiffness at follow-up in CPAP-treated severely obese patients relative to subjects without OSA. Despite improved arterial stiffness, this remained elevated in those compliant with CPAP treatment relative to non-OSA patients. Regression analysis showed that CPAP treatment in severe obesity was associated with a greater reduction in arterial stiffness compared to the non-OSA patients. Although these findings support the importance of CPAP in severe obesity with OSA, it was also found that in these patients, CPAP treatment may not completely reverse the increased arterial stiffness in patients with severe obesity and OSA.

These findings extend our knowledge of changes in arterial stiffness with CPAP in severe obesity and are consistent with previous lines of evidence that show that CPAP improves arterial stiffness in subjects with lesser degrees of obesity (Vlachantoni et al., 2013). In the present study, despite significant changes in values from baseline, there was still an increased AP and decreased SEVR in the CPAP-treated group compared with non-OSA patients. The increased AP may be due to increased ventricular pressure waves and peripheral wave reflection with arterial stiffness (Chirinos et al., 2005). The severity of OSA is associated with increased augmentation pressure and is improved with CPAP treatment (Noda et al., 2008). The SEVR provides an indication of myocardial perfusion (Chemla et al., 2008) and decreased values may be an index of impaired coronary flow (Tsiachris et al., 2012). The findings of an improved SEVR in the CPAP group, albeit still lower levels than non-OSA patients, contrast with those reported by Butt et al (2011) who examined PWA measures in patients with moderate-severe OSA (AHI>15) before and after 26 weeks of CPAP. In that study, although Aix and AP improved following CPAP, the SEVR was found to be significantly lower (Butt et al., 2011). However, it is possible that this difference may be a reflection of heterogeneity in the patient groups recruited given the lower BMI of patients in that trial compared with the patients in the present study with severe obesity.

In terms of change from baseline between the two groups, it was found that the change in systolic BP and mean arterial pressure (MAP) was significantly different between CPAP and non-OSA patients, with reduced pressor responses in the CPAP group. The evidence from studies indicates that CPAP treatment lowers BP in patients with more severe OSA (Montesi et al., 2012b). Additionally, the effect on blood pressure may be influenced by the presence of daytime sleepiness and adherence to CPAP therapy (Barbé et al., 2012). It may be questioned that given the association between BP and arterial stiffness (Payne et al., 2010), with a significant reduction in BP in the CPAP-treated group, a reduction in arterial stiffness might be expected. Nevertheless, regression

modelling did not demonstrate that BP was associated with the change in arterial stiffness. However, the reduction in BP may be an underlying mechanism for the improved arterial stiffness that was observed.

It was noted that there was a significant difference in the change from baseline in ESS scores, with reduced sleepiness reported in the CPAP group. This would be expected since the CPAP group comprised patients who were established on their treatment. Although neck circumference was increased in the CPAP group compared with the non-OSA group at follow-up, there was no significant difference in the change from baseline between the groups. Even though neck circumference was found to improve model fit, surprisingly, an inverse relationship with arterial stiffness was found. It is possible that this variable may have been a confounder explained by the significantly increased neck circumference in the CPAP group at follow-up. Therefore, this variable was not included in the final regression model.

Previous research has demonstrated that OSA is independently associated with metabolic syndrome (Coughlin et al., 2004), but does not improve with CPAP treatment in an RCT (Coughlin et al., 2007). In the present study, there was no difference in metabolic syndrome between CPAP and non-OSA patients at follow-up. A sub-analysis of patients with metabolic syndrome showed that this was attributable to a combination of lower blood pressures in the CPAP group and several non-OSA patients meeting the metabolic syndrome criteria. However, this may have been due to the small sample size and given the observational nature of this study, no cause-and-effect inferences regarding CPAP treatment and metabolic syndrome can be made. In relation to arterial stiffness, metabolic syndrome was included as a candidate variable when modelling Aix but did not improve the model fit and therefore was not included in the final model.

Subjects were included based on their compliance >4hrs per night given that there is evidence linking the effects of CPAP to compliance with therapy (Steiropoulos et al., 2007). However, in a meta-analysis of studies investigating CPAP and arterial stiffness, it was found that the duration of CPAP use did not alter the effect of CPAP on arterial stiffness (Vlachantoni et al., 2013).

As patients were recruited from a weight management service during this time and some would have received weight management care during their CPAP treatment, it is possible that weight loss may have occurred between baseline and follow-up. However, patients were also recruited from the sleep service who were not attending weight management clinic. This may explain the non-

significant difference in BMI, body fat composition and other measures of obesity at follow-up. It has been previously reported that in obese individuals with OSA, randomised treatment with weight loss, CPAP therapy, or combination therapy for 24 weeks did not appreciably reduce large artery stiffness (Chirinos et al., 2013). However, it remains plausible that combination treatment with weight loss and CPAP may have potential effects on arterial stiffness in the severely obese population in the long-term.

The change in arterial stiffness in a cohort of patients with severe obesity was evaluated over a relatively long period of time (~13 months) and the findings in this study are a useful addition to the literature given the limited knowledge available relating to subjects with severe obesity. This is relevant given the growing prevalence of severe obesity that is a major public health concern in many nations. It is hoped that this may encourage further research in this patient population group.

There are several limitations in this study. With the lack of a placebo CPAP group, it was not possible to make inferences on cause-effect and to demonstrate that our observations were indeed CPAP effects. However, it was envisaged that conducting such a placebo-controlled randomised trial over this length of time would be questionable from an ethical point of view given the duration of follow-up. The study subjects all volunteered to undergo treatment in a clinical setting and the aim was to investigate the changes in real-life settings.

Repeat polysomnography was not conducted to prove if the AHI was sufficiently reduced on CPAP but the patients were managed according to local 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.

Conclusion

Severely obese patients with OSA on CPAP treatment have increased arterial stiffness relative to non-OSA patients at follow-up at 13.5 months, and may be at increased cardiovascular risk. Regression analysis showed that CPAP was a significant predictor in influencing the change in arterial stiffness. The findings suggest that although CPAP treatment may improve arterial stiffness, it may not be sufficient to produce comparable results with non-OSA patients, and there may be a need to consider a multifaceted approach when managing such patients in order to ameliorate their cardiovascular risk. There may be a role for CPAP and other treatment modalities such as lifestyle

interventions that may have an effect on arterial stiffness. Further investigations are needed to evaluate this in severe obesity.

Chapter 4

Serum urate and obstructive sleep apnoea in severe obesity

In chapter 3, arterial stiffness was discussed that may have potential implications for cardiovascular risk for OSA patients with severe obesity. In this chapter, the relation between serum urate and OSA in severe obesity is explored. The evidence from this chapter lends further support for the importance of OSA assessment in obesity.

Abstract

Objectives

Obstructive sleep apnoea (OSA) may increase the risk of hyperuricaemia and predispose to gout. The evidence for the effects of OSA on serum urate in severe obesity is limited. This study investigated whether OSA was associated with serum urate in severe obesity, and whether continuous positive airway pressure (CPAP) treatment was associated with a fall in urate levels.

Methods

Severely obese subjects without known OSA or gout were recruited. Baseline assessments included urate, metabolic parameters, spirometry and overnight polysomnography. OSA patients were initially naïve to treatment and were offered CPAP. At follow-up, change in urate was compared between CPAP-treated and non-CPAP treated subjects. A high urate was defined as greater than the median. Logistic regression was performed to identify associations between 1) OSA and high urate at baseline and 2) use of CPAP and change in urate at follow-up.

Results

92 subjects were recruited (61 OSA, 31 non-OSA). Median urate was 345µmol/L. OSA was associated with high urate in females at baseline after adjusting for confounders (OR_{adj} 10.2 [95%CI 1.1-93.5]).

At follow-up (14 months), 58 subjects (28 on CPAP, 30 not on CPAP) were reassessed. CPAP was significantly associated with fall to a low urate category at follow-up (P=0.017). Regression revealed a trend for fall in urate category in the CPAP-treated group (OR_{adj} 9.3 [95%CI 0.8-97]).

Conclusions

Serum urate is associated with OSA in severely obese females and CPAP may reduce levels in patients with OSA. There may be a need to consider and assess for OSA in obese patients with hyperuricaemia and recurrent attacks of gout.

Introduction

Obstructive sleep apnoea (OSA) is a common condition associated with metabolic dysregulation and cardiovascular disease. Epidemiological studies have suggested an association between gout and OSA (Roddy et al., 2013) (Cantalejo Moreira et al., 2013). Urate levels may be influenced by cell apoptosis secondary to apnoea-induced episodes of hypoxia (Jelic et al., 2009). Furthermore, increased adenosine triphosphate degradation in recurrent hypoxia may increase uric acid levels (Saito et al., 2002). Given that these events occur intermittently throughout the sleep cycle, urate levels may fluctuate during sleep, and may contribute to an increased risk of gout attacks. This may explain the increased predilection for the nocturnal onset of many gout attacks.

Elevated levels of serum urate have been associated with OSA and increased cardiovascular risk (Feig et al., 2008). This may reflect shared risk factors of increased body weight associated with gout and OSA (Huang et al., 2008). Although several studies have shown that urate levels are raised in OSA, most studies were cross-sectional in design and did not control for obesity or gender that may confound this relationship (Garcia et al., 2006, Wiener and Shankar, 2012), Additionally, the association between urate and OSA has not been investigated in patients with severe obesity (BMI>35kg/m^2). Therefore, the aim of this study was to explore, in a severely obese population, whether the presence of OSA was associated with urate. In addition, the study aimed to identify whether use of CPAP treatment in OSA was associated with fall in serum urate.

Methods

Ethics Statement

Research approval was granted by the local research ethics committee (NRES 12/NW/123). The research was performed in accordance with the Declaration of Helsinki 2008. All patients gave written informed consent.

Participants

Subjects were recruited from weight management and sleep clinics at University Hospital Aintree from March 2012 to January 2013 and follow-up visits were from September 2013 to February 2014. Patients were eligible if they were ≥21 years old and had BMI≥35kg/m^2. Exclusion criteria were patients who were being treated or had prior treatment for OSA; those with known cardio-respiratory disease; current smokers or those with more than 10 pack years smoking history; kidney and liver disease; acute illnesses and pregnancy. No patients had a history of gout or were on urate-

lowering medications. At follow-up assessment, subjects with OSA who were non-compliant with CPAP (usage<4hrs per night) and those who had bariatric surgery were excluded.

Protocol

At baseline subjects attended between 0800-1000hrs and underwent a detailed history and physical examination. Body composition measurements, anthropometry, serum urate and biochemical tests were performed. An overnight sleep study was performed and patients were then grouped according to their sleep status.

Blood Pressure

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

Body measurements

All measurements were done 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.

Spirometry Assessment

Spirometry was performed at baseline with a Spiro Air LT system (Medisoft, Sorinnes, Belgium), supervised by an experienced technician. This was performed to assess for coexisting lung pathology.

Sleep Diagnostic Assessment

Diagnosis was confirmed by overnight multichannel respiratory limited polysomnography (PSG) (Somnoscreen Digital PSG acquisition system, Version 2.0, SomnoMedics, Germany). Sleep studies were independently assessed by experienced sleep physiologists using software (Domino PSG analysis software (version 2.5.0), SomnoMedics, Germany). Apnoea was defined as a cessation of airflow for >10secs. 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 ≥5. The oxygen desaturation index (ODI) which is measure of the hourly average number of desaturation episodes during sleep was also recorded for each patient.

Biochemical Measurements

Biochemical tests including serum urate were measured using standard laboratory assays (Roche, UK). Blood gases were analysed with a Cobas Blood Gas Analyser (Roche, UK).

Assessment of urate

The category "High urate" was defined as a value above the baseline median urate measure. This was necessary given that all subjects did not have a history of gout and it was expected that their urate levels were unlikely to exceed the upper reference range.

To compare the change in urate with treatment, a binomial variable of 'change in urate' category was created. This represented a change from a high urate category at baseline to a low urate category at follow-up.

Metabolic Syndrome

Subjects were assessed for metabolic syndrome according to the National Cholesterol Education Program (NCEP) guidelines (Cleeman et al., 2001). Patients had metabolic syndrome if three or more risk factors were present: waist circumference (males>102cm; females>88cm), triglycerides≥1.7mmol/l, HDL cholesterol (males<1.04 mmol/l; females<1.3mmol/l), blood pressure ≥130/≥85 mmHg, and fasting glucose ≥6.1mmol/l.

CPAP therapy

OSA patients received standard therapy with S8/S9 Escape machines (ResMed, Abingdon, UK). CPAP compliance was based upon usage in hours per night (hrs/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 clinic. Adequate compliance was defined as a usage time of >4hrs/night on>70% of nights in the treatment group (Engleman and Wild, 2003).

Follow-up

Patients were reassessed after 12-14 months with repeat urate measurement. Change in urate levels compared to baseline measures was recorded. Study participants with OSA who were compliant

with CPAP treatment (CPAP-treated group) were compared to non-OSA participants and OSA participants who chose not to undergo CPAP treatment (non-CPAP group).

Statistical Analysis

Statistical analyses were performed using Stata 13 (StataCorp. 2013. *Stata Statistical Software: Release 13*. College Station, TX: StataCorp LP). Normal distribution of data was confirmed using the Shapiro-Wilks test. 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 of demographics 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 frequencies.

Multivariate logistic regression analysis was performed at baseline exploring associations between high urate and OSA adjusting for age, previous smoking status, hsCRP and BMI. As urate levels are higher in males, gender stratified analysis was conducted.

In addition multivariate logistic models were generated to explore if CPAP was associated with a fall in urate category.

Results

Ninety-two consecutive patients with severe obesity were recruited at baseline (61 with OSA, 31 without OSA) (**Figure 4.1**). All subjects were of white European ethnicity. Baseline descriptors are summarised in **Table 4.1**. The median urate for all 92 subjects was 345µmol/L [IQR 288-414]. At baseline, the presence of OSA was significantly associated with high urate (35(57%) of the OSA group had a high urate compared to 11(35%) in non-OSA group). However, when urate was analysed as a continuous variable no significant association was observed with OSA. In addition, subjects with OSA had significantly increased systolic BP, hsCRP and metabolic syndrome compared with the non-OSA group. BMI was higher in the OSA group but did not reach significance. Therefore, this covariate was adjusted for in the multivariate analysis.

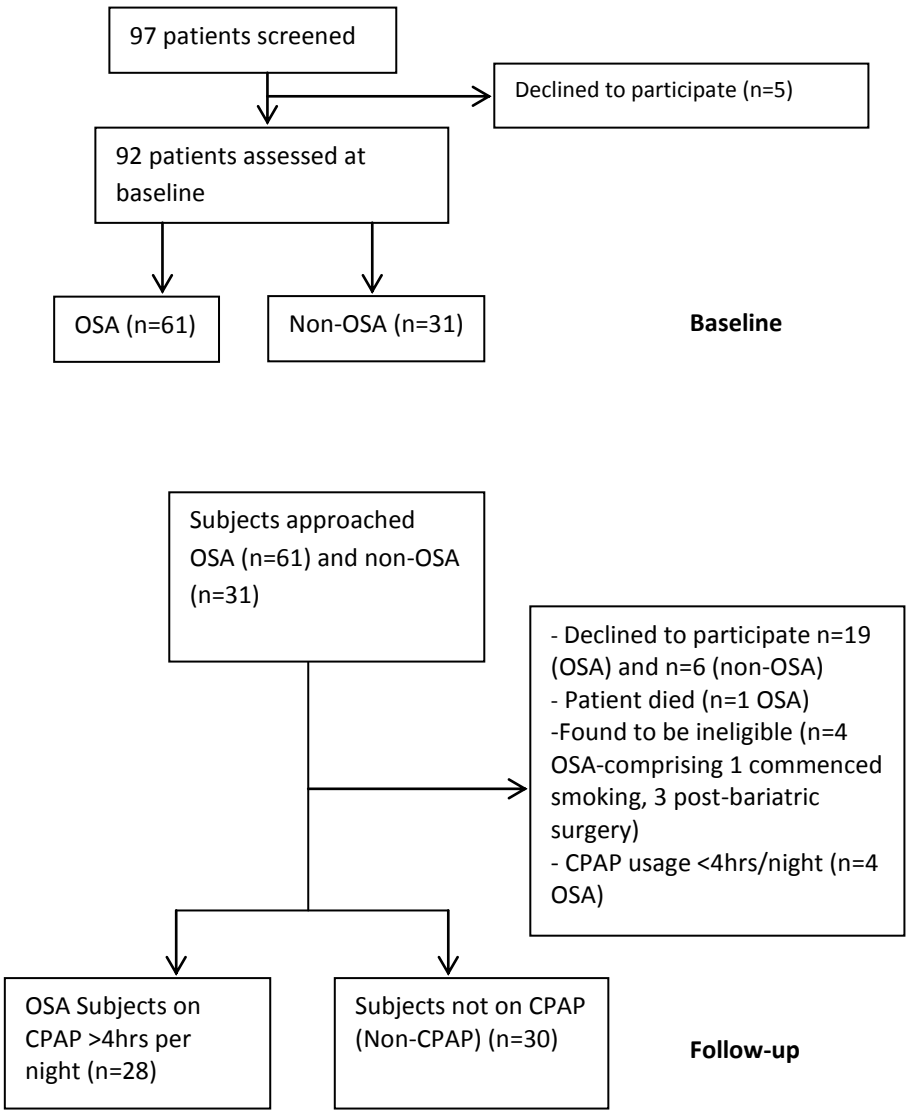


Figure 4.1 Study profiles at baseline and follow-up. OSA: Subjects with obstructive sleep apnoea. Non-OSA: subjects without obstructive sleep apnoea. CPAP: Continuous positive airway pressure treatment

Baseline Characteristics				**By Gender**					
	Non-OSA (n = 31)	OSA (n = 61)	p-value	Male non-OSA(n=16)	Male OSA (n=30)	P-value	Female non-OSA (n=15)	Female OSA (n=31)	p-value
Age (years)	49 (10)	48 (9)	0.325	49(9)	48(8)	0.497	49(11)	48(10)	0.650
Gender: Female	15 (48%)	31 (51%)		-	-	-	-	-	-
Male	16 (52%)	30 (49%)	0.825						
Smoked in past:	7 (23%)	16 (26%)	0.802	4(25%)	4(13%)	0.320	3(20%)	12(39%)	0.317
Alcohol Intake:									
<8 units per week	23(74%)	43 (70%)		10(63%)	17(57%)		13(87%)	26(84%)	
≥8 units per week	8 (26%)	18 (30%)	0.809	6(37%)	13(43%)	0.702	2(13%)	5(16%)	0.805
BMI (kg/m^2)	40[35, 43]	43[36, 46]	0.086	36[35,41]	37[35,44]	0.156	42[39,46]	45[39,47]	0.185
Neck Circumference (cm)	42.4(4.4)	44.2 (4.3)	0.054	45.4(3.6)	46.6(3.9)	0.355	39.1(2.3)	42.0(3.6)	**0.006**
Waist:Hip Ratio	0.96 (0.1)	0.98 (0.1)	0.485	1.0(0.1)	1.1(0.1)	0.164	0.88(0.1)	0.89(0.1)	0.396
type 2 Diabetes	6 (19%)	13 (21%)	0.827	4 (25%)	6 (20%)	0.720	2 (13%)	7 (23%)	0.696
Clinical Parameters									
Urate (µmol/L)	340 (87)	357 (80)	0.300	396(72)	383(59)	0.539	281(58)	331(89)	0.056
Subjects with a high urate category	11 (35%)	35 (57%)	**0.047**	10 (63%)	22 (73%)	0.447	1 (7%)	13 (42%)	**0.015**
hsCRP (mg/L)	3.3 [1.5, 5.3]	5.2 [2.5, 7.3]	**0.032**	1.9[0.9,3.3]	3.2[1.9,6.4]	**0.047**	4.1[3.0,6.9]	5.8[3.7,5.8]	0.266
Systolic BP (mmHg)	128 (12)	137 (13)	**0.004**	131(13)	138(12)	0.056	126(12)	136(14)	**0.025**
Diastolic BP (mmHg)	83.0 (10.2)	87.3 (11)	0.105	83(13)	89(10)	0.247	83(6)	85(12)	0.446
Creatinine (micromol/l)	77 (16)	73 (15)	0.355	86(14)	83(12)	0.423	67(11)	63(10)	0.398
MDRD-GFR (mL/min/1.73m^2)	90 (17)	95 (17)	0.221	90(19)	94(17)	0.503	90(16)	96(16)	0.259
Total Cholesterol (mmol/l)	4.7 (1.1)	5.0 (1.0)	0.247	4.4(1.1)	4.88(1.2)	0.204	5.0(1.1)	5.1(0.9)	0.855
Fasting Glucose (mmol/l)	5.2 [4.9, 5.6]	5.3 [4.8, 6.0]	0.778	5.4[5.0,5.9]	5.3[4.8,5.7]	0.481	5.2[4.9,5.6]	5.4[4.8,6.3]	0.751
Metabolic Syndrome	4 (13%)	35(57%)	**<0.001**	4 (25%)	20 (67%)	**0.012**	0 (0%)	15 (48%)	**0.001**
AHI	2.6 (1.1)	24.0 (18.6)	**<0.001**	2.4 (1.2)	29.2 (21.2)	**<0.001**	2.7 (1.1)	18.9 (14.3)	**<0.001**
ODI	4.5 (2.7)	23.5 (17.9)	**<0.001**	4.8 (2.4)	27.8 (20.3)	**<0.001**	4.3 (3.1)	19.2 (14.2)	**<0.001**

O2 saturations (%)	97(2)	97(2)	0.959	97(1)	97(2)	0.463	97(2)	96(1)	0.520
pCO_2 (kPa)	5.4 (5.0, 5.6)	5.4 (5.1, 5.6)	0.411	5.2[5.0,5.4]	5.4[5.2,5.6]	0.554	5.5[5.2,5.6]	5.3[5.1,5.5]	0.426
FEV1% predicted	94.1 (12.1)	94.3 (14.9)	0.987	95.4(11.2)	96.6(12.7)	0.963	92.7(13.2)	92.1(16.7)	0.906
FVC% predicted	103.9 (13.6)	101.7 (15.6)	0.577	103.7(13.5)	104.3(14.5)	0.927	104.1(14.2)	99.2(16.5)	0.325
FEV1:FVC	76.4(4.1)	77.5(5.1)	0.274	76.1(4.3)	76.1(4.9)	0.998	76.8(4.0)	79.0(5.0)	0.186

Table 4.1 Patient demographics at baseline are summarised by OSA prior to CPAP vs non OSA groups as means (Std Dev) (or median [IQR] if non-normal) for continuous variables and frequencies (%) for categorical variables. Significant P-values are highlighted in bold. A high urate was defined as a urate greater than the median urate (345µmol/L). Metabolic syndrome assessed according to National Cholesterol Education program guidelines.
AHI: Apnoea-Hypopnoea Index. BMI: Body Mass Index. BP: Blood Pressure. FEV1: Forced Expiratory Volume in 1 sec. FVC: Forced Vital Capacity. hsCRP: highly-sensitive C-reactive protein. MDRD-GFR: Glomerular Filtration Rate using Modification of Diet in Renal Disease Equation. ODI: Oxygen desaturation index. OSA: Obstructive Sleep Apnoea. Non-OSA: subjects without OSA. pCO_2: Partial pressure of carbon dioxide.

Gender stratification revealed that more females with OSA had a high urate level 13(42%) compared with females without OSA 1 (7%) (P=0.015) **(Table 4.1)**. Systolic BP in females with OSA (P=0.025) and hsCRP in males with OSA (P=0.047) were significantly different compared with controls. Metabolic syndrome was significantly increased in males (P=0.012) and female (P=0.001) OSA subjects compared with non-OSA subjects.

Logistic regression showed a significant association between OSA and a high urate [Odds ratio (OR) 10.1 (95%CI 1.2, 86.8) only in female participants **(Table 4.2)**. This remained significant after adjusting for confounding variables age, previous smoking status, hsCRP and BMI [OR_{adj} 10.2 (95%CI 1.1,93.5)]. However, this association was not significant in males [(OR_{adj} 0.8 (95%CI 0.1,4.1)] and when both genders were combined [OR_{adj} 2.3 (95%CI 0.8,6.7)] **(Table 4.2)**.

		Univariate		**Multivariate**	
	n	**Odds Ratio**	**95%CI**	**Odds Ratio Adjusted ***	**95% CI**
All subjects	92	2.4	1.0-5.9	2.4	0.8, 6.7
Males	46	1.6	0.4-6.0	0.8	0.2, 4.2
Females	46	10.1	1.2-86.8	10.2	1.1, 93.5

Table 4.2 Baseline regression analysis – association between OSA and high urate
*** Multivariate regression adjusted for age, BMI, previous smoking history, hsCRP.**

The median follow-up period was 14 months [IQR 13-15]. At follow-up, 58 subjects were reassessed (28 on CPAP, 30 not on CPAP).The characteristics of the follow-up cohort are presented in **Table 4.3**. Subjects were grouped into those who received CPAP and those who were not treated with CPAP. Mean CPAP use was 14 months (SD 1.5). Mean CPAP duration per night was 4.7hrs/night (SD 0.6). Mean CPAP pressures were 10cmH_20 (SD 1.0). Metabolic syndrome (P=0.036) was more prevalent in CPAP users.

At follow-up, CPAP-treated patients had similar mean urate (mean 345 µmol/L (SD 61) to non-CPAP users (mean 342µmol/L (SD 81). However, in CPAP-treated subjects, 7(25%) had a change from a high urate at baseline to a low urate level and this was significant compared with 1(3%) for non-CPAP subjects (P=0.017) **(Table 4.3)**.

Patient Characteristics at follow-up			
	Subjects not on CPAP (Non-CPAP) (n = 30)	Subjects on CPAP (n = 28)	p-value
Age (years)	53 (8)	50 (7)	0.117
Gender: Female	17 (57%)	12 (43%)	
Male	13 (43%)	16 (57%)	0.293
Smoked in past	8 (27%)	7 (25%)	0.885
Alcohol Intake: <8 units per week	21 (70%)	19 (68%)	
≥8 units per week	9 (30%)	9 (32%)	0.860
BMI (kg/m^2)	40 [36,44]	42 [35,47]	0.347
Neck Circumference (cm)	42.2 (4.1)	44.4 (4.6)	0.057
Waist:Hip Ratio	0.93 (0.1)	0.98 (0.1)	0.087
type 2 Diabetes:	8 (27%)	10 (36%)	0.457
Clinical Parameters			
Urate (µmol/L)	342(81)	345(61)	0.858
Median change in urate from baseline (µmol/L)	-6 [-37,43]	-19 [-49,16]	0.154
Subjects with a change from high urate to low urate category	1 (3.3%)	7 (25%)	**0.017**
hsCRP (mg/L)	2.9[0.9,5.8]	2.9[1.8,5.8]	0.474
Systolic BP (mmHg)	128 (11)	129 (12)	0.957
Diastolic BP (mmHg)	82 (8)	80 (7)	0.229
Creatinine (micromol/l)	76(13)	78(15)	0.495
MDRD-GFR (mL/min/1.73m^2)	89(14)	89(15)	0.472
Total Cholesterol (mmol/l)	4.7(1.0)	4.6(1.0)	0.687
Fasting Glucose (mmol/l)	5.4[4.9,5.9]	5.5[4.7,6.0]	0.726
Metabolic Syndrome	8 (27%)	15 (54%)	**0.036**
O2 saturations (%)	96(2)	97(1)	0.685
pCO2 (kPa)	5.3[5.1,5.6]	5.3[5.1,5.4]	0.322

Table 4.3 Patient demographics at follow-up were summarised by non-CPAP vs CPAP groups as means (Std Dev) (or median [IQR] if non-normal) for continuous variables and frequencies (%) for categorical variables. Significant p-values are highlighted in bold. A high urate was defined as a urate greater than the median urate (345µmol/L). Metabolic syndrome assessed according to National Cholesterol Education program guidelines. BMI: Body Mass Index; BP: Blood Pressure; hsCRP: Highly-sensitive C-reactive protein; MDRD-GFR: Glomerular Filtration Rate using Modification of Diet in Renal Disease Equation; CPAP: continuous positive airway pressure; pCO_2: Partial pressure of carbon dioxide.

For CPAP-treated subjects, the mean urate significantly fell between baseline 357µmol/L (95%CI 339-392) and follow-up 345µmol/L (95%CI 321,368) (P=0.016). This was non-significant in non-CPAP subjects (baseline 340µmol/L [95%CI 307-376] and follow-up 342µmol/L [95%CI 311-372] (P=0.980). In the CPAP-treated group, there was no correlation between change in urate and hours of CPAP use per night (r=0.035, P=0.860), and between change in urate and follow-up duration (r=0.012, P=0.952). Regression analyses stratified by gender did not demonstrate significant differences for change in urate.

Using the category of high urate (above 345µmol/L). Seven (25%) CPAP users had urate levels that fell from a high to a low category; 20 (72%) CPAP-treated subjects remained in the same category; 1 (3%) subject had a change in urate from a low to a high category. Effective CPAP was significantly associated with fall from high urate into a low urate category at follow-up (P=0.017). Multivariate logistic regression analysis revealed a trend for CPAP use with the fall in urate category but the result did not reach significance (OR_{adj} 9.3 [95%CI 0.8, 97]).

Discussion

In this study, there was an association between OSA and urate in severely obese females. Additionally, a trend towards a fall in urate levels in OSA patients treated with CPAP was observed. The evidence from this study may suggest an explanation for the onset of nocturnal gout flares in some patients. It may also be argued that the treatment of OSA with CPAP may potentially influence urate responses to gout treatment for patients with hyperuricaemia and OSA. Thus there may be a need to consider OSA in severely obese subjects who have hyperuricaemia or recurrent gout as there may be a possible role for CPAP in influencing urate levels.

These findings in this study are in agreement with previous evidence of an association between the severity of OSA and increased urate levels (Schafer et al., 2002, Hirotsu et al., 2013). However, at baseline, there was no association between urate and OSA in male participants. This may be explained by the lower BMI in this group compared with female subjects, and it is conceivable that a difference in adiposity may explain the disparity in findings in the analysis stratified by gender, given that obesity is linked with uric acid levels (Garcia et al., 2006).

At follow-up, the mean CPAP use was 4.7hrs/night, which is comparable to a previous study with compliance of 4.6hrs/night (Steiropoulos et al., 2009). There was a significant fall in mean urate in

OSA patients at follow-up with CPAP therapy compared with baseline and a trend for a fall in urate category with effective CPAP. However, multivariate regression analysis did not show a significant association. A randomised controlled trial (RCT) in males with type 2 diabetes and OSA did not demonstrate a significant change in serum urate with either 3 months of therapeutic or placebo-CPAP(Prudon et al., 2013). Conversely, two studies have previously reported reduced levels of urate following CPAP treatment (Sahebjami, 1998, Steiropoulos et al., 2009). However, these studies were not placebo-controlled.

There were differences in several characteristics between the groups at baseline, and between the groups at follow-up. At baseline, OSA patients had higher systolic BP and hsCRP; and more had metabolic syndrome compared with the non-OSA group. At follow-up, more patients on CPAP had metabolic syndrome. Previous work in our group has demonstrated an association between OSA and metabolic syndrome in a RCT (Coughlin et al., 2004). There is also evidence that OSA is associated with hypertension (Kohler and Stradling, 2010). It has been suggested that hyperuricaemia is associated with metabolic syndrome (Feig et al., 2008). Thus, it is conceivable that there may be an interaction between these factors that may potentiate changes in serum urate. It is unclear whether OSA is independently associated with increased CRP as it is known that obesity induces chronic low-grade inflammation and may be associated with CRP (Garvey et al., 2009).

It has been postulated that intermittent hypoxia in OSA leads to increased adenosine triphosphate degradation and associated purine catabolism that may lead to increased levels of urate (Saito et al., 2002, Marinchev, 2013). Additionally, it has been suggested that elevated oxidative stress in OSA may be linked with uric acid production, and there is evidence implicating serum urate as an oxidative stress marker (Glantzounis et al., 2005, Van Hoorenbeeck et al., 2012). It is known that OSA is associated with cardiovascular disease and hypertension through mechanisms including intermittent hypoxia, leading to oxidative stress and inflammation (Kohler and Stradling, 2010); and given our findings of an association between serum urate and OSA in females with severe obesity, there may be a possible role of uric acid in cardiovascular risk (Feig et al., 2008).

There are several limitations in this study. Without a placebo-CPAP group, it was not possible to categorically make inferences on cause-effect and to demonstrate that our observations at follow-up were indeed CPAP effects. However, it would have been ethically questionable to conduct a placebo-controlled randomised trial in symptomatic patients with OSA over such a prolonged duration (>1 year) as in our study and our aim was to investigate the changes in real-life settings. Direct matching

of patients in the groups was not performed as this would have restricted eligible patients who were willing to participate, potentially introducing a selection bias; and pragmatically it was important to try to recruit as many patients as possible for both groups.

In this study, urate was measured early morning and not at repeated times throughout the night. Therefore this may potentially reflect a random urate level. It is possible that the time of sampling may not directly reflect the fluctuations in urate during nocturnal hypoxic episodes. Although multiple sampling of urate at the time of apnoeic episodes sleep may have provided further precise information, this would have made sleep difficult and therefore a pragmatic approach for blood sampling was adopted.

Although the study found a trend that approached significance for an association between CPAP and fall in urate, this may have been attributable to the small patient numbers at follow-up. Thus further studies are needed to investigate these observations with larger sample sizes. It is also conceivable that a combination of several modalities of treatment may be needed for patients with hyperuricaemia including lifestyle and weight loss, where appropriate, in combination with CPAP may have a synergistic effect on serum urate levels in severely obese populations or patients with recurrent gout. These will also need to be explored in future work.

Conclusion

To conclude, serum urate levels are associated with OSA in severely obese females and CPAP treatment may influence serum urate. Further study is necessary to investigate the effects of a combination of several modalities of treatment including lifestyle and weight loss, where appropriate, in combination with CPAP on serum urate levels in severely obese cohorts; and also to ascertain if the duration of CPAP therapy has a role in influencing urate outcomes. Intervention to treat nocturnal hypoxia in OSA may be a consideration for patients with hyperuricaemia and recurrent attacks of gout.

Chapter 5

Assessing for obstructive sleep apnoea in diabetes patients

This chapter describes a questionnaire study that was performed to explore OSA assessment in clinical practice. The findings would suggest that more work needs to be done to improve OSA assessment in patients with type 2 diabetes, thus leading into the next chapter describing the urinary proteomics studies.

Abstract

Background

In 2008, the International Diabetes Federation (IDF) Taskforce on Epidemiology and Prevention released a consensus statement that recommended targeted screening for Obstructive Sleep Apnoea (OSA) in people with obesity and type 2 diabetes with classic OSA symptoms, and screening for diabetes, hypertension and dyslipidaemia in those with OSA.

Methods

A research survey was performed to gain a greater understanding of current practice in relation to the IDF recommendations for the assessment of patients in diabetes clinics in the United Kingdom. An on-line questionnaire was made accessible to diabetes health care professionals with the support of the websites of national diabetes organisations including Diabetes UK, Association for British Clinical Diabetologists and the Young Diabetologists and Endocrinologists Forum.

Results

Most (approximately two-thirds) of the 62 diabetes healthcare professionals who responded to this survey were not aware of the IDF recommendations either for diabetes screening in OSA patients or for OSA assessment in type 2 diabetes and obesity. Participants indicated that their local diabetes guidelines did not incorporate assessment for OSA in those deemed to be at risk. Furthermore, most participants perceived OSA investigations to be primarily the domain of the respiratory team and not the diabetes team.

Conclusions

The observations from this survey provide a better understanding of the application and impact of the IDF guidance in diabetes clinics. Diabetes teams are encouraged to take a more active role in identifying patients who may be at risk of OSA or other sleep breathing problems.

Introduction

OSA is associated with a clustering of clinical cardio-metabolic manifestations including hypertension, metabolic syndrome, cardiovascular disease, and type 2 diabetes, which are associated with cardiovascular risk. In recent years, the prevalence of type 2 diabetes and obesity worldwide have increased, with obesity linked with the increased OSA prevalence (Peppard et al., 2013). Cross-sectional estimates that up to 40% of OSA patients will have diabetes (Meslier et al., 2003), and the prevalence of OSA may be up to 23% in patients who are known to have diabetes (West et al., 2006). Prevalence estimates of OSA in severe obesity have been reported to be between 40-90% (Schwartz et al., 2008). Patients who attend diabetes clinics may be unaware of the association between OSA and type 2 diabetes and the symptoms and signs of OSA may not be perceived relevant to their diabetes care. OSA may remain unreported and undiagnosed.

The relation of OSA with type 2 diabetes has important implications for improving health outcomes given the substantial increase in the worldwide prevalence of diabetes mellitus in recent years; estimates indicate that this may reach 4.4% and patient numbers expected to reach 366 million by 2030 (Wild et al., 2004). Despite the absence of RCT data supporting cardiovascular risk reduction with continuous positive airway pressure (CPAP) treatment, with cardiovascular disease risk increased in OSA (Seicean et al., 2013), efforts at identifying patients with type 2 diabetes who are at risk of OSA may enable important steps to be taken towards managing cardiovascular risk irrespective of whether CPAP treatment is given. There is also evidence that OSA may be associated with microvascular complications such as diabetes retinopathy (West et al., 2010), nephropathy (Tahrani et al., 2013) and neuropathy (Tahrani et al., 2012). Taken together, the identification of patients with diabetes at high risk of obstructive sleep apnoea may allow targeted further investigations that would complement other aspects of diabetes care.

In 2008, the International Diabetes Federation (IDF) Taskforce on Epidemiology and Prevention released a consensus statement that recommended a targeted approach to screen individuals with type 2 diabetes and obesity for SDB (Shaw et al., 2008) (International Diabetes Federation, 2008). Briefly, the IDF recommends that healthcare professionals should consider the possibility of OSA in patients with type 2 diabetes, working in tandem with the local sleep service to provide a clinically appropriate process of assessment, referral and intervention for patients (International Diabetes Federation, 2008).

The purpose of this research survey was to gain a greater understanding of current practice in relation to the IDF recommendations with regards to the assessment of OSA in patients in diabetes clinics.

Methods

A national on-line survey open to all health professionals caring for patients with diabetes was conducted in the United Kingdom for four months from December 2013 to March 2014. Data were collected using a questionnaire consisting of seven questions designed in light of the IDF statement **(Table 5.1)**. The survey was publicly announced on the ABCD (Association for British Clinical Diabetologists) website, Diabetes UK website and Diabetes UK professional newsletter (Update December 2013 issue), and the Young Diabetologists and Endocrinologists Forum (YDEF) website, that provided the links to the on-line webpage to access the survey. In order to maintain confidentiality, all responses were anonymous.

Demographical data (location of provision of diabetes care, and the role of the respondent) were determined. The remaining five questions related to the study objectives. Questions 1 and 2 aimed to study current awareness of the IDF guidance; question 3 aimed to assess whether local diabetes pathways have adopted the IDF recommendations; questions 4 and 5 aimed to assess the perceived roles for investigating OSA in diabetes patients.

Table 5.1

Please tick the relevant boxes.

Location: Teaching/University Hospital ☐ District General Hospital ☐ GP Practice ☐

Role: Consultant ☐ Registrar ☐ Diabetes Specialist Nurse ☐ Other

		Y	N	Don't Know
1.	I know IDF guidance to screen for diabetes in OSA?	☐	☐	☐
2.	I know IDF guidance to screen for OSA in high risk patients with diabetes & obesity?	☐	☐	☐
3.	Our local diabetes guidelines recommend OSA screening in diabetes patients at risk of OSA?	☐	☐	☐
4.	Local people with diabetes suspected of OSA are investigated by diabetes team?	☐	☐	☐
5.	Local people with diabetes suspected of OSA are investigated by respiratory team?	☐	☐	☐

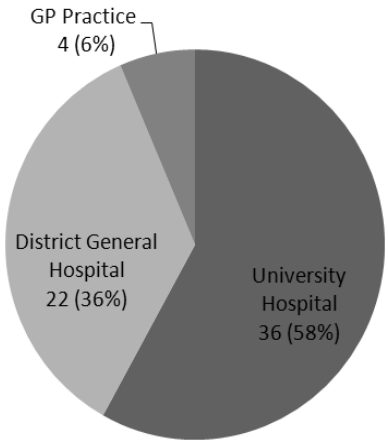
Table 5.1 Questionnaire on OSA assessment

Results

A total of 62 responses were received, mainly from hospital-based physicians (**Figure 5.1),** and the responses to questions 1-5 **(Table 5.2)** showed that a minority of respondents were aware of the IDF guidelines and their implications for practice, but 78% of respondents noted that diabetes patients with suspected OSA are investigated by the respiratory team (**Table 5.2). Table 5.3** shows questionnaire responses according to role and location. It is noteworthy that some respondents did not answer all the questions.

Figure 5.1

Location of work



Role

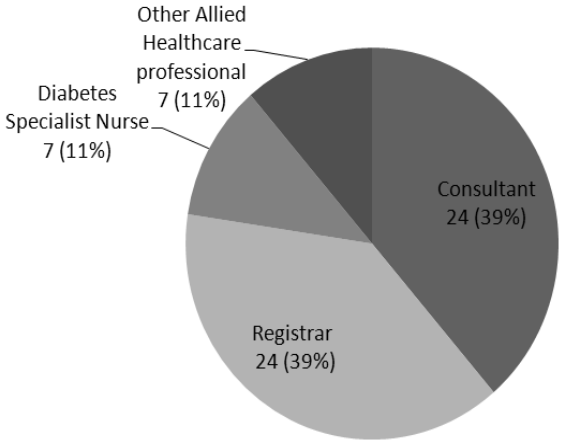


Figure 5.1 Questionnaire results showing number and percentage of responses (in brackets)

Question		Responses		
		Yes	No	Don't know
1	I know IDF guidance to screen for diabetes in OSA?	32% (n=19)	38% (n=23)	30% (n=18)
2	I know IDF guidance to screen for OSA in high risk patients with diabetes & obesity?	34% (n=21)	38% (n=23)	28% (n=17)
3	Our local diabetes guidelines recommend OSA screening in diabetes patients at risk of OSA?	19% (n=12)	45% (n=28)	36% (n=22)
4	Local people with diabetes suspected of OSA are investigated by diabetes team?	12% (n=7)	67% (n=40)	21% (n=13)
5	Local people with diabetes suspected of OSA are investigated by respiratory team?	78% (n=48)	3% (n=2)	19% (n=12)

Table 5.2 Responses to questions 1-5. Total number of respondents = 62
2 participants skipped question 1; 1 participant skipped question 2; 2 participants skipped Question 4

Discussion

This study has several key findings. Firstly, it was observed that the majority (approximately two-thirds) of diabetes healthcare professionals who responded to this survey were not aware of the IDF recommendations either for diabetes screening in OSA patients or for OSA assessment in type 2 diabetes and obesity. Secondly, most participants indicated that their local diabetes guidelines did not incorporate assessment for OSA in those deemed to be at risk. Thirdly, for the vast majority of participants, assessments were deemed to be primarily the domain of the respiratory team and not the diabetes team.

A beneficial effect of OSA treatment with CPAP in terms of blood pressure reduction was found in patients with type 2 diabetes (Myhill et al., 2012), although the evidence in relation to the influence of CPAP therapy on glucose homeostasis have thus far yielded mixed findings (Surani and Subramanian, 2012). Nevertheless, it has been proposed that there may be a role for a multifaceted approach for these individuals in order to manage their cardio-metabolic risks (Pepin et al., 2012). A recent observational study of OSA patients with type 2 diabetes assessed clinical outcomes and cost-

effectiveness of CPAP treatment compared with non-treatment. It was found that CPAP use was associated with significantly lower blood pressure, improved glycaemic control, and was more cost-effective than no treatment with CPAP (Guest et al., 2014). The identification of patients with diabetes at high risk of OSA remains a promising strategy that may allow targeted further investigations that would complement other aspects of diabetes care. Diabetes teams are encouraged to take a more active role in identifying patients who may be at risk of OSA or other sleep breathing problems. In addition, it is important to increase awareness of IDF recommendations in healthcare professionals caring for diabetes patients.

In a previous review, a strategy was proposed to identify, screen and diagnose patients with type 2 diabetes and obstructive sleep apnoea (Idris et al., 2009). When approaching patients, the need for diagnostic investigation is a clinical decision that should take account all available clinical information such as symptoms including snoring, apnoeic events during sleep and excessive somnolence; quality of life and comorbidities such as obesity, metabolic syndrome and cardiovascular disease. The Epworth Sleepiness scale is a validated questionnaire to assess the severity of sleepiness symptoms may be a simple screening tool that could be used for patients suspected of SDB. However, it should be borne in mind that although hypersomnolence symptoms may relate to micro-arousals and to changes in sleep architecture, it is non-specific and not always associated with OSA. Therefore it is not sufficiently discriminating to diagnose OSA (Strohl and Redline, 1996). Depending on services available, a referral for sleep studies or to the relevant sleep team for further assessment may be necessary. Lifestyle recommendations such as weight reduction for overweight or obese patients, smoking cessation, avoidance of sedatives, decreasing alcohol consumption and proper sleep hygiene may be recommended.

The treatment of OSA aims to reduce daytime sleepiness and CPAP is recommended as a treatment option for individuals with moderate or severe symptomatic OSA given the effects on blood pressure, implications for quality of life and driving safety (Giles et al., 2006). There is evidence that non-sleepy OSA patients treated with CPAP have not shown effective decreases in blood pressure and it is possible that non-sleepy asymptomatic OSA patients may face a different level of risk from those who are sleepy (Montserrat et al., 2007).

This study has several limitations. The findings that were observed relate to responses from participants. We relied on public announcements of the survey by engaging the help of organisations including the ABCD, the YDEF and Diabetes UK. It is envisaged that most diabetes professionals

would be members of at least one of these three organisations, but it is possible that there were individuals who remained unaware of this survey. For this reason, the estimated response rate would be difficult to ascertain as this would be a function of those who were aware of the study but instead chose not to participate. For example, Diabetes UK has an estimated 6,000 professional members (Richard Elliot, Personal Communication, 17 June 2014), a potential audience reach, but not everyone would have visited the website at that point in time or read the relevant issue of Update. Thus, there is likely to be a significant non-response bias given the limited sample size. A higher response rate using a validated questionnaire would increase confidence in the generalisability of the findings.

Conclusion

To date, the role of the IDF guidance in UK diabetes clinics has not been previously investigated and this study has sought to try to gain an understanding of its application on the care of patients with diabetes. The identification of patients with diabetes at high risk of obstructive sleep apnoea will allow targeted further investigations that would complement other aspects of diabetes care. Diabetes teams are encouraged to take a more active role in identifying patients who may be at risk of obstructive sleep apnoea or other sleep-related breathing problems.

Chapter 6

Urinary proteomics in obstructive sleep apnoea and obesity

In chapters 3 and 4, studies on arterial stiffness and serum urate were described in relation to OSA and severe obesity. In chapter 5, it was observed that more research needs to be done to improve the assessment of OSA. This led to the exploration of the use of urinary proteomics in the identification and characterisation of OSA patients with severe obesity. In this chapter, two studies are described relating to the study of urinary profiles in OSA and severe obesity. The first study was at baseline and the second study at follow-up of the patients. Finally, a comparison of the urinary peptide profiles between these two time points (baseline and follow-up) was performed.

Abstract

Background

Obstructive sleep apnoea (OSA) is a common complication of obesity and can have a substantial negative impact on a patient's quality of life and risk of cardiovascular disease. The aim of this case-control study was to undertake discovery profiling of urinary peptides using capillary electrophoresis-mass spectrometry (CE-MS) in obese subjects with and without obstructive sleep apnoea, without a history of coronary artery disease.

Methods

Urinary samples were analysed by CE-MS. Body composition and blood pressure measurements were recorded. Overnight polysomnography was conducted to confirm or refute OSA. OSA patients were naïve to continuous positive airway pressure treatment.

Results

Sixty-one subjects with OSA (age 47±9years, BMI 43±8kg/m^2) and 31 controls (age 49±10years, BMI 40±5kg/m^2) were studied; p=ns for age and BMI. Apnoea-hypopnoea Index was higher in patients with OSA (24±18.6) than controls without OSA (non-OSA) (2.6±1.1; p<0.0001). Metabolic syndrome was present in 35 (57%) of those with OSA compared with 4 (13%) of controls (p<0.0001). 24 polypeptides were candidates for differential distribution (p<0.01), although these differences did not reach significance after multiple testing. Sequences were determined for 8 peptides demonstrating origins from collagens and fibrinogen alpha.

Conclusion

In this study, urinary proteomic profile analyses using CE-MS in OSA and non-OSA obese groups is reported for the first time. The differences in urinary proteomic profiles prior to adjustment for multiple testing, with increased metabolic syndrome in obese OSA subjects, suggests that there may be a role for CE-MS in characterising urinary profiles in severely obese populations with OSA.

Introduction

The high prevalence of OSA poses a demanding challenge to healthcare providers in order to provide sufficient resources and facilities for patient diagnosis and treatment. There is effective treatment for OSA in the form of continuous positive airway pressure (CPAP), and patients who are untreated may have an increased risk of morbidity and mortality (McArdle et al., 2007).

In OSA, recurrent episodes of upper airway narrowing, intermittent hypoxia and sleep fragmentation may influence cardio-metabolic risk as a result of alterations in sympathetic activity (Somers et al., 1995), effects on endocrine (Meston et al., 2003) and hypothalamic–pituitary–adrenal axes (Vgontzas et al., 2007); oxidative stress and inflammatory responses (Lavie, 2009); and changes in adipokines that may alter glucose metabolism (Drager et al., 2010c). The kidney may be sensitive to these effects and has a role in blood pressure regulation (Fletcher, 1993), as this is often raised in patients with OSA (Kohler and Stradling, 2010). It has been previously shown that severely obese patients with OSA showed histological changes in glomeruli including focal glomerulosclerosis on renal biopsies that may have resulted from altered blood pressures and vascular flow; a possible effect on the kidneys as a consequence of the episodic hypoxia in OSA (Fletcher, 1993). Another study found that there was evidence of renal impairment in patients with severe OSA without diabetes and hypertension, and there was a positive relationship between impaired glomerular filtration and desaturation frequency, suggesting an association between OSA and chronic kidney impairment (Chou et al., 2011). Microalbuminuria has been associated with coronary artery disease and increased cardiovascular risk (Klausen et al., 2004). Tsioufis et al (2008) found that urine albumin excretion was correlated with OSA severity and oxygen desaturation in hypertensive subjects with OSA compared with those without OSA (Tsioufis et al., 2008), that may be related to underlying haemodynamic effects of OSA on blood pressure and endothelial dysfunction (Oflaz et al., 2006). Taken together it is conceivable that the sensitivity of renal tubular cells to hypoxia may influence the urinary proteome and the physiological changes associated with intermittent hypoxia in OSA may be reflected in urinary proteome changes.

There is evidence that molecular profiling using proteomics may be a powerful tool in the study of obstructive sleep apnoea, that may form the basis for new clinical tests (Arnardottir et al., 2009). Urinary proteomics is ideal as it allows non-invasive sampling and can be easily obtained (Ahmed, 2009). Capillary electrophoresis coupled to mass spectrometry (CE-MS) is a sensitive proteomic analysis technique that can be easily scaled to a high throughput clinical diagnosis platform and is

robust with high reproducibility of results in an acceptable time frame (Ahmed, 2009) (Mischak and Schanstra, 2011). 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 (Mischak and Schanstra, 2011). The accuracy, precision, selectivity, sensitivity, reproducibility, and stability of the CE–MS measurements have been previously demonstrated (Theodorescu et al., 2005). Furthermore, there have been studies using CE-MS that have developed validated biomarker panels composed of groups of urinary peptides for conditions such as coronary artery disease (Delles et al., 2010) and chronic kidney disease (Rossing et al., 2008, Good et al., 2010) that have been recorded in a large database (>13000 samples), that may indicate the presence of the relevant condition (Coon et al., 2008) (Siwy et al., 2011).

Understanding the influence of OSA on urinary profiles is important as molecular research in OSA may increase our understanding of complex sleep mechanisms and provide a platform for future therapeutic interventions (Caylak, 2011). For example, in recent years, genomic studies have been performed to identify susceptibility and candidate genes for OSA (Caylak, 2009). The molecular changes in OSA may potentially influence the urinary proteome (Arnardottir et al., 2009).

The use of urinary CE-MS analyses in adult OSA with obesity has not been previously investigated. The aim of this research was to undertake a study of urinary peptides from obese subjects with OSA in comparison to urinary samples from obese non-OSA patients using CE-MS. This study sought to investigate the hypothesis that the urinary peptide patterns were different between the two groups.

Research Design

Although this was broadly discussed in the methods chapter (chapter 2), the methods specifically pertaining to the proteomics studies presented in this chapter are described below.

This was a preliminary experimental case-control study that involved patient recruitment and sampling, and subsequent CE-MS urinary proteome analyses.

Ethics Statement

The study was approved by the local research ethics committee (NRES 12/NW/123). The study was performed in accordance with the Declaration of Helsinki. All patients gave written informed consent.

Reporting Statement

Reporting of this study conforms to Strengthening the Reporting of Observational Studies in Epidemiology (STROBE)(von Elm et al., 2007) (Vandenbroucke et al., 2007) (Gallo et al., 2011) and the broader Enhancing the QUAlity and Transparency Of health Research (EQUATOR)(Simera et al., 2010) guidelines.

Participants

Severely obese patients from multidisciplinary weight management clinics and sleep clinics at University Hospital Aintree were studied. Patients were recruited from March 2012 to January 2013. Inclusion and exclusion criteria were assessed according to the clinical history, physical examination and analysis of the medical notes.

Patients were eligible if they were ≥21 years old and had a BMI≥35kg/m². Exclusion criteria were patients who were being treated or had prior treatment for OSA; those with known 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, and other comorbidities.

The OSA and non-OSA patients were not deliberately matched per se as this would have restricted eligible patients who were willing to participate, potentially introducing a selection bias; and it was important to try to recruit as many patients as possible for both groups. Furthermore, the OSA status of patients could only be known after they had their sleep studies, thus making direct matching of patients less practicable.

Power calculation

In order to do a proper power calculation, samples must be taken from the population of interest. However, no previous studies using CE-MS in OSA with obesity have been performed, however, proteomic studies in paediatric OSA involving urine samples have investigated sample sizes of n=11 to 30 per group (Gozal et al., 2009b) (Krishna et al., 2006) (Snow et al., 2010a). As the OSA group included patients with AHI>5, that included both mild and moderate-severe OSA patients, a larger sample size for the OSA group was needed. Therefore, this exploratory study was performed with a target sample size of n=60 for the OSA group and n=30 controls.

Protocol

All patients attended a study visit day between 08:00-10:00hrs and underwent a detailed history and physical examination. Body composition measurements, anthropometry, fasting blood and urine biochemical tests, venous blood gases (to assess for hypercarbia), and spirometry testing were performed. Overnight polysomnography was performed and patients were then grouped according to their sleep status. This ensured that the researcher was blinded to the OSA status of the patients. To ensure comparability and uniformity of assessments of patients, body composition, anthropometry and sampling were carried out at approximately the same time at each study visit. Spirometry and sleep studies were performed by independent respiratory physiologists/technicians in line with local practice and published standards.

Blood Pressure

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

Body measurements

All measurements were done 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 (Jebb et al., 2000). Additionally, we measured body fat composition by air displacement plethysmography using BodPod (Life Measurement Inc, Concord, CA) whole body air-displacement plethysmography. In this test, subjects wore minimal skin-tight clothing whilst seated within the BodPod plethysmography chamber (Life Measurement Inc, Concord, CA) for 2-4 minutes. Body volume was determined by subtraction of the chamber volume when empty and the corresponding pressure change was measured. Each subject's thoracic gas volume was either measured during normal tidal breathing using a tube connected to the breathing circuit or based on a predicted estimate based on age, sex, and height where an accurate

measurement was not possible (Nunez et al., 1999). The measured body volume was used in estimating body density and percentage body fat was then computed by the software (Life Measurement Inc, Concord, CA) based on a standard algorithm (Fields et al., 2002).

Spirometry Assessment

Spirometry was performed with a Spiro Air LT system (Medisoft, Sorinnes, Belgium), supervised by an experienced technician.

Sleep Diagnostic Assessment

Daytime somnolence was assessed using an Epworth Sleepiness Scale Questionaire (ESS) where a score >10 indicated increased sleepiness (Johns 1993). Diagnosis was confirmed by overnight multichannel respiratory limited polysomnography (Somnoscreen Digital PSG & EEG acquisition system, Version 2.0, SomnoMedics, Germany) using a montage of pulse oximetry, chest and abdominal excursion, airflow by oronasal thermistry, single bipolar electrocardiogram and body position. Sleep studies were independently assessed by experienced sleep physiologists using software (Domino PSG analysis software (version 2.5.0), SomnoMedics, Germany). Apnoea was defined as a cessation of airflow for >10secs. 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 ≥5 (Flemons *et al*., 1999).

Biochemical Measurements

Serum samples were collected using standard phlebotomy vials and immediately sent to the local pathology laboratory for analysis in accordance with local protocol and standards. Serum urea, creatinine and electrolytes, fasting lipids, thyroid chemistry, and fasting glucose were measured using standard laboratory assays (Roche, UK). Blood gases were analysed with a Cobas Blood Gas Analyser (Roche, UK).

Metabolic Syndrome

Subjects were assessed for metabolic syndrome according to the National Cholesterol Education Program (NCEP) guidelines (Cleeman et al., 2001). Patients had metabolic syndrome if three or more risk factors were present: waist circumference (males>102cm; females>88cm), triglycerides≥1.7mmol/l, HDL cholesterol (males<1.04 mmol/l; females<1.3mmol/l), blood pressure ≥130/≥85 mmHg, and fasting glucose ≥6.1mmol/l.

CE-MS materials & methods

Urine sample preparation

Spontaneously voided urine samples for CE-MS analyses were collected at the study visit using urine Monovettes (Sarstedt AG & Co, Nümbrecht, Germany). Second void samples were collected at the same time each morning (~09:00 hours) after an overnight fast for 8 hours, at their study visit prior to the administration of any medication. The samples were stored at -80^{o}C in urine Monovettes until the sample preparation stage. Sample preparation for proteomic analysis was performed as previously described (Albalat et al., 2013). In this process, each 0.7 mL aliquot of urine was thawed immediately before use and diluted with 0.7 mL of 2M urea, 10mM NH_4OH containing 0.02% SDS. Each sample was ultrafiltered (2000g, 60 mins, 4^{o}C) to remove higher molecular mass proteins, such as albumin and immunoglobulin G using Centrisart ultracentrifugation filter devices (20kDa MW) (Sartorius stedim Biotech, Goettingen, Germany) at 2000g 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% NH4OH in HPLC-grade H_2O to decrease matrix effects by removing urea, electrolytes, and salts, and to enrich polypeptides present. Finally, all samples were lyophilized, stored at 4^{o}C.

CE-MS Analysis

Samples were re-suspended in HPLC-grade H_2O shortly before CE–MS analyses, as described (Albalat et al., 2013). 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) (Albalat et al., 2013). 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 seconds, over a range of m/z 350 to 3000 over 60 minutes (Albalat et al., 2013). The accuracy, precision, selectivity, sensitivity, reproducibility, and stability of the CE-MS measurements have been previously demonstrated (Theodorescu et al., 2005).

CE-MS Data Processing

Mass spectra were processed using MosaiquesVisu software (Mosaiques Diagnostics, Hannover, Germany), including peak picking, deconvolution and deisotoping (v. Neuhoff et al., 2004). 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, normalized 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 (Jantos-Siwy et al., 2009). 'Housekeeping' peptides that consistently appear in urine samples and are unaffected by disease states provided ideal reference mapping points for CE migration times (Coon et al., 2008). 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 = 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 (Albalat et al., 2013). The resultant peak list included molecular mass (Da), normalised CE migration time (min) and normalised signal intensity for each peptide. All results were entered into a Microsoft SQL database. Non-OSA and OSA-specific polypeptide patterns were generated using support vector machine (SVM)-based MosaCluster software (Mosaiques Diagnostics, Hannover, Germany) for comparison.

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 (Coon et al., 2008) (Zurbig et al., 2006). These human urinary peptide sequences can be accessed at (http://mosaiques-diagnostics.de/diapatpcms/mosaiquescms/front_content.php?idcat=257).

Statistical Analyses

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 t test has been shown to be well-suited for proteomics data sets (Dakna et al., 2010), and was used to determine significance in peptide abundance between the groups. Statistical significance was assigned at $P<0.01$. 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.

Results

Ninety-seven consecutive patients were recruited. 92 completed the protocol (61 obese OSA, 31 obese non-OSA) **(Table 6.1)**. Five subjects withdrew before their sleep studies and thus were excluded from the analyses. All subjects were of white European ethnicity.

Table 6.1 Patient demographics were summarised by OSA group as means (SD) (or median (IQR) if non-normal) for continuous variables and frequencies (%) for categorical variables. Significant P-values are highlighted in bold.

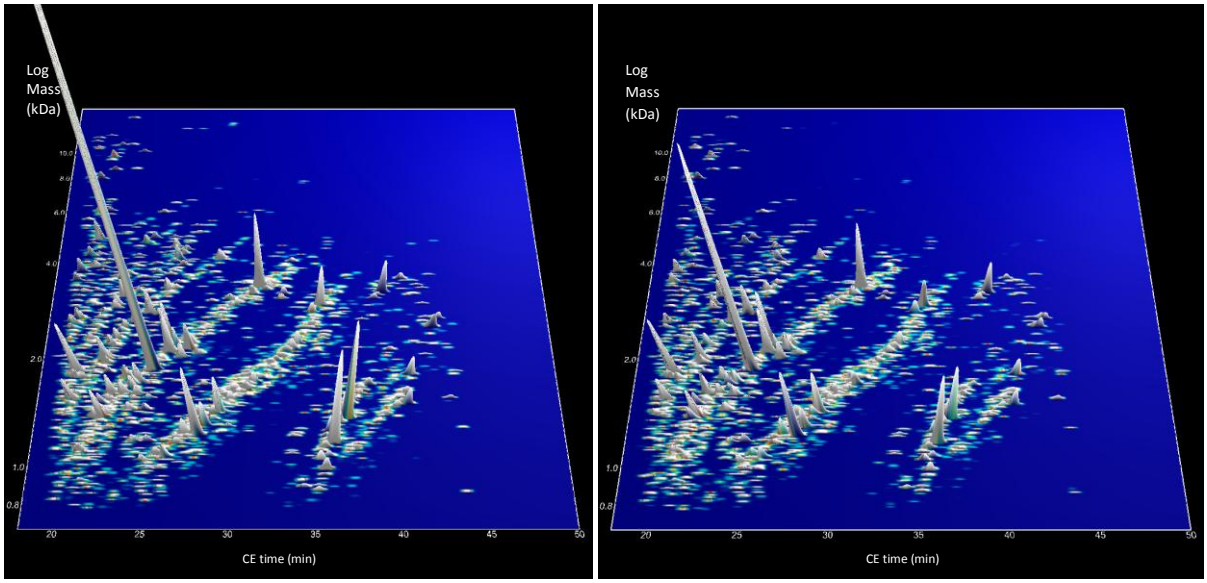
Patient Characteristics			
	Non-OSA (n = 31)	OSA (n = 61)	p-value
Age (years)	49.6 (10.3)	47.9 (9.0)	0.3252
Gender:			
Female	15 (48%)	31 (51%)	
Male	16 (52%)	30 (49%)	0.8254
Smoked in past:			
No	24 (77%)	45 (74%)	
Yes	7 (23%)	16 (26%)	0.8021
Alcohol Intake:			
<8 units per week	23(74%)	43 (70%)	
≥8 units per week	8 (26%)	18 (30%)	0.8090
BMI (kg/m^2)	40 (5)	43 (8)	0.0857
Neck Circumference (cm)	42.4(4.4)	44.2 (4.3)	0.0544
Waist:Hip Ratio	0.96 (0.1)	0.98 (0.12)	0.4847
Body Fat (%):			
TANITA	42 (34, 52)	46 (39, 51)	0.1359
BODPOD	43 (34, 52)	45 (39, 51)	0.2062
Diabetes:			
No	25 (81%)	48 (79%)	
Yes	6 (19%)	13 (21%)	0.8265
Hypertension:			
No	25 (81%)	47(77%)	
Yes	6 (19%)	14(23%)	0.7932
BP-lowering medications:			
No	25 (81%)	47 (77%)	
Yes	6 (19%)	14 (23%)	0.7932
Glucose-lowering therapy:			
No	25 (81%)	48 (79%)	
Yes	6 (19%)	13 (21%)	0.8265
Lipid-lowering medications:			
No	27 (87%)	52 (85%)	
Yes	4 (13%)	9 (15%)	0.8096
Clinical Parameters			
	Non-OSA (n = 31)	OSA (n = 61)	p-value
Systolic BP (mmHg)	128.6 (12.6)	137.5 (13.8)	**0.0039**
Diastolic BP (mmHg)	83.0 (10.2)	87.3 (11)	0.1051
ACR (mg/mmol)	0.4 (0.2, 1.1)	0.7 (0.3, 1.5)	0.1203
Creatinine (micromol/l)	77 (16)	73 (15)	0.3547
MDRD-GFR (mL/min/1.73m^2)	90 (17)	95 (17)	0.2214
LDL (mmol/l)	2.8 (1.0)	2.9 (0.9)	0.5712
HDL(mmol/l)	1.3 (0.4)	1.2 (0.3)	0.4743
HbA1c (mmol/mol)	39 (35, 41)	40 (37, 48)	0.1746
Total Cholesterol (mmol/l)	4.7 (1.1)	5.0 (1.0)	0.2472
Fasting Glucose (mmol/l)	5.2 (4.9, 5.6)	5.3 (4.8, 6.0)	0.7784
TSH (mU/l)	2.1 (1.6, 3.3)	2.0 (1.5, 2.8)	0.3421
Metabolic Syndrome:			
No	27 (87%)	26(43%)	
Yes	4 (13%)	35(57%)	**<0.0001**
Mean Arterial Pressure (mmHg)	98.5 (9.8)	105 (10.6)	**0.0069**
Triglycerides (mmol/l)	1.3 (1.1, 2.2)	1.6 (1.1,2.7)	0.2319
Bicarbonate (mmol/l)	24.5 (1.1)	24.8 (1.0)	0.0930
Heart Rate (beats per min):	72 (12)	75 (10)	0.1792
Epworth Sleep Score:	6 (3, 10)	10 (7, 15)	**0.0009**
AHI	2.6 (1.1)	24.0 (18.6)	**<0.0001**
O2 Saturations (%):	97.0 (1.5)	97.0 (1.5)	0.9596
PCO2 (kPa)	5.4 (5.0, 5.6)	5.4 (5.1, 5.6)	0.4110
FEV1% predicted	94.1 (12.1)	94.3 (14.9)	0.9868
FVC% predicted	103.9 (13.6)	101.7 (15.6)	0.5771
hsCRP (mg/L)	3.3 (1.5, 5.3)	5.2 (2.5, 7.3)	**0.0317**
Urate (μmol/L)	340.1 (86.7)	356.7 (79.9)	0.2999

There were significant differences in systolic (P=0.0039), mean arterial pressures (P=0.0069), ESS scores (P=0.0009) and hsCRP values (P=0.0317) between groups. Apnoea-hypopnoea Index was higher in OSA patients (24±18.6) than controls (2.6±1.1; p<0.0001). Furthermore, metabolic syndrome was present in more OSA subjects than non-OSA subjects 35 (57%) OSA subjects versus 4 (13%) controls; p<0.0001). The remainder of the variables did not show any significant difference between the groups.

Urine Peptide Analysis

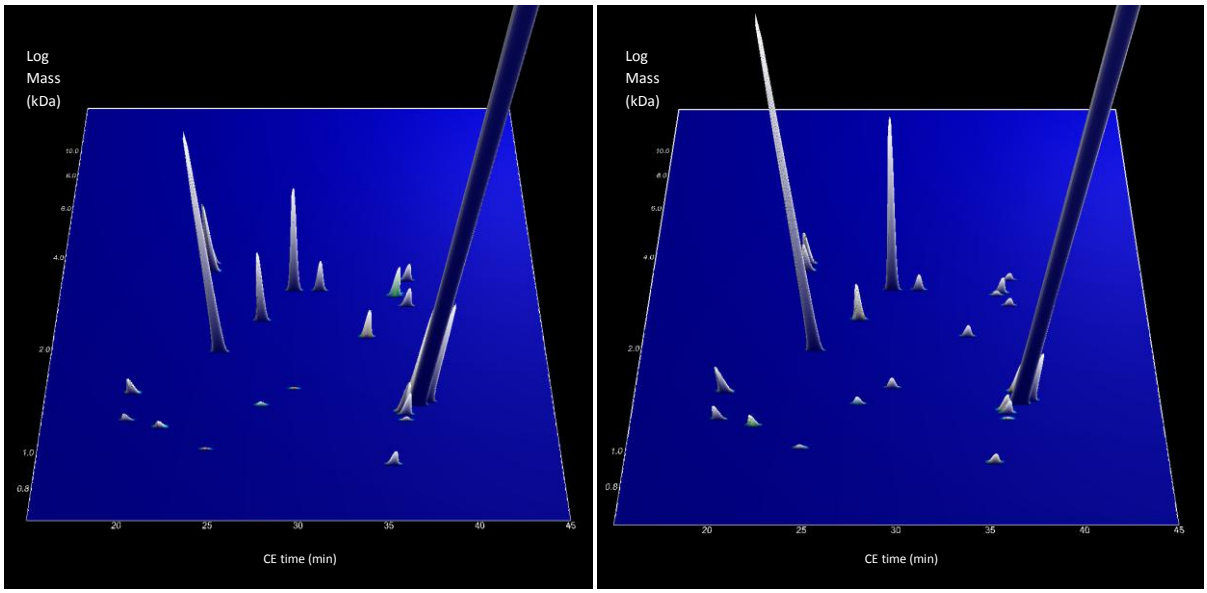
Peptide identification

In each sample that was analysed, 1247 different peptides were detected. The compiled urinary proteomic data from OSA and non-OSA patients is shown in **Figure 6.1**. 24 peptides were found to be candidates for differential distribution (all p<0.01) (**Figure 6.2**). Although our initial analysis appeared to show a significant differential distribution in these peptides, post-hoc analyses indicated that these did not reach significance upon adjustment for multiple testing.



(A) non-OSA **(B)** OSA

Figure 6.1 Compiled 3-dimensional depiction of urinary peptide profile for non-OSA (A) and OSA (B). 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.



(A) non-OSA **(B)** OSA

Figure 6.2 3-dimensional profile of 24 peptide markers that showed significant change in non-OSA vs OSA 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 24 peptides, figures 6.1 and 6.2 are not of similar scale.

Sequences were determined for 8 peptides demonstrating origins from collagens and fibrinogen alpha. The 24 peptides are presented as in **Table 6.2**. 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.

Table 6.2

Peptide ID	Peptide Mass (Da)	CE time (min)	Unadjusted Wilcoxon p-value	Area Under the Curve	Benjamini & Hochberg p-value	Sequence	Protein Name	Start amino acid	Stop amino acid	Swissprot Name	Accession number	Frequency OSA	Mean amplitude OSA (SD)	Frequency non-OSA	Mean amplitude non-OSA (SD)
x8792	950.2903	35.38838	0.0099307878	0.69	0.51557341							0.83	140 (129)	0.96	225 (165)
x24723	1204.597	21.94117	0.0082573722	0.69	0.489937405							0.90	160 (113)	0.63	93 (116)
x28018	1255.587	19.96208	0.0056890349	0.70	0.64441251							0.96	210 (209)	0.76	103 (120)
x28222	1259.418	36.26792	0.0071807828	0.66	0.559203476							0.20	24 (75)	0.53	55 (85)
x31247	1310.371	36.26037	0.0061211904	0.70	0.54478591							0.80	195 (164)	0.96	329 (206)
x32874	1333.416	36.10802	0.0080298181	0.69	0.500257661							0.80	237 (238)	0.94	463 (397)
x35979	1390.442	36.94585	0.0060445212	0.70	0.57934409							1.0	38860 (20324)	0.93	66804 (47599)
x37650	1422.545	37.5732	0.0074315015	0.69	0.487350013							0.63	681 (688)	0.87	1306 (992)
x43691	1510.682	20.16625	0.0073663365	0.69	0.509914148	SEADHEGTHSTKRG	Fibrinogen alpha chain					0.97	380 (310)	0.55	219 (274)
x43851	1513.443	36.78923	0.0072890730	0.69	0.534246174							0.70	418 (781)	0.83	1150 (1583)
x70635	2013.905	25.19463	0.0027106836	0.72	0.675502453	NSGEpGApGSKGDTGAKGEpGP	Collagen alpha-1(I) chain	432	453	CO1A1_HUMAN	gi124056487	1.0	2971 (777)	1.0	2293 (918)
x82708	2235.045	34.16645	0.0015772180	0.72	0.655071209	GRTGDAGPVGPPGPpGppGpPGPPS	Collagen alpha-1(I) chain	1169	1193	CO1A1_HUMAN	gi124056487	0.36	152 (290)	0.77	370 (353)
x96716	2525.195	27.73635	0.0057840266	0.70	0.600574804	LRGGAGPpGPEGGKGAAGPpGPpGAAGTpG	Collagen alpha-1(III) chain	694	723	CO3A1_HUMAN	gi124056490	0.56	466 (476)	0.87	872 (574)
x110430	2810.355	36.74954	0.0066259949	0.69	0.550399318							0.50	96 (146)	0.77	240 (274)
x119068	3031.427	36.03612	0.0035078399	0.69	0.54634608							0.33	52 (145)	0.58	376 (511)
x121772	3092.439	36.29566	0.0006342353	0.75	0.395128592	TGEVGAVGPpGFAGEKGPSGEAGTAGPpGTpGPQG	Collagen alpha-2(I) chain	831	865	CO1A2_HUMAN	gi124056488	0.73	203 (178)	0.90	18 (88)
x123969	3158.439	29.71371	0.0031096402	0.71	0.55351592	GERGSpGGpGAAGFpGARGLpGpPGSNGNPGPpGp	Collagen alpha-1(III) chain	861	895	CO3A1_HUMAN	gi124056490	0.97	1813 (874)	0.94	1191 (1024)
x124172	3165.462	31.32057	0.0046730947	0.70	0.646964041							0.67	202 (245)	0.90	385 (287)
x132725	3416.602	36.84899	0.0016322842	0.73	0.508456466							0.60	86 (130)	0.81	215 (193)

Peptide ID	Peptide Mass (Da)	CE time (min)	Unadjusted Wilcoxon p-value	Area Under the Curve	Benjamini & Hochberg p-value	Sequence	Protein Name	Start amino acid	Stop amino acid	Swissprot Name	Accession number	Frequency OSA	Mean amplitude OSA (SD)	Frequency non-OSA	Mean amplitude non-OSA (SD)
x140570	3677.766	24.49859	0.0088570397	0.69	0.479820515							0.63	341 (501)	0.81	810 (823)
x14548	1042.439	24.65243	0.0088265281	0.68	0.499902449							0.63	60 (72)	0.81	22 (38)
x35877	1388.638	27.79624	0.0047007556	0.69	0.585714198	GRpGEVGPpGPpGPA	Collagen alpha-1(I) chain	917	931	CO1A1_HUMAN	gi124056487	0.67	105 (103)	0.26	47 (107)
x45950	1551.699	29.735	0.0005468558	0.71	0.681382327	GTGGPpGENGKpGEpGP	COL3A1 Isoform 1 of Collagen alpha-1(III) chain					0.56	149 (204)	0.20	17 (64)
x145889	3891.752	24.52856	0.0030294039	0.71	0.629106231							0.93	381 (278)	0.48	243 (379)

Table 6.2 The table lists the peptide identification number (Peptide ID), experimental mass (in Da) and CE migration time (in min) for the 24 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. (SD=standard deviation)

As subjects in the study were grouped based on their AHI values obtained from their sleep studies and their clinical diagnosis, it was investigated whether the urinary proteome would be significantly different based on their oxygen desaturation index (ODI) values. The ODI is a measure of the hourly average number of desaturation episodes during sleep. An (ODI) ≥5 that was recorded during the sleep study was the diagnostic threshold for OSA. **Table 6.3** shows the significant 52 peptides in the OSA panel identified based on the ODI with Wilcoxon testing. However, there was no significant difference in the urinary proteome between the OSA and non-OSA groups after correction for multiple testing.

Table 6.3

Peptide ID	Peptide Mass (Da)	CE time (min)	Unadjusted Wilcoxon p-value	Benjamini & Hochberg p- value	Sequence	Protein Name	Start amino acid	Stop amino acid	SwissProt Name	Accession number
420	810.404	21.7045	0.005	1						
3696	882.392	21.5358	0.007	1						
10884	975.422	34.0982	0.009	1						
11760	984.467	23.0613	0.001	1						
17241	1090.48	25.221	0.004	1						
18980	1115.47	28.3642	0.01	1						
19965	1132.53	22.5241	0.002	1						
20170	1136.53	20.015	0.001	1						
22452	1166.56	22.561	0.001	1	HVGDEDFVHL	Cystatin-B	58	67	CYTB_HUMAN	gi1235678
28018	1255.59	19.9621	0.006	1						
33135	1338.6	23.9878	0.01	1						
33284	1341.58	29.9772	0.003	1	SpGEAGRpGEAGLp	Collagen alpha-1(I) chain	522	535	CO1A1_HUMAN	gi124056487
34403	1362.62	19.9866	0.007	1						
35877	1388.64	27.7962	0.002	1	GRpGEVGPpGPpGPA	Collagen alpha-1(I) chain	917	931	CO1A1_HUMAN	gi124056487
35979	1390.44	36.9459	0.001	1						
40243	1451.66	29.1716	0.007	1	SpGSpGPDGKTGPPGp	Collagen alpha-1(I) chain	543	558	CO1A1_HUMAN	gi124056487
40294	1452.66	23.6065	0.001	1	DEPPQSPWDRVK	Apolipoprotein A-I				
43691	1510.68	20.1663	0.006	1	SEADHEGTHSTKRG	Fibrinogen alpha chain				

Peptide ID	Peptide Mass (Da)	CE time (min)	Unadjusted Wilcoxon p-value	Benjamini & Hochberg p- value	Sequence	Protein Name	Start amino acid	Stop amino acid	SwissProt Name	Accession number
45950	1551.7	29.735	0.009	1	GTGGPpGENGK pGEpGP	COL3A1 Isoform 1 of Collagen alpha-1(III) chain				
46725	1564.72	28.37	0.008	1						
49713	1604.73	30.3377	0.001	1	TGLSmDGGGSP KGDVDP	Sodium/potassium-transporting ATPase subunit gamma	2	18	ATNG_HUMAN	gi20141251
56132	1708.75	23.3747	0.004	1						
58798	1762.57	38.3386	0.007	1						
59056	1767.78	19.81	0.006	1	EAGSEADHEGT HSTKRG	Fibrinogen alpha chain				
61855	1832.85	31.9399	0.004	1	lGPpGPAGApG DKGESGPSGP	Collagen alpha-1(I) chain	769	789	CO1A1_HUMAN	gi124056487
62256	1841.75	35.6598	0.001	1						
65746	1911.05	24.9809	0.01	1	SGSVIDQSRVLN LGPITR	Uromodulin	589	606	UROM_HUMAN	gi137116
68670	1968.96	35.3522	0.007	1	LLSPYSYSTTAVV TNPKE	Transthyretin	130	147	TTHY_HUMAN	gi136464
70457	2008.9	38.582	0.001	1						
76415	2117.93	32.9682	0.004	1	SNGNpGpPGPS GSPGKDGPpGp AG	Collagen alpha-1(III) chain	886	909	CO3A1_HUMAN	gi124056490
78283	2158.01	33.1661	0.001	1						
84300	2261.03	27.1527	0.007	1	LQGLpGTGGPp GENGKpGEpGP KG	Collagen alpha-1(III) chain	640	663	CO3A1_HUMAN	gi124056490
87740	2329.06	27.1698	0.005	1						
89857	2367.06	27.632	0.007	1						
99804	2584.2	28.768	0.005	1						
104195	2663.2	23.5069	0.009	1	NRGERGSEGSP GHPGQPGPPpGp pGApGP	Collagen alpha-1(III) chain	1168	1195	CO3A1_HUMAN	gi124056490
107313	2739.22	28.4297	0.004	1						
111966	2852.41	37.0846	0.001	1						

Peptide ID	Peptide Mass (Da)	CE time (min)	Unadjusted Wilcoxon p-value	Benjamini & Hochberg p- value	Sequence	Protein Name	Start amino acid	Stop amino acid	SwissProt Name	Accession number
117649	2999.29	22.2454	0.009	1	GESGREGApGAEGSpGRDGSpGAKGDRGETGP	Collagen alpha-1(I) chain	1010	1041	CO1A1_HUMAN	gi124056487
118311	3015.44	36.0019	0.001	1						
119068	3031.43	36.0361	0.002	1						
119795	3047.39	35.7446	0.002	1						
124172	3165.46	31.3206	0.006	1						
126512	3245.38	20.4607	0.004	1	FQFHFHWGSTNEHGSEHTVDGVKYSAEL	Carbonic anhydrase 1				
129302	3324.46	39.4544	0.006	1						
132629	3414.57	21.5724	0.01	1						
132720	3416.59	32.0106	0.001	1	GPpGADGQPGAKGEpGDAGAKGDAGPpGPAGPAGPpGPIG	Collagen alpha-1(I) chain				
Peptide ID	Peptide Mass (Da)	CE time (min)	Unadjusted Wilcoxon p-value	Benjamini & Hochberg p- value	Sequence	Protein Name	Start amino acid	Stop amino acid	SwissProt Name	Accession number
132725	3416.6	36.849	0.005	1						
133168	3432.59	32.0468	0.003	1						
134448	3473.6	33.0091	0.007	1						
136534	3551.67	26.2271	0.005	1						
137132	3575.75	32.3571	0.007	1						

Table 6.3 The table lists the peptide identification number (Peptide ID), experimental mass (in Da) and CE migration time (in min) for the 52 peptides in the OSA panel based on ODI. 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.

Discussion

In this study, the results of urinary proteomic profile analyses using CE-MS in OSA and non-OSA subjects with obesity are reported for the first time. 24 peptides were found to be statistically different between the groups (assessed using their AHI) prior to adjustment for multiple testing. Sequences for 8 of these peptides were identified that comprised collagen alpha chain subtypes and fibrinogen. However, post-hoc correction for multiple testing did not provide sufficient evidence to indicate a significant difference in peptide profiles between the groups. Notwithstanding this finding, the trends observed in urinary proteomic profiles between the obese OSA and non-OSA groups prior to adjustment for multiple testing, in the presence of increased metabolic syndrome in obese OSA subjects, suggests that there may be potential for such methods for the study of obstructive sleep apnoea in obesity.

The increasing demand for sleep studies, coupled with the relative complexity, costs and resource utilisation associated with overnight polysomnography, has led to research into novel methods to aid more timely assessment and treatment of OSA, in order to reduce the risk of OSA-associated comorbidities (Seetho and Wilding, 2013). To date, the study of urinary proteomics using CE-MS to distinguish adult OSA with obesity, has not been investigated previously. There have been several studies involving urinary protein profiles that have been carried out in the paediatric OSA setting, In a two-dimensional gel-based analysis, Gozal et al. (2009) studied 30 OSA subjects and 30 controls, and identified concentrations of uromodulin, urocortin-3, orosomucoid-1 and kallikrein-1 as having favourable predictive properties (sensitivity 95% and specificity 100%) that were specific for OSA (Gozal et al., 2009b). Krishna et al. (2006) examined urine samples from 11 children with OSA and 11 controls using gel electrophoresis coupled with matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI TOF-MS), identifying two differentially expressed proteins in OSA: gelsolin and heparan sulphate proteoglycans (Krishna et al., 2006). In another study, Snow et al. (2010) used surface-enhanced laser desorption ionisation MS (SELDI-TOF MS) to discover a specific increase in urocortins in OSA in the paediatric setting (30 OSA subjects compared with 25 controls [sensitivity 93% and specificity 97%]) (Snow et al., 2010a).

It is known that urine contains different fragments of peptides such as collagen and other proteins such as alpha-1-antitrypsin, haemoglobin, serum albumin, fibrinogen and uromodulin, and the resultant effects of proteolytic activity may be reflected in the pattern of peptide fragments observed in CE-MS analysis (Coon et al., 2008). The mechanisms underlying the trends observed for peptides differences between the groups that were found in this preliminary study remain unclear,

although it remains conceivable that the observed differences in peptide excretion may be associated with intermittent hypoxia and systemic hypertension. In a study of rodents treated with a high fat diet exposed to chronic intermittent hypoxia, histological changes were found in the thoracic aorta, myocardium, liver and kidney compared with controls, suggesting a pathophysiological effect of intermittent hypoxia (Wang et al., 2013). It has been previously suggested that there may be an association between OSA and proteinuria (Fletcher, 1993) although several studies have not demonstrated this (Mello et al., 2004). Altered glomerular capillary pressures and flows associated with raised pressor responses in OSA have been proposed as another potential mechanism, leading to glomerular haemodynamic adaptations that may potentially induce renal anatomical changes (Fletcher, 1993).

Although there was no significant difference in the urinary proteome between the two groups based on the ODI, it is possible for one to question the role of intermittent oxygen desaturation and hypoxia in relation to the urinary proteome. Nevertheless, it may be argued that other factors such as sympathetic arousal from apnoeic events may also be present (Somers et al., 1995). In addition, it is possible that inflammatory changes (Jelic et al., 2010) and oxidative stress (Lavie, 2009) that occur in OSA may be underlying mechanisms for the trends in the urinary proteome observed.

In this study, more subjects with OSA had the metabolic syndrome, higher blood pressures and hsCRP. These findings would be consistent with previous studies that have demonstrated associations between OSA and the metabolic syndrome (Coughlin et al., 2004), hypertension (Kohler and Stradling, 2010) and inflammatory responses (Jelic et al., 2010). Although it is plausible that these covariates may influence the peptides observed, these parameters were not specifically controlled for as the aim was to study a cohort of participants that would be similar to other patients seen in daily clinical practice, thus allowing for generalisability of the findings. Although the Epworth Sleepiness Scale (ESS) scores were significantly higher in the OSA group, the severity of somnolence symptoms may not necessarily correlate with the severity of OSA as determined by sleep studies (Osman et al., 1999).

This study has several limitations. Firstly, given the non-significant findings after correction for multiple testing, we were not able to proceed on to the validation of our findings in independent test sets, which is an important step in the process towards identification and qualification of proteomic biomarkers in order to ascertain clinical relevance (Mischak et al., 2010). Nevertheless, it must be emphasised that this was primarily a preliminary discovery study, with the aim to study

urinary profiles of obese OSA subjects using CE-MS. The findings provide unique insights into urinary profile patterns in obese patients with and without OSA.

It is likely that the excretion of urinary peptides may vary during the day, because of physical activity, diet, or medications taken. In order to control for this, the sampling all participants was at the same time each morning after an overnight fast, at their study visit prior to the administration of any medication. It is envisaged that a urinary polypeptide panel consisting of an array of defined peptides would need to reflect real life clinical patients for it to be relevant and applicable. Controlling for every potential confounding factor would not have been practicable and would have limited recruitment of potential participants. The study did not deliberately attempt to match OSA and non-OSA patients per se as this would have restricted eligible patients who were willing to participate, potentially introducing a selection bias; and it was important to try to recruit as many patients as possible for both groups.

As this study was based on observed findings from samples collected from patients at a specific point in time, a cause-and-effect relationship between the urinary proteome and OSA itself cannot be established. It is also important to note that all subjects in this study were of white European ethnicity and this may impose limitations on the generalisability of the findings.

In summary, this study has provided useful insights into the human urinary proteome in OSA with obesity using CE-MS, a proteomic method of analysis. Despite the limitations of this study, the differences in urinary proteomic profiles prior to adjustment for multiple testing, suggests that, with continued research, there remains potential for this approach in the further study of obese patients with OSA.

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

Abstract

Background

Obstructive sleep apnoea (OSA) is common in obesity and is associated with cardiovascular and metabolic complications. Patients are treated with continuous positive airway pressure (CPAP). The effects of 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±7years, BMI 43±7kg/m^2) and 25 controls without OSA (age 52±9years, BMI 39±4kg/m^2) were studied; p=ns for age and BMI. The mean CPAP use for OSA patients was 14.5±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 two groups ($p<0.01$). Although correction for multiple testing did not reach significance, nevertheless, 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 characterised for the first time in OSA with CPAP. The effects of CPAP on OSA may lead to changes in the urinary peptides. There is a potential role for urinary proteomics in characterising urinary peptide profiles in OSA.

Introduction

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 (Kohler and Stradling, 2010). The kidney has a role in blood pressure regulation and may be sensitive to the effects of intermittent hypoxia (Fletcher, 1993). Nocturnal hypoxia may be associated with loss of kidney function (Ahmed et al., 2011), and a positive relationship has been observed between impaired glomerular filtration and desaturation frequency, suggesting an association between OSA and chronic kidney impairment (Chou et al., 2011). Histological changes in renal glomeruli have been reported in severely obese patients with OSA (Fletcher, 1993), that may be related to pressor responses and endothelial dysfunction in OSA (Oflaz et al., 2006).

In the first study that was described earlier in this chapter, CE-MS was used 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 (Seetho et al., 2014b). 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 CPAP treatment.

CPAP therapy can improve glomerular hyperfiltration in OSA patients (Kinebuchi et al., 2004), perhaps by reducing renal renin-angiotensin system (RAS) activity, suggesting a potential effect of CPAP on kidney function (Nicholl et al., 2014). 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, endothelial dysfunction (Kuzniar and Klinger, 2014).

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. This study sought to investigate the

hypothesis that the urinary peptide patterns were different between the two groups. To achieve this goal, the urinary proteome was characterised in severely obese subjects with OSA who were receiving CPAP compared to subjects without OSA.

Methods

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.

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.

Participants

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

Patients were eligible if they were ≥21 years old and had BMI≥35kg/m^2. 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 on 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 the aim was to study urinary proteome in subjects who were receiving CPAP treatment (CPAP≥4hrs per night) and non-OSA subjects.

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.(Gozal et al., 2009b) (Krishna et al., 2006) Therefore, the study aimed to recruit a minimum of 25 subjects per group.

Protocol

All patients attended a study visit day between 0800-1000hrs 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.

Blood pressure, body measurements, biochemical measurements

These were performed as previously described in the initial study in this chapter.

Metabolic Syndrome

Subjects were assessed for metabolic syndrome according to the National Cholesterol Education Program (NCEP) guidelines (Cleeman et al., 2001) as previously described earlier in this chapter.

Baseline Sleep Studies & Spirometry

All subjects previously had overnight multichannel respiratory limited polysomnography (PSG) (Somnoscreen Digital PSG acquisition system, Version 2.0, SomnoMedics, Randersacker, Germany) and spirometry (Spiro Air LT system, Medisoft, Sorinnes, Belgium) to confirm or refute OSA.

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 (hrs/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 hrs/night on >70% of nights in the treatment group.

CE-MS materials & methods

Urine sample preparation, CE-MS Analysis and CE-MS data processing were performed. These steps were previously described in the first study in this chapter.

Statistical Analysis

Statistical analyses were performed as previously described earlier in this chapter.

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) **(Figure 6.3)**. 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 hours (SD 0.6). Mean CPAP pressures was 10cmH_20 (SD 1.0).

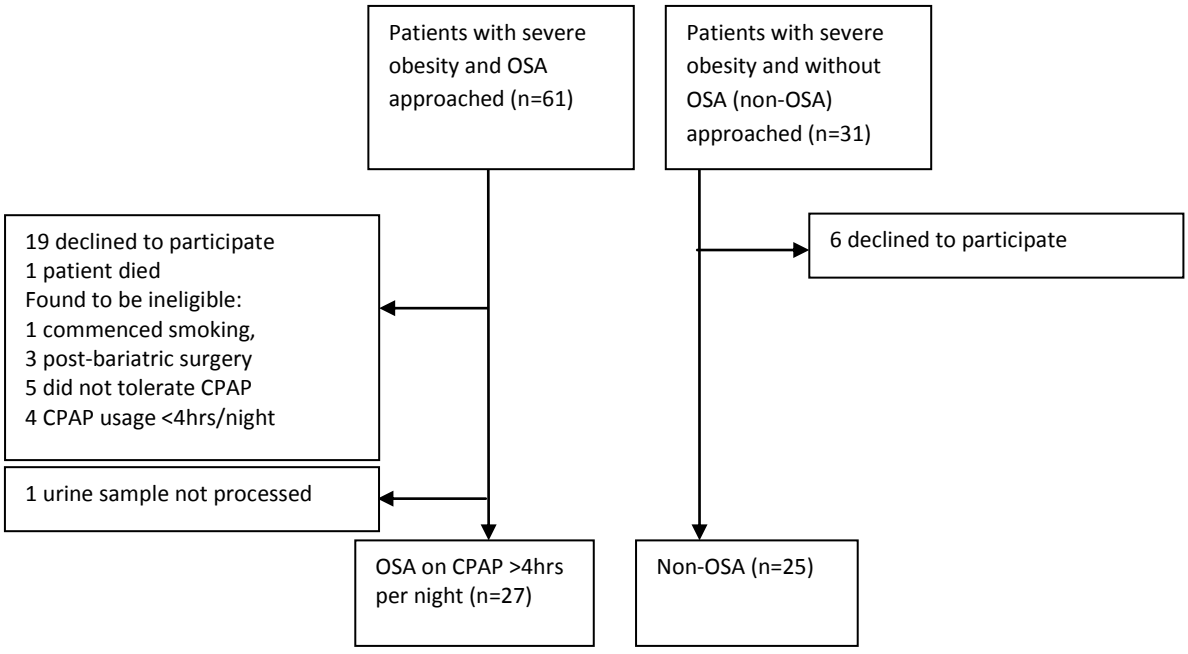


Figure 6.3 Study profile

Clinical and biochemical parameters of the 52 patients according to those without OSA (non-OSA) and OSA-on-CPAP groups are presented in **Table 6.4**. 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.

Table 6.4 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.

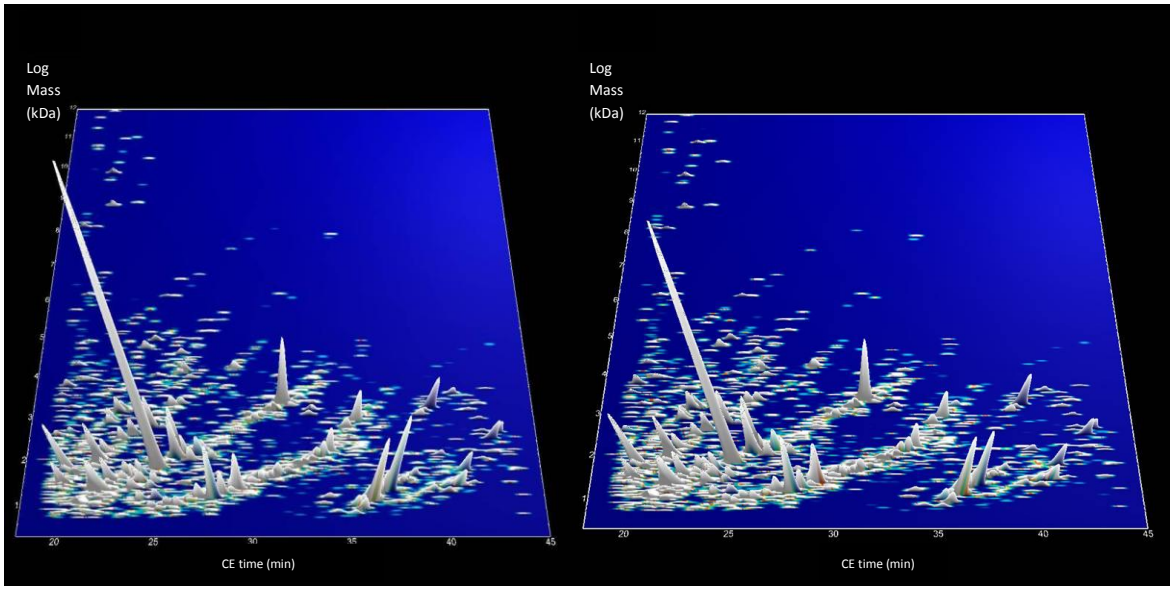
Patient Characteristics			
	Non-OSA (n = 25)	OSA on CPAP (n = 27)	p-value
Age (years)	52(9)	49(7)	0.2252
Gender:			
Female	12 (48%)	12 (44%)	
Male	13 (52%)	15 (56%)	0.7972
Smoked in past:			
No	19 (76%)	20 (74%)	
Yes	6 (24%)	7 (26%)	0.8727
Alcohol Intake:			
<8 units per week	17(68%)	19 (70%)	
≥8 units per week	8 (32%)	8 (30%)	0.8532
BMI (kg/m^2)	39(4)	43(7)	0.1860
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 (%):			
TANITA scales	42.8 (8.8)	44.4 (8.4)	0.5029
BODPOD	42.0 (9.0)	44.0 (9.0)	0.4986
Diabetes:			
No	20 (80%)	18 (67%)	
Yes	5 (20%)	9 (33%)	0.2788
Hypertension:			
No	20 (80%)	21 (78%)	
Yes	5 (20%)	6 (22%)	0.8446
BP-lowering medications:			
No	20 (80%)	21 (78%)	
Yes	5 (20%)	6 (22%)	0.8446
Glucose-lowering therapy:			
No	20 (80%)	18 (67%)	
Yes	5 (20%)	9 (33%)	0.2788
Lipid-lowering medications:			
No	17 (68%)	18 (67%)	
Yes	8 (32%)	9 (33%)	0.9184
Metabolic Syndrome:			
No	19 (76%)	13(48%)	
Yes	6 (24%)	14(52%)	**0.0391**
Cardio-Respiratory parameters			
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)	0.1610
Heart Rate (beats per min):	71 (10)	74 (10)	0.2775
CPAP use per night (hrs)	-	4.6 (0.6)	-
CPAP pressures (cmH20)	-	10 (1.0)	-
Epworth Sleep Score:	4 (3, 9)	3 (2, 11)	0.5015
O2 Saturations (%):	97 (96,99)	97 (96,98)	0.9180
PCO2 (kPa)	5.4 (5.1, 5.6)	5.3 (5.1, 5.5)	0.3882
Metabolic parameters			
ACR (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/1.73m^2)	87 (13)	89 (15)	0.5420
LDL (mmol/l)	2.7 (1.1)	2.6 (0.9)	0.7637
HDL(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
TSH (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
hsCRP (mg/L)	3.3 (1.1, 6.8)	3.0 (1.6, 5.8)	0.8189

Significant P-values are highlighted in bold.

Urine Peptide Results

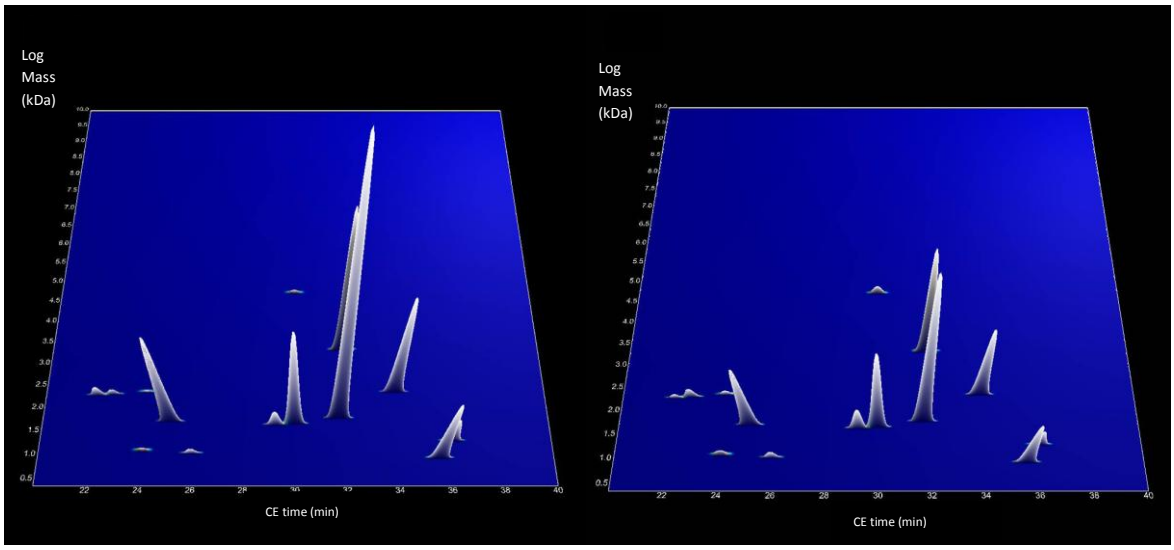
Peptide identification

The CE-MS analysis 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 **(Figure 6.4)**. 15 peptides were found with significant unadjusted Wilcoxon p-values (all $p \leq 0.01$) **(Figure 6.5)**. Although the initial data analysis showed a significant differential distribution in these peptides, it did not pass the more strict adjustment for multiple testing.



(A) non-OSA **(B)** OSA-on-CPAP

Figure 6.4 Compiled 3-dimensional depiction of urinary peptide profile for non-OSA (A) and OSA-on-CPAP (B). 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.



(A) non-OSA **(B)** OSA-on-CPAP

Figure 6.5 3-dimensional profile of 15 peptides that showed significant differences between non-OSA and OSA-on-CPAP 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, figures 6.4 and 6.5 are not of similar scale.

Sequences were determined for 8 peptides demonstrating origins mainly from collagen alpha chains. The 15 peptides are presented as in **Table 6.5**. 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.

Table 6.5

Peptide ID	Peptide Mass (Da)	CE time (min)	Unadjusted Wilcoxon p-value	Area Under the Curve	Benjamini & 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							1.0	1557 (656)	1.0	2616 (1196)
55756	1700.721	29.93699	0.00096	0.76	0.499525	GmpGSpGGpGSDGKpGPpG	Collagen alpha-1(III) chain	537	555	CO3A1_HUMAN	gi124056490	1.0	913 (265)	1.0	1200 (292)
130747	3359.578	31.89787	0.001473	0.75	0.511072	PpGADGQPGAKGEpGDAGAKGDAGPpGPAGPAGPpGPIG	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							0.7	69 (58)	0.3	24 (38)
15593	1066.478	25.95367	0.003683	0.72	0.645249	GDRGEpGPpGP	Collagen alpha-1(I) chain	800	810	CO1A1_HUMAN	gi124056487	0.77	82 (88)	0.4	68 (205)
11249	980.2951	35.57253	0.004019	0.73	0.645249							0.8	529 (405)	1.0	808 (295)
54269	1675.708	29.20317	0.004713	0.72	0.645249	GpPGPpGTSGHpGSpGSpG	Collagen alpha-1(III) chain	180	198	CO3A1_HUMAN	gi124056490	1.0	259 (87)	1.0	200 (112)
131294	3375.574	31.91691	0.005008	0.72	0.645249							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	KGNSGEPGApGSKGDTGAKGEpGPVG	Collagen alpha-1(I) chain	430	455	CO1A1_HUMAN	gi124056487	0.8	102 (62)	0.6	63 (88)
87411	2321.165	22.05962	0.007984	0.70	0.645249	DKKREEAPSLRPAPPPISGGGY	FGB protein (Fragment)					0.4	42 (69)	0.7	113 (125)
32874	1333.416	36.10802	0.008054	0.71	0.645249							0.7	186 (155)	0.8	321 (184)
19969	1132.569	24.0363	0.008187	0.70	0.645249	PGKEGPpGPQGP	Collagen alpha-1(XXII) chain	1221	1232	COMA1_HUMAN	gi296434458	0.7	49 (48)	0.4	20 (29)

Peptide ID	Peptide Mass (Da)	CE time (min)	Unadjusted Wilcoxon p-value	Area Under the Curve	Benjamini & 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)
91249	2401.078	24.0189	0.008734	0.70	0.645249							0.7	46 (50)	0.3	16 (26)
57584	1738.76	25.0428	0.009298	0.71	0.645249	FVHARTPHAEDmAEL	Cadherin-13					0.8	711 (477)	1.0	1115 (396)

Table 6.5 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.
SD:standard deviation. FGB: fibrinogen beta chain. OSA-on-CPAP: subjects with obstructive sleep apnoea receiving CPAP. Non-OSA: subjects without obstructive sleep apnoea

Discussion

CE-MS was used 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. A urinary peptide pattern consisting of 15 peptides was identified. These peptides 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. The identified sequences for 8 of these peptides 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, a panel of peptides was observed in severely obese subjects with and without OSA that was described earlier in this chapter (Seetho et al., 2014b). 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 (Kohler and Stradling, 2010).

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 (Fish and Neerman-Arbez, 2012). 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 (Parker-Duffen et al., 2013). 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 metalloproteinases) may account for the excretion of urinary collagen fragments (Lopez et al., 2010). Furthermore, it is known that alterations of the myocardial collagen matrix may occur in cardiovascular disease that may result in perturbations in vascular function (Lopez et al., 2010). In this context, it is known that OSA is associated with cardiovascular disease (Monahan and Redline, 2011), 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 (Kumar and Prabhakar, 2008). Multiple lines of evidence underscore the importance of CPAP treatment in lowering BP in patients with severe OSA (Fava et al., 2014). Additionally, OSA-mediated hypertension may be influenced by the effects of CPAP on reducing renin-angiotensin system activity with changes in renal perfusion (Kinebuchi et al., 2004) (Nicholl et al., 2014). 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 (Coon et al., 2008).

As pointed out in the first study earlier in this chapter, a limitation of urinary peptide analysis is that the excretion of urinary peptides may vary during the day because of physical activity, diet, or medications taken. In order to control for this, sampling of all participants was performed at the same time each morning, at second void, after an overnight fast at their study visit prior to the administration of any medication. It was envisaged that it would be 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.

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

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.

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.

Analysis of peptide profiles over time

Background

The urinary proteome findings from the two studies allowed a comparison to be made of the urinary peptide profiles at baseline and follow-up for OSA patients and non-OSA patients respectively. It was important to study how the peptide panel changed during this period of time.

Methods

Peptide profiles at baseline and at follow-up (median 15 months [IQR 13,16]) were examined for OSA patients and controls.

Results

The patient characteristics are summarised in **Tables 6.6 (OSA patients) and 6.7 (non-OSA patients)**.

Table 6.6

OSA patient characteristics			
	OSA (baseline) (n = 61)	OSA-on-CPAP (follow-up) (n = 27)	p-value
Age (years)	48 (9)	49 (7)	0.397
BMI (kg/m^2)	43 (8)	43 (7)	0.741
Neck Circumference (cm)	44.2 (4.3)	44.2 (4.6)	0.996
Waist:Hip Ratio	0.98 (0.1)	0.98 (0.1)	0.835
Body Fat (%):			
TANITA scales	46 (39,51)	44.4 (39,52)	0.899
BodPod	45 (39,51)	44.0 (39,52)	0.903
Cardio-Respiratory parameters			
Systolic BP (mmHg)	137.5 (13.8)	129 (11)	**0.006**
Diastolic BP (mmHg)	87.3 (11)	79 (7)	**0.002**
Mean Arterial Pressure (mmHg)	105 (10.6)	98 (93,101)	**<0.001**
Heart Rate (beats per min):	75 (10)	74(10)	0.794
Epworth Sleep Score:	10 (7, 15)	3 (2, 11)	**0.001**
O2 Saturations (%):	97 (96,98)	97 (96,98)	0.413
PCO2 (kPa)	5.4 (5.1, 5.6)	5.3 (5.1, 5.5)	0.152
Metabolic parameters			
ACR (mg/mmol)	0.7 (0.3, 1.5)	0.5 (0.3, 0.8)	0.204
Creatinine (micromol/l)	73 (15)	78 (15)	0.136
MDRD-GFR (mL/min/1.73m^2)	95 (17)	89 (15)	0.129
LDL (mmol/l)	2.9 (0.9)	2.6 (0.9)	0.285
HDL(mmol/l)	1.2 (0.3)	1.2 (0.3)	0.940
HbA1c (mmol/mol)	40 (37, 48)	38 (35, 48)	0.348
Total Cholesterol (mmol/l)	5.0 (1.0)	4.6 (1.0)	0.126
Fasting Glucose (mmol/l)	5.3 (4.8, 6.0)	5.4 (4.7, 6.0)	0.924
TSH (mU/l)	2.0 (1.5,2.8)	2.1 (1.2, 3.0)	0.859
Triglycerides (mmol/l)	1.6 (1.1, 2.7)	1.6 (1.0,2.6)	0.730
Bicarbonate (mmol/l)	24.8 (1.0)	24.2 (2.1)	0.063
hsCRP (mg/L)	5.2 (2.5, 7.3)	3.0 (1.6, 5.8)	0.146

Table 6.6 Patient demographics were summarised by OSA (baseline) and OSA on CPAP (follow-up) groups as means (SD) (or median (IQR) if non-normal) for continuous variables and frequencies (%) for categorical variables. Significant p-values are in bold.

Table 6.7

Non-OSA patient characteristics			
	Non-OSA (baseline) (n = 31)	Non-OSA (follow-up) (n = 25)	p-value
Age (years)	49(10)	52(9)	0.298
BMI (kg/m^2)	40(5)	39(4)	0.552
Neck Circumference (cm)	42.4(4.4)	42.1(4.3)	0.841
Waist:Hip Ratio	0.96 (0.1)	0.94 (0.1)	0.610
Body Fat (%):			
TANITA scales	42 (34,52)	42.8 (34,51)	0.662
BodPod	43 (34,52)	42.3 (33,51)	0.789
Cardio-Respiratory parameters			
Systolic BP (mmHg)	128 (12)	128 (11)	0.953
Diastolic BP (mmHg)	83 (10)	82 (8)	0.875
Mean Arterial Pressure (mmHg)	98.5 (89,108)	100 (91,104)	0.993
Heart Rate (beats per min):	72 (12)	71(10)	0.836
Epworth Sleep Score:	6 (3, 10)	4 (3, 9)	0.512
O2 Saturations (%):	97 (96,99)	97 (96,99)	0.448
PCO2 (kPa)	5.4 (5.0, 5.6)	5.4 (5.1, 5.6)	0.817
Metabolic parameters			
ACR (mg/mmol)	0.4 (0.2, 1.1)	0.4 (0.2, 0.5)	0.560
Creatinine (micromol/l)	77 (16)	77 (13)	0.828
MDRD-GFR (mL/min/1.73m^2)	90 (18)	87 (13)	0.436
LDL (mmol/l)	2.8 (1.0)	2.7 (1.1)	0.984
HDL(mmol/l)	1.3 (0.4)	1.3 (0.4)	0.185
HbA1c (mmol/mol)	39 (35, 41)	37 (34, 40)	0.134
Total Cholesterol (mmol/l)	4.7 (1.1)	4.7 (1.1)	0.977
Fasting Glucose (mmol/l)	5.2 (4.9, 5.6)	5.4 (5.1, 5.9)	0.614
TSH (mU/l)	2.1 (1.6, 3.3)	2.2 (1.5,3.2)	0.318
Triglycerides (mmol/l)	1.3 (1.1, 2.2)	1.2 (0.8,2.0)	0.223
Bicarbonate (mmol/l)	24.5 (1.1)	24.0 (1.8)	0.224
hsCRP (mg/L)	3.3 (1.5, 5.3)	3.3 (1.1, 6.8)	0.843

Table 6.7 Patient demographics were summarised by non-OSA (baseline) and non-OSA (follow-up) groups as means (SD) (or median (IQR) if non-normal) for continuous variables and frequencies (%) for categorical variables.

In the OSA group, 399 peptides were found to be significantly different between baseline and at follow-up. In the non-OSA (control) group, 267 peptides were significantly different between the patients at baseline and at follow-up. The peptides in both panels represented the change in peptide profiles over time. **(Figure 6.6)**

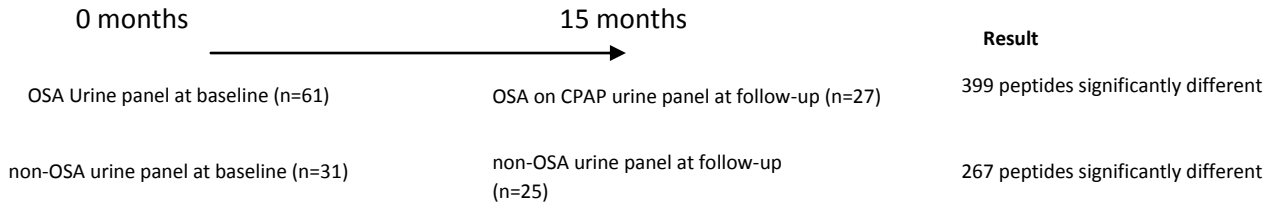


Figure 6.6 Comparison of peptide profiles at baseline and follow-up for OSA patients (399 peptides) and non-OSA patients (267 peptides).

Both panels were compared to exclude overlapping peptides that appeared in both groups. 216 peptides were found to overlap in the OSA-over-time and control group-over-time peptide profiles. Therefore, 183 peptides significantly changed in the OSA group over time and in the control group over time, 51 peptides were significantly different after correction for multiple testing. **(Figure 6.7)**

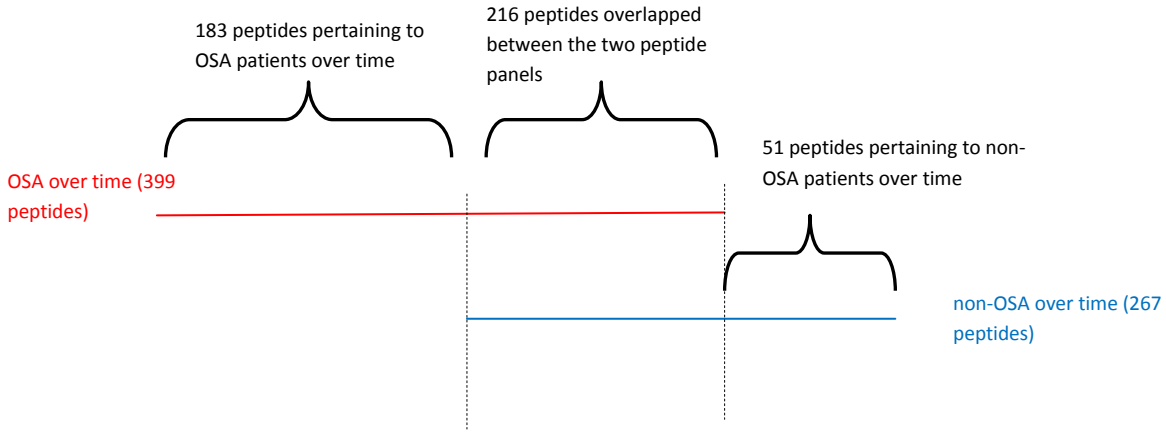
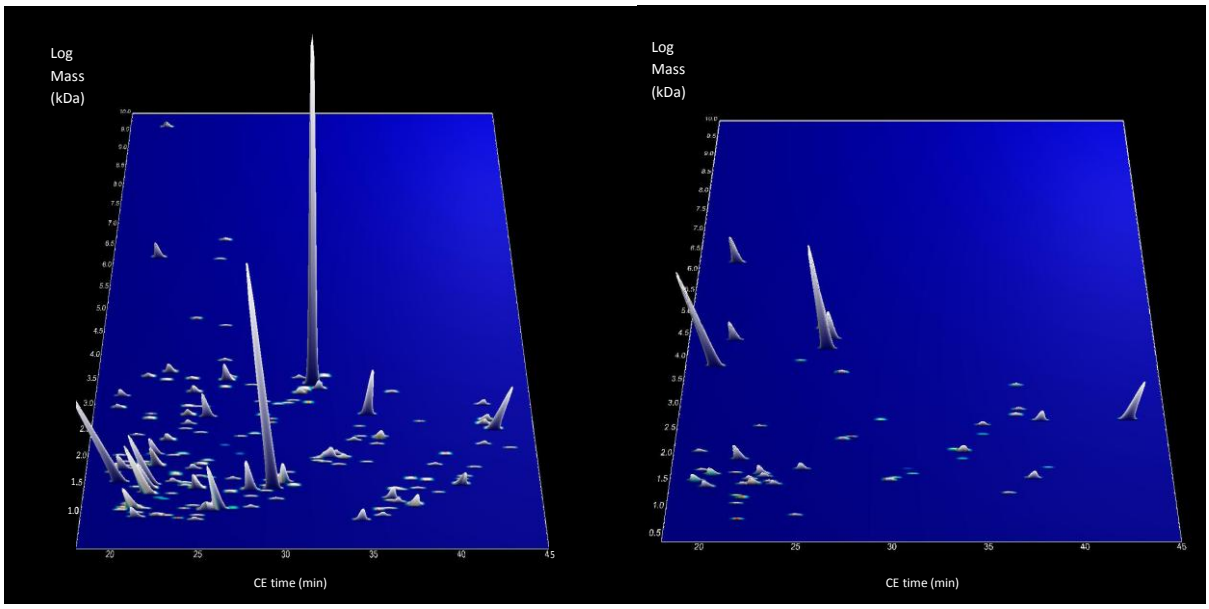


Figure 6.7 Illustrating the comparison for overlapping peptides in the peptide panels for OSA patients over time (399 peptides) [red line] vs non-OSA patients (controls) over time (267 peptides) [blue line].



(A) OSA patients over time showing 183 peptides (B) controls over time 51 peptides

Figure 6.8 Compiled 3-dimensional depiction of significantly different urinary peptides for (A) OSA patients before (baseline) and after CPAP (follow-up) and OSA-on-CPAP; (B) non-OSA patients. 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.

The peptides are presented in the tables below**. Table 6.8** below lists the 183 significantly different peptides in OSA patients before and after CPAP (OSA over time). Following this, **Table 6.9** shows the 51 significantly different peptides for non-OSA patients between baseline and follow-up. It should be noted that the sequencing of all peptides in the panel may not be feasible due to post-translational modifications and resistance of larger peptides to fragmentation (Frantzi et al., 2014).

Table 6.8

peptide ID	Mass	CE time	Amplitude	Sequence	Protein name	Start Amino Acid	Stop Amino Acid	SwissProt name	Accession number
3048	868.3865	27.80894	342.7287						
5199	906.406	24.70353	78.94503						
5661	911.2648	34.34517	1619.454						
7408	935.4465	23.68105	174.3233	GRpGPpGPpG	Collagen alpha-1(I) chain	563	572	CO1A1_HUMAN	gi124056487
8342	944.5103	21.24885	391.3036						
11249	980.2951	35.57253	127.6192						
11413	981.5851	24.79552	545.9605	VLNLGPITR	Uromodulin	598	606	UROM_HUMAN	gi137116
11760	984.4667	23.06132	50.29906						
11989	988.5208	22.44416	218.1374						
14071	1032.498	21.2104	749.1479						
15474	1063.437	20.27573	117.5974						
16908	1083.487	25.5576	263.5282	ApGEKGEGGPpG	Collagen alpha-1(III) chain	829	840	CO3A1_HUMAN	gi124056490
16976	1084.428	25.23275	1432.578						
16979	1084.449	35.40236	68.98887						
17622	1095.504	23.99375	45.551						
17649	1096.334	35.93955	123.8788						
17694	1096.483	26.07573	4464.49	ApGDRGEpGpP	Collagen alpha-1(I) chain	798	808	CO1A1_HUMAN	gi124056487
17901	1098.506	21.62882	197.0547						
18164	1103.302	36.0893	45.81509						
18393	1107.511	26.83722	132.6135	SEPQEVLHIG	PDZ and LIM domain protein 1				
18448	1108.534	20.38335	163.2336						
19648	1126.471	21.18499	283.4154						
19828	1129.46	27.91491	215.6868						
20659	1139.486	20.96505	1342.152						
20750	1141.515	24.50651	231.9815	IHEDPGPPPPS	ACVR2B Activin receptor type-2B				
21747	1157.537	37.44405	1573.432	GPPGPpGppGPPS	Collagen alpha-1(I) chain	1181	1193	CO1A1_HUMAN	gi124056487
22835	1173.529	37.49036	321.0378	GPpGPpGPpGPVT	Collagen alpha-1(XVII) chain	1036	1048	COHA1_HUMAN	gi146345399
23224	1178.392	20.71175	257.7512						
23356	1179.52	37.4916	947.583	GPpGPpGPSSNQG	Collagen alpha-6(IV) chain	1277	1289	CO4A6_HUMAN	gi116241307
24990	1210.386	36.48163	470.6391						
26654	1235.372	36.10898	360.9464						
28342	1261.559	22.76938	60.53939	TPAQFDADELR	Annexin A1				
28850	1270.548	29.38075	184.8586						
29677	1283.366	36.12409	554.2863						
30295	1294.6	22.45243	101.2303						
30942	1304.524	27.88006	851.3366						
34267	1359.608	23.19466	138.6576						

peptide ID	Mass	CE time	Amplitude	Sequence	Protein name	Start Amino Acid	Stop Amino Acid	SwissProt name	Accession number
34339	1361.627	21.98741	380.5203						
35550	1383.637	38.94024	308.4563						
36156	1392.623	21.75213	3423.801						
36350	1396.624	26.67312	130.6586						
36500	1399.623	28.74437	170.2496						
38174	1428.391	36.74784	2388.916						
38798	1438.667	27.87549	3000.847	GLpGTGGPpGENGKpG	Collagen alpha-1(III) chain	642	657	CO3A1_HUMAN	gi124056490
39064	1442.626	27.6328	383.2681	SpGENGApGQMGPRG	Collagen alpha-1(I) chain	291	305	CO1A1_HUMAN	gi124056487
39129	1444.363	36.37433	1150.989						
40133	1450.559	37.72479	365.1717	PGEPGmQGEpGPpGP	Collagen alpha-3(IV) chain	1366	1380	CO4A3_HUMAN	gi134035067
40243	1451.656	29.17162	22054.55	SpGSpGPDGKTGPPGp	Collagen alpha-1(I) chain	543	558	CO1A1_HUMAN	gi124056487
41431	1466.659	21.8713	2383.136						
41514	1467.807	24.68522	1136.244	DQSRVLNLGPITR	Uromodulin	594	606	UROM_HUMAN	gi137116
41665	1470.684	21.08042	242.7755	DGLAHLDNLKGTFA	Hemoglobin subunit beta				
42683	1492.666	28.97724	502.216	GSpGSPGPDGKTGPpGP	Collagen alpha-1(I) chain	542	558	CO1A1_HUMAN	gi124056487
42776	1494.661	30.3993	353.7276	EpGDAGAKGDAGPpGPA	Collagen alpha-1(I) chain	828	844	CO1A1_HUMAN	gi124056487
43339	1505.614	28.77205	653.2408						
43538	1508.663	23.99497	310.0396	GApGKNGERGGpGGpGP	Collagen alpha-1(III) chain	588	604	CO3A1_HUMAN	gi124056490
44592	1523.671	21.96611	4018.63	AEPGDPRAMSGRSPP	Beta-1,3-galactosyltransferase 6				
44750	1525.669	30.3927	596.6625	YKTTPPVLDSDGSF	Ig gamma-1 chain C region	274	287	IGHG1_HUMAN	gi121039
45445	1539.735	40.30862	2837.09	GpEGPpGEPGpPGPPGP	Collagen alpha-2(V) chain	1209	1225	CO5A2_HUMAN	gi143811378
45564	1541.73	25.78398	121.8796	AVGSpGVNGApGEAGRD	Collagen alpha-2(I) chain	911	927	CO1A2_HUMAN	gi124056488
45597	1542.692	23.96014	259.5157	DGQpGAKGEpGDAGAKG	Collagen alpha-1(I) chain	820	836	CO1A1_HUMAN	gi124056487
46139	1555.719	40.1404	109.3591						
46649	1563.701	29.46172	1345.828	SpGSPGPDGKTGPpGPAG	Collagen alpha-1(I) chain	543	560	CO1A1_HUMAN	gi124056487
46928	1568.709	29.89318	445.8234	EGSPGHPGQpGPpGPpG	Collagen alpha-1(III) chain	1175	1191	CO3A1_HUMAN	gi124056490
48089	1579.684	20.05599	5948.412						
48106	1579.713	29.81744	2923.505	SpGSpGPDGKTGPPGpAG	Collagen alpha-1(I) chain	543	560	CO1A1_HUMAN	gi124056487
48394	1583.701	23.26777	383.4824	NDGApGKNGERGGpGGp	Collagen alpha-1(III) chain	586	602	CO3A1_HUMAN	gi124056490
48686	1591.709	38.27637	117.0876						
49608	1602.743	28.97183	377.1098						
50126	1612.614	38.2826	102.0367						
50593	1619.788	40.3982	232.798	VGPpGPpGPPGPPGPPSAG	Collagen alpha-1(I) chain	1177	1195	CO1A1_HUMAN	gi124056487
50791	1622.722	26.79132	248.3684	DNGASTDDSAAEKKGGT	Plexin domain-containing protein 2				
51779	1634.652	37.33193	280.8534						
53287	1656.747	25.39242	100.8085						
53435	1659.748	29.33067	495.3625	GPpGPpGTSGHPGSpGSpG	Collagen alpha-1(III) chain	180	198	CO3A1_HUMAN	gi124056490
53800	1667.793	40.56083	621.9006	GPPGFTGPpGPpGPPGPPG	Collagen alpha-1(IV) chain	197	215	CO4A1_HUMAN	gi125987809
53838	1668.711	29.63573	123.7109	GmPGSpGGPGSDGKpGpPG	Collagen alpha-1(III) chain	537	555	CO3A1_HUMAN	gi124056490
54448	1679.953	24.7908	2950.083						
55523	1696.727	29.51782	128.9522	SpGSNGApGQRGEpGPQG	Collagen alpha-1(III) chain	358	375	CO3A1_HUMAN	gi124056490
56132	1708.752	23.37465	173.253						
57870	1745.894	23.29239	136.013	LpGLkGHNGLQGLpGIAG	Collagen alpha-2(I) chain	1019	1036	CO1A2_HUMAN	gi124056488

peptide ID	Mass	CE time	Amplitude	Sequence	Protein name	Start Amino Acid	Stop Amino Acid	SwissProt name	Accession number
58008	1748.797	30.07475	238.0238						
58222	1752.783	19.61447	148.2035						
61221	1817.694	20.23435	3290.374						
61510	1823.988	24.39771	1380.493	GSVIDQSRVLNLGPITR	Uromodulin	590	606	UROM_HUMAN	gi137116
61984	1835.709	19.91313	1015.238						
62226	1840.836	41.17953	310.093						
63391	1864.807	20.42988	152.1199						
63910	1876.866	22.20126	1448.42	DDGEAGKPGRPGERGpPGp	Collagen alpha-1(I) chain	231	249	CO1A1_HUMAN	gi124056487
64431	1885.651	38.81996	160.4808						
64442	1885.874	21.26479	221.7097	GSpGRDGSpGAKGDRGETGP	Collagen alpha-1(I) chain	1022	1041	CO1A1_HUMAN	gi124056487
64621	1889.868	33.0683	190.3908						
65243	1899.861	21.32026	350.5332	SpGRDGSpGAKGDRGETGPA	Collagen alpha-1(I) chain	1023	1042	CO1A1_HUMAN	gi124056487
65257	1899.853	24.83469	171.7328	SGEpGApGSKGDTGAKGEpGP	Collagen alpha-1(I) chain				
67362	1936.734	20.24288	479.9166						
67382	1936.874	34.748	145.3868						
67386	1936.878	32.24218	369.8674	GEKGPSGEAGTAGPpGTpGPQG	Collagen alpha-2(I) chain	844	865	CO1A2_HUMAN	gi124056488
67462	1938.881	21.38561	266.9038	kGNDGApGKNGERGGpGGpGP	Collagen alpha-1(III) chain	584	604	CO3A1_HUMAN	gi124056490
68415	1962.875	31.81242	311.4506	QGLpGTGGPpGENGKpGEpGP	Collagen alpha-1(III) chain	641	661	CO3A1_HUMAN	gi124056490
68663	1968.879	25.19977	393.2712	GEpGApGSKGDTGAKGEpGPVG	Collagen alpha-1(I) chain	434	455	CO1A1_HUMAN	gi124056487
70633	2013.893	31.75577	117.4104	AGpPGPPGppGTSGHpGSpGSpG	Collagen alpha-1(III) chain	176	198	CO3A1_HUMAN	gi124056490
71029	2022.894	33.36646	248.8853						
71312	2025.873	32.22548	860.3418	SEGSpGHpGQpGpPGPPGApGp	Collagen alpha-1(III) chain	1174	1195	CO3A1_HUMAN	gi124056490
71602	2030.93	32.60772	1249.692	PpGEAGKpGEQGVpGDLGAPGP	Collagen alpha-1(I) chain	651	672	CO1A1_HUMAN	gi124056487
73006	2058.864	21.44346	221.9235						
73010	2058.937	23.15082	559.8969						
73238	2063.924	19.9221	128.3866						
74420	2085.931	22.06718	12774.36	EGSpGRDGSpGAKGDRGETGPA	Collagen alpha-1(I) chain	1021	1042	CO1A1_HUMAN	gi124056487
74902	2087.966	32.91176	1532.091	GPpGEAGkPGEQGVPGDLGApGP	Collagen alpha-1(I) chain	650	672	CO1A1_HUMAN	gi124056487
74942	2088.875	23.6675	128.1062						
74984	2089.939	27.12544	214.3377	ANDRESVENLAKSSNSGQQG	Nidogen-1				
74987	2089.958	39.51667	179.6785						
81019	2207.073	43.4589	291.3098	EAEDLQVGQVELGGGPGAGSLQP	Insulin	57	79	INS_HUMAN	gi|124617
83021	2242.998	26.19505	1075.983	GNSGEpGApGSKGDTGAkGEpGPVG	Collagen alpha-1(I) chain	431	455	CO1A1_HUMAN	gi124056487
84736	2269.62	35.40606	191.6505						
85177	2279.071	41.85022	199.4272						
88282	2339	34.00721	1177.717	GANGApGNDGAKGDAGApGApGSQGApG	Collagen alpha-1(I) chain	698	725	CO1A1_HUMAN	gi124056487
88299	2339.085	22.66349	251.7059	KGNSGEPGApGSKGDTGAKGEpGPVG	Collagen alpha-1(I) chain	430	455	CO1A1_HUMAN	gi124056487
88850	2346.016	29.52018	380.7082						
89233	2355.085	22.74837	1486.563	KGNSGEpGApGSKGDTGAKGEpGPVG	Collagen alpha-1(I) chain	430	455	CO1A1_HUMAN	gi124056487
90588	2383.662	35.56059	159.8052						
92029	2414.626	35.61674	565.8539						
92147	2418.163	34.42614	95.20189						
92171	2419.075	27.13582	77.84179	pGSRGEDGPEGPKGRTGPTGDpGpP	Collagen alpha-2(XI) chain				
93270	2443.13	20.83575	197.4544	PpGKNGDDGEAGKPGRpGERGPpGP	Collagen alpha-1(I) chain	225	249	CO1A1_HUMAN	gi124056487
94345	2472.121	27.98832	224.9151	pPGADGQPGAKGEPGDAGAKGDAGPpGp	Collagen alpha-1(I) chain	816	843	CO1A1_HUMAN	gi124056487

peptide ID	Mass	CE time	Amplitude	Sequence	Protein name	Start Amino Acid	Stop Amino Acid	SwissProt name	Accession number
95084	2490.231	24.68039	140.5386						
98720	2565.143	23.74013	406.9312	REQGHQKERNQEmEEGGEEEH	Retinitis pigmentosa GTPase regulator				
99021	2570.19	42.56018	5231.009						
100416	2601.105	34.37743	143.5772						
103198	2654.193	23.92493	301.0248	ERGEAGIpGVpGAKGEDGKDGSpGEpGA	Collagen alpha-1(III) chain	448	475	CO3A1_HUMAN	gi124056490
104376	2668.25	41.97426	556.2366						
105105	2687.219	28.98551	158.6293	KDGEAGAQGPpGPAGPAGERGEQGPAGSpG	Collagen alpha-1(I) chain	612	641	CO1A1_HUMAN	gi124056487
105620	2701.259	38.064	122.586						
107452	2742.251	42.14319	670.2255						
108189	2758.254	28.99336	103.869	KNGETGPQGPpGPTGpGGDKGDTGPpGPQG	Collagen alpha-1(III) chain	610	639	CO3A1_HUMAN	gi124056490
108327	2761.315	21.49226	2446.863	ERGSPGpAGPKGSpGEAGRpGEAGLpGAKG	Collagen alpha-1(I) chain	510	539	CO1A1_HUMAN	gi124056487
109164	2777.316	21.5225	388.2919	ERGSpGPAGpKGSpGEAGRpGEAGLpGAKG	Collagen alpha-1(I) chain	510	539	CO1A1_HUMAN	gi124056487
110052	2799.085	25.06936	3196.757						
111239	2832.352	28.46226	196.0313						
111426	2837.361	23.86843	1801.296	IPVKQADSGSSEEKQLYNKYPDAVAT	Osteopontin	17	42	OSTP_HUMAN	gi129260
112082	2854.294	21.54826	331.4307						
112106	2854.363	34.86364	2589.894						
116812	2977.179	19.52319	460.2419						
117318	2987.351	24.86474	147.2873						
117371	2989.451	24.42708	514.459						
117823	3002.238	23.80085	186.7636						
119292	3035.191	42.02093	532.4753						
119798	3047.436	29.36741	244.5695						
120157	3057.395	29.96413	189.728						
121241	3076.463	31.24033	146.1622	ADGQPGAKGEpGDAGAKGDAGPpGPAGPAGPPGPIG	Collagen alpha-1(I) chain	819	854	CO1A1_HUMAN	gi124056487
121716	3091.436	28.39639	453.6313						
122623	3114.426	30.29016	327.1601						
125263	3205.273	19.65755	887.6813						
127575	3271.49	30.70446	1186.011	NTGApGSpGVSGpKGDAGQpGEKGSPGAQGPPGAPGp	Collagen alpha-1(III) chain	910	946	CO3A1_HUMAN	gi124056490
128145	3287.506	36.4383	188.0146						
128249	3290.503	24.14338	887.018						
129131	3318.546	30.99198	199.3411	GTSLSPPPESSGSPQQPGLSAPHSRQIPAPQGAV	Metastasis-suppressor KiSS-1				
129985	3334.54	31.0159	332.1059						
130482	3350.549	31.01736	166.9587						
130747	3359.578	31.89787	1406.8	PpGADGQPGAKGEpGDAGAKGDAGPpGPAGPAGPpGPIG	Collagen alpha-1(I) chain	816	854	CO1A1_HUMAN	gi124056487
130947	3366.563	30.99609	142.7641						
131635	3386.569	26.0506	192.623						
132980	3426.31	27.69928	153.1036						
133849	3457.612	31.49392	16180.89	NTGAPGSpGVSGpKGDAGQpGEKGSpGAQGppGAPGPLG	Collagen alpha-1(III) chain	910	948	CO3A1_HUMAN	gi124056490
134470	3473.596	31.47644	3508.754	NTGApGSpGVSGpKGDAGQpGEKGSpGAQGpPGAPGpLG	Collagen alpha-1(III) chain	910	948	CO3A1_HUMAN	gi124056490
135979	3530.427	22.324	83.44129						
135984	3530.645	26.12897	762.2011						

peptide ID	Mass	CE time	Amplitude	Sequence	Protein name	Start Amino Acid	Stop Amino Acid	SwissProt name	Accession number
136697	3556.588	23.95474	1230.333	FAEEKAVADTRDQADGSRASVDSGSSEEQGGSSRA	Polymeric-immunoglobulin receptor	605	639	PIGR_HUMAN	gi150421625
137053	3572.6	30.6681	206.5417						
137920	3583.632	26.30391	191.4182						
138508	3602.688	32.25433	113.8074						
138813	3605.571	21.1852	265.9208						
139630	3634.699	32.22291	251.753						
140823	3687.708	22.49438	246.2683						
141168	3703.717	22.51526	1264.578						
146717	3932.864	25.93393	419.6563						
165737	4670.149	25.85822	491.8343						
168919	4833.145	23.92431	2505.421						
177734	6185.419	25.23224	1847.474						
177971	6236.907	21.066	2358.224						
179035	6650.641	25.53712	250.3089						
184886	9625.366	20.68655	456.2157						

Table 6.8 Significantly different 183 peptides in OSA patients before and after CPAP (OSA over time). The table lists the peptide identification number (Peptide ID), experimental mass (in Da) and CE migration time (in min) for the 183 significantly different peptides. 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.

Table 6.9

peptide ID	Mass	CE time	Amplitude	Sequence	Protein name	Start Amino Acid	Stop Amino Acid	SwissProt name	Accession number
97	801.4409	21.80364	57.03727						
3806	884.3214	24.85187	457.2986						
17973	1099.499	21.67177	181.2622						
24723	1204.597	21.94117	208.3972						
27229	1245.551	21.62387	217.3369						
30136	1292.393	36.15933	198.0948						
37420	1417.635	20.02529	447.9929						
40294	1452.66	23.60651	269.9552	DEPPQSPWDRVK	Apolipoprotein A-I				
40487	1457.601	21.93479	153.4954	FHDDGFLAFPGHV	Basement membrane-specific heparan sulfate proteoglycan core protein	4206	4218	PGBM_HUMAN	gi218512120
41476	1467.665	22.51989	668.6269	GPPGkNGDDGEAGKPG	Collagen alpha-1(I) chain	224	239	CO1A1_HUMAN	gi124056487
41833	1474.674	22.44045	675.0306						
42833	1495.684	23.3582	172.3582						
44146	1518.604	19.37145	636.0246						
45384	1538.69	29.77393	312.2466	PpGEAGKpGEQGVpGD	Collagen alpha-1(I) chain	651	666	CO1A1_HUMAN	gi124056487
45944	1551.655	22.28557	263.9911						
48354	1583.687	30.07762	219.5918	DRGESGpAGpAGApGpAG	Collagen alpha-1(III) chain				
48417	1584.506	37.49593	417.5619						
49295	1594.726	23.05294	330.2448	ApGGKGDAGApGERGPpG	Collagen alpha-1(III) chain	670	687	CO3A1_HUMAN	gi124056490
52100	1638.728	20.22988	740.7142	AGSEADHEGTHSTKRG	Fibrinogen alpha chain	607	622	FIBA_HUMAN	gi1706799
52219	1640.699	31.01427	155.4492						
53328	1657.722	22.89732	339.3599						
53957	1669.689	21.46473	417.1889	DEAGSEADHEGTHSTK	Fibrinogen alpha chain	605	620	FIBA_HUMAN	gi1706799
56662	1720.686	19.66568	216.8555						
57584	1738.76	25.0428	449.4016	FVHARTPHAEDmAEL	Cadherin-13				
57775	1743.761	30.74729	176.9393						
58798	1762.57	38.33862	194.6434						
67152	1932.902	21.63847	511.1817	LQKGNDDHWIVDTDYD	Retinol binding protein 4, plasma				
67217	1933.877	21.62452	669.0764	GDDGEAGKPGRpGERGPpGP	Collagen alpha-1(I) chain	230	249	CO1A1_HUMAN	gi124056487
67696	1945.004	33.70855	223.2444						
74703	2087.844	19.42125	762.7224						
75128	2093.924	33.77695	241.6734	PGppGDQGPPGPDGPRGApGPpG	Collagen alpha-4(IV) chain				
76370	2116.964	33.26193	173.9895						
87740	2329.062	27.16976	242.7122						
89857	2367.061	27.63196	138.8182						
92231	2420.998	34.86081	110.1795						
100020	2589.056	22.56216	180.6334						
100949	2611.23	34.8716	413.7747						

peptide ID	Mass	CE time	Amplitude	Sequence	Protein name	Start Amino Acid	Stop Amino Acid	SwissProt name	Accession number
105661	2702.213	38.0799	327.4308						
106067	2713.234	29.22274	201.6525	PpGADGQpGAKGEpGDAGAKGDAGPpGPAGP	Collagen alpha-1(I) chain	816	846	CO1A1_HUMAN	gi124056487
106667	2726.283	42.93932	3380.374						
110430	2810.355	36.74954	137.6011						
114702	2923.432	36.91513	351.9374						
132725	3416.602	36.84899	109.2565						
141007	3696.761	26.94283	342.6135	ARGNDGATGAAGPpGPTGPAGppGFpGAVGAKGEAGPQGPRG	Collagen alpha-1(I) chain				
144826	3839.813	19.70303	4139.322						
146964	3944.712	24.55436	178.4225						
154329	4217.975	26.05299	4504.634	EEKAVADTRDQADGSRASVDSGSSEEQGGSSRALVSTLVPLG	Polymeric-immunoglobulin receptor	607	648	PIGR_HUMAN	gi150421625
159259	4404.842	20.66586	1781.454						
160089	4436.083	26.31772	2812.614						
165400	4654.08	25.83388	2852.955						
177848	6211.741	20.28513	707.2819						

Table 6.9 Significantly different 51 peptides for non-OSA patients between baseline and follow-up. The table lists the peptide identification number (Peptide ID), experimental mass (in Da) and CE migration time (in min) for the 51 significantly different peptides for non-OSA patients. 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.

Discussion

The 183 peptides in the OSA group panel that were significantly different between baseline and follow-up may be related to changes with CPAP treatment. However, in the control group panel over time, there were 51 peptides that were significantly different between controls at baseline and at follow-up. One possible explanation for these findings may be that the differences in peptide profiles each reflect the relevant groups being compared; for example, the panel determined from the baseline study was specifically for OSA (prior to CPAP) and controls, while the peptide panel differences that were observed between baseline and follow-up may specifically relate to differences between controls over time and OSA patients over time per se.

It may be argued that the large number of significantly different peptides in the control group over time may lead to questions concerning the reproducibility of the urinary proteome. Nevertheless, given the fewer number of significantly different peptides in non-OSA subjects (51 peptides) relative to the OSA patients over time (183 peptides), and the large number of overlapping peptides between both groups (216 peptides), it may be speculated that inherent differences and similarities in the urinary proteome exist between OSA patients and controls. Furthermore, although the variability of polypeptides (as noted in the controls over time) may represent a limitation in terms of reproducibility of the urinary proteome, the observations may need to be taken within the context that the excretion of some polypeptides may vary over time. Hence reproducibility at the level of a single/individual peptide may be of limited value (Coon et al., 2008). As such, a proteome with one or two peptides may only have modest value in characterising a complex condition such as OSA. However, a polypeptide panel consisting of a combination of peptides may be more robust towards changes in individual peptides within the proteome (Coon et al., 2008). With the possibility of high biological variability influencing the urinary profile, a panel containing multiple peptides may be useful as each peptide may potentially contribute information pertaining to the condition or disease even in the presence of biological variability (Mischak et al., 2013).

Chapter 7

Discussion

This chapter brings together the work in this thesis, exploring what is to be done based on the findings, limitations of methods used and a discussion of future research.

One of the key contributions of this work has been in increasing our understanding of the complexities of OSA and obesity, as well as the investigation into the use of a novel proteomic tool (CE-MS) in the study of OSA. As discussed in chapter 1, it is clear that OSA has important cardiovascular and metabolic effects. However, the evidence available pertaining to severely obese subjects is limited. The studies on arterial stiffness and urate in severe obesity have attempted to address this deficiency and increase our knowledge of the effects of OSA in these patients. More importantly, they highlight the importance of assessing for OSA. A national survey using a questionnaire on assessing for OSA in diabetes practice was the next study that identified areas that could be improved in clinical practice. The rising prevalence of obesity has led to a growing demand for OSA assessment and diagnostic studies and treatments. As such, there has been increasing emphasis on the need to screen and assess patients with obesity and diabetes that may be at risk of OSA. In chapter 1, the concept of novel screening methods was discussed. Although assessment tools are available for SDB, there has been increasing interest in methods to develop new ways to try to improve the assessment of OSA in patients (Montesi et al., 2012a). In particular, the role of urinary proteomics as a potential tool for classifying patients with OSA has been suggested. The final studies in this thesis relate to work performed using CE-MS in order to explore the urinary proteome in OSA and severe obesity.

Arterial Stiffness studies

In terms of arterial stiffness and OSA, the aims of the studies were to evaluate arterial stiffness in severe obesity with and without OSA using pulse wave analysis. In the initial study, it was hypothesized that the presence of OSA in severe obesity, even in the absence of an antecedent history of cardiovascular disease, would affect measurements derived from PWA. It was demonstrated that severely obese OSA patients have increased arterial stiffness, and that OSA severity was significantly associated with arterial stiffness at baseline. Therefore, OSA may potentially influence cardio-metabolic risk, in severely obese patients even without a history of cardiovascular problems. This has implications for the clinical assessment of severely obese patients. Early testing of symptomatic individuals for OSA may be important so that treatment in the form of CPAP may be offered and applied to relieve daytime sleepiness.

In this study, OSA and non-OSA patients were not deliberately matched for every characteristic as this would have restricted eligible patients who were willing to participate, potentially introducing a selection bias; and it was important to try to recruit as many patients as possible for both groups. Furthermore, the OSA status of patients could only be known after they had their sleep studies, thus making direct matching of patients less practicable.

The second aim of the arterial stiffness studies was to investigate the changes in arterial stiffness and other indices of pulse wave analysis following CPAP in the severely obese cohort at follow-up. In the second study, significant improvements in arterial stiffness were found at follow-up in CPAP-treated severely obese patients relative to subjects without OSA. However, this remained elevated in those compliant with CPAP treatment relative to non-OSA patients. CPAP treatment in severe obesity was associated with a greater reduction in arterial stiffness compared to the non-OSA patients. Nevertheless, CPAP treatment did not completely reverse the increased arterial stiffness in patients with severe obesity and OSA to levels found in the non-OSA group.

With the lack of a placebo CPAP group, it was not possible to make inferences on cause-effect and to demonstrate that the observations were indeed CPAP effects. However, it was envisaged that conducting such a placebo-controlled randomised trial over this length of time would be questionable from an ethical point of view given the duration of follow-up. The study subjects who were evaluated all volunteered to undergo treatment in a clinical setting and the aim was to investigate the changes in real-life settings in order for the findings to be clinically relevant. Repeat polysomnography was not conducted to prove if the AHI was sufficiently reduced on CPAP but the patients were managed according to local 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.

At follow-up, the change in arterial stiffness was evaluated in a cohort of patients with severe obesity over a relatively long period of time (~13 months), which is a strength of the study, and the findings represent an important addition to the literature given the limited knowledge available relating to subjects with severe obesity. This is relevant given the growing prevalence of severe obesity that is a major public health concern in many nations.

In relation to the use of PWA for both studies, the assessment of arterial stiffness may be performed non-invasively using PWA or pulse-wave velocity (PWV). Although PWV is the gold standard for measuring arterial stiffness (Laurent *et al*., 2006), the choice of radial tonometry using PWA over PWV was a pragmatic decision and based on previous work that highlighted challenges in measurements at the carotid and femoral arteries (Bakker *et al*., 2011). The use of PWV in patients with abdominal obesity may make distance measurements difficult with inaccuracies in pressure waveform recordings (Laurent *et al*., 2006); and the technique is hindered by the intimate nature of femoral pulse acquisition. Nevertheless, there may be limitations with the use of PWA at the radial pulse in relation to the determinants of Aix such as age, height, vascular properties, use of brachial artery pressures for calibration of central pressures (Laurent *et al*., 2006).

There is evidence that severe OSA in patients without known cardiovascular disease is associated with arterial stiffness and cardiac remodelling (Drager *et al*., 2007b). Although cardiac catheterisation and echocardiography would have provided further precise information regarding circulatory and structural changes, the use of PWA provided a validated and reproducible non-invasive approach (Wilkinson *et al*., 1998), to explore the use of this tool to aid risk assessment and to further our understanding of OSA in severe obesity.

Although a causal link between OSA and arterial stiffness could not be established, as measured by PWA, it was demonstrated that arterial stiffness may potentially be a useful early marker of cardiovascular disease risk in this patient group. Given that CPAP significantly predicts changes in arterial stiffness in severe obesity, it would be important to encourage its use in these patients with OSA. However, there is also a need to consider a multifaceted approach when managing such patients in order to ameliorate their cardiovascular risk.

There may be a role for CPAP and other treatment modalities such as lifestyle interventions that may have an effect on arterial stiffness. Further studies will be required to investigate how a combination of lifestyle changes with and without CPAP, may influence arterial stiffness and the potential mitigating effects on cardiovascular risk. Furthermore, all subjects in the studies were of white European ethnicity and it would be important to explore arterial stiffness changes in severe obesity in other ethnic groups.

OSA & Urate

In the work done to investigate serum urate in severe obesity in OSA, the aims were firstly, to explore whether the presence of OSA was associated with serum urate, and secondly, to determine whether use of CPAP treatment in OSA was associated with a fall in serum urate. An association between OSA and urate in severely obese females was found. Furthermore, a trend towards a fall in urate levels in OSA patients treated with CPAP was observed. Thus there may be a need to consider OSA in severely obese subjects who have hyperuricaemia or recurrent gout as there may be a possible role for CPAP in influencing urate levels.

The enzyme xanthine oxidase may have a role in cardiovascular dysfunction in OSA (Dopp et al., 2007). Vascular remodelling in OSA has been linked with mechanisms including increased sympathetic activity, oxidative stress and vascular inflammation (Kohler and Stradling, 2010). In relation to oxidative stress in particular, it has been suggested that episodes of intermittent hypoxia result in the production of superoxide radicals by xanthine oxidase in the vascular endothelium, that may influence nitric oxide bioavailability and consequently impair vasodilation (Price et al., 2000) (Dopp et al., 2011). It has been previously shown in rodents that exposure to intermittent hypoxia impairs vasodilation in skeletal muscle vasculature and these changes may be prevented with allopurinol (Dopp et al., 2011). A RCT was performed in humans that compared allopurinol in relation to placebo demonstrated that allopurinol improves endothelial dysfunction as assessed using hyperaemia-induced flow mediated vasodilation in patients with moderate-to-severe OSA (El Solh et al., 2006). Although these studies did not show a reduction in oxidative stress per se, they suggest a role for xanthine oxidase in intermittent hypoxia in OSA that may lead to cardiovascular effects. Taken together, it may be speculated that there may be a link between urate, OSA and cardiovascular disease.

It was not possible to make inferences on cause-effect and to demonstrate that the observations at follow-up were indeed CPAP effects. However, a randomised controlled trial using sham/placebo CPAP over a prolonged duration such as in this study would have raised ethical concerns. Although there was a trend for an association between CPAP and fall in urate that approached significance, this may have been attributable to the small patient numbers at follow-up. The numbers of patients at follow-up was determined by the patient numbers who were assessed at baseline as it was important to follow the original cohort over a period of time (at least 12 months). Even though it may have been possible to recruit additional patients at follow-up, these individuals would have had

shorter periods of follow-up, potentially confounding the findings. Nevertheless, the findings will need to be explored further in future studies with larger sample sizes.

The work performed investigated the effects of CPAP on urate levels. The subjects who participated did not have a history of gout and were not on allopurinol treatment. It would be interesting to explore the effects of CPAP on urate levels in OSA patients with previous known gout or recurrent gout episodes who are on allopurinol treatment, in order to study the effects of xanthine oxidase inhibition (allopurinol) and the treatment of intermittent hypoxia (CPAP). It would also be important to understand the effects of a combination of different modalities of treatment for patients with hyperuricaemia with OSA that includes lifestyle and weight loss, where appropriate, in combination with CPAP, that may have a synergistic effect on serum urate levels in severely obese populations or patients with recurrent gout. These will also need to be explored in future work.

Questionnaire on Assessment of OSA in diabetes clinics

The objective of this study was to obtain an understanding of current practice in relation to the International Diabetes Federation (IDF) 2008 recommendations with regards to the assessment of OSA in patients in UK diabetes clinics. This was the first study to study the impact of the IDF statements in diabetes clinical practice in the UK. By understanding current practice, it was hoped that awareness of the importance of OSA could be increased through the publication of the findings. The key finding was that the majority (approximately two-thirds) of the diabetes healthcare professionals who responded to the survey were not aware of the IDF recommendations, and most participants indicated that their local diabetes guidelines did not incorporate assessment for OSA in those deemed to be at risk. This was rather disappointing given that the recommendations were released in 2008. However, given the limitations attributable to the use of questionnaires, as highlighted in chapter 5, the findings only pertain to those who responded to the questionnaire, and it is not possible to make definitive conclusions concerning clinical practice as a whole. Nevertheless, the findings did suggest that it was important to encourage more awareness of the importance of OSA assessment in high-risk patients with type 2 diabetes.

Diabetes clinics and not weight management clinics were chosen for the study because of the close association of type 2 diabetes and obesity, and the IDF recommendations have placed an emphasis on patients with type 2 diabetes. From my clinical experience, it is usual clinical practice to assess patients for OSA in weight management clinics, working in tandem with the local sleep service. Therefore, it was important to consider how OSA was evaluated in diabetes clinics.

Proteomics studies

In the studies that were undertaken in urinary proteomics, the aims were firstly, to undertake discovery profiling of urinary peptides using capillary electrophoresis-mass spectrometry (CE-MS) in severely obese subjects with and without OSA. Secondly, to characterise the urinary proteome in severely obese adult subjects with OSA receiving CPAP compared with severely obese subjects without OSA. These studies in urinary proteomics are the first to demonstrate the urinary proteome in adult OSA and severe obesity with and without CPAP, compared with severely obese subjects without OSA. Using CE-MS, in the first study, 24 peptides were found to be statistically different between the OSA and non-OSA groups prior to adjustment for multiple testing. The sequences for 8 of these peptides were identified that comprised collagen alpha chain subtypes and fibrinogen. However, post-hoc correction for multiple testing did not provide sufficient evidence to indicate a significant difference in peptide profiles between the groups. In the second study, after a median follow-up period of 15 months, a urinary peptide pattern of 15 peptides was identified that were differentially distributed between the OSA on CPAP group and the non-OSA group, with significant unadjusted Wilcoxon p-values, though these differences did not reach significance when multiple testing was accounted for. Sequences for 8 of these peptides that comprised breakdown products of collagen, as well as fibrinogen and a cadherin subtype were identified. The trends in the peptide panels in both studies suggest that there may be inherent differences between OSA and non-OSA groups, even with a period of effective CPAP treatment.

Since it was expected that multiple hypotheses (>1000 peptides) would be tested for, correction for multiple testing was performed to control for the false discovery rate. The Benjamini-Hochberg test (Benjamini and Hochberg, 1995) has been shown to best conserve statistical power in the context of multiple testing of proteomics data sets (Dakna et al., 2010). One possible explanation for the non-significance after correction for multiple testing in both studies may be ascribed to individual heterogeneity in the groups that were studied. Despite matching of multiple variables, there were more OSA subjects with metabolic syndrome and high blood pressures in the first study, as well as metabolic syndrome in the second study. Clearly, exact matching of the groups (for example, by excluding patients with metabolic syndrome) would have made recruitment difficult, and a pragmatic approach was necessary so that the patients mirrored those seen in real life situations.

Although single proteomic biomarkers generally do not allow for the sufficient accuracy for the detection of pathophysiology (Albalat et al., 2011), it is important to point out that the peptide

classes found in both proteomic studies were generally similar in class (collagen peptide fragments and fibrinogen in the first study) and (collagen peptide fragments, fibrinogen-beta chain peptide and T-Cadherin in the second study). These urinary peptides may be related to changes associated with underlying cardiovascular disease. Therefore, it may be speculated that the urinary proteome in OSA may be mediated by changes in cardiovascular risk.

The urinary proteome in coronary artery disease has been previously characterised using CE-MS (Zimmerli et al., 2008) (Delles et al., 2010). In that study, the panel comprised 15 peptides, including five peptides that were identified as collagen type fragments (Zimmerli et al., 2008). In the proteomic studies in this thesis, collagen fragments were also consistently found in the peptide profiles before and after CPAP that had significant unadjusted p-values. The other peptides were also associated with altered vascular function. Collagen fragments may be markers of collagen synthesis and turnover and it is possible that altered vascular function, for example in atherosclerosis, may increase synthesis of collagen and other structural peptides. One exclusion criteria in these studies was a history of coronary artery disease but the proteomic profiles in the subjects with OSA and OSA with CPAP compared with non-OSA patients suggests that patients with OSA may be at greater risk of altered vascular function, which may be linked with the mechanistic pathways that stem from intermittent hypoxia and periodic arousals. An alternative explanation may be the presence of heightened cardiovascular risk given that there were more OSA subjects who had metabolic syndrome. Therefore, it would be important to identify if there are any associations with the mechanisms linked with cardiovascular disease in OSA such as oxidative stress, endothelial dysfunction or inflammation markers. This will need to be investigated in studies in severely obese subjects with OSA, with and without cardiovascular disease.

In the first study, the subjects with OSA were analysed as a whole and were not stratified according to the severity of their OSA. Given the heterogeneity of the patient population, in the first instance, it was important to explore the urinary proteome in all subjects with OSA and without OSA, with any observed differences potentially attributable or reflecting the presence OSA. In this regard, it was felt that a peptide panel as a potential biomarker of disease would first have to distinguish the presence or absence of OSA first before disease stratification studies could be carried out. It was felt that this method of analysis would be relevant to common clinical practice, for example, when deciding which patients to send for further sleep assessment. Although it is plausible that a peptide panel may vary according to the severity of the condition, this would ultimately depend on the nature of the disease and the peptides identified. Based on the novel findings from the two

proteomic studies, it would be important to determine whether the peptide patterns vary according to OSA severity, and how this is influenced by CPAP in further studies.

In the second proteomic study, subjects with OSA on CPAP and non-OSA subjects were studied. The control group was not 'OSA subjects who were not on CPAP' because the numbers in this group were too small (n=5) for meaningful proteomic comparisons. Furthermore, it would not have been ethically possible to withhold CPAP treatment to the OSA subjects for such a long time (>1 year). It should be noted that during the design of the study protocol, this group was considered for analysis if substantial numbers were present. This 'third arm' however yielded too few subjects. Likewise, a 'fourth arm' was also anticipated during the design of the study, that comprised subjects who did not have sufficient hours of CPAP use per night (non-compliers) but again, this group did not have sufficient numbers (n=4) for meaningful proteomic analysis. Therefore, the comparison was between OSA subjects on CPAP and non-OSA subjects. This comparison may also be justified given the evidence of renal effects associated with OSA and the use of CPAP in potentially mitigating these effects, and this allowed the characterised urinary proteome to be compared with the peptide pattern from the first study.

It was important to ascertain if the urinary proteome in OSA with CPAP was similar to non-OSA that may suggest 'reversibility' of changes in urinary proteome. The differential urinary profiles between severely obese OSA vs non-OSA groups in the first study, and between severely obese CPAP-treated OSA vs non-OSA groups in the second study suggest that CPAP treatment may potentially produce urinary proteome changes associated with OSA but may not necessarily alter the peptide profile to a pattern that is comparable to severely obese non-OSA subjects. It is suggested that this is likely to be related to underlying cardio-metabolic changes in OSA and the presence of coexisting conditions such as metabolic syndrome which was more common in the OSA groups in both studies.

In chapter 1, the concept of research into novel tools to assist in the early identification and treatment of OSA was introduced. One of the original questions that arose during the design of the proteomic studies was whether CE-MS could distinguish OSA from non-OSA subjects. The findings from the two proteomic studies do not provide sufficient evidence to indicate that CE-MS can distinguish OSA from non-OSA, or OSA on CPAP from non-OSA. Nevertheless, these are novel findings. Moreover, the significant differences evidenced by the unadjusted p-values and the nature of peptide profiles identified in both studies suggest that there is a potential role for CE-MS in the

study of urinary profiles in OSA; and these urinary panels may be related to mechanisms involved in cardiovascular function.

The results of the analysis over time profiles support further research using CE-MS, with a fewer number of significantly different peptides in non-OSA subjects (51 peptides) over time relative to the OSA patients before and after CPAP (183 peptides), it is conceivable that inherent differences in the urinary proteome exist between OSA patients before and after treatment over time. But it remains to be elucidated whether the changes in peptide expression do indeed represent a cause or a consequence of OSA and/or CPAP. Although this work has been a step forward in sleep-disordered breathing research, the proteomic findings illustrate the challenges of urinary biomarker discovery that may be influenced by patient heterogeneity with diverse phenotypes in OSA. It is conceivable that there may be changes in gene expression that may translate into actual changes in proteins that may evolve over the course of disease and exposure to CPAP - for example, obesity-related genes may have potential to impact on the pathogenesis of OSA (Polotsky and O'Donnell, 2007). Even so, there will be obese individuals who do not have OSA. Thus there is a need for further systematic analyses to decipher mechanisms at both genomic and proteomic levels.

These are still early days in the study of urinary proteomics in adult OSA and severe obesity. Definitive conclusions concerning the feasibility of its translation to real life OSA practice can only be made with further research, in particular, the need for validation of the findings in independent test sets, which is an important step in the process towards identification and qualification of proteomic biomarkers in order to ascertain usefulness in clinical practice (Mischak et al., 2010). In this context, the identification of ubiquitous urinary peptide panels present in obesity and OSA remains an exciting concept because it may potentially help define patients and mechanisms that may be amenable to target for treatment.

In previous studies that examined the urinary proteome in paediatric OSA, several proteins were identified with different functions. These included orosomucoid (acute phase protein), kallikrein-1 (vasodilation) and a phorbol ester–inducible co-activator (signalling protein)(Gozal et al., 2009b); gelsolin (actin modulating protein) and heparan sulphate proteoglycans (component of glycosaminoglycans linked with urinary tract disease)(Krishna et al., 2006); and urocortins (activated in stress responses) (Snow et al., 2010b). These proteins may be markers of paediatric OSA but are relatively non-specific. The findings from the paediatric OSA studies were different from the adult OSA peptides identified in this thesis possibly because of differences in proteomic methods used and

because of differences in populations and context at the time of testing (Becker et al., 2014). In this regard, the effects of aging on morphological change on the kidneys may influence the proteome (Albalat et al., 2011). Interestingly, in a CE-MS study of the urinary proteome with aging, it was found that many urinary markers changed significantly during puberty, and 49 peptides were found to represent aging-related peptide excretion patterns (Zurbig et al., 2009). Therefore, it is possible that paediatric OSA and adult OSA urinary proteomes may yield different peptides.

Future directions

Important lessons have emerged from the studies in this thesis that provide a platform for further research. For the first time, the urinary proteome has been characterised in adult OSA and severe obesity, with and without CPAP. The next steps would be to validate the findings in larger studies with independent test sets and to relate the findings with mechanistic pathways that may lead to cardio-metabolic dysregulation. For example, the urinary proteome could be studied in relation endothelial dysfunction using high-resolution ultrasonographic measurements of the brachial artery at rest, during reactive hyperaemia (increased flow causing endothelium-dependent dilatation), and after sublingual glyceryl trinitrate administration (causing endothelium-independent dilatation) (Celermajer et al., 1992). The studies on urinary proteomics and arterial stiffness may also be investigated in the context of assessment of sympathetic nerve activity and this may be evaluated using microelectrode measurements at the muscle (Ogawa et al., 2003) (Grassi et al., 2014).

As the observed associations between OSA and its vascular effects may be complicated by the presence of obesity (Kohler and Stradling, 2010), therefore, it would be important to investigate the use of CE-MS in characterising the urinary profiles of non-obese subjects with and without OSA, before and after CPAP to determine the effects of being lean on the urinary proteome; and to study if there is a change in urinary proteome in severely obese patients with OSA before and after bariatric surgery, that may be compared with the findings from this work. These studies would potentially allow the effects of obesity on the proteome to be investigated.

One area that could potentially be explored would be the role of urinary proteomics in other sleep – breathing disorders such as obesity hypoventilation syndrome (OHS). In OHS and obesity, chronic daytime alveolar hypoventilation develops from an interaction between sleep-disordered breathing, diminished respiratory drive, impaired respiratory mechanics, abnormal central ventilatory control, sleep-disordered breathing and neurohormonal changes (leptin resistance or insulin resistance) may occur (Piper and Grunstein, 2011) (Marik, 2012). Patients typically have chronic CO_2 retention and

compensated respiratory acidosis on blood gas (Marik, 2012). Patients with OHS were not studied in the studies in this thesis primarily because these patients tended to be very ill with multiple comorbidities such as chronic obstructive pulmonary disease that did not meet recruitment criteria, with poor performance levels, and were unlikely to cope with the study protocol. There were also difficulties in identifying suitable OHS patients for recruitment because many had commenced non-invasive ventilation (NIV) and would not have been naïve to treatment at baseline. Nevertheless, it would be interesting to evaluate this group of patients in relation to pulse wave analysis and urinary proteomics, perhaps in those who are already on NIV, as hypoxia-related disorders such as pulmonary hypertension and cor pulmonale are seen in many patients (Piper, 2011).

A question that has not been explored in this thesis has been the role of non-alcoholic fatty liver disease (NAFLD) in OSA. This was because liver disease was a component of the exclusion criteria for the studies, and patients with known liver pathology were not recruited in order to avoid potential confounding effects of liver disease on the urinary proteome. NAFLD refers to hepatic damage ranging from simple steatosis, steatohepatitis, advanced fibrosis and cryptogenic cirrhosis (Farrell and Larter, 2006). OSA is associated with an increased risk of NAFLD (Musso et al., 2013). OSA and NAFLD are associated with obesity (Mirrakhimov and Polotsky, 2012) (Farrell and Larter, 2006); and with insulin resistance, hyperlipidaemia and cardiovascular disease (Farrell and Larter, 2006). Studies have investigated the possible role of OSA as a risk factor for liver injury and in the progression of NAFLD (Mirrakhimov and Polotsky, 2012). Prevalence estimates of obesity and NAFLD are 30-100%, and the prevalence of type 2 diabetes and NAFLD is 10-75% (Byrne et al., 2009). At present, the prevalence of OSA in NAFLD is unknown (Mirrakhimov and Polotsky, 2012). However, the evidence from studies suggests that it may be common (Turkay et al., 2012) (Minville et al., 2014). In a study by Singh et al (2005), almost half of the NAFLD patients who completed a Modified Berlin Sleep Apnoea Questionnaire had symptoms suggestive of OSA (Singh et al., 2005). More recently, in a study of 226 subjects who were referred for assessment for OSA, more than half (61%) had moderate or severe steatosis; and a relationship between the severity of nocturnal hypoxia and liver injury was found in severe obesity (Minville et al., 2014). Intermittent hypoxia has been associated with NAFLD in severe obesity (Aron-Wisnewsky et al., 2012); and hence a potential mechanism that may be related to cardio-metabolic risk (Pepin et al., 2012). Therefore, it will be important to explore the urinary peptide profiles in severely obese OSA subjects with NAFLD, with and without CPAP. Such studies may potentially incorporate imaging techniques, such as magnetic resonance imaging and proton magnetic resonance spectroscopy that may be used to assess for hepatic steatosis (van Werven et al., 2010).

Relevance to clinical practice

The focus of this thesis has been on characterising various aspects of OSA in people with severe obesity, before and after treatment with CPAP**. Figure 7.1** below was introduced in Chapter 1. The common unifying theme that links the studies has been the importance of OSA in relation to cardiovascular risk **(Figure 7.1)**. It should be noted that there were differences in follow-up durations for the proteomics studies (15 months) and arterial stiffness studies (13.5 months). This is because of the different patient numbers in both sets of studies with the arterial stiffness studies excluding subjects with hypertension.

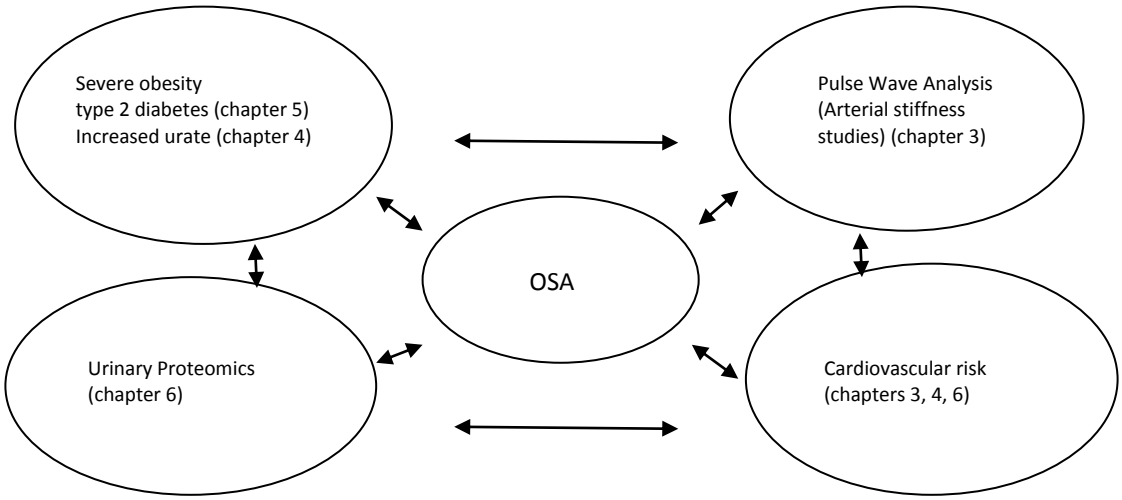


Figure 7.1 How the work in this thesis is linked. In chapter 1, the main themes pertaining to the work in this thesis were introduced. The main findings suggest an underlying link with vascular change and cardiovascular risk. Although OSA has been placed in the centre, it must be emphasised that all circles are equally important. The arterial stiffness work (Chapter 3) highlights the importance of assessing for OSA in severe obesity because of possible effects on arterial stiffness relating to cardiovascular risk. The urate study (Chapter 4) suggests that CPAP treatment may potentially influence urate levels in severe obesity, and hence there may be a need for assessment of OSA in patients with hyperuricaemia. The questionnaire study (Chapter 5) suggested that more work needs to be done to try to increase awareness of the importance of assessment for OSA in type 2 diabetes. Finally, given the multifaceted nature of obesity and OSA, and the complex heterogeneity of patients, urinary proteomic profiling was performed (chapter 6) as a possible approach to try to identify urinary factors integral to both conditions, that suggested links with altered vascular function.

In the context of clinical practice, the assessment and treatment for OSA is often incorporated into the patient assessments when they attend the weight management clinic. It is acknowledged that there will be differences in individual responses to CPAP, with some who may decline CPAP, due to

reasons of non-tolerability and perceived lack of effect. Although this may not be ideal, it should nevertheless be remembered that the clinical management of severely obese patients also includes other multidisciplinary approaches including lifestyle interventions, dietary methods, behavioural therapy, pharmacotherapy and surgical interventions. Each method would allow goals to be set for weight management that has the potential to improve glycaemic control and cardiovascular risk (Inzucchi et al., 2012). Other benefits would include an improved quality of life, confidence and self-esteem; and reduced risk of potential complications relating to obesity such as cardiovascular disease.

For individuals who are not attending a weight management service, it will also be important to consider assessment for OSA where clinically appropriate and to try to halt the progression or worsening of their obesity with different options for weight management; for example, dietician advice or lifestyle changes and physical activity, and medication. Hence there is a need to place an emphasis on a holistic approach when caring for patients with obesity.

Conclusion

These are exciting times for obesity and OSA research. The work in this thesis has contributed to our understanding of the effects of OSA in severe obesity. It has explored arterial stiffness, the role of urate and applied a novel basic science approach using capillary electrophoresis-mass spectrometry, to explore the potential of urinary peptide patterns in characterising OSA in severely obese patients. Pertinent lessons have been learnt that have shown how cardiovascular risk may be increased in severe obesity and OSA. More importantly, the work contributes to the way forward, to direct further research in our efforts to improve health outcomes and lives of our patients.

Publications, abstracts and presentations in support of this work

1. Serum urate and obstructive sleep apnoea in severe obesity.
Seetho IW, Parker RJ, Craig S, Duffy N, Hardy KJ, Wilding JP, Goodson NJ
Chronic Respiratory Disease. 2015 May 19. pii: 1479972315586197. [Epub ahead of print]

2. Effect of CPAP on arterial stiffness in severely obese patients with obstructive sleep apnoea.
Seetho IW, Asher R, Parker RJ, Craig S, Duffy N, Hardy KJ, Wilding JPH.
Sleep & Breathing 6 Feb 2015. http://dx.doi.org/10.1007/s11325-015-1131-0.

3. Sleep-disordered breathing, type 2 diabetes and the metabolic syndrome.
Seetho IW, Wilding JP.
Chronic Respiratory Disease 2014 Nov;11(4):257-75. doi: 10.1177/1479972314552806. Epub 2014 Oct 3.

4. Urinary proteomics in obstructive sleep apnoea and obesity.
Seetho IW, Siwy J, Albalat A, Mullen W, Mischak H, Parker RJ, Craig S, Duffy N, Hardy KJ, Burniston JG, Wilding JP.
European Journal of Clinical Investigation 2014 Nov;44(11):1104-15. doi: 10.1111/eci.12346.

5. Obstructive sleep apnoea in diabetes - assessment and awareness
Seetho IW, O'Brien SV, Hardy KJ, Wilding JPH.
British Journal of Diabetes & Vascular Disease 2014; 14(3):105-108.

6. Obstructive sleep apnea is associated with increased arterial stiffness in severe obesity.
Seetho IW, Parker RJ, Craig S, Duffy N, Hardy KJ, Wilding JP.
Journal of Sleep Research 2014 Dec;23(6):700-8. doi: 10.1111/jsr.12156. Epub 2014 Apr 15.

7. Screening for obstructive sleep apnoea in obesity and diabetes--potential for future approaches.
Seetho IW, Wilding JP.
European Journal of Clinical Investigation 2013 Jun;43(6):640-55. doi: 10.1111/eci.12083. Epub 2013 Apr 16.

Presentations

1. Urinary Proteomics in Obstructive Sleep Apnoea with Obesity
Oral presentation at the European Congress of Obesity, 28-31 May 2014, Sofia, Bulgaria

2. Urate is associated with Obstructive Sleep Apnoea in obese women
Poster presentation at the British Society of Rheumatology 2014, Liverpool, UK

3. Arterial Stiffness is associated with Obstructive Sleep apnoea in severe obesity
Poster presentation at the European Congress of Obesity 2013, Liverpool, UK

Appendix 1

Epworth Sleepiness Scale

Epworth Sleepiness Scale

The Epworth Sleepiness Scale is used to determine the level of daytime sleepiness. A score of 10 or more is considered sleepy. A score of 18 or more is very sleepy. If you score 10 or more on this test, you should consider whether you are obtaining adequate sleep, need to improve your sleep hygiene and/or need to see a sleep specialist. These issues should be discussed with your personal physician.

Use the following scale to choose the most appropriate number for each situation:

0 = would *never* doze or sleep.
1 = *slight* chance of dozing or sleeping
2 = *moderate* chance of dozing or sleeping
3 = *high* chance of dozing or sleeping

Print out this test, fill in your answers and see where you stand.

Situation	**Chance of Dozing or Sleeping**
Sitting and reading	_____
Watching TV	_____
Sitting inactive in a public place	_____
Being a passenger in a motor vehicle for an hour or more	_____
Lying down in the afternoon	_____
Sitting and talking to someone	_____
Sitting quietly after lunch (no alcohol)	_____
Stopped for a few minutes in traffic while driving	_____
Total score (add the scores up) (This is your Epworth score)	_____

Appendix 2
Chapter 5 questionnaire responses as analysed by role of participant & location

Location of work and role of participants

Cons DGH	9
Cons Uni Hosp	15
Registrar DGH	10
Registrar University Hosp	14
DSN DGH	1
DSN University Hospital	4
DSN GP Practice	2
AHP University Hospital	3
AHP DGH	2
AHP GP Practice	2
total	62

Responses to Q1 Awareness of IDF guidance to screen for diabetes in OSA

Q1	**Yes**	**No**	**Don't Know**	**Skipped**
Cons DGH	5	2	2	0
Cons Uni Hospital	7	3	4	1
Registrar DGH	1	7	1	1
Registrar Uni Hosp	2	5	7	0
DSN DGH	0	0	1	0
DSN Uni Hosp	1	2	1	0
DSN GP Prac	1	0	1	0
AHP Uni Hosp	0	2	1	0
AHP DGH	2	0	0	0
AHP GP Prac	0	2	0	0
total	19	23	18	2

Responses for Q 2 Awareness of IDF guidance to screen at-risk patients with diabetes & obesity for OSA

Q2	Yes	No	Don't Know	Skipped
Cons DGH	5	2	2	0
Cons Uni Hosp	4	5	5	1
Registrar DGH	2	6	2	0
Registrar Uni Hosp	4	6	4	0
DSN DGH	0	0	1	0
DSN Uni Hosp	2	1	1	0
DSN GP Prac	2	0	0	0
AHP Uni Hosp	0	2	1	0
AHP DGH	2	0	0	0
AHP GP Prac	0	1	1	0
total	21	23	17	1

Responses for Q 3 Do local diabetes guidelines recommend OSA screening in diabetes patients at-risk of OSA?

Q3	Yes	No	Don't Know	Skipped
Cons DGH	1	5	3	0
Cons Uni Hosp	1	9	5	0
Registrar DGH	2	6	2	0
Registrar Uni Hosp	5	4	5	0
DSN DGH	0	1	0	0
DSN Uni Hosp	0	2	2	0
DSN GP Prac	1	0	1	0
AHP Uni Hosp	1	0	2	0
AHP DGH	1	0	1	0
AHP GP Prac	0	1	1	0
total	12	28	22	0

Responses for Q 4 Are patients with diabetes suspected of OSA investigated by the diabetes team?

Q4	Yes	No	Don't Know	Skipped
Cons DGH	2	6	0	1
Cons Uni Hosp	1	14	0	0
Registrar DGH	0	8	2	0

Registrar Uni Hosp	3	8	2	1
DSN DGH	0	1	0	0
DSN Uni Hosp	1	1	2	0
DSN GP Prac	0	0	2	0
AHP Uni Hosp	0	0	3	0
AHP DGH	0	1	1	0
AHP GP Prac	0	1	1	0
total	7	40	13	2

Responses for Q 5 Are patients with diabetes suspected of OSA investigated by the respiratory team?

Q5	**Yes**	**No**	**Don't Know**	**Skipped**
Cons DGH	7	2	0	0
Cons Uni Hosp	13	0	2	0
Registrar DGH	9	0	1	0
Registrar Uni Hosp	12	0	2	0
DSN DGH	1	0	0	0
DSN Uni Hosp	2	0	2	0
DSN GP Prac	1	0	1	0
AHP Uni Hosp	1	0	2	0
AHP DGH	1	0	1	0
AHP GP Prac	1	0	1	0
total	48	2	12	0

Key: DGH: District General Hospital, Cons: Consultant, Uni Hosp: University Hospital, AHP: allied health professional, GP Prac: GP Practice, DSN: Diabetes Specialist Nurse.

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