# DOCTOR OF PHILOSOPHY

## Factors associated with the severity of Apnoea Hypopnoea Index (AHI) in Obstructive sleep apnoea (OSA)

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# FACTORS ASSOCIATED WITH THE SEVERITY OF APNOEA HYPOPNOEA INDEX (AHI) IN OBSTRUCTIVE SLEEP APNOEA (OSA)

by

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## Declaration

This work has not been previously accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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*This thesis was supervised by Dr Hans-Peter Kubis and Dr Jonathan Moore of the School of Sport, Health and Exercise Sciences, Bangor University and Dr Damian McKeon Respiratory Consultant at Ysbyty Gwynedd and Honorary lecturer at the School of Medical Sciences, Bangor University and Cardiff University.*

**Summary**

Obstructive sleep apnoea (OSA) is the most prevalent sleep disorder, characterised by repetitive episodes of complete or partial obstructions of the upper airway during sleep. These apnoeic related events have been associated with intermittent hypoventilation, hypoxemia, hypercapnia, recurrent arousals in sleep and the activation of the sympathetic nervous system. Questions arise over the feasibility of a risk factor intervention strategy in reducing the incidence of mild to moderate OSA. Currently the only adequately supported intervention is weight loss. The long term objective of this thesis will be to guide the design of future interventions which are focussed on the specific symptomatology of OSA.

The general theme of this thesis is to identify which physiological factors most strongly contribute to the pathogenesis of OSA. A particular interest is first given to the potential effects of the exposure to intermittent hypercapnia and hypoxia during sleep, while newly developed techniques for assessment of chemosensitivity towards carbon dioxide ($CO_2$) and oxygen ($O_2$) was piloted in scuba divers and controls. Following this, the implications of the baroreflex-chemoreflex interactions are assessed before reviewing inflammatory markers present within the patients with OSA. Finally, with the understanding that the occlusive airway and subsequent apnoeas associated with OSA may lead to increased inspiratory efforts whereby the inspiratory muscles are overloaded during sleep, alongside the environment of nocturnal bouts of hypoxia and hypercapnia and systemic inflammation, the prevalence of inspiratory muscle fatigue in OSA is also investigated.

The general introduction **(Chapter 1)** provides the background information and proposes the aims of the research presented in the thesis. Following this, the first study **(Chapter 2)** investigates the ventilatory response to $CO_2$ amongst scuba divers using a novel methodology. Different populations have displayed altered ventilatory responses to $CO_2$; scuba divers are an example in a healthy population. Investigating scuba divers enabled us to develop and test methodology designed to assess the relative contribution of adaptations to the peripheral and/or central chemoreceptors to their ventilatory response to $CO_2$. Their ventilatory response

was also compared to the patients with OSA in chapter 3. The same methodology was then applied in the first OSA Study **(Chapter 3)** to assess the ventilatory response amongst patients with OSA with the theory that the exposure to intermittent hypercapnia and hypoxia during apnoeic related events may cause a similar modification in the ventilatory response seen in scuba divers. The third study **(Chapter 4**) assessed the implications of baroreflex sensitivity on the severity of OSA and the ventilatory response to $CO_2$ observed in the third chapter, to increase our understanding of the strength of the baroreflex-chemoreceptor interaction previously reported in the research literature. To increase our knowledge of the inflammatory processes involved in the pathogenesis of OSA, the forth study **(Chapter 5)** investigates cytokines related to obesity through the quantification of adiponectin, c-reactive protein, leptin and the endocannabinoids (2-arachidonoylglycerol and arachidonoylethanolamide) on the severity of OSA. The endocannabinoids have been shown to mediate anti-inflammatory properties in addition to playing a significant role in the regulation of energy metabolism within adipose tissue. The final study **(Chapter 6)** assesses the neuromuscular properties of the breathing apparatus with particular interest in studying the fatigability of the inspiratory muscles. This chapter involves the development of an entirely novel protocol which is designed to elicit inspiratory muscle fatigue through submaximal loading. The final chapter **(Chapter 7)** integrates the findings of all the studies to propose a novel regression model which can be designed to predict the severity of OSA from the physiological processes investigated.

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I would first like to express my gratitude to my supervisor and friend, Dr Hans-Peter Kubis who I met back in 2004. Since then he has supervised me through the completion of three successful projects including an undergraduate and master's dissertation and now this PhD thesis. In each of those projects he has captivated me with his enthusiasm for research in the field of Physiology. It is through his patience, expertise and high teaching standards that I have developed this fascination for the subject.

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## Publications

I was involved in all aspects of design, data collection, data analyses and preparation of manuscripts for publication and the following thesis chapters. Additionally, I gratefully acknowledge input from the other named authors for each publication. The following is a list of publications arising from the material presented in this thesis.

**Full papers:**

Earing, C.M.N, McKeon, D.J. and Kubis, H-P. (2014). Divers revisited: The ventilatory response to carbon dioxide in experienced scuba divers. *Respiratory Medicine*, 108, 758-765.

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Gwynedd BSAC Annual General Meeting. (2013) $CO_2$ and the Scuba Diver

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## List of Abbreviations

| | |
|---|---|
| 2-AG | 2-arachidonoylglycerol |
| 5-HT | 5-hydroxytryptamine |
| AASM | American Academy of Sleep Medicine |
| AEA | Arachidonoylethanolamide |
| AHI | Apnoea hypopnoea index |
| ANCOVA | Analysis of Covariance |
| ANOVA | Analysis of variance |
| APAP | Auto-titrating continuous positive airway pressure |
| BE | Base Excess |
| BiPAP | Bi-level positive airway pressure |
| BRS | Baroreflex sensitivity |
| BSA | Body surface area |
| BTPS | Body temperature and pressure saturation |
| CB | Cannabinoid receptor |
| CBD | Carotid body denervated |
| CCHS | Congenital central hypoventilation syndrome |
| CPAP | Continuous Positive Airway Pressure |
| CRP | C-reactive protein |
| CSA | Central sleep apnoea |
| ELISA | Enzyme-linked immunosorbent assay |
| FAAH | Fatty acid amidohydrolase |
| $FEV_1$ | Forced Expiratory Volume in one second |
| FVC | Forced vital capacity |
| HCO3std | Standard bicarbonate |
| HDL | High density lipoprotein |
| HIF | Hypoxia inducible factor |
| IL-1 | Interleukin 1 |
| IL-6 | Interleukin 6 |
| $IP_{14}$ | Inspiratory pressure at a RPE of 14 |
| IPAQ | International Physical Activity Questionnaire |
| LC-MS | Liquid chromatography–mass spectroscopy |

| | |
|---|---|
| LDL | Low density lipoprotein |
| MSNA | Muscle sympathetic nerve activity |
| NF-κB | Nuclear factor kappa B |
| NREM | Non-rapid eye movement |
| NTS | Nucleus of the solitary tract |
| ODI | Oxygen Desaturation Index |
| OEA | Oleylethanolamide |
| OSA | Obstructive Sleep Apnoea |
| $PaCO_2$ | arterial $pCO_2$ |
| $PaO_2$ | Arterial $pO_2$ |
| PAP | Positive Airway Pressure |
| PAV | Proportional assist ventilation |
| $P_{crit}$ | Critical pressure |
| $Pdi_{max}$ | Maximal transdiaphragmatic pressure |
| PEA | Palmitoylethanolamide |
| PHD | Prolyl-hydroxylases |
| PHOX2B | Paired-like Homeobox 2B |
| PiMax | Maximal inspiratory pressure |
| PPARγ | peroxisome proliferator-activated receptor |
| RDI | Respiratory disturbance index |
| RERA | Respiratory effort-related arousal |
| ROS | Reactive oxygen Species |
| ROS | Reactive oxygen species |
| RPE | Rating of perceived effort (or exertion) |
| RTN | Retrotrapezoid nucleus |
| RVLM | Rostral ventrolateral medulla |
| THBC | Total haemoglobin |
| THC | Delta (9)-tetrahydrocannabinol |
| TNFα | Tumor necrosis factor alpha |
| TRAF6 | Receptor-associated factor 6 |
| VIF | Variance Inflation Factor |
| VR1 | Vanilloid receptor type 1 |

# Chapter 1 General Introduction:

## Definition:

Obstructive sleep apnoea (OSA) is a chronic condition characterised by partial or complete narrowing of the upper respiratory airways (pharyngeal airway) during sleep associated with concomitant hypoxic and hypercapnic episodes related to the cessation of ventilation (Carter & Watenpaugh 2008, Punjabi 2008). Clinically, OSA is defined by the occurrence of daytime sleepiness, loud snoring, witnessed breathing interruptions, or awakenings due to gasping or choking in the presence of at least five obstructive respiratory events per hour of sleep. Alternatively, the presence of fifteen or more obstructive respiratory events per hour of sleep in absence of sleep related symptoms can be applied (Epstein et al. 2009).

## Epidemiology:

OSA is the most common sleep disorder (Al Lawati, Patel & Ayas 2009) conservatively estimated to affect 5% of the general population (Young, Peppard & Gottlieb 2002), with moderate to severe symptoms found in 9% of middle-aged men and 4% of women (Al Lawati, Patel & Ayas 2009). Certain subgroups of the population are known to be of an increased risk of developing OSA with factors involving age, gender, obesity, family history, menopause, craniofacial abnormalities and certain health related behaviours such as cigarette smoking and alcohol use (Punjabi 2008). The diagnosis of OSA is time consuming, labour intensive and costly, so it is not surprising the majority of people affected remain undiagnosed (Punjabi 2008, Young, Peppard & Gottlieb 2002).

## Diagnosis and symptoms of OSA:

Presently, the standard diagnostic test for sleep apnoea involves an overnight polysomnogram which involves simultaneous recordings of multiple physiological signals during sleep (Punjabi 2008). Two respiratory events are associated with OSA, hypopnoeas and apnoeas. The latest rules for the scoring of respiratory events in adults during sleep as defined by the American Academy of Sleep Medicine (AASM) defines a hypopnoea as a peak signal excursion drop by ≥30% of pre-event baseline using nasal pressure or an alternative sensor with ≥3% arterial oxygen desaturation or an arousal. An apnoea on the other hand, is scored when there is a drop in peak signal excursion by ≥ 90% of the pre-event baseline using an

oronasal thermal sensor or alternative apnoea sensor. Both types of event must last for a duration of at least 10 seconds (Berry et al. 2012).

Different forms of sleep apnoea also exist. OSA is where there is an absence in air flow despite respiratory muscle movement, whereas central sleep apnoea involves a complete cessation of air flow accompanied by a lack of respiratory muscle movement. Finally, mixed sleep apnoea involves a combination of OSA and central apnoeic related events being observed. The scoring of hypopnoeas as either obstructive or central is now listed as optional by the AASM task force (Berry et al. 2012).

Typically, the severity of OSA is assessed using the apnoea-hypopnoea index (AHI) which is the amount of apnoeas and hypopnoeas per hour of sleep (Epstein et al. 2009, Berry et al. 2012). To be classified as a mild OSA patient, an AHI of ≥5 and <15 with reported symptoms related to sleepiness is present. To be classified as a moderate OSA patient, a recording of AHI ≥15 and <30 is required. A severe OSA patient has an AHI ≥30 (Epstein et al. 2009). An alternative classification of the degree of severity of sleep apnoea is using the respiratory disturbance index (RDI); this is similar to AHI however also considers the number of respiratory effort-related arousal (RERA) episodes. RERA episodes are sequences of breaths characterised by increased respiratory effort or flattening of the nasal pressure waveforms leading to arousal from sleep but does not meet the criteria of an apnoea or hypopnoea (Iber et al. 2007). Many sleep centres including Ysbyty Gwynedd do not score RERAs (Berry et al. 2012). As with AHI, a RDI≥5 and <15 with reported symptoms related to sleepiness is defined as mild OSA whereas moderate OSA a RDI ≥15 and <30 is present and in severe OSA a RDI ≥30 is required (Iber et al. 2007).

The main symptoms of OSA are reduced nocturnal sleep quality causing excessive daytime sleepiness, reduced neurocognitive function, impaired work performance along with decrements in health-related quality of life and increased likelihood of cardiovascular disease and motor vehicle accidents (Punjabi 2008, Young, Peppard & Gottlieb 2002, Young et al. 2009, Tregear et al. 2009).

**Possible causes of OSA:**

*Collapsibility of the upper airway:*

The collapsibility of the retropalatal and retroglossal regions of the pharynx in OSA has often been assessed by measuring critical closing pressure (Lurie 2011a). This is a concept which arises from modelling the oropharynx as shown in figure 1-a. The model is based on the Starling resistor model. Ernest Starling was a British physiologist who during his heart-lung preparation connected the aorta to a thin-walled collapsible tube traversing into a chamber which had the pressure surrounding the tube controlled. This device became known as a Starling resistor and with the work of Banister and Torrance (1960) and Permutt et al. (1962) later it became apparent that the Starling resistor may serve as a model to explain some of the pressure-flow relationships of pulmonary circulation (Lopez-Muniz et al. 1968). The model has since been applied to the upper airway of patients with OSA  it involves a collapsible segment which is subjected to surrounding or critical pressure ($P_{crit}$) that governs its collapsibility (Schwartz et al. 2010).

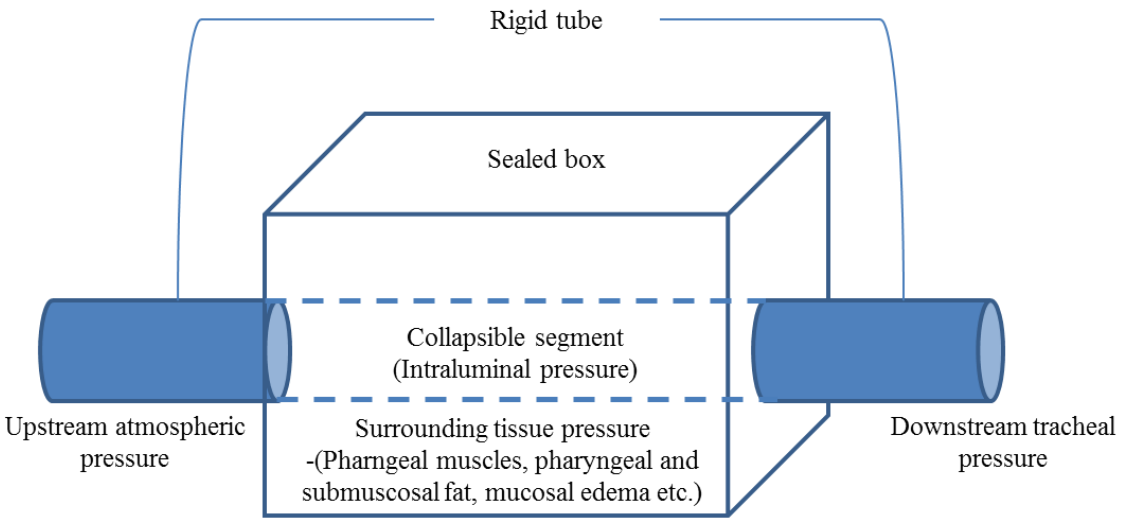


**Figure 1-a.** using the Starling resistor model to explain upper airway collapsibility in OSA modified from Lurie (2011b). Once downstream (e.g. epiglottic or tracheal pressure at the thoracic inlet) and intraluminal pressures in the collapsible midsection drops below the surrounding tissue pressure, flow limitation occurs (Owens et al. 2014).

The model provides a mechanism that has been useful in explaining snoring, hypopnoeas and obstructive apnoeas and the benefit of CPAP in the treatment of OSA (in CPAP the flow is increased by raising the upstream pressure positive to the atmospheric pressure) (Owens et al. 2014). Recently however, Owens et al. (2014) has been critical of relating the upper airway to the classical Starling resistor model stating that if the upper airway does behave as the resistor model suggests, then flow-limited inspiratory airflow should be relatively constant, i.e. have a characteristic "flattened" pattern when the downstream pressure falls below $P_{crit}$. However airflow traces from clinical sleep studies often show an initial peak before the plateau. Owens et al. (2014) compared the pressure-flow curves for slow vs. fast reductions in downstream pressure. It was reported that the initial peak is not transient but a distinct mechanical property of the upper airway. In reality, in contrast to the single peri-airway pressure portrayed in the classical Starling resistor model, the upper airway is more complex with multiple elements involved (Owens et al. 2014).

Based on the Starling model, the collapsibility due to mechanical properties is termed passive $P_{crit}$ and this can be measured in conditions of no neuromuscular control such as in anesthetized patients (Lurie 2011a, Eastwood et al. 2002). There is also a large but not complete reduction in neuromuscular control during sleep (Patil et al. 2007). Active $P_{crit}$ on the other hand, represents the sum of passive $P_{crit}$ and the neuromuscular response to the passive structural/anatomical loads (Lurie 2011a). Patil et al. (2007) findings suggest that increased airway mechanical loads and blunted neuromuscular responses are both required in the development of OSA. The effect of obesity on mechanical loads and the interaction with compensatory neuromuscular responses may explain the increased prevalence of OSA associated with obesity (Horner 2007, Young, Peppard & Taheri 2005)

*Anatomy of the airway and implications of increased body mass:*

Walsh et al. (2008) emphasises that the understanding of the pharyngeal morphology of OSA is advantageous, as it bears directly on the pathogenesis of the condition, on its clinical assessment and on the likely efficiency of surgical and non-surgical treatments. Narrowed pharyngeal airways which are more susceptible to collapse with the presence of a sleep-related loss of the compensatory dilator muscle activity have been demonstrated in patients with OSA (Walsh et al. 2008, Pillar & Shehadeh 2008). Along with size, shape of the airway may also be considered an important contributor (Pillar & Shehadeh 2008). Walsh et al. (2008) evaluated the shape and size of the pharyngeal airways using anatomical optical

coherence tomography, concluding individuals with OSA have a smaller velopharyngeal cross sectional area but similar shape than BMI, gender and age matched controls, suggesting an abnormality in size rather than shape is the most important predictor of OSA.

Almost all cross-sectional clinical and population-based studies have found significant associations between OSA and measures of excess body weight (Young, Peppard & Gottlieb 2002). BMI, visceral fat and neck circumference are seen as major predictors of OSA (Carter & Watenpaugh 2008). It has been proposed that males could exhibit a 2 to 4 fold greater risk compared to females of developing OSA due to their increased disposition of adipose tissue around the neck and abdomen (Carter & Watenpaugh 2008). Simpson et al. (2010) investigated the effects of regional fat distribution on the severity of OSA in both genders using traditional anthropometric measures and dual-energy absorptiometry. It was found in females' fat around the neck region and BMI together explained 33% of the variance in AHI, while in males the percentage of fat in the abdominal region and neck to waist ratio together accounted for 37% of the variance in AHI.

The major respiratory complications of obesity include heightened demand for ventilation, elevated work of breathing and decreased functional residual volume and expiratory reserve volume (Parameswaran, Todd & Soth 2006). A reduction in lung volume has also been shown to further increase the collapsibility of the upper airway (Tagaito et al. 2007) and OSA severity (Heinzer et al. 2006). Some studies have also reported altered muscle structure due to fatty deposits within the muscles lining the upper airways (Pillar & Shehadeh 2008, Whittle et al. 1999, Carrera et al. 2004).

The potential affects of fat mass on the anatomy of the upper airway structures may cause disturbance of the balance between respiratory drive and load compensation (Young, Peppard & Gottlieb 2002). The apnoeic related events are associated with hypoventilation, hypoxemia and hypercapnia (Cooper et al. 2005). Its possible exposure to periods of intermittent hypercapnia and hypoxia during sleep may alter the ventilatory response to hypercapnia and hypoxia.

*Alteration of the ventilatory response to hypoxia and/or hypercapnia:*

Ventilation is controlled via an important feedback control system, consisting of the central and peripheral chemoreceptors. These work to keep $PaCO_2$ and plasma pH remarkably constant with the presence of normal kidney function (Miyamoto et al. 2004, Ogoh et al. 2008). Based on the reaction theory for ventilatory control, $CO_2$ stimulates the central chemoreceptors through liberating $H^+$ ions resulting in decreased pH (Loeschcke 1982). Based on this theory, the central chemoreceptors detect the resulting acidosis and respond by increasing ventilation to help maintain arterial $pCO_2$ within a few mm Hg of the steady-state (~40 mm Hg) regardless of the metabolic production of $CO_2$ and level of vigilance (Nattie & Li 2009, Nattie 1999, Feldman, Mitchell & Nattie 2003, Guyenet, Stornetta & Bayliss 2010). It is recognised that the carotid bodies (peripheral chemoreceptors) are polymodal receptors that are responsible for detecting a variety of circulating stimuli including $O_2$, $CO_2$ and $H^+$ ions in addition to $K^+$, noradrenaline, temperature, osmolality, glucose and insulin (Dempsey & Smith 2014, Kumar & Bin-Jaliah 2007). The peripheral chemoreceptors particularly respond to hypoxia when $PaO_2$ falls below 70 mm Hg (Nattie 2006). It is now more widely accepted that the peripheral and central chemoreceptors do not act as entirely separate entities but interact with each other. The stimulation of the peripheral chemoreceptors enhances the slope of the central $CO_2$ ventilatory response and inhibition of the carotid bodies reduces the slope of the central $CO_2$ response (Dempsey & Smith 2014, Blain et al. 2010).

The frequent exposure to nocturnal bouts of hypoxia and hypercapnia during apnoeic related events has been implicated to induce alterations in the response of the central and peripheral chemoreceptors (Cooper et al. 2005). One theory is this is a progressive adaptation involving "resetting" of the receptors of the integrative neurons in the brainstem to a different sensitivity threshold (Verbraecken et al. 1995, Guilleminault & Cummiskey 1982). It is also plausible that there is a pathogenic role of inflammation which mediates the upregulation of the renin-angiotensin system in the carotid body causing over activity in the chemoreflex (Fung, Tipoe & Leung 2014, Lam et al. 2012).

Other populations have displayed altered ventilatory responses to $pCO_2$ which have been attributed to frequent exposure to $CO_2$. Examples of a healthy population include breath hold divers and scuba divers (Florio, Morrison & Butt 1979, Kerem, Melamed & Moran 1980). In terms of clinical populations abnormal breathing patterns, with $CO_2$ retention during waking and especially in sleep has been documented in neurodegenerative diseases such as

Parkinson's disease, amyotrophic lateral sclerosis, post-polio syndrome with bulbar involvement and multiple system atrophy All have been linked to deficits in neurons within the pre-Bötzinger complex, pontine raphe and adjacent areas (Dempsey & Smith 2014, Schwarzacher, Rüb & Deller 2011). These are all areas believed to play roles in chemoreception, which are discussed in more detail in the introduction of the second chapter. Patients with Chronic Obstructive Pulmonary Disease (COPD) have also been reported to have an attenuated ventilatory response to hypercapnia/hypoxia (Fahey & Hyde 1983, Xu et al. 2007). Unfortunately however in COPD it is difficult to determine whether the reduced ventilatory response is due to impaired respiratory central drive, as the ventilatory response is correlated with the mechanical limitations of COPD (Xu et al. 2007, Cherniack & Snidal 1956).

An alteration in the ventilatory response may cause an increase in the collapsibility of the upper airway. An increase in the ventilatory drive would activate the upper airway muscles and promote patency whereas a reduction in ventilatory drive would relax the upper airway muscles and facilitate closure (Wellman et al. 2008). Other researchers also speculate that a heightened responsiveness may contribute to respiratory control instability potentially leading to periodic breathing and further airway obstruction via increased loop gain whereas blunted responsiveness may prolong apnoea duration due to lowering of the arousal reflexes (Verbraecken et al. 1995). The concept of loop gain is explained in greater detail in the introduction of the third chapter.

Studies which have investigated the ventilatory response to hypercapnia and/or hypoxia in patients with OSA have found inconsistent results (Sin, Jones & Man 2000, Radwan et al. 2000). Such studies are summarised in table 1-a.

**Table 1-a**. Summary of studies which have investigated the ventilatory response in OSA

| Study | Sample size | Inclusion criteria | Methodology | Main Findings |
|---|---|---|---|---|
| Foster et al. 2009 | 8 patients with OSA; 10 gender matched controls. | 18-50 years of age, BMI <35 kg/m$^2$, no prescribed medications or history of diabetes/cardio-respiratory disease. | Ventilatory response to $CO_2$ assessed before and after 4-6 weeks of CPAP. Dyanmic end-tidal forcing used to allow for 10 minutes of isocapnic euoxia followed by euoxic hypercapnia for 20 minutes. | Patients with OSA had normal ventilatory responses to hypercapnia. |
| Trombetta et al. 2013 | 24 patients with OSA and metabolic syndrome; 22 patients with metabolic syndrome; 11 age matched controls. | No prescribed medications or cardiovascular disease, no history of smoking or excessive alcohol consumption. | Isocapnic hypoxia (10% $O_2$/$N_2$). The central chemoreflex was assessed by 7% $CO_2$/93% $O_2$. | Patients with OSA and metabolic syndrome had increased ventilatory response to $CO_2$ but not hypoxia. |
| Narkiewicz et al. 1999 | 16 patients with OSA; 12 age and body mass matched controls. | Normotensive, not on any medications, free of other diseases. | Isocapnic hypoxia (10% $O_2$/$N_2$). The central chemoreflex was assessed by 7% $CO_2$/93% $O_2$. | Only the hypoxic response is increased in patients with OSA. |

| | | | | |
|---|---|---|---|---|
| Gold et al. 1993 | 35 normocapnic sleep apneoa patients; 17 age, body mass matched controls | Not on a prescribed diet. | Progressive hyperoxic hypercapnia was used using a modification of the Reads rebreathing method. Patient breathed from an $O_2$ filled 7-L bag with 6% $CO_2$. | Lower ventilatory response to $CO_2$ in sleep apneoa patients. |
| Verbraecken et al. 1995 | 14 patients with OSA; 11 hypercapnic patients with OSA; 11 normocapnic overlap patients; 14 controls. | FEV ≥ 84% of predicted and TLC ≥90% of predicted. | Progressive hyperoxic hypercapnia using Reads rebreathing method involving a small rebreathing bag (4-6 litres) with a gas mixture of 7% $CO_2$ in $O_2$. | Depressed ventilatory response to $CO_2$ only in hypercapnic patients with OSA. |
| Sin et al. 2000 | 115 patients with OSA defined as AHI >15; 104 controls defined as AHI <15. | Not taking any hypnotics and major tranquilizers. | Modification of Reads rebreathing method 5.5% $CO_2$ in $O_2$. | OSA not associated with blunted ventilatory response to $CO_2$. |

The conflicting findings between studies may be the result of the variability of the inclusion/exclusion criteria and methodology applied between different studies. OSA is heavily associated with the development of numerous comorbidities with 60% of patients with metabolic syndrome also experiencing OSA (Drager et al. 2010, Trombetta et al. 2013, Trombetta et al. 2010). Foster et al. (2009) recruited 8 men with OSA and found the ventilatory, cerebrovascular and cardiovascular responses to hypercapnia were normal. In Foster et al. (2009) publication it is questioned whether their sample size was sufficient to reveal whether there is a change in the ventilatory response. The low recruitment of the study is likely due to the strict inclusion criteria used the patients were 18-50 years of age, BMI <35 $kg/m^2$, not prescribed any medications and had no history of diabetes or cardio-respiratory disease including hypertension (defined as blood pressure >140/90 mm Hg).Upon personal experience of patients attending sleep clinics in both Ysbyty Gwynedd, Wales and the Countess of Chester Hospital, England patients with OSA which meet all the inclusion criteria used in Foster et al. (2009) study are very rarely seen in clinic.

Knowing which conditions are a consequence of OSA and which conditions OSA contributes to the development of is debatable (Pillar & Shehadeh 2008) and therefore justification for inclusion and exclusion criteria is difficult. Hypertension (Trzebski et al. 1982), obesity (Burki & Baker 1984) and age (Kronenberg & Drage 1973) have all been shown to significantly influence chemoreflex sensitivity and the effects of treatment with medications are unpredictable. Furthermore, undiagnosed OSA and misdiagnosis can lead to apparently normal control subjects which may inadvertently affect results (Narkiewicz et al. 1999).

Another reason for the conflicting findings between studies may be due to the different methodologies used. Sin et al. 2000 for example investigated the ventilatory response using the closed-circuit rebreathing technique of Read but with a lower 5.5% $CO_2$ in $O_2$ instead of 7% $CO_2$. Additionally, in Sin et al. (2000) study, an AHI ≥15 was used to dichotomize between OSA from those without OSA. We feel any adaptation occurring to the ventilatory response to $CO_2$ may occur early in the pathogenesis of OSA. Our previous research with healthy populations assessed the ventilatory response to $CO_2$ in experienced scuba divers. As with other studies with scuba divers, no correlation was found between the lowered ventilatory response to $CO_2$ and the number of dives previously performed suggesting that the adaptation may have occurred early on in the participation of scuba diving or the response is

inherit with those who are less prone to hypercapnia related symptoms staying in the diving population (Florio, Morrison & Butt 1979, Kerem, Melamed & Moran 1980, Froeb 1961). Our previous investigations with healthy non-divers and scuba divers also identified 6% inspired $CO_2$ should be used to identify whether the ventilatory response has been altered in scuba divers. The previously mentioned studies start $CO_2$ rebreathing at high concentrations which may not allow enough time between the accumulations of $CO_2$ to assess the ventilatory response appropriately in patients with OSA. Furthermore the concentration of $CO_2$ used may be too high in that it results in strong simulation of ventilation in individuals who have a low sensitivity to $CO_2$.

Previous research with patients with OSA has not tested the interaction between the central and peripheral chemoreceptors when attempting to investigate the ventilatory response to $CO_2$. This is possibly because it was previously not well acknowledged. It is now widely recognised that the peripheral and central chemoreceptors do not act as entirely separate entities but interact with each other (Dempsey & Smith 2014, Duffin 2007). Therefore Duffin (2007) recommends in order to investigate the contribution of the peripheral chemoreceptors on the ventilatory response, the $CO_2$ concentration of interest should be compared at a high constant $O_2$ tension (isoxic hyperoxic ventilatory response to $CO_2$) and a low constant $O_2$ tension (isoxic hypoxic ventilatory response to $CO_2$). The hyperoxic response measures the central chemoreflex whereas the hypoxic response measures the sum of the central and peripheral responses. The contribution of the peripheral chemoreflex is represented by the difference between the responses. That said, there is evidence which does observe that when carotid body denervation is performed the hypoxic ventilatory response is eliminated (as expected) but the central hyperoxic $CO_2$ response is also markedly depressed (Dempsey & Smith 2014, Dahan, Nieuwenhuijs & Teppema 2007, Rodman et al. 2001).

The development of leptin resistance is regarded as a leading cause in the onset of obesity (Koch et al. 2014). Produced in the white adipose tissue, leptin is an adipokine which was initially considered just to reduce food intake and increase energy expenditure (Koch et al. 2014, Friedman & Halaas 1998). In rodents it has been shown that leptin prevents respiratory depression (O'donnell et al. 1999) and prolonged treatment with leptin attenuates respiratory complications associated with the obese phenotype (Tankersley et al. 1998).

In studies with rodents the importance of leptin has been vividly demonstrated by the profound obesity exhibited by the ob/ob mouse (C57BL/6J $Lep^{ob}$) which are unable to

produce functional leptin, unlike the wildtype mouse or the obese db/db mouse (diabetic) which are leptin deficient (O'Donnell et al. 2000). In humans it is likely the relationship between leptin and respiratory control is more complicated than the initial observations in ob/ob mice. Furthermore, it has been proposed that the relationship between leptin and the control of the muscles of the upper airway warrants further investigation however caution is required when applying the results of studies with leptin and mice. This is because the cross-sectional area of the upper airway of a mouse may produce mechanical stability, since according to the law of Laplace, the wall tension required to maintain a given transmural pressure decreases proportional to the radius. Additionally, the neuronal circuitry controlling the upper airway collapsibility or $CO_2$ retention and the distribution of leptin receptors in humans in areas of the central nervous system (CNS) may also be different to mice (O'Donnell et al. 2000).

Phipps et al. (2002) suggests leptin may be one of many predictors for hypercapnia in the obese population with hyperleptinaemia found to be associated with hypercapnic respiratory failure in obese humans. Furthermore, hyperleptinaemia has been found to be associated with a reduction in respiratory drive and hypercapnic response, irrespective of the amount of body fat in obese participants (Redolfi et al. 2007).

Some studies investigating patients with OSA have reported higher circulating leptin levels compared to BMI-matched control subjects (Ip et al. 2000, Phillips et al. 2000, Kapsimalis et al. 2008) with the severity of nocturnal hypoxemia associated with leptin levels independent of obesity (Kapsimalis et al. 2008). Although Redolfi et al. (2007) observed that in a small number of patients with hypoventilation syndrome without OSA (n= 6) there was an increase in leptin levels following non-invasive ventilation (Redolfi et al. 2007).

Some authors do however still consider the higher levels of leptin to be mostly related to obesity rather than OSA (Barceló et al. 2005) with some studies finding no significant association between OSA and leptin levels after controlling for body fat and/or BMI (Schäfer et al. 2002, Patel et al. 2004). CPAP treatment has been shown to reduce circulating leptin levels in patients with OSA despite unchanged BMI during the study period (Ip et al. 2000, Harsch et al. 2003). Although decreased leptin levels with CPAP have only been reported in non-obese patients with OSA (Barceló et al. 2005) or to be more pronounced in patients with OSA and a BMI <30 $kg/m^2$ (Harsch et al. 2003, Lurie 2011b).

*Alteration in baroreflex sensitivity in the development of OSA:*

The arterial baroreceptors are mainly located in the carotid sinuses and aortic arch, they are mechanoreceptors innervated by the glossopharyngeal and vagus nerves (Cortelli et al. 2012). Baroreflex sensitivity refers to the response in heart beat interval to a change in blood pressure expressed in ms/mm Hg (Westerhof et al. 2004). Baroreflex sensitivity is an important mechanism in the regulation of arterial blood pressure (Freet, Stoner & Tang 2013) and its evaluation is regarded as an established tool for the assessment of autonomic control (La Rovere, Pinna & Raczak 2008). A reduction in arterial baroreflex sensitivity has been associated with increased sympathetic nerve activity (Grassi et al. 1998) and higher blood pressure (Trombetta et al. 2010, Wustmann et al. 2009).

There is little doubt that considerable interaction exists between the chemoreceptors and the baroreceptor reflexes (Cooper et al. 2005, Somers, Mark & Abboud 1991). Chemoreflex activation elicits an increased vascular sympathetic outflow via the efferent limb of the reflex arc causing blood pressure to rise due to increased systemic vascular resistance (Olson & Somers 2013). On the other hand, activation of the arterial baroreceptors has an inhibitory influence on the chemoreflex. However, this can be impaired such as in conditions like heart failure which can lead to a counterproductive cycle of increased sympathetic activity resulting in augmented peripheral chemoreflex sensitivity and further increased sympathetic outflow (Olson & Somers 2013, Heistad et al. 1972).

As previously mentioned, Trombetta et al. (2013) reported an increased ventilatory response to hypercapnia in patients with OSA with comorbid metabolic syndrome. It has been suggested this may in part be explained by an increase in the sympathetic peripheral and central chemoreflex because greater muscle sympathetic nerve activity (MSNA) was observed. This study did not measure baroreflex sensitivity so could not observe whether a reduction in arterial baroreflex sensitivity may also contribute to the resulting chemoreflex-mediated sympathetic outflow in the patients. In a previous study, however, Trombetta et al. (2010) did find MSNA is inversely associated with arterial baroreflex sensitivity in patients with metabolic syndrome with comorbid OSA. Increased blood pressure and sympathetic drive was found in patients with OSA and metabolic syndrome which was theorised to be linked at least in part to the diminished baroreflex sensitivity also reported.

Patients with OSA have a greater risk of a number of cardiac pathologies including heart failure (Malone et al. 1991, Johnson et al. 2008), coronary artery disease (Mooe et al. 2001) and stroke (Dyken et al. 1996). Data from a previous study known as the Akershus sleep apnea project (n= 514) has recently demonstrated an independent association between circulating cardiac troponin concentrations (a marker of myocardial injury) and OSA (Einvik et al. 2014). Recently OSA and depression has been reported to be independently associated with refractory angina in patients with coronary artery disease (Geovanini et al. 2014). Furthermore, a large multicentre observational study known as the Sleep and Stent Study with a recruitment target of 1600 patients is currently underway. With the results expected to be presented in 2016, this study aims to assess the effects of severity of OSA on cardiovascular outcomes in patients treated with a percutaneous coronary intervention (Loo et al. 2014).

Cross sectional studies have demonstrated that the prevalence of hypertension increases with the severity of OSA (Grote, Hedner & Peter 2001, Bixler et al. 2000). One of the largest cross-sectional studies to date is the Sleep Heart Health Study which consisted of 6132 participants. AHI was associated with markers of hypertension among normal and overweight individuals of both sexes and in young and older age groups (Nieto et al. 2000). A depressed baroreflex sensitivity has been observed in patients with severe OSA especially in stage two of non-rapid eye movement (NREM) sleep and during nocturnal wakefulness with a significant improvement following 6 weeks of CPAP therapy (Ryan et al. 2007). Furthermore, this finding is supported by a randomised controlled trial which found daytime baroreflex sensitivity is significantly increased in patients treated with therapeutic CPAP compared to subtherapeutic CPAP (Kohler et al. 2008). Investigating children with OSA Crisalli et al. (2012) found an improvement in baroreflex sensitivity following adenotonsillectomy during both sleep and wakefulness.

*Implications of inflammation on the development of OSA*

It has been recognised that intermittent hypoxia may contribute to the comorbidities associated with OSA including hypertension, obesity, dyslipidemia, insulin resistance (Lavie 2009), diabetes (Punjabi et al. 2004) and metabolic syndrome (Trombetta et al. 2013). It is believed that the development of systemic inflammation plays a key role in the pathogenesis of metabolic dysfunction though the precise mechanisms are not completely understood (Drager, Jun & Polotsky 2010).

Inflammation potentially plays a large role in the pathogenesis of OSA through a number of mechanisms. Firstly intermittent hypoxia which occurs as a result of apnoeic events has been shown in cell culture models to lead to a selective and preferential activation of inflammatory pathways mediated by the transcription factor, nuclear factor kappa B (NF-κB) (Ryan, Taylor & McNicholas 2005). NF-κB serves as a key component in the regulation of inflammatory cytokines involved in the development of various conditions such as atherosclerosis and insulin resistance (Lurie 2011b) and has been reported to play a dual role in the modulation of cell apoptosis (Abe 2007, Han et al. 2013). Secondly, the intermittent changes in blood oxygen saturation levels in OSA have been considered similar to the hypoxia and reoxygenation demonstrated in conditions characterised by ischemia and reperfusion associated with increased production of reactive oxygen species (ROS) (Lavie 2009). Although this may be seen as controversial as some studies have failed to demonstrate increased oxidative stress with OSA (Oztürk et al. 2003, Wali et al. 1998), ROS are normal by-products of cellular metabolism, which when overproduced, overwhelms antioxidant capabilities relating to pathogenic oxidative stress and inhibition of cellular mechanisms and cellular injury (Valko et al. 2007). It has been speculated that an increased production of ROS may trigger expression of multiple proinflammatory genes via activation of the oxidant-sensitive transcription factor NF-κB (Htoo et al. 2006).

Adipose tissue is now regarded as one of the main sources of inflammatory mediators. In particular circulating levels of interleukin 6 (IL-6) secreted from adipose tissue is the most strongly correlated to adiposity and type 2 diabetes of all the cytokines (El-Kadre & Tinoco 2013). IL-6 along with interleukin 1 (IL-1) regulates at the post –transcriptional level CRP production (Artemiou et al. 2012).

CRP is an acute-phase reactant synthesised by the liver (Lurie 2011b) which is associated with an increased risk of atherosclerosis and cardiovascular disease though its relative importance has been questioned in a large study (Danesh et al. 2004). Moreover, low-grade systemic inflammation as observed by elevated CRP levels has been suggested as one potential mediator of insulin resistance in OSA (Kokturk et al. 2005, Hargens et al. 2013, Kelly et al. 2010). Studies assessing the relationship between OSA severity and CRP have found conflicting results. Whilst some research have revealed an independent association from body mass parameters between the severity of OSA and CRP (Yokoe et al. 2003, Lui et al. 2009, Guven et al. 2012), other studies have found CRP production is more related to

obesity than OSA (Akashiba et al. 2005, Ryan et al. 2007). Arnardottir et al. (2012) studied patients with moderate to severe OSA (n = 454) from five sites in Iceland (the Icelandic Sleep Apnoea Cohort) and found the association of OSA and the inflammatory biomarker CRP depends on obesity. A correlation between OSA severity, CRP and IL-6 levels was only found in obese males with a BMI$\geq$ 30 kg/m$^2$.

The effects of CPAP treatment on CRP are also unclear. CPAP has been shown to cause a reduction in CRP levels independent of BMI (Yokoe et al. 2003) however these findings have been challenged by studies reporting no reduction in CRP levels (Akashiba et al. 2005, Ryan et al. 2007). The effects of CPAP cessation has also been investigated with Phillips et al. (2007) studying the effects of short term (1 week) withdrawal of CPAP finding a marked increase in sympathetic activity without concomitant elevation of CRP and other vascular inflammatory markers.

As previously mentioned, the relationship between leptin concentration and OSA remains to be clarified (Lurie 2011b). Secreted from the adipose tissue, leptin has also been associated with atherosclerosis (Konstantinides et al. 2001), as well as thrombosis (Bodary et al. 2002) and hypertension (Rahmouni et al. 2005). It has been suggested that a relationship exists between tumor necrosis factor alpha (TNFα) and leptin production (Kirchgessner et al. 1997, Fawcett et al. 2000) which may provide a mechanism by which TNFα can modulate inflammation (Pickup, Chusney & Mattock 2000).

The adipose tissue also releases adiponectin, an insulin sensitising hormone which decreases hepatic glucose output and increases fatty acid oxidation by the muscle (Kelly et al. 2010). Adiponectin protects against chronic inflammation with reduced adiponectin levels being related to increased endothelial inflammatory responses, the presence of coronary heart disease, dyslipidemia, insulin resistance and type 2 diabetes in humans (Wolk et al. 2005). Despite being produced in adipose tissue plasma, adiponectin levels have been found to be decreased with obesity (Arita et al. 1999, Yang et al. 2001). The mechanisms of this paradoxical finding are unknown but because there is a high prevalence of obesity in sleep apnoea, it has been theorise OSA may influence adiponectin level. It has been reported adiponectin concentrations in patients with sleep apnoea are lower than in normal subjects (Wolk et al. 2005). Furthermore, Kelly et al. (2010) has revealed in obese pubertal children OSA severity is negatively associated with adiponectin levels even after adjustment for BMI.

Many studies have used different durations of CPAP treatment to demonstrate an increased adiponectin concentration following CPAP intervention (Nakagawa et al. 2008, de Lima et al. 2010, Carneiro et al. 2009). A randomised controlled trial revealed no change in adiponectin levels following 3 months of CPAP compared to sham CPAP treatment (West et al. 2007) and similar results were found in another randomised controlled study which used 4 weeks follow up from either therapeutic or sub-therapeutic levels of CPAP (Kohler et al. 2009). It has though been acknowledged that many studies with negative findings have had participants with very poor CPAP compliance (a use of less than 4 hours a night) or have not monitored or reported CPAP compliance information (Lurie 2011a).

A key discovery enhancing our understanding of the control of adipose tissue and in particular the regulation of energy metabolism within adipose tissue, was finding the expression of functional cannabinoid receptors in the adipocytes which were up-regulated during adipogenesis (André & Gonthier 2010, Matias et al. 2006, Roche et al. 2006). Briefly, the endocannabinoids are produced on demand and consist of cannabinoid receptors, the fatty acid signalling molecules that bind to and activate these receptors, and enzymes that synthesize and catabolise the endocannabinoid receptors (Crowe et al. 2014). For a more detailed description of the endocannabinoid system, the reader is directed to the introduction section of chapter 5. The cannabinoids have received much greater research attention in the recent years, due to development of genetic research models and highly selective pharmaceutical tools and greater appreciation of their effects on pain, inflammation, emotion, memory, sleep and metabolic function among other physiological processes (Crowe et al. 2014).

The endocannabinoids are generally regarded as having anti-inflammatory properties (Crowe et al. 2014). *In vivo* changes in endocannabinoid concentrations have been observed in inflammation related pathologies. Higher 2-arachidonoylglycerol (2-AG) and arachidonoylethanolamide (AEA) concentrations have been found in animal models of several neuropathology's that include an inflammatory component such as multiple sclerosis (mice model (Baker et al. 2001)) and Parkinsons disease (non-human primate model (Stelt et al. 2005)).

To date, only two studies have investigated the influence of OSA on the circulating endocannabinoids. In Engeli et al. (2012) the OSA group were found to have significantly higher concentration of endocannabinoids and AEA was found to positively correlated with

RDI. Additionally Engeli et al. (2012) reported the nightly decrease in mean oxygen saturation in the patients with OSA correlated with all three endocannabinoids tested. However, after adjustments for BMI, waist circumference, body mass, fasting insulin and glucose, and glucose infusion rate, all three correlations with the endocannabinoids were diminished. Jumpertz et al. (2010) also reported no significant difference in endocannabinoids between sleep apnoea patients and controls after adjustment for confounders including BMI, fasting insulin, high density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol. Jumpertz et al. (2010) however did find a significantly higher oleylethanolamide (OEA) concentration in patients with sleep apnoea which remained significant after adjustment for BMI, fasting insulin, LDL and HDL cholesterol and after direct comparison with BMI matched groups. OEA is a cannabinoid receptor-inactive and biosynthetically related congener of AEA (Côté et al. 2007). OEA in cerebrospinal fluid has been shown to be elevated in volunteers following 24 hours of sleep deprivation (Koethe et al. 2009). Unfortunately Jumpertz et al. (2010) does not make any description as to whether the sleep apnoea group consisted of predominately central or obstructive sleep apnoea.

*Alterations in inspiratory muscle function in OSA:*

Referring back to Starling's model illustrated in Figure 1-a it has been theorised that increased airway mechanical loads and blunted neuromuscular responses are both required in the pathogenesis of OSA (Patil et al. 2007). Furthermore, it has been reported the inspiratory efforts generated at the end of apnoeas are often very large, such that when transdiaphragmatic pressure and the tension-time index of the diaphragm are determined, end-apnoeic values in some patients approach or surpass the threshold of fatigue described in normal subjects (Vincken et al. 1987, Kimoff et al. 1994, Montserrat et al. 1997). Moreover, these efforts are also occurring under conditions of hypoxemia, hypercapnia and declining cardiac output (Garpestad et al. 1992).

Whether hypercapnia impairs respiratory muscle function is debatable (Jonville, Delpech & Denjean 2002) but acute hypoxia is associated with inflammation and the presence of obesity is associated with low grade systemic inflammation (Petelin et al. 2014). It has been proposed that inflammation may further contribute to abnormalities in muscle function. Skeletal muscle (both respiratory and limb) abnormalities are common and profound in patients with chronic inflammatory disorders including COPD (Kim, Mofarrahi & Hussain

2008, MacIntyre 2006) and congestive heart failure (Strassburg, Springer & Anker 2005, Chien et al. 2013).

Impaired inspiratory muscle contractility has been observed in patients with OSA by means of the pleural pressure relaxation rate during voluntary sniff manoeuvres being prolonged in the morning compared to preceding night of sleep (Griggs et al. 1989). Montserrat et al. (1997) however investigated patients with severe OSA to identify if diaphragmatic fatigue occurred during the large inspiratory efforts at the end of apnoeas during stage two of sleep at the beginning and at the end of the night. Montserrat et al. (1997) documented no support for the development of diaphragmatic fatigue however a relatively small number of participants were studied (n = 7).

More recently, Chien et al. (2010) investigated muscle strength, endurance and the fatigability of the inspiratory muscles and the knee extensors in patients with OSA compared to age and BMI matched controls. Chien et al. (2010) used simultaneous surface electromyography to identify a significantly lower function of performance in both the inspiratory muscles and knee extensors in the OSA group in response to magnetic stimulation. A higher fatigability was seen only in the inspiratory muscles of patients with severe OSA. Chien et al. (2010) used peripheral muscle (knee extensors) as a control because they are not considered to be overloaded during sleep. A significantly lower strength and endurance was found in the knee extensors during magnetic stimulation. These findings are suggested to support that the systemic effects of chronic intermittent hypoxia and reoxygenation on skeletal muscles in patients with OSA cannot be completely ruled out (Chien et al. 2010).

Although a variety of methods do exist aimed at inducing inspiratory muscle fatigue, they have mainly only been successfully implemented with healthy individuals. There is currently no consensus regarding an optimal protocol to induce and assess the fatigability of the inspiratory muscles (Janssens et al. 2013). This may be a potential contributor for the lack of research assessing whether inspiratory muscle fatigue occurs in OSA and is discussed in detail in chapter 6.

***Current Treatments:***

OSA should be approached as a chronic disease requiring long-term and multidisciplinary management. The current treatment of choice for OSA of all severities is positive airway

pressure and this is usually offered to all patients with alternative therapies provided depending on the severity of OSA, patients' anatomy, risk factors and preferences (Epstein et al. 2009). Alternative treatments for OSA include: upper airway surgery, oral appliances and weight loss (Quan 2009).

*Positive airway pressure (PAP):*

There are three different types of PAP devices commonly used, continuous positive airway pressure (CPAP), bilevel positive airway pressure (BiPAP) and auto-titrating positive airway pressure (APAP). PAP works by delivering an intraluminal pressure that is positive with reference to atmospheric pressure, believed to stretch the upper-airway structures and increase the upper-airway size (Kakkar & Berry 2007). CPAP delivers a predetermined constant pressure during both inspiration and exhalation. Whereas BiPAP which is particularly effective in some forms of restrictive lung disease or hypoventilation syndromes which are associated with hypercapnia (Kushida et al. 2006a), delivers separately adjustable lower expiratory PAP and higher inspiratory PAP. BiPAP may be more tolerable than CPAP in patients who have difficulty exhaling (Kakkar & Berry 2007).

CPAP and APAP are the most commonly used in the treatment of OSA. An APAP device has the advantage of varying the pressure delivered depending on changes in airflow resistance such as with factors including changes in nasal congestion or posture (Ip et al. 2012). Despite this however, the advantages of APAP over CPAP are continually debated because treatment effects are similar and so the therapy of choice mostly depends on factors such as patient preference, specific reasons for non-compliance and cost (Ip et al. 2012). Excluding add-on expenses such as the masks, the cost of a CPAP machine for the National Health Service (NHS) in 2009, was estimated to be £280 and that of an APAP machine is £420 (McDaid et al. 2009).

The benefits of CPAP are wide-ranging. Research evidence reports CPAP being beneficial in reducing fatigue (Chotinaiwattarakul et al. 2009), nocturia (Margel et al. 2006), renal resistance index (Buchner et al. 2011) and risk of ischemic stroke (Tosun et al. 2008). Furthermore, CPAP has been shown to reduce insulin resistance, leptin levels, low density lipoprotein levels, total cholesterol (Cuhadaroğlu et al. 2009), elevated c-reactive protein (Ishida et al. 2009) and hypertension (Jaimchariyatam, Rodriguez & Budur 2010). It is however difficult to obtain an exact estimate from the literature of rates of patient adherence

to CPAP treatment (McDaid et al. 2009). Epidemiological data show that on average 25% of patients with OSA do not accept CPAP and of those who do undertake CPAP therapy, only 30-60% can be considered adherent (Catcheside 2010, Lo Bue et al. 2014). Numerous strategies have been applied to increase compliance such as patient educational training, information at the start of therapy, timely approach to resolution of possible causes of non-adherence, structured follow up and motivational support (Ballard, Gay & Strollo 2007, Aloia et al. 2007, Smith & Lasserson 2009). However, each of these interventions increase time and cost demands for the NHS.

*Oral appliances:*

Dental devices (also known as oral appliances) represent the main alternative group of treatments to CPAP (McDaid et al. 2009). Mandibular repositioning devices, also termed mandibular repositioning appliances, mandibular advancement devices, mandibular advancement appliance or mandibular advancement splint, form the most common type of oral appliance for the treatment of OSA. These tend to be designed to prevent upper airway collapse by protruding the mandible (Chan & Cistulli 2009, Lurie 2011c).

According to the AASM, the use of oral appliances is indicated for use in patients with mild to moderate OSA after consideration of CPAP. CPAP should be used whenever possible with patients with severe OSA (Kushida et al. 2006b). While a significant amount of patients have a near to complete control of the apnoea and snoring when using an oral appliance, a significant proportion also do not respond or display only a partial response (Ngiam et al. 2013) therefore CPAP is usually considered the primary option (Epstein et al. 2009). Recently, Marklung and Franklin (2014) reported no difference in the effectiveness between elderly and younger patients using a mandibular advancement device. Unfortunately, a poor retention and high compliance failure rate has been reported in thermoplastic non-custom-made devices compared to custom-made devices in the treatment of mild sleep apnoea (Vanderveken et al. 2008, Marklund, Verbraecken & Randerath 2012). Some studies have observed greater compliance with the use of a mandibular advancement device over CPAP (Gagnadoux et al. 2009, Randerath et al. 2002). However Randerath et al. (2002) reports even in patients with mild to moderate OSA, CPAP is more effective long term. Other studies have reported similar compliance to CPAP using an oral appliance (Marklund, Verbraecken & Randerath 2012, Ferguson et al. 1996, Ferguson et al. 1997).

*Surgery:*

The AASM acknowledges different types of common surgery for the treatment of OSA. These include nasal, oral, oropharyngeal and nasopharyngeal, hypopharyngeal, laryngeal and global airway procedures such as bariatric surgery. The consensus-derived AASM guidelines suggest that surgery should be considered a secondary treatment for OSA where use of PAP or oral appliances is inadequate. Its use depends on the severity, patients' eligibility, general sleep evaluation, and presence of medical, psychological or social comorbidities along with the determination of patients to undergo surgery. Surgery may be considered as a primary treatment in patients who have mild OSA and severe obstructing anatomy that is considered surgically correctible (Epstein et al. 2009).

*Weight loss:*

Health initiatives with clinical support hold promise in the elimination of OSA. Currently the only adequately supported intervention is weight loss (Young, Peppard & Gottlieb 2002). Peppard et al. (2000) conducted a longitudinal study assessing the association between change in weight and sleep disordered breathing. 609 US Wisconsin residents were recruited; after adjustment for sex, age and cigarette smoking for each 1% decrease or increase in body weight, an approximate 3% decrease or increase in AHI was expected. Furthermore, using the same adjustment and population, a 10% increase in body weight was reported to predict a 32% increase in AHI with a 10% weight loss predicted a 26% decrease in AHI.

A randomised controlled trial has found that a very low calorie diet (600–800 kcal/day) combined with active lifestyle counselling is effective in the majority of patients with mild OSA (Tuomilehto et al. 2009). Furthermore, Johansson et al. (2009) investigated the effects of a very low energy diet using a standard 550 kcal/day liquid energy intake protocol (Cambridge diet, Cambridge, Northants, UK) followed by two weeks of gradual introduction to normal food to reach 1505 kcal/day at week 9. In the obese group with moderate and severe OSA, it was reported at week 9, that 5 of the 30 participants were disease free (AHI <5) with 15 to 30 (50%) having mild OSA with the mean weight in the intervention group 20 kg lower than the control group. The effectiveness of bariatric surgery however has consistently been shown to be superior to dieting in reducing AHI (Greenburg, Lettieri & Eliasson 2009). There is currently a need for long term treatment studies to validate weight loss as a primary treatment strategy in OSA (Johansson et al. 2009). As a systematic review and meta-analysis

of randomised controlled trials on bariatric surgery reveals that, a substantial number of patients do experience a poor weight loss outcome with a post-operative regain of weight, suggesting that post-operative behavioural management has the potential to facilitate optimal long-term weight loss following surgery (Rudolph & Hilbert 2013).

*Limitations of current treatment:*

There is a distinct lack of interventions focusing on the specific physiological pathways altered by the progression of OSA. Furthermore, CPAP and oral appliances struggle with compliance issues (Catcheside 2010, Lo Bue et al. 2014, Vanderveken et al. 2008, Marklund, Verbraecken & Randerath 2012, Ferguson et al. 1996, Ferguson et al. 1997). Recently Rossi et al. (2014) reported that in 71 % of cases of the 125 patients with OSA recruited, OSA recurred after 4 nights of withdrawal from CPAP. The current emerging therapies which may offer novel treatment approaches include nasal expiratory PAP, oral negative pressure devices, bariatric surgery and upper airway muscle stimulation (Freedman 2014). Future treatment interventions need to be designed which are focused on altering the specific parameters which are associated with the development of the disease.

**Implications for research thesis:**

There is a distinct lack of treatment interventions focusing on the specific physiological pathways altered by the progression of OSA. Furthermore, CPAP and oral appliances struggle with compliance issues (Catcheside 2010, Lo Bue et al. 2014, Vanderveken et al. 2008, Marklund, Verbraecken & Randerath 2012, Ferguson et al. 1996, Ferguson et al. 1997). In this thesis we aim to investigate the pathophysiological mechanisms as highlighted in this general introduction. The long term objective of this thesis is to help guide the design of future interventions which are focussed on the specific symptomatology of OSA.

**Aims of the thesis:**

Firstly the potential effects of the exposure to intermittent hypercapnia and hypoxia during sleep on the central and/or peripheral chemoreceptors is assessed through seeing if an alteration in the ventilatory response occurs using a novel methodology performed first with experienced scuba divers. Scuba divers represent an excellent population with which to test the methodology. Following this, the implications of the baroreflex-chemoreflex interactions

are assessed using a Finometer MIDI which was donated by Ysbyty Gwynedd's League of Friends for use in this project. After this, the inflammatory markers present within patients with OSA are investigated along with two anti-inflammatory endocannabinoids 2-AG and AEA through collaboration with Hannover Medical School, Germany. Finally, the design and implementation of an entirely novel method of eliciting inspiratory muscle fatigue is used with patients with OSA.

The orginal aim of the thesis was to study all aspects of the thesis together within a single group of subjects with untreated OSA and then construct a regression model around this. Unfortunately, though this was not feasible due to numerous reasons. Accordingly, studies preceeded as was possible and to remain faithful to the orginal intent, a regression model was calculated following the completion of the studies. It is however accepted that the introduction of bias is possible.

Some partcipants did perform more than one of the sub-studies. The partcipants in all the studies were all newly diagnosed patients with OSA. Patients were diagnosed with OSA using unattended home respiratory polygraphy (Embletta® Gold, Embla Systems, USA). Measures of pulse oximetry, nasal airflow, thoracic and abdominal movements were analysed using RemLogic software. Diagnosis of OSA was performed by either an experienced RCCP (Registration Council for Clinical Physiologists) registered Clinical Physiologist or an experienced Sleep Technologist. Patients were excluded in all studies if they had a BMI≥50 $kg/m^2$ and if they were on medications known to affect their respiratory drive (i.e. opiate based painkillers). Partcipants were excluded in the ventilatory response and inspiratory muscle fatigue study if COPD was found to be present. Table 1-b states the number of partcipants taking part in each study of this thesis.

This PhD thesis represents the first collaborative research between Ysbyty Gwynedd's Pulmonary Function Department, the School of Sport, Health and Exercise Sciences, Bangor University and Hannover Medical School, Germany. It is hoped that the completion of this research facilitates the development of future research projects.

**Table 1-b.** Number of patients with OSA recruited in each study of this thesis.

| Chapter/study | Patients with OSA recruited: |
|---|---|
| Chapter 3: Ventilatory control to $CO_2$ in patients with OSA | 32 patients |
| Chapter 4: Baroreflex sensitivity in patients with OSA and its association with chemosensitivity to $CO_2$ | 33 patients |
| Chapter 5: Association of metabolic and inflammatory makers with the severity of OSA | 61 patients |
| Chapter 6: Development of a protocol to measure inspiratory muscle fatigue in OSA patients | 24 patients |

## Chapter 2 The ventilatory response to $CO_2$ of experienced scuba divers and non-diving controls.


### Abstract:

*Purpose:* To investigate the ventilatory response to $CO_2$ amongst experienced scuba divers and matched controls with a particular interest to establish whether an adaptation to the peripheral chemoreception is likely to play a dominant role in the adaptational ventilatory response to $CO_2$ observed amongst experienced scuba divers. This investigation also acts as a pilot study to test the methodology which will be applied in the next chapter with patients with OSA.

*Methods:* The ventilatory response in scuba divers (n=10) and matched controls (n=10) were assessed whilst breathing four different gas mixtures balanced with $N_2$ (ambient air; 25% $O_2$/6% $CO_2$; 13% $O_2$; 13% $O_2$/6% $CO_2$) to assess the combined response to hypercapnia and moderate hypoxia.

*Results:* The divers revealed a lower ventilatory response to hypercapnia with inhalation of the four gas mixtures revealing the tested oxygen pressures caused no significant alteration in the ventilatory sensitivity to $CO_2$ in divers and controls.

*Conclusions:* Experienced scuba divers possess a lower ventilatory response to $CO_2$ which was not affected by the tested oxygen pressures suggesting a possible dominant adaptation of central $CO_2$ sensitivity.

**Introduction**:

The rhythmogenesis of breathing is the role of the brainstem central pattern generator which receives inputs from many divergent sources related to the state of the organism, including emotional, sleep-related, environmental and motor activity states, and more basic sensory inputs from mechano- and chemoreceptors located in lung and airway tissues and the bloodstream (Bellingham 1998). The chemoreceptors include the central and peripheral chemoreceptors which function as part of a feedback control system which is responsible for keeping arterial $pCO_2$ ($PaCO_2$) and pH remarkably constant with the presence of normal kidney function (Miyamoto et al. 2004, Ogoh et al. 2008).

*The central chemoreceptors:*

Based on the reaction theory for ventilatory control, $CO_2$ stimulates the central chemoreceptors through liberating $H^+$ ions resulting in decreased pH (Loeschcke 1982). The central chemoreceptors detect the resulting acidosis and respond by increasing ventilation to help maintain arterial $pCO_2$ within a few mm Hg of the steady-state (~40 mm Hg) regardless of the metabolic production of $CO_2$ and level of vigilance (Nattie, Li 2009, Nattie 1999, Feldman, Mitchell & Nattie 2003, Guyenet, Stornetta & Bayliss 2010).

Findings from investigations conducted in the 1960's found acidification of the ventral surface of the brain in anesthetised animals' stimulated breathing (Loeschcke 1982). The actual identity of these neurons that early investigators proposed were exclusively located on the ventral medullary surface though has never been determined (Richerson 2004). The most plausible thoughts are three types of neurones: the retrotrapezoid nucleus (RTN), raphe serotonergic neurons and the locus coeruleus (Guyenet 2010). It is also possible that the central chemoreceptors are acid-sensitive glial cells or possibly vascular cells that regulate activity of surrounding neurons via paracrine mechanisms (Guyenet, Stornetta & Bayliss 2010).

The superficial presence of acid sensitive RTN neurons have been shown to be vigorously activated by raising arterial $CO_2$ *in vivo*, consistent with the theory that central chemoreception resides around the ventral medullary surface (Guyenet 2010, Mulkey et al. 2004). Furthermore these RTN neurones express Phox2b, a transcription factor where mutation causes congenital central hypoventilation syndrome (CCHS). CCHS is characterised

by a reduction or absence of respiratory automaticity during sleep and a large reduction in the central chemoreflex response (Guyenet 2008).

There is evidence that other sites in addition to the ventral surface of the medulla oblongata may also participate in central chemoreception (Nattie, Li 2012). Indeed historically, topical acidification was first used at the ventral medullary surface due to its accessibility (Guyenet, Stornetta & Bayliss 2010). More recently, acidification of many brainstem or cerebellar regions with dialysis probes (nucleus of the solitary tract (NTS), RTN, ventral respiratory column, midline medulla, fastigial nucleus) has been found to activate breathing to some degree and in some cases simultaneous stimulation of two regions produced additive effects (Guyenet, Stornetta & Bayliss 2010). A hypothesis has been formulated whereby the overall sensitivity of the respiratory control system " relies on an additive or greater effect" of multiple central chemoreceptor sites meaning the different central chemoreceptor sites act in a state-dependent fashion so multiple sites are necessary (Hodges et al. 2004, Nattie 2000).

It has been known that the serotonergic neurons are located in many parts of the medullar with most located in the midline (Raphé Nuclei) (Richerson 2004, Jacobs & Azmitia 1992). Recently, Iceman, Richerson and Harris (2013) have demonstrated for the first time that $CO_2$ stimulated and unstimulated serotonin 5-hydroxytryptamine (5-HT) neurons are present in the intact raphé of rodents *in situ*. Dysfunction of the 5-HT neurons has been suggested to be involved in the pathogenesis of Sudden Infant Death Syndrome, CCHS, sudden unexplained death in Epilepsy, Prader-Willi Syndrome, panic disorder, neurodegenerative diseases as well as sleep apnoea (Iceman, Richerson & Harris 2013, Sowers et al. 2013, Hilaire et al. 2010, Kinney 2009, Richerson et al. 2001). However, some researchers have argued that if the central chemoreceptors could directly detect $pCO_2$, there would not be such a large difference in response times between the central and peripheral chemoreceptors (Nattie 2006).

*The peripheral chemoreceptors:*

The peripheral chemoreceptors have locations in the carotid and aortic bodies (Piskuric & Nurse 2013). In comparison to the carotid bodies, the aortic bodies have been poorly studied. Responding to $O_2$ changes, the aortic bodies are thought to have a dual function as a circulatory and respiratory $O_2$ monitor generating a circulatory chemoreflex for $O_2$ homeostasis. This is in contrast to the carotid bodies which primarily function to monitor respiratory blood gases and hence, initiate chemoreflexes that control respiration (Lahiri et al.

1981). Additionally, the carotid body are known to play a role in cardiovascular reflexes resulting in bradycardia and peripheral vasoconstriction (Piskuric & Nurse 2013, Alsberge, Magno & Lipschutz 1988, Kumar 2009).

The principle cells of the carotid body are the glomus cells or type I cells (Nurse 2005). The carotid body acts as a polymodal sensor, capable of detecting reduced $pO_2$ (hypoxia) and increases in $pCO_2/H^+$ (hypercapnic acidosis) along with other sensory modalities including low glucose and temperature (Piskuric & Nurse 2013, Nurse 2010, Lopez-Barneo 2003, Kumar & Bin-Jaliah 2007).

*Interactions between the peripheral and central chemoreceptors:*

A synergistic effect of $pO_2$ and $pCO_2$ on ventilation has been found (Lahiri & Delaney 1975, Lahiri & Forster 2003). It is therefore important not to view the central and peripheral chemoreceptors as entirely separate entities. The RTN neurons have powerful excitatory inputs from the carotid bodies via a short and presumably disynaptic pathway (Guyenet 2010, Guyenet 2008). The central respiratory chemoreceptors have been described as normally operating together with the peripheral chemoreceptors (Smith et al. 2006) with their assumed interactions summarised in figure 2-a.

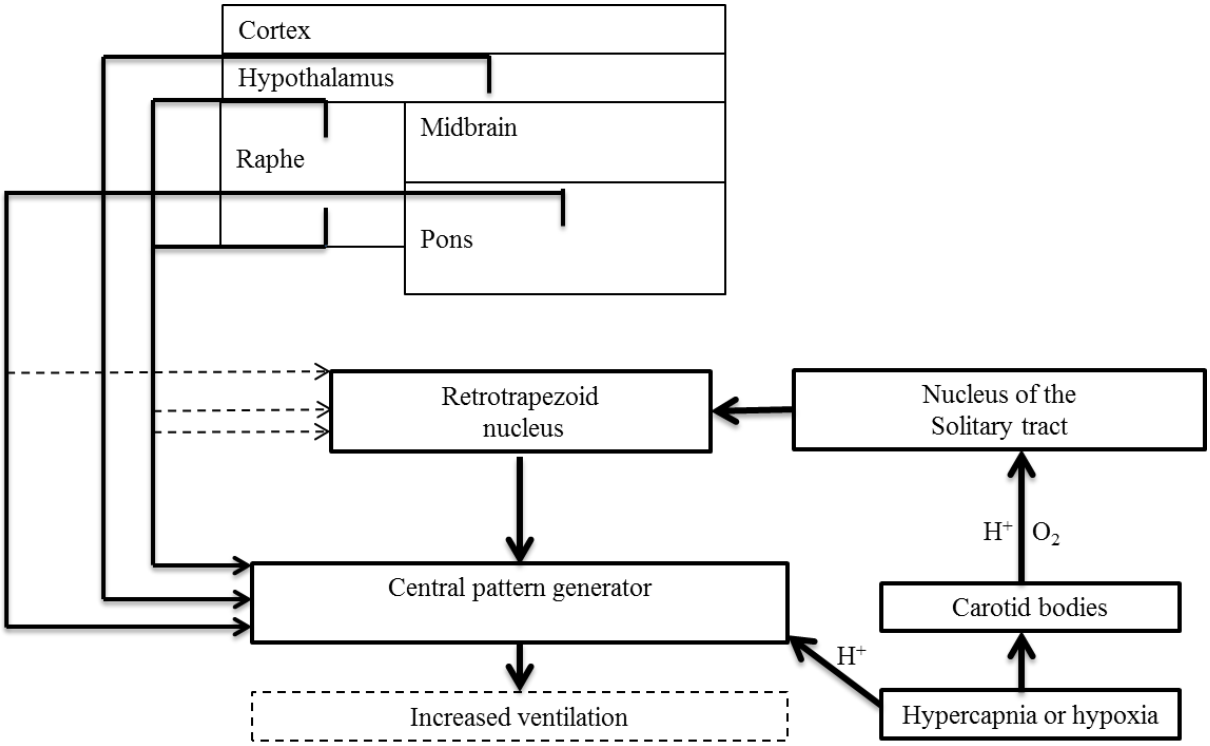


**Figure 2-a.** Showing the interaction between the central and peripheral chemoreceptors operating through a common respiratory controller located in the RTN. Adapted from Guyenet (2010).

It has been reported that the response time of the carotid chemoreceptors is one-half times faster than the central chemoreceptors to step increases or decreases in $PaCO_2$ (Dempsey 2005). Findings though have remained controversial despite decades of research as many studies have used carotid body denervated (CBD) preparations to find the relatively slow response time on the order of 30-35 seconds in the central chemoreceptors (Smith et al. 2006). It has been reported that the RTN neurons receive powerful excitatory inputs from the carotid bodies via a short, presumably disynaptic pathway and therefore it is possible CBD reduces the gain of the central respiratory control system (Guyenet 2010, Guyenet 2008). Furthermore, other studies have shown functional deficits in the medullary raphe and pre-Bötzinger complex after CBD (Smith et al. 2006, Hodges et al. 2005). The pre- Bötzinger complex contains six basic types of respiratory neurons that participate in respiratory rhythmogenesis as a function of the central pattern generator (Bellingham 1998). Lastly, following CBD in ponies it has been shown the aortic chemoreceptors become functional in a time-dependent manner (Bisgard, Forster & Klein 1980).

Investigations with intact carotid bodies include Smith et al. (2006) which investigated the differences between the central and peripheral $CO_2$ sensitivity and speed of response in

anaesthetised dogs. Smith et al. (2006) found the central chemoreceptors accounted for ~63% of steady-state ventilatory sensitivity to hypercapnia with ~37% being due to the carotid chemoreceptors. However, this relative contribution was highly variable amongst the dogs. It was also found that the ventilatory response to abrupt increases in $CO_2$ was delayed by ~11 seconds when only the central chemoreceptors were recruited by maintaining normal blood gas values at the carotid body chemoreceptors. These findings may suggest that the relatively slow time of the central chemoreceptors is due to a central site for $CO_2$ reception which requires diffusion from the blood through the interstitial fluid (Smith et al. 2006). A high gain has been demonstrated in the central chemoreceptors. For example in man at rest, ventilation has been reported to approximately double for a 1.5 mm Hg rise in alveolar (presumed arterial) $pCO_2$ (Guyenet, Stornetta & Bayliss 2010, Haldane & Priestley 1905). It has been theorised that the central chemoreceptors are therefore responsible for detecting interstitial pH and monitoring the balance of arterial $CO_2$, cerebral blood flow and cerebral metabolism whereas the peripheral chemoreceptors are involved in detecting $PaCO_2$ and pH and monitoring alveolar ventilation (Nattie 2006).

*Ventilatory response to $CO_2$ amongst scuba divers:*

Different populations have displayed altered ventilatory responses to $pCO_2$. Examples of a healthy population include breath hold divers and scuba divers (Florio, Morrison & Butt 1979, Kerem, Melamed & Moran 1980). Awareness of having a low ventilatory response to $CO_2$ has important implications on the safety of a dive as hypercapnia related symptoms ranging from increased depth and rate of breathing, breathlessness (air hunger), headache, dizziness, mental disorientation to complete unconsciousness are potential risks associated with diving (Fothergill, Taylor & Hyde 1998, Cheshire & Ott 2001). Furthermore, during scuba diving, exposure to even mild levels of hypercapnia has been shown to substantially increase the risks of developing central nervous system oxygen toxicity (Arieli et al. 2001, Eynan, Arieli & Adir 2005).

Previous studies have found a lowered ventilatory response to $CO_2$ amongst scuba divers (Florio, Morrison & Butt 1979, Kerem, Melamed & Moran 1980). In our previous experiments we recruited male experienced scuba divers (completed 1045 ± 1083 dives over 15.5 ± 9.0 years) and non-divers. The participants' level of physical activity was collected and assessed through the use of a physical activity questionnaire and the groups were matched for age; body mass, height and physical activity. We tested the ventilatory response to

progressive hyperoxic hypercapnia achieved using a breath by breath metabolic cart (3B Metalyser®, Cortex Biophysik, Germany) with its volume transducer and gas sampling port attached to a closed circuit rebreathing loop including a Douglas bag filled with 100% oxygen. The amount of oxygen required in the Douglas bags was estimated based on the body characteristics of the participant using the Harris and Benedict (Amirkalali et al. 2008) and Weir equation (Weir 1990) enabling a test duration of 15 to 20 minutes during the resting $CO_2$ rebreathing. It was revealed the accumulation of 6% inspired $CO_2$ induced the greatest change in minute ventilation amongst the non-diving controls in comparison to scuba divers who significantly increase their minute ventilation with the accumulation of 7% $CO_2$ in the rebreathing loop (Figure 2-b).

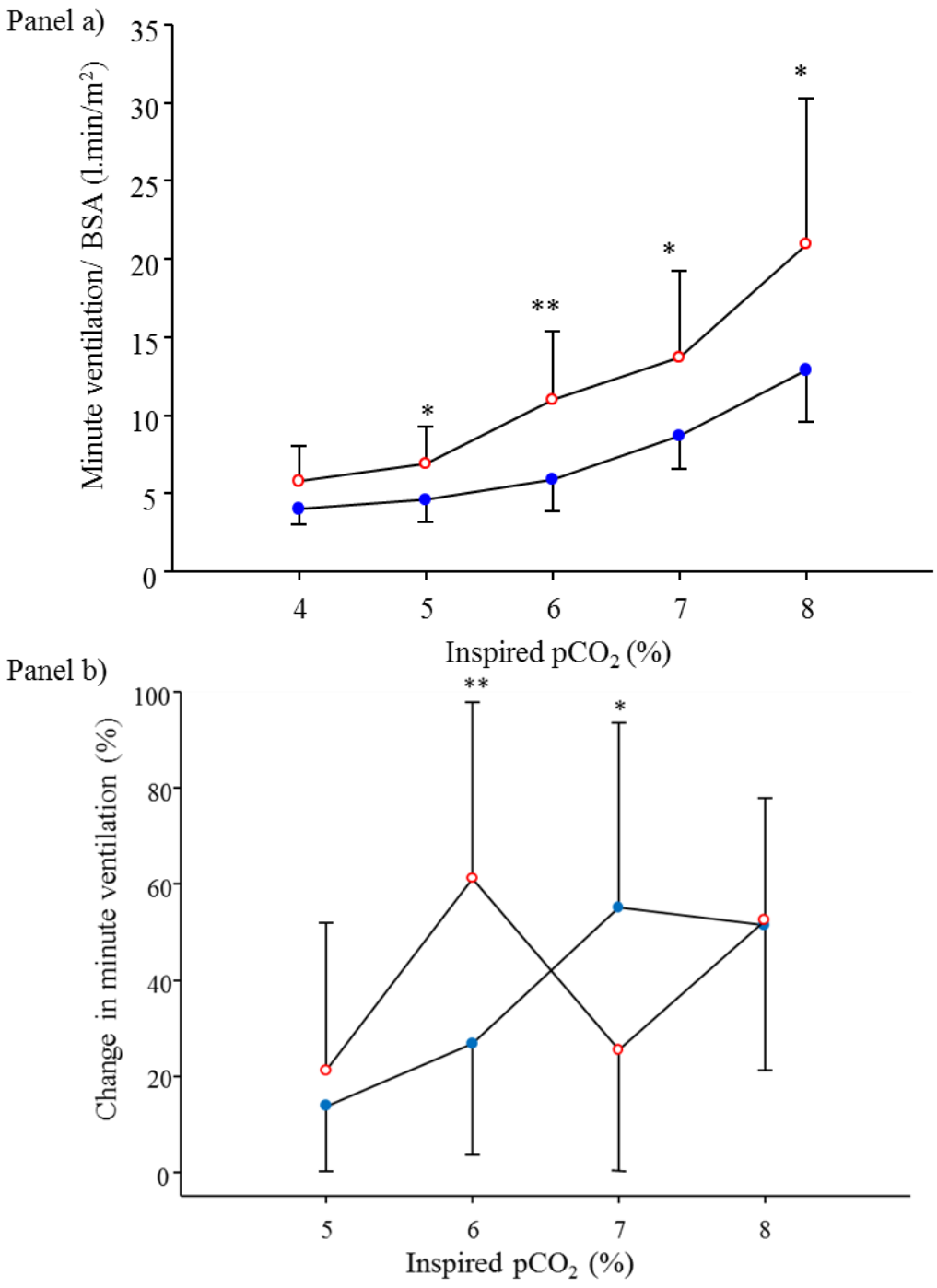


**Figure 2-b.** Panel a) Absolute minute ventilation/body surface area with inspired $pCO_2$ (BSA). Panel b) Percentage change in minute ventilation with the accumulation of $CO_2$ (%). ● = scuba diving group and ○ = control group. Values represent mean ± SD where significantly different between groups, ** = $p<0.01$ *= $p<0.05$.

The analysed capillary blood samples revealed significant $CO_2$ retention in the scuba divers during $CO_2$ rebreathing but not on room air (normocapnic and normoxic condition). The two groups were also found to not differ in their $CO_2$ retention with the accumulation of more than 7% inspired $CO_2$, suggesting that both groups had reached their peak ventilatory response to the $pCO_2$ (Table 2-a).

**Table 2-a.** Capillary blood gas parameters during ambient and resting $CO_2$ rebreathing.

| | Ambient | 5% | 6% | 7% |
|---|---|---|---|---|
| pH | | | | |
| Scuba | 7.40 ± 0.02 | 7.36 ± 0.02** | 7.34 ± 0.01** | 7.32 ± 0.02 |
| Control | 7.40 ± 0.02 | 7.38 ± 0.02 | 7.37 ± 0.02 | 7.33 ± 0.3 |
| $pCO_2$ (mm Hg) | | | | |
| Scuba | 43.18 ± 2.40 | 47.73 ± 2.28** | 49.43 ± 2.23** | 53.20 ± 2.20 |
| Control | 41.55 ± 1.57 | 44.72 ± 1.74 | 45.80 ± 1.48 | 51.14 ± 4.22 |
| HCO3std (mmol/L) | | | | |
| Scuba | 26.01 ± 0.91 | 25.45 ± 0.80 | 24.97 ± 0.81 | 25.46 ± 1.29 |
| Control | 25.71 ± 0.95 | 25. 70 ± 0.71 | 25.56 ± 0.78 | 25.33 ± 0.93 |
| BE (mmol/L) | | | | |
| Scuba | 1.44 ± 1.16 | 0.57 ± 1.03 | -0.04 ± 1.03 | 0.60 ± 1.64 |
| Control | 1.05 ± 1.21 | 0.90 ± 0.91 | 0.70 ± 0.99 | 0.42 ± 1.19 |
| THbc (g/dL) | | | | |
| Scuba | 13.91 ± 0.80 | 13.93 ± 0.85 | 14.26 ± 0.91 | 14.42 ± 1.03 |
| Control | 13.93 ± 0.85 | 13.65 ± 1.01 | 13.88 ± 0.98 | 14.06 ± 0.84 |

Where significantly different means between the two groups, ** = $p$ <0.01. Abbreviations: standard bicarbonate (HCO3std) , base excess (BE), total haemoglobin (THbc).

The influence of exercise designed to simulate the workload of scuba diving (7 METS: (Ainsworth et al. 2011)) on the ventilatory response to $CO_2$ was also investigated. The exercise was found to have no effect on the lowered ventilatory response to $CO_2$ amongst the experienced scuba divers (Figure 2-c, panel b).

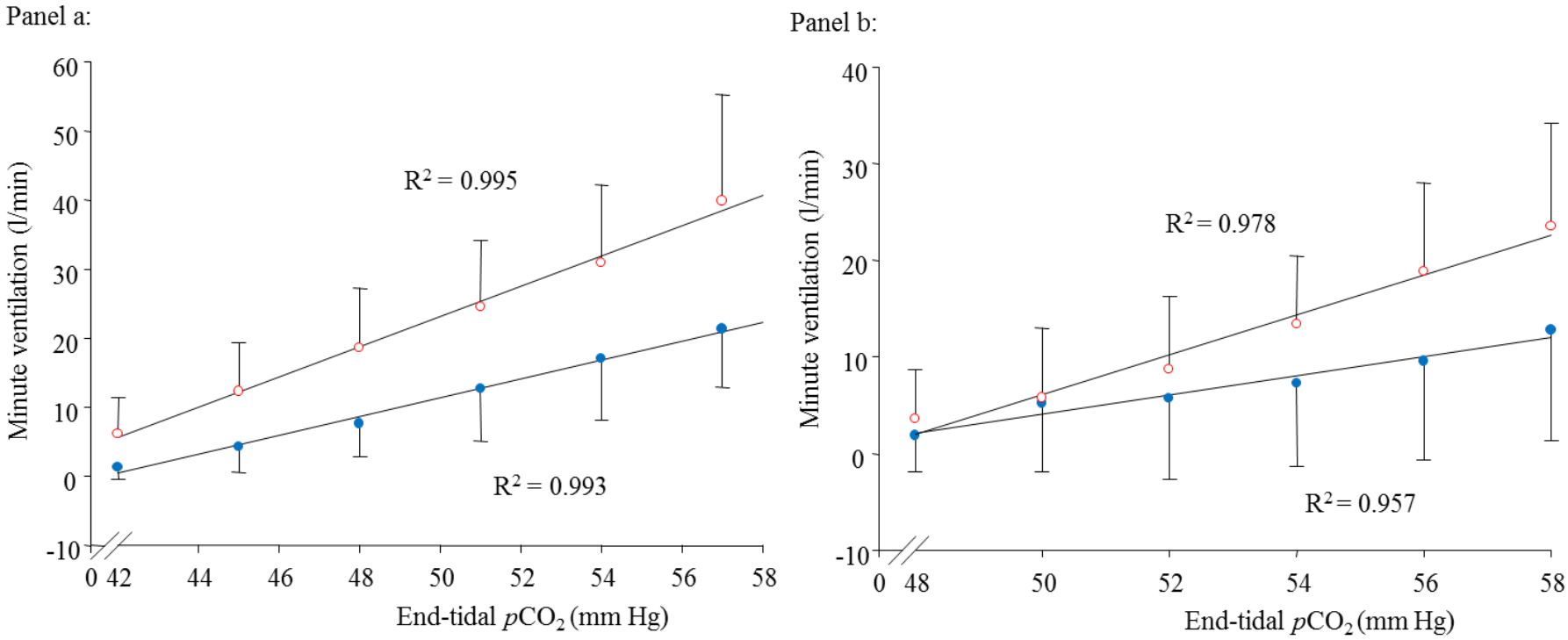


**Figure 2-c.** Panel a. Change in minute ventilation from baseline resting minute ventilation vs. end-tidal $pCO_2$ during $CO_2$ rebreathing at rest. Panel b. Change in minute ventilation from baseline exercising minute ventilation with ambient air. Where ● = scuba diving group and ○ = control group. The scuba divers had a significantly lower ventilatory response slope during both rest and exercise ($p<0.05$). Values represent mean ± SD.

To date, no research with scuba divers has investigated whether these previous findings can be attributed to an alteration involving the peripheral chemoreceptors. Hypoxic stimulation of the peripheral chemoreceptors results in an increase in the peripheral chemoreflex sensitivity to $CO_2$ via changes in $H^+$ ions at the carotid body (Kumar & Bin-Jaliah 2007, Duffin 2007, Torrance 1996). Duffin (2007) recommends in order to investigate the contribution of the peripheral chemoreceptors on the ventilatory response, the $CO_2$ concentration of interest should be compared at a high constant $O_2$ tension (isoxic hyperoxic ventilatory response to $CO_2$) and a low constant $O_2$ tension (isoxic hypoxic ventilatory response to $CO_2$). The hyperoxic response measures the central chemoreflex whereas the hypoxic response measures the sum of the central and peripheral responses. The contribution of the peripheral chemoreflex is represented by the difference between the responses.

This current study serves two purposes; firstly it investigates whether experienced scuba divers have a lowered ventilatory response to $CO_2$ and whether this is partly due to an adaptation involving the peripheral chemoreceptors. This is achieved by comparing the ventilatory response to breathing 6% $CO_2$ with hyperoxia (25%$O_2$ / 6% $CO_2$) to breathing 6% $CO_2$ with hypoxia (13% $O_2$/ 6% $CO_2$). The second purpose of this study is as a pilot study to allow the design and testing of a methodology to allow the investigation of the central and peripheral chemoreflex amongst patients with OSA.

**Method:**

*Participants:*

This study was approved by the Ethics Committee of Bangor University (Gwynedd, Wales) and was carried out in accordance with the Declaration of Helsinki for research on human subjects. Written informed consent was obtained from all subjects prior to testing. Male experienced scuba divers and non-divers were recruited. To be eligible for the scuba diving group participants' were required to have performed at least 200 dives. For inclusion in the control group, participants were required not to had any experience in scuba or breath hold diving. The participants level of physical activity was collected and assessed through the use of a physical activity questionnaire. Categories of physical activity levels (low, moderate, high) were converted into scores for comparison between the two groups. The groups were matched for physical activity, age, body mass, and height.

*Experimental System:*

The volume transducer and gas sampling port of a metabolic cart (MetaMax® 3B, Cortex Biophysik, Germany) was attached to a two way valve allowing gases to be inspired from the Douglas bag and expired into the atmosphere as illustrated in Figure 2-d. The metabolic cart was calibrated prior to testing with a premixed gas composition of 13% $O_2$ with 6% $CO_2$. Calibration of the volume transducer was carried out while connected to the tubing and valve system with the Douglas bag disconnected, allowing adjustment for any resistance generated by the system. A 250l Douglas bag was filled prior to each experiment with the required premixed gas balanced with $N_2$ (BOC Ltd, England). These gas mixtures were Mixture 1: ambient air; Mixture 2: 25% $O_2$/6% $CO_2$, Mixture 3: 13% $O_2$ and Mixture 4: 13% $O_2$ /6% $CO_2$. 6% $CO_2$ was used as a result of our previous $CO_2$ rebreathing study, as around 6% $CO_2$ was found to induce the greatest increase in minute ventilation amongst the non-diving controls (Figure 2-b, Table 2-a). Furthermore, 6% $CO_2$ was implemented in the method in Eynan et al. (2003) study, which performed a test procedure that was later adapted by the IDF Medical Corps of the Israel Naval Medical Institute (Eynan et al. 2003). The hypoxic gas mixture of 13% $O_2$ was chosen to obtain a mean end-tidal $pO_2$ of 56.5± 3.99 mm Hg regarded as moderate hypoxia (Goodall, Ross & Romer 2010) and fitting closely with Duffin's (2007) recommendation of a hypoxic $pO_2$ of 50 mm Hg being used to add the peripheral response. The 25% $O_2$ was chosen as equivalent to $pO_2$~150 mm Hg (25%$O_2$ / 6% $CO_2$) supported to effectively silence the peripheral chemoreflex to $CO_2$ (Duffin 2007, Mohan & Duffin 1997) and avoid stimulatory effects (Duffin 2007, Becker et al. 1996).

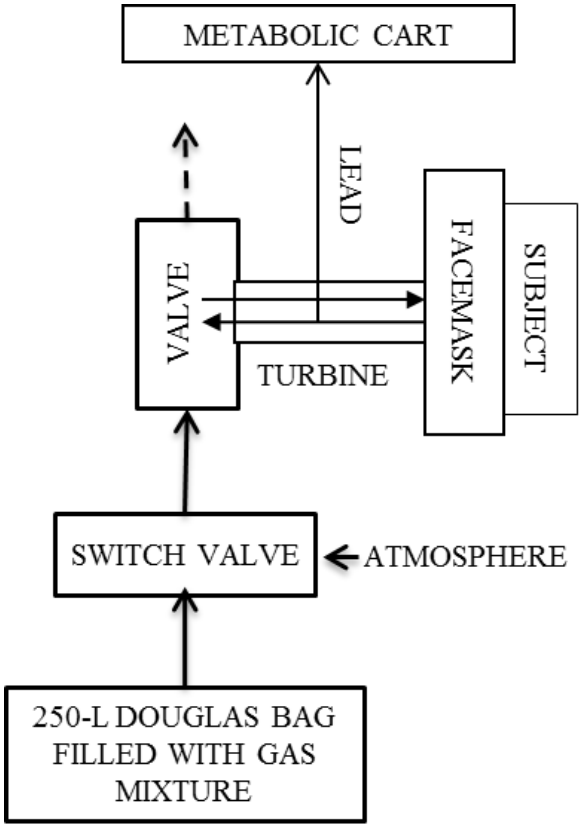


**Figure 2-d**. Set up of the breathing system

*General Procedures:*

The study was performed at room temperature (18-22°C) with humidity (<70%) with $FEV_1$ and FVC measured first whilst seated using a portable, handheld spirometer (MicroLoop Spirometer, MicroMedical Ltd., Basingstoke,UK) following ATS/ERS guidelines (Miller et al. 2005). After spirometry the participants were set up on the breathing system with baseline seated minute ventilation on ambient air measured first. The participant then breathed the first test gas mixture until a plateau in minute ventilation was achieved (~5 minutes). Participants were blinded to the order of tests and given adequate time between tests to allow ventilation, blood pressure and heart rate to return to resting baseline conditions. Participants were also instructed to raise a hand to end the test early if needed. During the testing, participants focused on a non-dramatic movie with questions being asked about the movie at the end of the study. This was done in order to avoid participants consciously controlling their ventilation (Eynan et al. 2003). The photo below (Figure 2-e) displays an example a participant connected to the breathing apparatus for the tests.

**Figure 2-e**. Photograph of Kevin Williams (Technician of the School of Sport, Health and Exercise Sciences, Bangor) connected to the experimental breathing apparatus (image used with permission).

*Data Analysis:*

All measurements are expressed in BTPS with mean ± SD and $p< 0.05$ considered statistically significant. Spida 5 version 2.0.8.-2 software automatically selected the highest measures, according to ATS/ERS guidelines for $FEV_1$ and FVC (Miller et al. 2005). Statistical analysis was carried out using the Statistical Package for Social Sciences Version 20 for Windows® (SPSS Inc., Chicago, IL), a one-way ANOVA compared change from ambient (baseline) minute ventilation between the two groups with each gas mixture. An independent *t*-test compared the change in minute ventilation with the hyperoxic hypercapnic gas mixture and the hyperoxic hypercapnic gas mixture between the groups. Furthermore, Pearson *r* was used to identify if a correlation existed between increased diving experience and the ventilatory response to the gas mixtures.

**Results:**

The scuba diving group included experienced divers only. The results of the diving experience questionnaires are displayed in Table 2-b. The two groups were adequately matched for physical activity, age, body mass, and height as tested. The physical characteristics of the two groups are shown in Table 2-c.

**Table 2-b.** Diving experience of the scuba diving group measured with a diving questionnaire in all studies. All divers used open-circuit breathing apparatus and regularly used enriched air nitrox gas mixtures. Values represent the median

| Parameter: | Value |
|---|---|
| N | 10 |
| Years diving | 14 |
| Number of dives | 990 |
| Max depth dived (m) | 46 |
| Common diving depth (m) | 30 |

**Table 2-c.** Physical characteristics of the two groups. For the categories of physical activity scores 1 = low, 2 = moderate, 3 = high activity. Values represent mean ± SD.

| Parameter | Divers | Controls |
|---|---|---|
| N | 10 | 10 |
| Age (yr) | 33.6 ± 8.9 | 31.2 ± 8.0 |
| Height (cm) | 177.7 ± 6.6 | 177.5 ± 5.2 |
| Mass (kg) | 75.8 ± 9.3 | 78.4 ± 7.3 |
| BSA ($m^2$) | 1.93 ± 0.14 | 1.96 ± 0.10 |
| FVC (l) | 5.45 ± 0.49 | 5.49 ± 0.55 |
| $FEV_1$ (l) | 4.47 ± 0.35 | 4.41 ± 0.62 |
| FEV1/FVC (%) | 82.02 ± 0.42 | 80.33 ± 0.59 |
| Physical activity scores | 2.1 ± 0.7 | 2.0 ± 0.7 |

The minute ventilation between the divers and controls was not significantly different whilst breathing ambient air (Scuba divers: 9.33 ± 2.94 l/min; Controls: 10.32 ± 1.97 l/min). There was no significant difference in the change in minute ventilation from baseline with the 13% $O_2$ mixture (Scuba divers: 9.76 ± 3.62 l/min; Controls: 11.58 ± 1.76 l/min). In both the hyperoxic hypercapnic gas condition (25% $O_2$/6% $CO_2$; Scuba divers: 18.54 ± 6.72 l/min; Controls: 25.76 ± 6.12 l/min) and the hypoxic hypercapnic gas condition (13% $O_2$ /6% $CO_2$; Scuba divers: 22.42 ± 7.92 l/min; Controls: 29.88 ± 8.05 l/min) the divers displayed a significantly lower increase in minute ventilation from baseline compared to the controls ($p$<0.05). The change in minute ventilation from resting (baseline) is displayed in Figure 2-f. Additionally, the minute ventilation with the 25% $O_2$ / 6% $CO_2$ gas mixture test for the most sensitive participant who was a non-diver and the least sensitive individual (a scuba diver) are displayed in Figure 2-g.

The tested oxygen concentration used in our study did not significantly alter the ventilatory response to $CO_2$ in both groups, suggesting the results are due to an adaptation modifying predominantly the central chemosensitivity of the scuba divers. No correlation between diving experience and the ventilatory response to $CO_2$ was found.

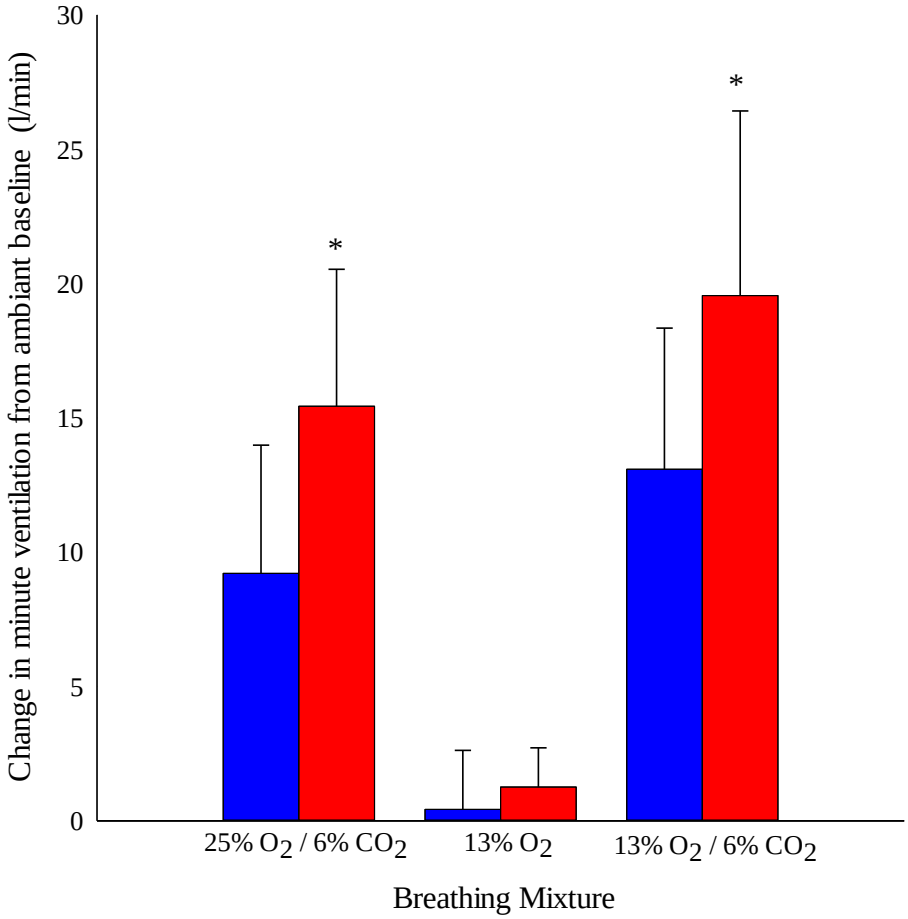


**Figure 2-f.** Change in minute ventilation from resting baseline (l/min) with breathing mixture. ■ = scuba divers ■ = controls, where significantly different between the groups with each gas mixture, * = $p<0.05$. Values represent mean ± SD.

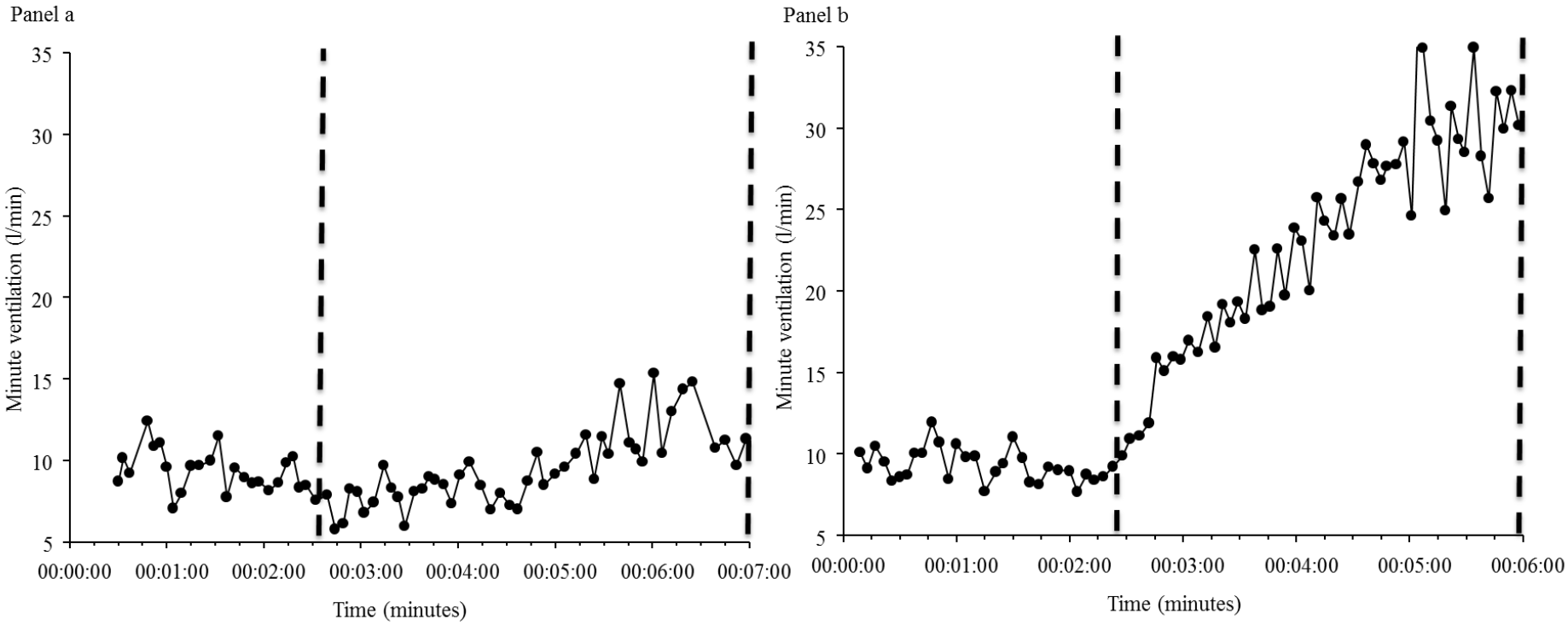


**Figure 2-g.** Panel a; results of the lowest ventilatory response to $CO_2$ (a scuba diver who reported experience of 400 dives over 6 years). Panel b; results of the highest ventilatory response to $CO_2$ (non-diving control). In both panels, between the two black dashed vertical lines, the participant breathed the 25% $O_2$ / 6% $CO_2$ gas mixture.

**Discussion:**

This is the first study to investigate a possible contribution of peripheral chemoreflex for the scuba divers' altered ventilatory response to $CO_2$. We observed that the ventilatory response to $CO_2$ was significantly lower in experienced scuba divers compared with matched non-diving controls even if hypercapnia was combined with moderate hypoxia or hyperoxia.

*Effects of moderate hypoxia on $CO_2$ sensitivity:*

Synergistic effects of $O_2$ and $CO_2$ on ventilation has been shown to be based on carotid body response (Lahiri & Delaney 1975). Even under euoxic/normocapnic ventilation the carotid body is suggested to play an important role in the control of ventilation and a hyper-additive peripheral-central interaction for the combined response to $O_2$ and $CO_2$ has been reported (Blain et al. 2009). We hypothesised that an alteration in gain of the carotid body chemoreflex could be an influencing factor for the adaptation of $CO_2$ response in divers. While there is so far no evidence for a central $O_2$-$CO_2$ interaction, we combined moderate hypoxia with hypercapnia to investigate a possible contribution of the carotid bodies for the reduced $CO_2$ response in divers. If divers would display a reduced gain for the combined response to $CO_2$ and $O_2$ of the carotid bodies, the ventilatory response to $CO_2$ in hypoxia versus hyperoxia should be reduced in divers compared with non-divers. However, in this study we could observe that the differences in ventilatory $CO_2$ response in the hypoxic and hyperoxic conditions between divers and controls were unchanged, suggesting that the altered ventilatory response in experienced divers is a central adaptation. Additionally, the finding that the ventilatory response to moderate hypoxia was not significantly different between the two groups supports this notion. This finding corresponds with Melamed and Kerem (1988) which also found no difference in the peripheral chemoreflex amongst non-divers, active $O_2$ divers and ex-$O_2$ divers with hypoxia.

*Potential mechanisms:*

No difference in the vital capacity was found between the scuba divers and the controls which is consistent with Froeb (1961) and Florio, Morrison and Butt (1979) findings. Potential mechanisms which may explain the ventilatory response to $CO_2$ amongst the scuba divers include the development of a conditioned breathing pattern when breathing through a mouthpiece (Kerem, Melamed & Moran 1980). However, the resting minute ventilation was

not significantly different between the two groups. Another possibility is that divers have a higher setting of chemostat. This implies however a higher resting eupneic $PaCO_2$ (Kerem, Melamed & Moran 1980). Furthermore, the analysed capillary blood samples performed prior to $CO_2$ rebreathing in a previous study which involved many of the same participants recruited in this current study revealed no significant difference between the groups when breathing atmospheric air. There is the possibility the decreased ventilatory response to $CO_2$ is due to a reduced $CO_2$ build-up around the chemoreceptors caused by vasodilation and higher cerebral blood flow amongst the divers (Kerem, Melamed & Moran 1980). Slosman et al. (2004) investigated 215 healthy recreational divers and reported a negative influence of dive depth on cerebral blood flow suggesting scuba diving may have long-term negative neurofunctional effects when performed in extreme conditions such as cold water, with more than 100 dives per year and with maximal dive depth below 40 metres. However, the divers in our study regularly dive below this stated 40 metre threshold.

*An inherited or acquired response?*

Whether the ventilatory response to $CO_2$ amongst the scuba divers is inherited or acquired through learning is still debated (Florio, Morrison & Butt 1979, Eynan, Arieli & Adir 2005, Froeb 1961). Kerem, Melamed and Moran (1980) compared ex-divers, active divers and non-divers measuring end-tidal $pCO_2$ in rest and exercise. They found hypercapnic values were almost indistinguishable between the ex-divers and the active divers. Kerem, Melamed and Moran (1980) also suggested either this characteristic was acquired through training and retained after cessation of diving or was an inherited feature prevalent within the diving population. There is also the possibility the response to $CO_2$ is an interaction between both acquired through learning and inherited.

In favour of an acquired component Wood, Fatemian and Robbins (2003) found repeated bouts of exercise paired with simultaneous $CO_2$ inhalations altered the ventilatory response to exercise, suggesting the ventilatory response to $CO_2$ may be influenced through learning and memory. In view of inheritance Saunders, Leeder and Rebuck (1976) found a significant relationship between $CO_2$ ventilatory sensitivity in young swimmers and their siblings. However Scoggin et al. (1978) found non-athletic parents and siblings of long-distance runners displayed a similar decreased ventilatory response to hypoxia but not hypercapnia. Furthermore, Eynan et al. (2005) studied novice divers who trained extensively for 1 year (~150 dives) using closed-circuit breathing apparatus with oxygen at shallow depths of 3-5

metres. It was found that the divers did not develop a tendency to retain $CO_2$ after this period suggesting $CO_2$ retention is not a trait that is acquired during diving in shallow water. These findings may not be applicable to deeper diving as there is an increased gas density with depth resulting in an elevation in the work of breathing and subsequent reduction in ventilation (Dean et al. 2003). Eynan et al. (2005) suggest that a conditioned breathing pattern may be developed in divers conducting deep dives which may save on the work of breathing but result in an increase in $CO_2$.

In all our experiments as well as those conducted by Froeb (1961), Florio, Morrison and Butt (1979), and Kerem, Melamed and Moran (1980) no correlation was observed between the number of dives performed and the ventilatory sensitivity to $CO_2$ amongst the scuba divers. This leads to the suggestion that the changes in $CO_2$ sensitivity are achieved in a comparably short time or that sensitivity is inherited with individuals who are sensitive to $CO_2$ leaving the diving population. Adaptations have been shown to occur in a short time amongst clinical populations which may also further increase our understanding of the modification of the ventilatory adaptation amongst scuba divers. Patients with OSAare frequently exposed to nocturnal bouts of hypoxia and hypercapnia implicated to induce alterations in the central and peripheral chemoreceptors (Cooper et al. 2005) and this is investigated in the next chapter. Likewise patients with Chronic Obstructive Pulmonary Disease (COPD) are also reported to process an attenuated ventilatory response to hypercapnia/hypoxia (Fahey & Hyde 1983, Xu et al. 2007). Unfortunately however, in COPD it is impossible to determine whether the reduced ventilatory response is due to an impaired respiratory central drive, as the ventilatory response is correlated with the mechanical limitations of COPD (Xu et al. 2007, Cherniack & Snidal 1956). In terms of a genetic association, Congenital Central Hypoventilation Syndrome is a rare neurodevelopmental disorder involving the inheritance of a mutation in the paired-like homeobox 2B (PHOX2B) gene (4p12) (Carroll et al. 2014) which is defined by a reduced physiological response to elevated $CO_2$ (Patwari et al. 2010).

**Conclusion:**

Scuba divers possess a lower ventilatory response to $CO_2$ which seems limited to adaptation of the central chemoreceptors as there is no change in the difference between divers and non-divers in ventilatory drive in the hypoxic and hyperoxic $CO_2$ response.

## Chapter 3 Ventilatory control to $CO_2$ within patients with OSA


**Abstract:**

*Purpose:* To investigate the ventilatory response to $CO_2$ amongst patients with OSA and to analyse whether possible alterations are related to central and/or peripheral alterations of chemosensitivity. Furthermore, the study identifies relationships between the ventilatory response to $CO_2$, the severity of OSA and physical characteristics.

*Methods:* The respiratory response of 16 patients with severe OSA (AHI≥30) is compared to 16 patients with mild/moderate OSA (AHI <30) whilst breathing four different gas mixtures balanced with $N_2$ (ambient air; 25% $O_2$/6% $CO_2$; 13% $O_2$; 13% $O_2$/6% $CO_2$) to assess the combined response to hypercapnia and moderate hypoxia.

*Results:* The patients with severe OSA revealed a significantly lower ventilatory response to hypercapnia ($p$<0.05) with inhalation of the four gas mixtures revealing the tested oxygen pressures caused no significant alteration in the ventilatory sensitivity to $CO_2$ in the two groups. AHI was found to be negatively correlated with ventilatory response in the hyperoxic hypercapnic condition ($r_s$= -0.51; $p$<0.01). Despite the lowered ventilatory response to $CO_2$ being associated with increased body mass, AHI was still found to be negatively correlated with the ventilatory response to $CO_2$ even after controlling for BMI ($r$ = -0.36, $p$ =0.047).

*Conclusions:* Patients with severe OSA possess a lower ventilatory response to $CO_2$ which was not altered by the tested oxygen pressures suggesting a dominant adaptation of central $CO_2$ sensitivity. This lowered ventilatory response to $CO_2$ is correlated with the significantly higher body mass and AHI found amongst the patients with severe OSA.

**Introduction:**

In obstructive sleep apnoea (OSA) the hyperventilation which may follow apnoeas and hypopnoeas can cause recurrent periods of decreased $PaCO_2$ (hypocapnia) causing a reduction in the central respiratory drive during sleep (Mateika & Ellythy 2003, Longobardo, Evangelisti & Cherniack 2002). Furthermore, patients with OSA are frequently exposed to nocturnal bouts of hypoxia and hypercapnia during sleep and this exposure has been implicated to induce alterations in the ventilatory response of the central and peripheral chemoreceptors (Cooper et al. 2005). One suggestion is the intermittent exposure to hypercapnia and hypoxia would lead to a progressive adaptation involving "resetting" of the receptors of the integrative neurons in the brainstem to a different sensitivity threshold (Verbraecken et al. 1995, Guilleminault & Cummiskey 1982). It is also plausible that there is a pathogenic role of inflammation which mediates the upregulation of the renin-angiotensin system in the carotid body causing over activity in the chemoreflex (Fung, Tipoe & Leung 2014, Lam et al. 2012).

It has been recognised that OSA and central sleep apnoea (CSA) share many pathophysiological entities and often coexist in the same patient (Leung et al. 2012). A central apnoea is defined as an apnoea with the absence of inspiratory effort throughout the entire period of absent airflow (Berry et al. 2012). It is not uncommon to observe periods of central apnoeas in patients with OSA particularly immediately following the establishment of upper airway patency using CPAP, often referred to as 'complex sleep apnoea' (Leung et al. 2012). The apnoeas and hypopnoeas that manifest in patients with CSA are due to destabilisation of ventilatory control, associated with high loop gain (Leung et al. 2012, White 2005, Burgess 2012). It has only recently become acknowledged, that ventilatory control instability plays a role in the pathogenesis of OSA (Plataki, Sands & Malhotra 2013).

Any system regulated by feedback loops has the potential to become unstable and this is best described in the context of 'loop gain' (White 2005). Breathing control is regulated by a negative feedback loop which works to keep $PaCO_2$ and pH remarkably constant (Leung et al. 2012, Ogoh et al. 2008, Miyamoto et al. 2004). The concept of loop gain is used to quantify the internal amplification of a system (Grodins, Buell & Bart 1967), it is a dimensionless value of the tendency of a system governed by feedback loops to develop unstable behaviour (Burgess 2012). The two primary variables which influence loop gain are known as controller gain and plant gain.

In OSA a modification in the ventilatory response may cause an increase in the collapsibility of the upper airway. An increase in the ventilatory drive would activate the upper airway muscles and promote patency whereas a reduction in ventilatory drive would relax the upper airway muscles and facilitate closure (Wellman et al. 2008). Other researchers also speculate that a heightened responsiveness may contribute to respiratory control instability potentially leading to periodic breathing and further airway obstruction via increased loop gain as observed in CSA (Leung et al. 2012, White 2005, Burgess 2012) whereas a reduced responsiveness may prolong apnoea duration due to lowering of the arousal reflexes (Verbraecken et al. 1995).

It has been observed that patients with severe OSA are more susceptible to unstable breathing despite the upper airway being stabilised. This has been interpreted in two ways. It's possible the instability is responsible for or contributes to the greater severity of OSA or the difference in severity of OSA are primarily related to difference in upper airway structure/function where the greater instability of the control of breathing is not a cause but a consequence of severe OSA, or some other factor that correlates with severity of OSA (Younes et al. 2001). Figure 3-a shows a simplified diagram of the concept of loop gain in the control of breathing during sleep.

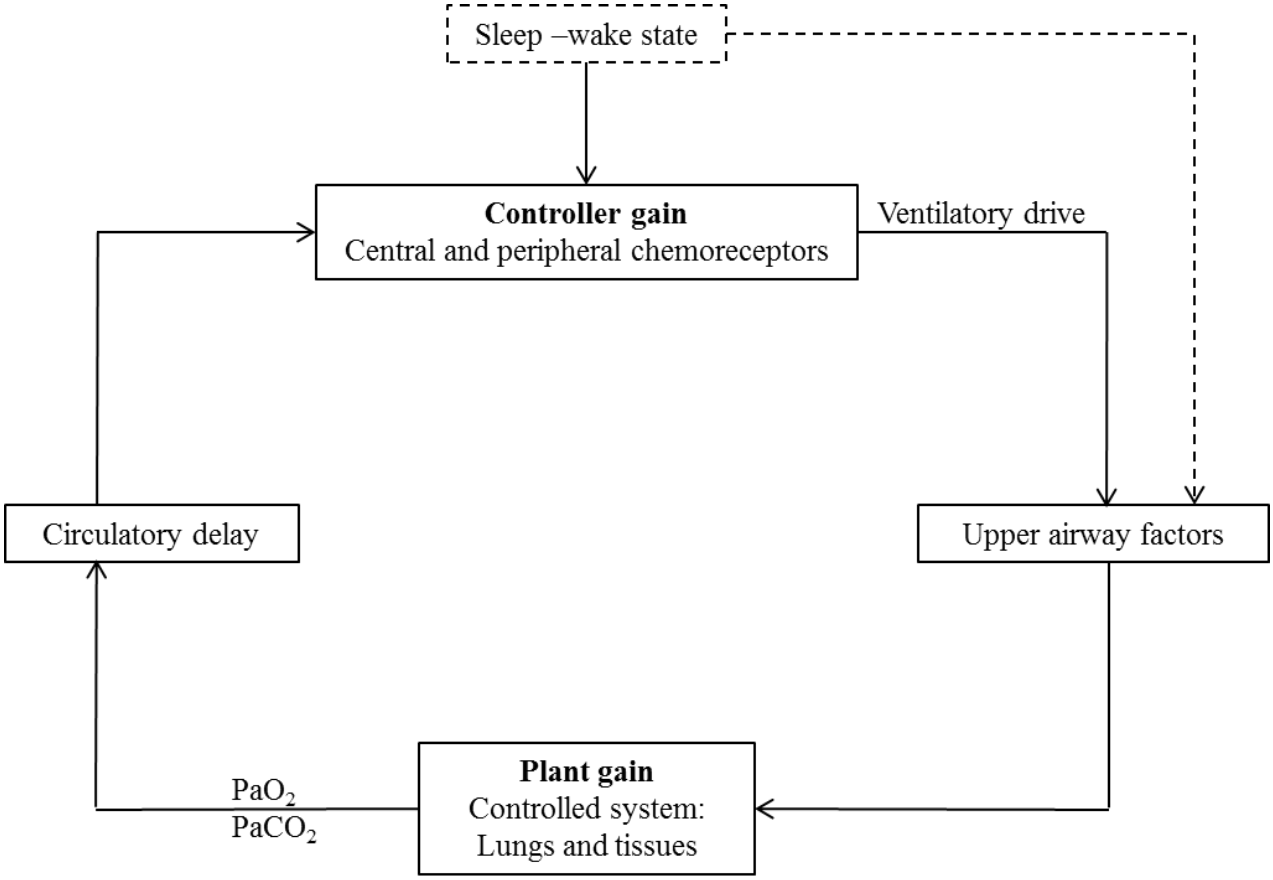


**Figure 3-a**. A simplified diagram of the control of breathing during sleep modified from Burgess (2012).

The controller gain relates to the response of the central and peripheral chemoreceptors to hypercapnia and hypoxia whereas plant gain primarily reflects the ability of a given level of ventilation to eliminate $CO_2$ referring to the lungs and tissues (White 2005, Burgess 2012). A high plant gain could be due to low functional residual capacity, low dead space, low metabolic rate and low cardiac output (White 2005). A circulatory delay effects the interaction between ventilation and the controller gain (Burgess 2012). There is an inherent delay between blood gas changes in the lung and the detection of changes at the sensor which is prolonged in conditions such as congestive heart failure (CHF) which further destabilises ventilation (White 2005). Unfortunately CHF is often prevalent amongst patients with OSA (Villa et al. 2003, Chan et al. 1997, Javaheri et al. 1995, Javaheri et al. 1998, Ancoli-Israel et al. 2003). Lastly, upper airway factors (e.g. resistance) affects the interaction between the controller gain and ventilation (Burgess 2012). In order for the system to become unstable, the loop gain would be greater than 1 as expressed by the equation:

$$\text{Loop gain} = \frac{\text{(response to disturbance)}}{\text{(the disturbance itself)}}$$

(White 2005)

For example, let us consider that the ventilatory response to hypercapnia doubles through adaptation. According to the equation, exposure to hypercapnia (the disturbance) brings about a greater response (response to disturbance) that is double the value of the disturbance, resulting in a loop gain of 2. The ventilatory response therefore overcompensates for the disturbance causing subsequent hypocapnia thus potentially leading to a further apnoea as a result. Furthermore, any delays in the feedback process would potentially cause further respiratory instability. This would cause a tendency for oscillation in response of the corrective action by the controller being delayed to the extent that it becomes out of phase with the preceding disturbance as observed during periodic breathing (Khoo 2010).

The higher the loop gain, the potentially more unstable the respiratory control system becomes (Burgess 2012). During sleep the influence of state of wakefulness on respiration is minimised making the presence of ventilatory instability more explicit (White 2005). An adaptation to the central and/or peripheral chemoreceptors could potentially alter controller gain by altering the chemosensitivity and therefore ventilatory response to hypercapnia and/or hypoxia potentially making patients with OSA more susceptible to periodic breathing (Leung et al. 2012, White 2005, Burgess 2012) or a prolonged apnoea duration (Verbraecken et al. 1995).

In support of the presence of increased loop gain as a contributor to the development of OSA or as a consequence of the severity of OSA or related factor, Younes et al. (2001) used proportional assist ventilation (PAV) to stimulate the ventilatory response to respiratory muscle activation and thereby increase the gain of the respiratory controller. This was performed comparing patients with mild/moderate OSA to patients with severe OSA after stabilising their upper airway patency during sleep using CPAP. In all the patients the presence of complex sleep apnoea was not observed (central apnoeas with CPAP) and therefore the loop gain must be less than one whilst on CPAP. Younes et al. (2001) found that the patients with severe OSA were more likely to develop periodic breathing with recurrent central apnoeas with the use of PAV compared to the patients with mild/moderate OSA. This occurred even when the patients with mild/moderate OSA were subjected to greater amplification of loop gain.

Studies which have investigated whether there is an alteration in the central or peripheral chemosensitivity amongst patients with OSA have found conflicting results (Sin, Jones & Man 2000, Radwan et al. 2000). Such examples of studies investigating the ventilatory response to hypercapnia include Verbraecken et al. (1995) which reports an increased ventilatory response slope in normocapnic patients with OSA with a depressed hypercapnic ventilatory response found in chronic hypercapnic patients with OSA. Gold (1993) on the other hand found a decreased ventilatory response to $pCO_2$ amongst normocapnic moderately obese patients with sleep apnoea and Narkiewicz (1999) found no change in the ventilatory response to $pCO_2$ in patients with OSA. These conflicting findings could be explained as a result of the variability in methodology applied and the inclusion/exclusion criteria used in different studies.

In Sin et al. (2000) study consisting of 104 patients with OSA and 115 controls the criteria of a AHI≥15 was used to dichotomize between patients with OSA from those without OSA. We feel any adaptation to the ventilatory response to $CO_2$ in the pathogenesis of OSA though, may occur early in the development of the disease. Our previous findings with experienced scuba divers found no correlation between the lowered ventilatory response to $CO_2$ and the number of dives performed suggesting that the adaptation either occurred early on in the participation of scuba diving or the response is inherit with those who are less prone to hypercapnia related symptoms staying in the diving population.

Based on our findings with experienced scuba divers we feel a concentration of 6% $CO_2$ should be used to identify the ventilatory response between groups. Many studies however researching the ventilatory response in patients with OSA have used a higher concentration of $CO_2$. This may result in a strong simulation of ventilation in all individuals including those with low sensitivity to $CO_2$. Moreover, many of the studies used progressive hypercapnia, starting at a high concentration of $CO_2$ which may not allow enough time between accumulations of $CO_2$ to assess the ventilatory response appropriately in the patients with OSA. Additionally some studies have not performed Respiratory Polygraphy on their control group and therefore, undiagnosed OSA or misdiagnosis can lead to apparently normal control subjects inadvertently affecting the results (Narkiewicz et al. 1999).

OSA is heavily associated with the development of numerous co-morbidities with 60% of patients with metabolic syndrome also experiencing OSA (Drager et al. 2010, Trombetta et al. 2013, Trombetta et al. 2010). Knowing which conditions are a consequence of OSA and

which conditions OSA contributes to the development of is debatable (Pillar & Shehadeh 2008). The most strict inclusion/exclusion criteria known is Foster et al. (2009) study which included patients who were 18-50 years of age, BMI <35 kg/$m^2$, no medications and no history of diabetes or cardio-respiratory disease including hypertension (defined as blood pressure >140/90 mm Hg). Foster et al. (2009) recruited 8 men with OSA and found the ventilatory, cerebrovascular and cardiovascular responses to hypercapnia were normal. In my personal experience patients who meet Foster et al. (2009) inclusion/exclusion criteria are rarely seen in clinic.

The relative contributions of the central and peripheral chemoreceptors in the control of ventilation, cardiac output and sympathetic responses to exercise are still debated (Dempsey 2005). These are complex research questions because while we can experimentally identify and isolate individual contributors underlying a given physiological response, each of these contributions almost always changes (increases or decreases), sometimes dramatically, when put together with all of the other influences inherent in the integrated response (Dempsey 2005). As with the previous study investigating the ventilatory response to $CO_2$ amongst scuba divers, the interaction between the central and peripheral chemoreceptors on the ventilatory response amongst patients with OSA has been overlooked. To our knowledge, all other studies have investigated the peripheral and central ventilatory response separately in patients with OSA discounting Duffin's (2007) recommendations to take into account the synergistic effects of hypoxia on the ventilatory response to $pCO_2$ (Lahiri & Delaney 1975, Lahiri & Forster 2003) in order to help understand the integrated response (Dempsey 2005). Furthermore obstructive apnoeic events are characterised by inspiratory flow obstruction associated with asphyxia (hypoxia and hypercapnia) and so to test the ventilatory response to a combined moderately hypoxic with hypercapnic gas mixture may provide a better simulation of OSA than hypoxia or hypercapnia alone (Cooper et al. 2004). Such a method of simulating OSA events has been implemented previously by Cooper et al. (2004), however, their research only tested healthy participants with no history of snoring or excessive daytime sleepiness and was designed to simulate the potential mechanisms which may contribute to the development of hypertension in patients with OSA.

In this chapter we investigate, using the methodology applied with the experienced scuba divers in the previous chapter, the ventilatory response to $CO_2$ in newly diagnosed patients with OSA awaiting CPAP treatment. Some similarities exist between the scuba divers

investigated in the previous chapter and the patients with OSA. Scuba divers are thought to be exposed to elevations of $CO_2$ during a scuba dive whereas the patients are exposed to intermittent episodes of hypercapnia and/or hypoxia during sleep (Cooper et al. 2005). Exposure to both hypercapnia and/or hypoxia has been implicated to induce changes in chemosensitivity amongst the patients with OSA (Cooper et al. 2005). Based on our previous findings with scuba divers we hypothesise that the exposure to intermittent hypercapnia during sleep will lower the ventilatory response to $CO_2$. However, unlike the scuba divers the patients are also frequently exposed to intermittent hypoxia during sleep which may also be associated with a further adaptation of the peripheral chemoreflex on the ventilatory response to $CO_2$ affecting the integrated response (Dempsey 2005). We apply relatively lenient inclusion/exclusion criterion in comparison to Foster et al (2009) study. This was thought to provide better external validity to help identify the greatest physiological implications in the 'typical' OSA patient attending Ysbyty Gwynedd's Sleep Clinic.

**Method:**

*Participants*

Male, newly diagnosed patients with OSA were recruited after consultation regarding their sleep study results in clinic at Ysbyty Gwynedd, Bangor. All participants were newly diagnosed with OSA and were tested within the two weeks prior to their treatment of CPAP. Written informed consent was obtained from all participants prior to testing. Patients were excluded if they had a BMI$\geq$50 kg/m$^2$. This was to avoid the recruitment of patients having predominantly obesity hypoventilation syndrome. Patients were also excluded if they were on medications known to affect their respiratory drive (i.e. opiate-based painkillers). Patients were diagnosed with OSA using an unattended home sleep study (respiratory polygraphy) (Embletta® Gold, Embla Systems, USA). Measures of pulse oximetry, nasal airflow, thoracic and abdominal movements were analysed using RemLogic software. Diagnosis of OSA was performed by either an experienced RCCP (Registration Council for Clinical Physiologists) registered Clinical Physiologist or an experienced Sleep Technologist. The physical characteristics of the two groups are displayed in Table 3-a unfortunately 2 medical records were unattainable. Medical notes were reviewed and patients with documented respiratory disease were excluded.

**Table 3-a.** Physical characteristics of the four groups where significantly different between patient groups ** = $p<0.01$. Values represent mean ± SD.

| Parameter | Mild/Moderate OSA | Severe OSA | Scuba divers | Non-diving controls |
|---|---|---|---|---|
| N | 16 | 16 | 10 | 10 |
| AHI | 12.9 ± 6.1** | 53.7 ± 18.8 | - | - |
| ODI | 14 0± 7.1** | 49.8 ± 22.4 | - | - |
| Age (yr) | 54.7 ± 9.9 | 50.9 ± 10.7 | 33.6 ± 8.9 | 31.2 ± 8.0 |
| Height (cm) | 173.9 ± 7.4 | 176.2 ± 7.8 | 177.7 ± 6.6 | 177.5 ± 5.2 |
| Mass (kg) | 100 0 ± 15.4** | 120.4 ± 21.1 | 75.8 ± 9.3 | 78.4 ± 7.3 |
| Neck (cm) | 42.9 ± 2.9** | 46.9 ± 3.9 | - | - |
| Waist (cm) | 109.0 ± 11.0** | 123.3 ± 13.6 | - | |
| Hip (cm) | 110.2 ± 11.0 | 117.7 ± 12.7 | - | - |
| BMI ($kg/m^2$) | 33.1 ± 4.6** | 38.9 ± 6.8 | 24.0 ± 2.5 | 24.9 ± 2.5 |
| BSA ($m^2$) | 2.19 ± 0.19** | 2.42 ± 0.23 | 1.93 ± 0.14 | 1.96 ± 0.10 |
| FEV1 (l) | 3.20 ± 0.43 | 3.21 ±0.74 | 4.47 ± 0.35 | 4.41 ± 0.62 |
| FVC (l) | 4.17 ± 0.61 | 4.09 ± 0.93 | 5.45 ± 0.49 | 5.49 ± 0.55 |
| FEV1/FVC (%) | 77.3 ± 7.9 | 79.1 ± 9.4 | 82.02 ± 0.42 | 80.33 ± 0.59 |

*General procedures*

This study was approved by the local North West Wales NHS Ethics Committee (Ref: Earing 11/WNo01/2) (Gwynedd, Wales) and carried out in accordance with the Declaration of Helsinki for research on human subjects. The study measurements were performed in a quiet room within the Pulmonary Function Department of Ysbyty Gwynedd. During the visit, participants breathed through the same breathing apparatus used with the healthy participants in the first study of this thesis, as shown in Figure 3-b. As with the first study the metabolic cart was calibrated prior to testing with a premixed gas composition of 13% $O_2$ with 6% $CO_2$ and calibration of the volume transducer was carried out while connected to the tubing and valve system with the Douglas bag disconnected.

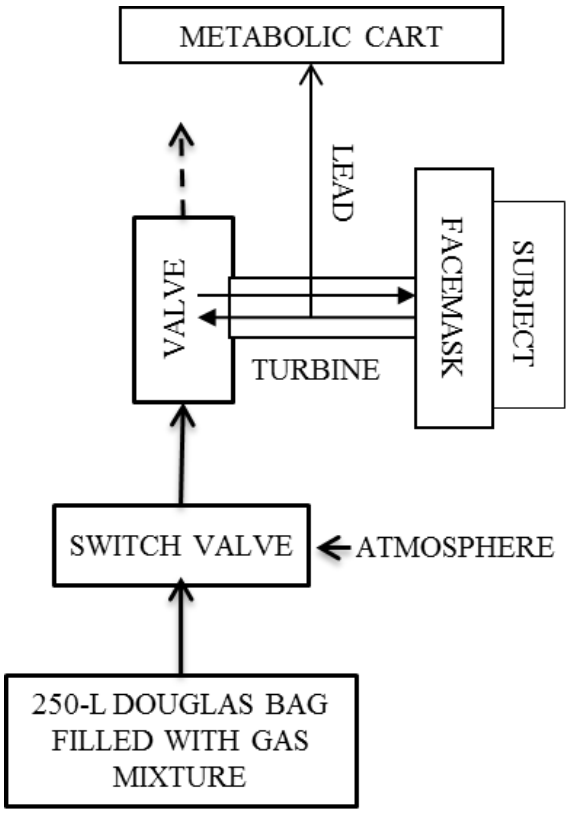


**Figure 3-b.** Set up of the breathing system

Participants' height, weight, neck, waist and hip circumference were measured first. Patients had previously performed spirometry in Ysbyty Gwynedd and so their previous results were used for this study. Each participant was then connected to a 3 lead electrocardiogram (ECG) of a Finometer® MIDI (Finapres Medical Systems, Netherlands) and finger cuff to allow ECG and blood pressure to be monitored during the entire study using BeatScope® Easy software (Finapres Medical Systems, Netherlands) situated on a standalone laptop. Additionally during the breathing of each gas mixture the patients' oxygen saturation levels were continuously monitored via pulse oximetry (Nonin Medical, The Netherlands). A drop to 75% oxygen saturation would end the test. This cut-off was decided as was previously used in Tun et al. (2000) study. Participants were also instructed to raise a hand to end the test early if needed.

During the tests, participants focused on a non-dramatic movie with questions being asked about the movie at the end of the study. This was done in order to avoid participants consciously controlling their ventilation (Eynan et al. 2003). Each participant breathed each gas mixture until a plateau in minute ventilation was achieved or three minutes of duration had passed. Participants were blinded to the order of tests, gas mixture 1 was breathed first which consisted of ambient air; this was used to obtain resting minute ventilation. Then the participants breathed each gas mixture in the following test order: mixture 2: 25% $O_2$/6% $CO_2$, Mixture 3: 13% $O_2$ and Mixture 4: 13% $O_2$ /6% $CO_2$. Participants were given adequate time between tests to allow ventilation and heart rate to return to resting baseline conditions.

*Data analysis*

All measurements are expressed in body temperature and pressure saturated units (BTPS) with mean ± SD and $p<0.05$ considered statistically significant. The patients with OSA were split into two groups dependent on their AHI. Group one: consisted of mild and moderate patients with OSA. To be classified as a patient with mild OSA an AHI of ≥5 and <15 with reported symptoms related to sleepiness was present (Epstein et al. 2009). To be classified as a patient with moderate OSA, a recording of AHI ≥15 and <30 was needed. Group two: consisted of patients with severe OSA (AHI ≥30).

ANOVA was used to compare the body characteristics and dynamic lung volumes between the groups. Furthermore, in order to normalise for anthropometric differences between the two groups as ventilation is proportional to metabolic rate and therefore body size, the participants minute ventilation with each gas mixture was divided by body surface area (BSA) (Menitove et al. 1984, Hirshman, McCullough & Weil 1975) estimated using the Mostellers equation as previously recommended as the most valid for use with obese individuals (Verbraecken et al. 2006).

A two factor ANOVA compared change from ambient (baseline) minute ventilation and change from ambient (baseline) minute ventilation/BSA between the two groups with each gas mixture. An independent *t*-test then compared the change in minute ventilation between the hypoxic hypercapnic gas mixture and the hyperoxic hypercapnic gas mixture in between each group to identify the contribution of the peripheral chemoreflex. Spearman's rho was used to test if any correlations existed between the participant's AHI (the marker of the severity of OSA), ventilatory response to each gas mixture and the body characteristics. Spearman's correlation coefficient ($r_s$) was used as AHI was not normally distributed (Field 2009).

A partial correlation was also performed on the variables change in minute ventilation and minute ventilation/BSA with the 25%$O_2$ / 6% $CO_2$ with AHI controlling for BMI. Log transformation was used to normalise the non-normally distributed variables in order to allow the partial correlation to be performed. Furthermore multiple regression was performed to predict AHI and ventilatory response to gas mixtures where significant correlations existed.

**Results:**

The patients with mild/moderate OSA had significantly lower body mass and related anthropometric measures which meant minute ventilation needed to be divided by BSA to normalise the participants minute ventilation for the anthropometric differences between the two groups (Menitove et al. 1984, Hirshman, McCullough & Weil 1975). The individual ventilatory response to the gas mixtures was highly variable. The difference between the most responsive participant (AHI = 6) and least responsive participant (AHI = 30) is presented in Figure 3-c.

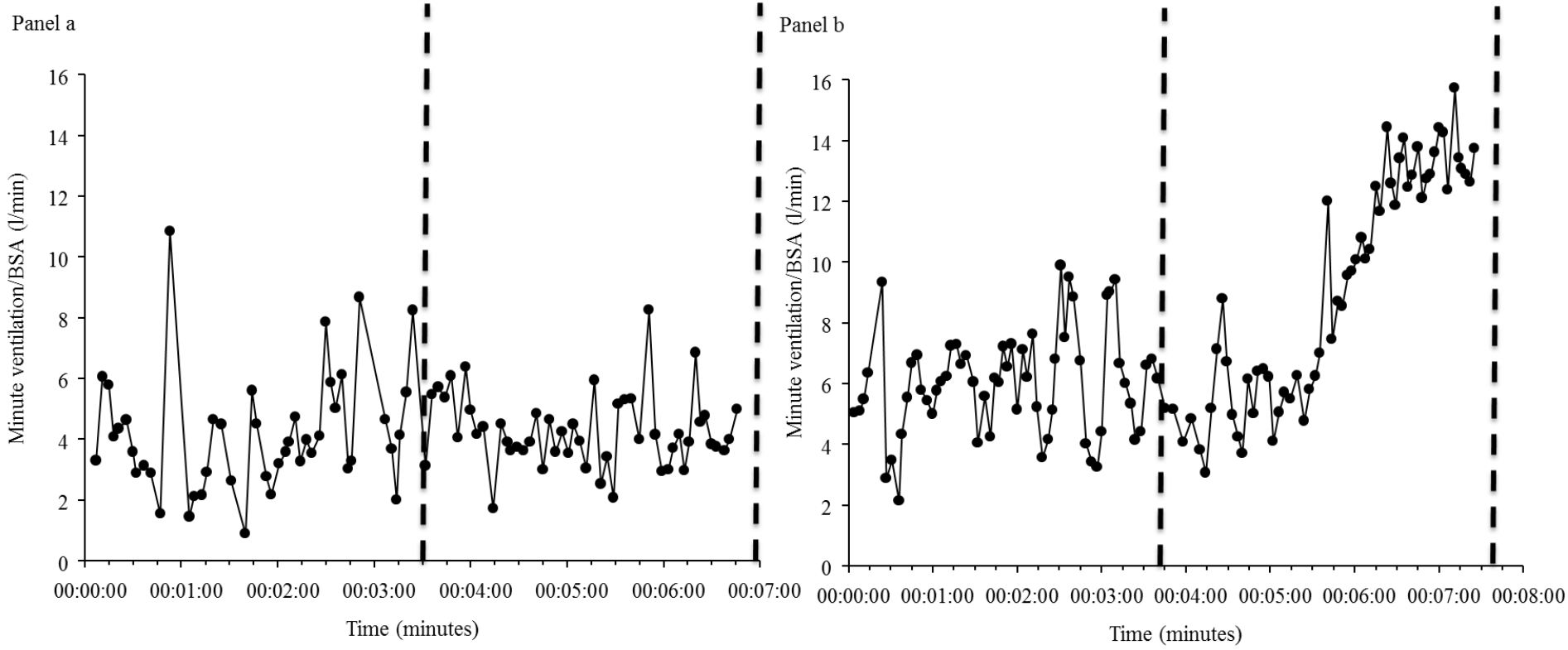


**Figure 3-c**. Panel a; Patient with a low ventilatory response to $CO_2$ (Patient with mild OSA AHI = 6). Panel b; example of a high ventilatory response (Patient with Severe OSA, AHI = 30). In both panels, between the two black dashed vertical lines the participant breathed the 25% $O_2$ / 6% $CO_2$ gas mixture.

The two factor ANOVA comparing ventilatory response between the two groups with each gas mixture (mild/moderate OSA vs. severe OSA) revealed no significant effect for group ($p$ = 0.06). A significant negative correlation however was found between the ventilatory response to the 25%$O_2$/6%$CO_2$ and AHI ($r_s$ = -0.39, $p$ = 0.027). The two factor ANOVA comparing the ventilatory response normalised by BSA however revealed the patients with severe OSA had a significantly lower ventilatory response change from baseline (ambient) with both the hyperoxic hypercapnic gas condition (25% $O_2$/6%$CO_2$: Mild/moderate OSA group: 5.40 ± 1.73 l/min/BSA, Severe OSA group: 3.12 ± 1.68 l/min/BSA, $p$ = 0.001) and the hypoxic hypercapnic gas condition (13% $O_2$/6% $CO_2$; Mild/moderate OSA group: 6.29 ± 2.62 l/min/BSA, Severe OSA group: 4.16 ± 2.19 l/min/BSA $p$ = 0.018). There was no significant difference in baseline (ambient) ventilation between the two groups (Mild/Moderate OSA group: 4.29 ± 1.55 l/min/BSA, Severe OSA group: 4.11 ± 0.85 l/min/BSA, $p$ = 0.688) and the change from baseline with the hypoxic gas mixture (13%$O_2$; Mild/moderate OSA group: 1.09 ± 0.82 l/min/BSA, Severe OSA group: 0.85 ± 0.85 l/min/BSA, $p$ = 0.432). The difference between the 25% $O_2$/ 6%$CO_2$ and the 13% $O_2$/ 6% $CO_2$ was not significant in both groups. The change in minute ventilation from resting (baseline) normalised by BSA with each gas mixture is displayed in Figure 3-d.

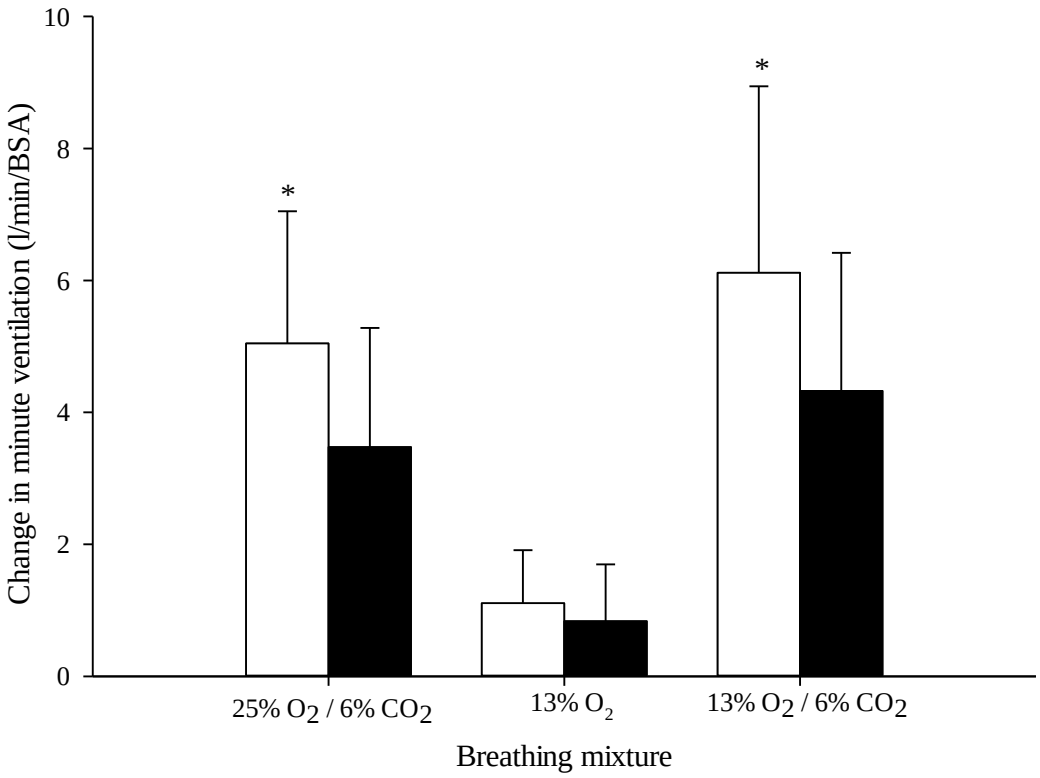


**Figure 3-d.** Change in minute ventilation from resting baseline (l/min/BSA) with each breathing mixture. □ = Patients with mild/moderate OSA ; ■= Patients with severe OSA, where significantly different between the groups with each gas mixture, * = $p$<0.05. Values represent mean ± SD.

Due to the variable AHI not meeting the assumption of normality, Spearman's rho investigated for correlations between body characteristics, ventilatory change from baseline (ambient) with each gas mixture normalised by BSA and AHI. Table 3-b presents the correlation matrix. AHI was found to be correlated with body mass and anthropometric measures. Additionally AHI was also found to be correlated with the ventilatory response to the 25%$O_2$/6%$CO_2$ normalised by BSA. Not presented on the correlation matrix, age was found to be significantly correlated with the ventilatory response to the hypoxic gas mixture (13% $O_2$) ($r_s$ = 0.42, $p$ =0.015).

In order to conduct a partial correlation to control for the effects of body mass on the correlation between AHI and ventilatory response to the 25% $O_2$/6%$CO_2$ normalised by BSA, the variables: AHI and BMI were first normalised using a log transformation. This was

performed as the variables AHI and BMI did not meet the assumption of normality using Kolmogorov-Smirnov (*D*) (AHI *D* (32), $p < 0.01$; BMI *D* (32) $p < 0.05$) and Shapiro-wilk (*W*) (AHI *W* (32), $p<0.01$; BMI *W* (32), $p<0.05$). A partial correlation was then conducted using the transformed variables and 25% $O_2$/6%$CO_2$ normalised by BSA. It was found controlling for BMI did weaken the correlation but did not remove the significance between AHI and the ventilatory response to 25% $O_2$/6%$CO_2$ normalised by BSA ($r = -0.36$, $p = 0.047$).

Multiple regression analysis was also performed to predict the ventilatory response to 25%$O_2$/ 6% $CO_2$/BSA with the log transformed variables BMI and AHI used as predictors. The residuals of the model met the assumption of normality and homoscedasticity. Tests to see if data met the assumption of collinearity indicated that multicollinearity was not a concern (Tolerance = 0.78, Variance Inflation Factor (VIF) = 1.29). Additionally multiple regression was used to predict AHI based on the variables neck circumference and ventilatory response to 25%$O_2$/ 6% $CO_2$/BSA. Neck was chosen as was most correlated with AHI in the correlation matrix displayed in Table 3-b. Residuals of this model though did not meet the association of normality. This was not the case however following log transformation of neck variable. The residuals of the model then met the assumption of normality and homoscedasticity. Tests to see if data met the assumption of collinearity indicated that multicollinearity was not a concern (Tolerance = 0.81, VIF = 1.24). The results of both regression models are displayed in Table 3-c and Table 3-d respectively. Both models were statistically significant with the model predicting AHI accounting for approximately 38% of variance and similarly the model predicting ventilatory response accounting for 40% percent of the variance.

**Table 3-b.** Spearman's rho correlation matrix of measured variables where * = $p<0.05$ and ** = $p<0.01$

| | AHI | Mass | Neck | Waist | BMI | 25%$O_2$/6%$CO_2$ | 13% $O_2$ | 13%$O_2$/6%$CO_2$ |
|---|---|---|---|---|---|---|---|---|
| AHI | 1.00 | | | | | | | |
| Mass (kg) | 0.49** | 1.00 | | | | | | |
| Neck (cm) | 0.54** | 0.67** | 1.00 | | | | | |
| Waist (cm) | 0.52** | 0.89** | 0.80** | 1.00 | | | | |
| BMI (kg/$m^2$) | 0.44* | 0.84** | 0.80** | 0.91** | 1.00 | | | |
| 25%$O_2$/6%$CO_2$ ΔVE (l/min/BSA) | -0.51** | -0.50** | -0.42* | -0.44* | -0.44* | 1.00 | | |
| 13% $O_2$ ΔVE (l/min/BSA) | -0.03 | 0.06 | 0.32 | 0.31 | 0.34 | 0.03 | 1.00 | |
| 13%$O_2$/6%$CO_2$ ΔVE (l/min/BSA) | -0.23 | -0.50** | -0.16 | -0.36* | -0.28 | 0.53** | 0.23 | 1.00 |

**Table 3-c.** Multiple regression to predict ventilatory response to 25%$O_2$/ 6% $CO_2$.

| | B | Standard Error (SE) B | β |
|---|---|---|---|
| Constant | 22.57 | 6.44 | |
| logAHI | -1.88 | 0.90 | -0.34* |
| logBMI | -10.14 | 4.47 | -0.38* |

$R^2$= 0.38, ($p$<0.01); * = $p$<0.05.

**Table 3-d.** Multiple regression to predict log transformed AHI.

| | B | Standard Error (SE) B | β |
|---|---|---|---|
| Constant | -4.93 | 2.70 | |
| logNeck | 3.99 | 1.60 | 0.40* |
| 25%$O_2$/6% $CO_2$ ΔVE (l/min/BSA) | -0.06 | 0.03 | 0.35* |

$R^2$ = 0.40, (p<0.01), * = $p$<0.05.

The participants who were involved in the previous study (Chapter 2) for the purposes of comparison of their minute ventilation with the patients with OSA had their minute ventilation normalised by their BSA estimated using the Dubois and Dubois equation (Dubois & Dubois 1989). Obviously the presence of sleep apnoea has not been investigated using respiratory polygraphy in the scuba divers or the non-diving controls however they are considered healthy according to the results of their health questionnaire. A one-way ANOVA with Bonferroni post hoc ($\varepsilon < 0.70$) revealed age was significantly different between the patients with OSA and the non-divers/scuba divers (scuba divers age: 33.6 ± 8.87; non-divers age: 31.2 ± 7.97; age of patients with mild/moderate OSA: 54.69 ± 9.88 $p = 0.000$; age of patients with severe OSA: 50.94 ± 10.68 $p = 0.00$). However, age cannot be used as a covariant within an ANCOVA as a significant interaction was found between change in minute ventilation/BSA and age ($p = 0.00$). Tukey post hoc test was performed ($\varepsilon > 0.70$) following the ANOVA revealing the non-diving controls of the previous study were found to have a significantly higher ventilatory response when breathing the 25%$O_2$ /6%$CO_2$ gas mixture compared to all groups (non-diving controls: 7.81 ± 2.41 l/min/BSA; scuba divers: 4.79 ± 2.56 l/min/BSA $p = 0.009$; patients with mild/moderate OSA: 5.40 ± 1.73 l/min/BSA $p = 0.025$; patients with Severe OSA: 3.12 ± 1.68 l/min/BSA $p = 0.00$). The scuba divers were found to not be significantly different to the patients with OSA with any of the gas mixtures. No significant differences between all groups were found with the hypoxic gas mixture (13% $O_2$). With the hypoxic hypercapnic gas mixture (13%$O_2$ / 6%$CO_2$), the non-diving controls of the previous study were found to have a significantly higher ventilatory response compared to both the patients with mild/moderate OSA and the patients with severe OSA (non-diving controls: 9.91 ± 3.26 l/min/BSA; patients with mild/moderate OSA 6.28 ± 2.62 l/min/BSA $p = 0.08$; patients with severe OSA: 4.16 ± 2.19 l/min/BSA $p = 0.00$). Figure 3-e shows the findings with and without minute ventilation being normalised by BSA. These findings may help to predict the results we would find if a group of matched controls were recruited in our study.

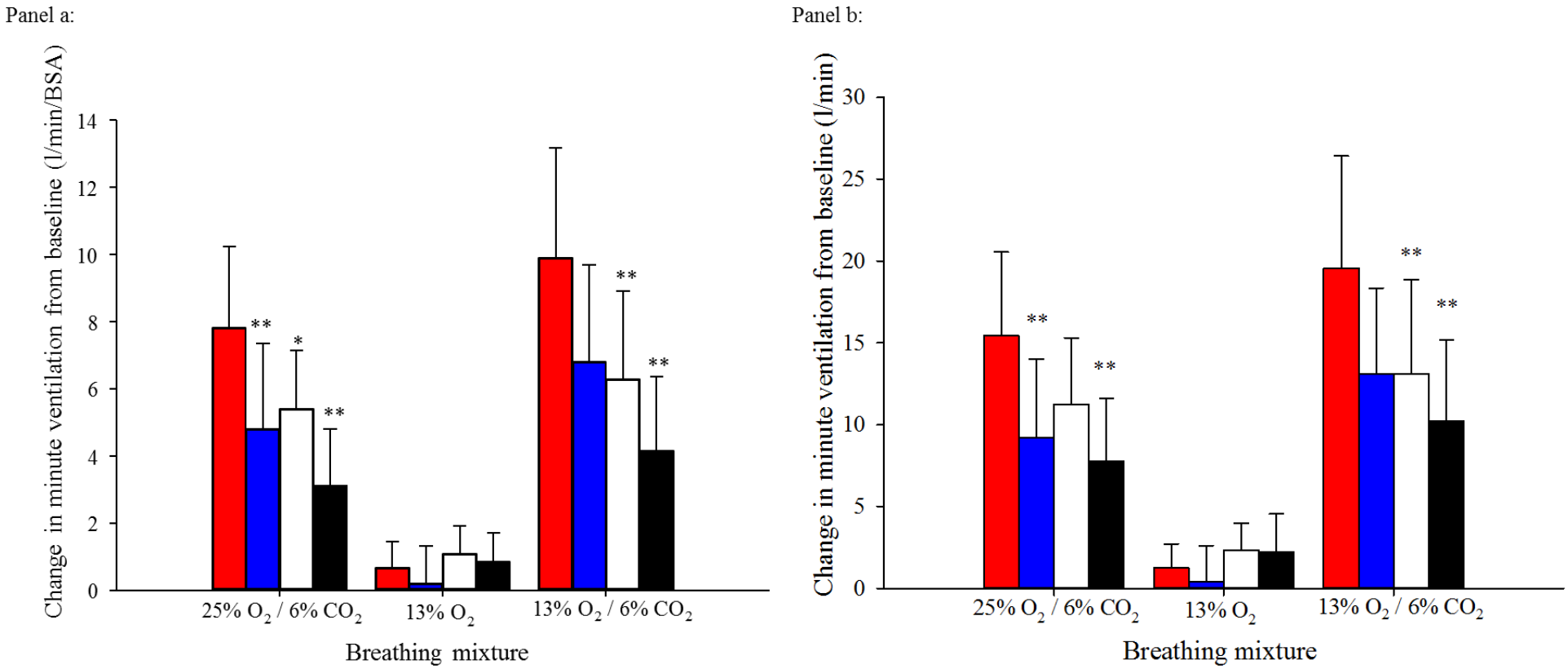


**Figure 3-e.** Panel a) Comparing change in minute ventilation normalised by BSA from resting baseline (l/min/BSA) with each breathing mixture. Panel b) Comparing change in minute ventilation between groups without correction for BSA. Where: ■ = non-diving controls from chapter two, ■ = scuba divers from chapter two, □= patients with mild/moderate OSA, ■ = patients with severe OSA. Values represent mean ± SD, * = $p$ <0.05, ** = $p$ <0.01 between non-diving control group and other groups.

**Discussion:**

The main aim of this study was to test the hypothesis that the pathogenesis of OSA involves a reduction in the ventilatory response to $CO_2$ which is observed during wakefulness. A novel approach was applied using a methodology previously described by Duffin (2007). We assessed for the first time the ventilatory response to $CO_2$ amongst patients with OSA with and without the presence of hypoxia. This method was used in order to identify if an alteration of the ventilatory response to $CO_2$ in patients with OSA exists and whether this involves an adaptation related to the central and/or peripheral chemoreceptors. Additionally, we investigated the influence of physical characteristics on the ventilatory response to $CO_2$ in the patients with OSA.

The patients with severe OSA in our study were found to display a significantly lower ventilatory response to $CO_2$ in both hypercapnic conditions; the difference between the two groups remained unchanged with the addition of hypoxia. These findings however were only present when ventilation was normalised by BSA. This may introduce some bias as the severe OSA patient group were signficiantly larger than the patients with mild/moderate OSA. A significant negative correlation was however found between the ventilatory response to the $25\%O_2/6\%CO_2$ and AHI ($r_s$ = -0.39, $p$ = 0.027) without normalisation for BSA. When ventilation was normalised by BSA a significant relationship was found between the severity of sleep apnoea (AHI) and the change in minute ventilation when breathing the hypercapnic hyperoxic gas mixture ($r_s$ = -0.51, $p$ <0.01) which wasstill present when BMI was taken into account using a partial correlation ($r$ = -0.36, $p$ =0.047). Interestingly this relationship was not present with the hypercapnic hypoxic condition which may provide some evidence to suggest a change in the hypoxic ventilatory response with OSA. Perhaps the ventilatory response to hypoxia is altered in an attempt to compensate for the lowered ventilatory response to hypercapnia. Recently Fiamma et al. (2013) have shown in decerebrate vagotomised, *in situ* rat preparations that central apnoeas can be overcome using peripheral chemoreceptor stimulation alone when $CO_2$ stimulation of the central chemoreceptors is diminished. Fiamma and colleagues suggest therapeutic targeting of the peripheral chemoreceptors may help to reduce the occurrence of central apnoea. To date, there is no evidence of the central chemoreceptors responding to an interaction between $O_2$-$CO_2$ and therefore our main findings

suggest with our tested oxygen pressures, that the altered ventilatory response to $CO_2$ is likely the result of an alteration that acts predominantly on the central chemoreceptors.

The peripheral chemoreceptors have been shown to behave more rapidly compared to the central chemoreceptors (Dempsey 2005) and as a result, it can be believed that the peripheral chemoreceptors play a vital role in the initiation of an apnoea in response to a single ventilatory overshoot. Once the apnoea is initiated though the ensuing $CO_2$ retention must also then influence the central as well as the peripheral chemoreceptors (Dempsey 2005). Indeed our study did only test the ventilatory response with 13% $O_2$ chosen to achieve moderate hypoxia (Goodall, Ross & Romer 2010) and a mean end-tidal $pO_2$ of 56.5± 3.99 mm Hg. In order to rule out any adaptation acting upon the peripheral chemoreceptors, a series of different hypoxic isoxic $CO_2$ responses at different $pO_2$ tensions needs to be measured to fully characterise the peripheral chemoreflex (Duffin 2007).

Recently, Iceman et al. (2013) have been able to confirm the presence of $CO_2$ stimulated 5-HT medullary raphe neurons in rats *in situ* and, for the first time, shown a subset of neurons identified to be TPH-ir (5-HT-synthesizing enzyme tryptophan hydrolase-immunoreactive) that are stimulated by moderate levels of hypercapnia. Dysfunction of the medullary 5-HT neurons has been thought to be involved in the pathogenesis of Sudden Infant Death Syndrome, Congenital Hypoventilation Syndrome, sudden unexplained death in Epilepsy, Prader-Willi Syndrome, panic disorder and other neurodegenerative diseases (Iceman, Richerson & Harris 2013, Sowers et al. 2013, Hilaire et al. 2010, Kinney 2009, Richerson et al. 2001).

*Implications of loop gain:*

Our data may support a reduction in loop gain via the central chemoreceptors becoming less responsive in OSA; this may lead to prolonged apnoea duration (Verbraecken et al. 1995). Interestingly however the correlation between AHI was diminished in the hypoxic hypercapnic condition, potentially hypoxia may compensate for the difference in the ventilatory response to $CO_2$ with different tested oxygen pressures modifying loop gain. Furthermore, the potential contribution of an alteration in plant gain on the ventilatory response to $CO_2$ in our data cannot be dismissed. Although $FEV_1$ and FVC were not significantly different between the two groups, other factors including changes in functional residual capacity, dead space, metabolic rate, cardiac output and diffusion capacity can all

lead to a change in plant gain (White 2005). Unfortunately diffusion capacity and total lung capacity could not be measured as the facilities were not available and so only spirometry data was present in this study. Furthermore, circulatory delay may be different amongst the participants particularly due to the potential cardiovascular comorbidities associated with OSA. The presence of circulatory delay will further effect the interaction between ventilation and the controller gain (Burgess 2012).

*Influence of obesity:*

The patients recruited in our study are a representative sample of the patients who were attending the local sleep clinic (Ysbyty Gwynedd) for treatment of OSA. The investigated study population is similar to that of Subramanian et al. (2012) research, which retrospectively reviewed patients with OSA referred to a sleep laboratory in the USA between 2006 and 2008. The 661 male patients that were reviewed required CPAP only, had an average BMI: 35.2 ± 7.14 kg/m$^2$. The patients of our study had a mean BMI of 36.0 ± 6.46 kg/m$^2$. It is acknowledged that many of these patients will have other comorbidities which may be undiagnosed at the time of study. One such condition which alters the ventilatory response to hypercapnia and hypoxia is Obesity Hypoventilation Syndrome (Jokic et al. 2000, Zwillich et al. 1975) often referred to as "Pickwickian syndrome" (Powers 2008).

Obesity Hypoventilation Syndrome is defined as obesity (BMI >30 kg/m$^2$) with hypoventilation ($PaCO_2$>45 mm Hg) without another coexisting pulmonary, chest-wall, or neuromuscular condition contributing to ventilatory impairment (Powers 2008). The prevalence of Obesity Hypoventilation Syndrome in sleep apnoea is thought to be 8-10% if BMI is between 30-34 kg/m$^2$ and 18- 25% if BMI > 40 kg/m$^2$ (Powers 2008, Mokhlesi, Kryger & Grunstein 2008). Early diagnosis of Obesity Hypoventilation Syndrome is rare. Mokhlesi et al. (2007) found 30% of the patients with OSA were later found to be diagnosed with Obesity Hypoventilation Syndrome. We did not rule out the presence of Obesity Hypoventilation Syndrome in our study population. The correlation matrix reveals the severity of sleep apnoea in our study is moderately correlated with increased body mass ($r_s$ = 0.49, $p$ = 0.004), waist circumference ($r_s$ = 0.52, $p$ = 0.003) and in particular neck circumference ($r_s$ = 0.54, $p$ <0.001). Partial correlation controlling for body mass did weaken the correlation between AHI and the ventilatory response to $CO_2$ with hyperoxia however despite this AHI was still significantly correlated ($r$ = -0.36, $p$ =0.047). Furthermore minute ventilation of each individual was normalised by their body surface area with an ANOVA

revealing the ventilatory response to $CO_2$ was significantly higher in the patients with mild and moderate OSA compared to the patients with severe OSA (Figure 3-d).

As a result of the relationship between body parameters and the ventilatory response to $CO_2$, two regression models were generated. The first was designed to predict the ventilatory response to $CO_2$ using the predictors BMI and AHI, this model is believed to predict 38% of the variance in ventilatory response to $CO_2$ ($p<0.01$). Whilst the second regression model designed to predict AHI used neck circumference as this was found to be the most correlated parameter with AHI. The other predictor was the ventilatory response to $CO_2$, it is believed this model is capable of predicting 40% of the variance in AHI ($p<0.01$) with both factors almost equally contributing to the model (logneck β 0.40, $p<0.05$; ventilatory response to $CO_2$ β 0.35; $p<0.05$).

*Ventilatory response to hypoxia:*

In terms of the ventilatory response to hypoxia, only age was found to be significantly related to the hypoxic gas mixture ($r_s = 0.42$, $p<0.015$). Ageing has been previously reported to increase the ventilatory response leading to the maintenance of arterial $O_2$ saturation in hypoxia (Lhuissier, Canouï-Poitrine & Richalet 2012) and a heighted peripheral chemoreceptor hypersensitivity is a feature of chronic heart failure (Ponikowski et al. 2001). A high prevalence of chronic heart failure exists in sleep apnoea patients (Villa et al. 2003, Chan et al. 1997, Javaheri et al. 1995, Javaheri et al. 1998, Ancoli-Israel et al. 2003). This is associated with abnormalities in the cardiorespiratory reflex control found to be responsible for heightened peripheral chemosensitivity and depressed baroreflex function (Ponikowski et al. 2001).

*Comparison with scuba divers:*

In comparison with the previous study conducted on experienced scuba divers and non-diving controls, the non-divers had a significantly higher ventilatory response to $CO_2$ compared to the patients with OSA and the scuba divers. The scuba divers however had no such significant difference with the patients with OSA. Comparable to the patients with OSA during sleep, the scuba divers are thought to be exposed to elevations of $CO_2$ during a scuba dive, the exposure to both hypercapnia and/or hypoxia has been implicated to induce changes in chemosensitivity (Cooper et al. 2005). Obviously we cannot rule out a presence of OSA

amongst the experienced scuba divers as this was not tested. However, based on the pre-study health questionnaires the scuba divers did not express any health complaints compared to the patients with OSA. These results may therefore provide some support for the exposure to hypercapnia during sleep implicating the changes in ventilatory response to $CO_2$ amongst the patients with OSA.

Limitations:

Other studies have identified the ventilatory response being altered in patients who are hypercapnic during wakefulness (Verbraecken et al. 1995). Unfortunately a blood gas analyser was not available during the study so exclusion of patients with daytime hypercapnia was not feasible. Although the presence of obesity hypoventilation syndrome was reduced by excluding individuals with a BMI >50 kg/$m^2$, it is acknowledged that obesity hypoventilation syndrome associated with daytime hypercapnia can be present with a lower BMI (Powers 2008, Mokhlesi, Kryger & Grunstein 2008). Given the significant correlations between body mass, hip, neck and waist circumference it is logical to assume the presence of obesity hypoventilation syndrome is going to be increased with the severity of OSA. This may potentially skew our data and explain the lowered ventilatory response being found amongst the patients with severe OSA. To control for the difference in body mass amongst groups normalisation of ventilation by BSA was performed this will also influence our results ideally an obesity matched control group would have been also investigated however this not feasible in the population of patients coming to Ysbyty Gwynedd's sleep clinic. Furthermore greater recruitment would potentially allow mild, moderate and severe groups to be compared as opposed to combining mild and moderate patients with OSA together as one group.

**Conclusion:**

Further research is needed to understand the impact of the comorbidities associated with OSA on the development of the ventilatory response to $CO_2$. It is well acknowledged the pathogenesis of OSA can be highly variable across individuals and therefore other factors in addition to or in absence of the alteration of chemosensitivity are also likely to be involved in the development of the ventilatory response to $CO_2$ observed in our study population.

In summary, the findings of this study provide a stimulus for further investigation; we have shown that the ventilatory response to $CO_2$ is altered with OSA during wakefulness. Using the

tested oxygen pressures in our study, the findings support that the alteration of the ventilatory response to $CO_2$ involves the central chemoreception only. This study further highlights the contribution of body characteristics on the pathogenesis of OSA and the ventilatory response to $CO_2$. Furthermore, with the comparison of the results with our previous study with scuba divers, the findings of this study provide potential support for the exposure to hypercapnia during sleep implicating the changes in ventilatory response to $CO_2$ amongst patients with OSA. Other comorbidities associated with OSA such as metabolic syndrome and cardiovascular conditions have previously been shown also to alter the ventilatory control (Trombetta et al. 2013). A broader investigation incorporating these other factors is needed in order to fully understand the contribution of the different mechanisms in the development of the ventilatory response to $CO_2$ in OSA.

## Chapter 4 Baroreflex sensitivity in patients with OSA and its association with chemosensitivity to $CO_2$

**Abstract**:

*Purpose:* The evaluation of baroreflex sensitivity is an established measurement for the assessment of autonomic control (La Rovere, Pinna & Raczak 2008). In this chapter, the baroreflex sensitivity of patients with OSA is investigated and correlational analysis is performed to assess whether an association exists between an adaptation of the baroreceptor sensitivity and the ventilatory response to hypercapnia and/or hypoxia.

*Methods:* The baroreflex sensitivity of 33 patients with OSA was investigated using the spontaneous method during wakefulness in the supine position. Baroreflex sensitivity was calculated using BeatScope Easy software which utilises a time domain assessment estimating baroreflex sensitivity based on the cross-correlation function of blood pressure and pulse interval. This method has been shown to have values which correlate strongly with and close to the EuroBaVar averages and yield more values per minute, with lower within-patient variance and measured baroreflex delay (Westerhof et al. 2004).

*Results:* Initially no correlations were found between baroreflex sensitivity, AHI, body characteristics and ventilatory response to $CO_2$ with and without control of hypertension using a partial correlation. Further analysis of the data revealed the large variability amongst the participants in the number of detected baroreflex measurement sequences (range = 8 to 185 measures). Stratification was therefore performed based on the median number of measures. Participants were placed into a low measures or a high measures group dependent upon whether their number of measures was greater or less than 100 (the median) detected baroreflex sensitivity measurements. It was found the low measures group had a significantly higher AHI ($p = 0.049$) providing possible evidence to suggest that the spontaneous method of assessing baroreflex sensitivity during wakefulness was not appropriate amongst the patients with more severe OSA.

*Conclusions:* Our study finds that baroreflex sensitivity is less strongly associated with AHI than other parameters like body mass or the ventilatory response to $CO_2$. Caution however has also arisen as to whether the spontaneous approach is appropriate in measuring baroreflex sensitivity during wakefulness in the severe OSA patient group.

**Introduction:**

The baroreceptors are stretch receptors located in the vessel wall of the carotid sinus and aortic arch which act as a negative feedback system designed to buffer beat-to-beat fluctuations in arterial blood pressure from an internal set point or baseline. Afferent baroreceptor discharge is relayed from the carotid sinus via the glossopharyngeal nerve and from the aorta via the vagus nerve to the nucleus tractus solitarius (NTS), which evoke changes in efferent sympathetic and parasympathetic outflow to the heart and blood vessels (Thomas 2011). Baroreflex sensitivity refers to the response in heart beat interval to a change in blood pressure expressed in ms/mm Hg (Westerhof et al. 2004). It is recognised as an important mechanism in the regulation of arterial blood pressure (Freet, Stoner & Tang 2013) and its evaluation is regarded as an established tool for the assessment of autonomic control (La Rovere, Pinna & Raczak 2008). A reduction in arterial baroreflex sensitivity has been associated with increased sympathetic nerve activity (Grassi et al. 1998) and higher blood pressure (Wustmann et al. 2009, Trombetta et al. 2010).

*Hypertension and OSA:*

Hypertension is the most extensively studied cardiovascular outcome related to OSA. However, most of the studies published prior to 1995 were limited by small sample sizes and possible confounding factors such as obesity; a factor strongly related to both hypertension and OSA (Levitzky & Redline 2010). As a result, the association between OSA and hypertension was previously disregarded by many clinicians and researchers (Silverberg, Oksenberg & Iaina 1997). Recently though, Tremea et al. (2014) has published plans for a novel randomised controlled trial which aims to test the use of diuretics to treat both hypertension and OSA. This intervention is designed to reduce the extravascular fluid shift from the legs which is believed to contribute to the increased neck circumference during sleep in the supine position and also to increase salt and water excretion causing a reduction in renal sympathetic activation (Cichelero et al. 2014, Esler et al. 2010).

Epidemiological evidence for a connection between hypertension and OSA now includes six large cohort studies (Sleep Heart Health Study (Nieto et al. 2000), Wisconsin Sleep Cohort (Peppard et al. 2000), Southern Pennsylvania (Bixler et al. 2000), Spanish Cohort (Durán et al. 2001), Outcomes of Sleep Disorders in Older Men Study (Mehra et al. 2007) and a multi-center study in China (He et al. 2010)). These studies each support an association

between OSA and hypertension however, since data from observational studies may never completely address residual confounding or precisely identify temporal associations, data from experimental studies such as randomised controlled trials are required (Levitzky, Redline 2010).

The relationship between treatment of OSA and the effects this has on hypertension has also been subject of debate (Kartali et al. 2013). Pepperell et al. (2002) conducted a randomised, parallel, double blind trial assessing 24 hour mean blood pressure changes in patients with OSA treated with either therapeutic nasal CPAP or subtherapeutic nasal CPAP to act as a placebo. It was found in the majority of the patients with severe OSA, a reduction in blood pressure occurred with the therapeutic CPAP after one month of use. These findings support Faccenda et al. (2001) who used an oral placebo tablet as a control for therapeutic nasal CPAP over 1 month's duration. Campos-Rodriguez et al. (2006) however also assessed the effect of CPAP on ambulatory blood pressure monitoring in patients with OSA. These patients were selected for having hypertension and were therefore all receiving antihypertensive treatment, unlike the Pepperell et al. (2002) study. Campos-Rodriguez et al. (2006) study was also randomised, double-blind and used subtherapeutic CPAP as a placebo. The results of the study revealed that four weeks of therapeutic CPAP did not significantly improve ambulatory blood pressure. Campos-Rodriguez et al. (2006) suggests that these findings may be because the patients had already achieved the maximum decrease in blood pressure possible with their pharmacologic treatment and so CPAP could only add a modest reduction in which four weeks of duration was not enough.

Hypertension is also a major risk factor for arterial stiffness yet limited studies have investigated whether there is a relationship between arterial stiffness and OSA (Kartali et al. 2013). Recently, Kartali et al. (2013) showed that hypertensive patients with OSA have a more pronounced arterial stiffness than normotensive controls with less severe OSA. Furthermore, Kartali et al. (2013) demonstrated that long-term CPAP (after 3 months) reduced blood pressure in the hypertensive patients and arterial stiffness with a favourable effect apparent even after the first night of CPAP implementation.

*Baroreflex sensitivity and OSA:*

The proposed mechanisms contributing to the development of hypertension with OSA include the changes in sympathetic activity during apnoeic episodes characterised by inspiratory flow

obstruction and associated with hypoxia and hypercapnia (Cooper et al. 2004). It is possible this may cause an alteration in baroreflex sensitivity or baroreceptor resetting to occur as a result of the frequent episodes of nocturnal hypertension (Cooper et al. 2004). A depressed baroreflex sensitivity has been observed in patients with severe OSA especially in stage two of non-rapid eye movement (NREM) sleep and during nocturnal wakefulness with a significant improvement following 6 weeks of CPAP therapy (Ryan et al. 2007). Furthermore, this finding is supported by a randomised controlled trial which found daytime baroreflex sensitivity is significantly increased in patients treated with therapeutic CPAP compared to subtherapeutic CPAP (Kohler et al. 2008).

Cooper et al. (2004) performed experiments inducing mild asphyxia ($12\%O_2/5\%CO_2$) and inspiratory gas flow obstruction separately and together with healthy subjects, examining their resulting carotid baroreflex sensitivity and 'set point'. These experiments were designed to simulate the increased inspiratory pressure and asphyxia exposure which occurs in OSA. The findings however are limited, as they simulated only the effects of a relatively mild episode of OSA and did not examine whether the responses persisted following the stimulus. Cooper et al. (2004) found breathing against an inspiratory resistance reduced baroreflex sensitivity whereas asphyxia alone did not change baroreflex sensitivity, however shifted the set point to work at higher arterial pressures. Additionally, the combination of inspiratory resistance and asphyxia resulted in a combination of the effects of two stimuli applied separately.

There is little doubt that considerable interaction exists between the chemoreceptors and the baroreceptor reflexes (Cooper et al. 2005, Somers, Mark & Abboud 1991). Chemoreflex activation elicits an increased vascular sympathetic outflow via the efferent limb of the reflex arc causing blood pressure to rise due to increased systemic vascular resistance (Olson & Somers 2013). On the other hand, activation of the arterial baroreceptors has an inhibitory influence on the chemoreflex. However, this can be impaired such as in heart failure which can lead to a counterproductive cycle of increased sympathetic activity resulting in augmented peripheral chemoreflex sensitivity and further increased sympathetic outflow (Olson & Somers 2013, Heistad et al. 1972).

Independently, hypoxic stimulation of the peripheral chemoreceptors has been shown to cause vasoconstriction and hypertension (Cooper et al. 2004, Hainsworth et al. 1983b, Hainsworth et al. 1983a), whereas central hypercapnia has been shown to increase vascular resistance (Cooper et al. 2004, Soladoye, Rankin & Hainsworth 1985) in anaesthetised dogs

with their cephalic circulation acutely perfused with $pCO_2$. The potential interactions between the baroreceptors and the chemoreceptors may be explained by findings from neurophysiological studies demonstrating interneuronal connections (Cooper et al. 2005, Somers, Mark & Abboud 1991, Miura & Reis 1972). Both the peripheral chemoreceptor and the arterial baroreceptor afferent fibres terminate in the NTS of the medulla (Miura & Reis 1972). There is however some speculation that stimulation of the baroreceptors or chemoreceptors afferences may activate different groups and ratio of fibers, which may then contribute to reported differences in the timing of synaptic processing at $2^{nd}$-order NTS neurons (Accorsi-Mendonca & Machado 2013).

Further interaction between sympathetic function and the central chemoreceptors may be facilitated by a selection of sympathetic preganglionic neurons responsible for roles as a vasoconstrictor, adrenal, renal and cardio-accelerator. These neurons receive dominant excitatory input from the rostral ventrolateral medulla (RVLM) and other excitatory inputs from the spinal cord interneurons, the caudalmost portion of the medulla oblongata, the raphe, and the hypothalamus. Inhibitory inputs arrive from the areas of the ventromedial medulla, the spinal cord and the raphe (Guyenet 2010). As previously discussed in chapter 2, introduction section, it is assumed that the central chemoreception resides around the ventral medullary surface (Guyenet 2010, Mulkey et al. 2004) however acidification of many other brainstem or cerebellar regions with dialysis probes has also been found to activate breathing to some degree and in some cases, simultaneous stimulation of two regions often produced additive effects (Guyenet, Stornetta & Bayliss 2010). This locality of neural connections is likely to be critical for blood pressure stability and blood gas regulation (Guyenet 2010, Guyenet 2006).

In the previous chapter, we observed that individuals with severe OSA (high AHI) have a lowered ventilatory response to $CO_2$. We therefore hypothesise that individuals may display an altered baroreflex sensitivity associated with their previous ventilatory response findings. In this chapter, the baroreflex sensitivity of patients with OSA is investigated using the spontaneous method. To our knowledge this method has only been used in Crisalli et al. (2012) and Ryan et al. (2007) research. Crisalli et al. (2012) investigated children with OSA and found an improvement in baroreflex sensitivity following adenotonsillectomy during both sleep and wakefulness. Ryan et al. (2007) study on adults with OSA focussed on baroreflex sensitivity also during sleep and nocturnal wakefulness.

In comparison to the spontaneous method, other methods of measuring baroreflex sensitivity are more invasive. These methods include the use of vasoactive drugs particularly α-adrenoreceptor agonist phenylephrine. Another method is the use of the Valsalva manoeuvre which produces a natural challenge for the baroreceptors by voluntarily increasing intrathoracic and abdominal pressure through straining this also has limited clinical applicability with patients with advanced heart disease. Finally, used in research laboratories for particular pathophysiological investigations, the neck chamber technique involves applying negative/positive pressure to the neck region, (La Rovere, Pinna & Raczak 2008).

The resting spontaneous method utilises weighted regression analyses to examine changes in sympathetic baroreflex sensitivity or heart rate cardiovagal baroreflex sensitivity as they relate to natural resting oscillations in diastolic arterial pressure or systolic arterial pressure respectively (Yang & Carter 2013). The sympathetic baroreflex sensitivity is estimated by responses in muscle sympathetic nerve activity (MSNA) to changes in diastolic arterial pressure, whereas the cardiovagal baroreflex sensitivity which is used in our study is estimated by responses in heart rate to changes in systolic arterial pressure (Yang & Carter 2013). The spontaneous baroreflex sensitivity has been shown to be reliable against more invasive pharmacological approaches (Yang & Carter 2013, Hart et al. 2010). A significant correlation has been demonstrated between spontaneous baroreflex sensitivity and the Valsalva manoeuvre (Yang & Carter 2013) and Hart et al. (2010) reported that spontaneous baroreflex sensitivity was significantly correlated with the modified Oxford technique, which is regarded as the gold standard pharmacological approach to estimating baroreflex sensitivity (Yang & Carter 2013) . Lastly, the spontaneous approach applied in this study needs the least additional equipment and therefore the least clinical resources compared to other more invasive methods.

The proposed study serves two purposes, firstly to investigate the baroreflex sensitivity during wakefulness amongst patients with OSA. The second purpose is entirely novel in which we will investigate whether an alteration of baroreflex sensitivity amongst patients with OSA is related to an alteration in the ventilatory response to hypercapnia with and without hypoxia. To achieve this we will test if a correlation exists between the ventilatory response data of the previous study and the baroreflex sensitivity identified amongst the patients with OSA.

**Method:**

*Participants*

Male patients newly diagnosed with OSA were recruited after consultation regarding their sleep study results in the clinic at Ysbyty Gwynedd. All participants were diagnosed with OSA using the same procedures stated in the previous chapter and were tested within the two weeks prior to their treatment of CPAP. Written informed consent was obtained from all subjects prior to testing. Patients were excluded if they had a BMI≥50 or if they were prescribed medications known to effect respiratory drive (i.e. opiate-based painkillers). The ventilatory response to hypercapnia and hypoxia data from the previous study was incorporated into this current study for correlational analysis.

*General procedures*

This study was approved by the local North West Wales NHS Ethics Committee (Ref: Earing 11/WNo01/2) (Gwynedd, Wales) and carried out in accordance with the Declaration of Helsinki for research on human subjects. The study was performed in a quiet room within the Pulmonary Function Department of Ysbyty Gwynedd. If participants were also performing the ventilatory response tests to the gas mixtures on the same day, the baroreflex sensitivity tests were always conducted first as Cooper et al. (2005) reports "hypercapnia appears to have a lasting effect after the removal of the stimulus". Participants were instructed to abstain from unaccustomed vigorous exercise within 24 hours prior to the visit, not to consume caffeine at least 8 hours prior to the visit and to refrain from consuming any food two hours prior to visit. This was stated on their study information sheet and also verbally during a phone conversation the day prior to the visit.

*Assessing the baroreflex sensitivity:*

Beat-to-beat blood pressure was recorded using a Finometer MIDI (Finapres Medical Systems, Amsterdam, Netherlands) which was generously funded by Ysbyty Gwynedd's League of Friends in 2011 for use in this project. Patients were positioned near supine on a reclining chair and electrocardiography (ECG) electrodes of the Finometer MIDI were attached to the patient. The Finometer MIDI uses an inflatable finger cuff with built-in photo-electric plethysmograph. From the finger pressure waveform, heart beats are detected and

systolic, diastolic and mean pressure and pulse rate are outputted in a beat-to-beat mode (Imholz et al. 1998). Participants' middle finger was placed in the required cuff size according to the user manual and the height sensor was calibrated prior to the recording which corrects for any artefacts related to any positional changes during the test.

Participants were measured for 20 minutes with the last five minutes being used in the study results. When available, participant's breathing rate was measured via thoracic and abdominal belts (Embletta® Gold, Embla Systems, USA) and analysed afterwards using RemLogic software. In all cases, measured breathing rate was 15 ± 1 breaths per minute which corresponds with the recommendations stated in Bernardi et al. (2011) review on methods for assessing cardiac autonomic function. Baroreflex sensitivity was calculated using BeatScope Easy software which is displayed in Figure 4-a. This software utilises a time domain assessment estimating baroreflex sensitivity based on the cross-correlation function of blood pressure and pulse interval. This method has been shown to have values which correlate strongly with and close to the EuroBaVar averages and yield more values per minute, with lower within-patient variance and measured baroreflex delay (Westerhof et al. 2004). The EuroBaVar study compared spontaneous baroreflex sensitivity estimates obtained from an identical set of data from eleven European centers using different methods and procedures (Laude et al. 2004).

The calculated baroreflex sensitivity data was exported into Microsoft Excel enabling the data of the last five minutes of each test to be averaged. The medical history of each participant was analysed to allow consideration of the presence of hypertension.

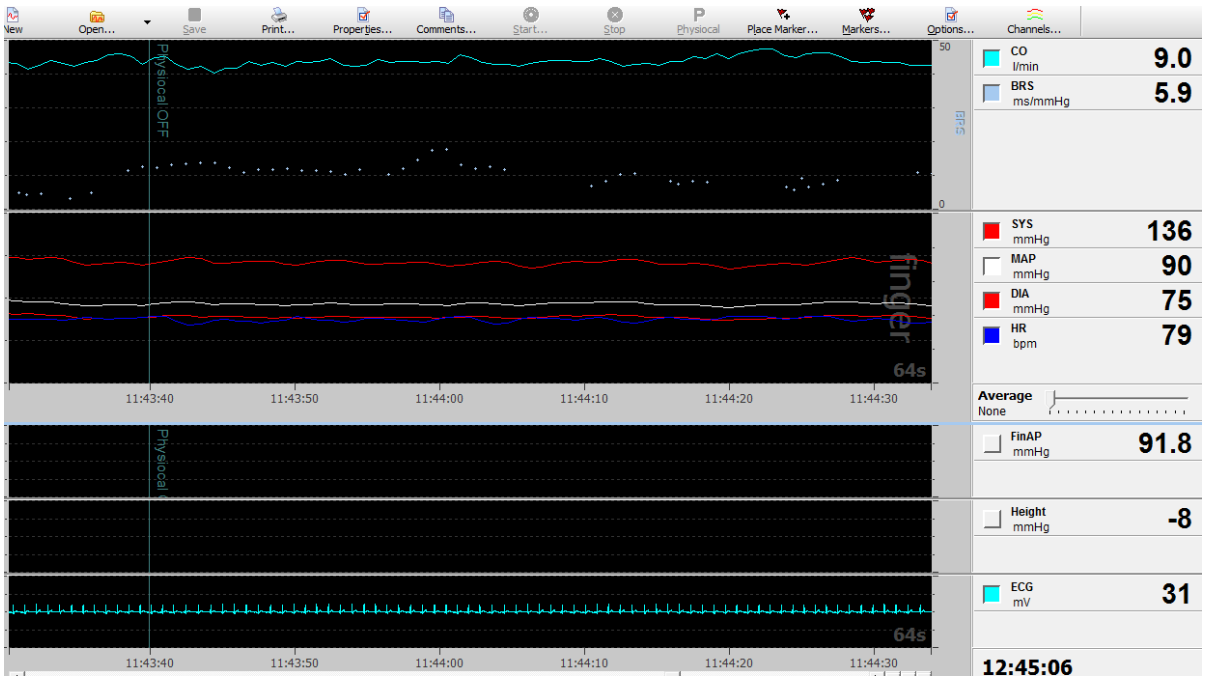


**Figure 4-a**. Screenshot of the data displayed when recording blood pressure beat-by-beat using BeatScope Easy software. The grey dots represent software calculated baroreflex sensitivity values.

*Data analysis*

All data are presented mean ± SD and $p<0.05$ is considered statistically significant. Previous respiratory data are expressed in BTPS. Spearman's rho was used to test if any correlations existed between the participant's AHI, ventilatory response to each gas mixture, body characteristics and baroreflex sensitivity. Spearman's correlation coefficient ($r_s$) was used as AHI was not normally distributed (Field 2009).

As with the previous study, patients with OSA were split into two groups dependent on their AHI. Group 1: consisted of mild and moderate patients with OSA and group 2: patients with severe OSA. To be classified as a mild OSA patient an AHI of ≥5 and <15 with reported symptoms related to sleepiness was present. To be classified as a moderate OSA patient, a recording of AHI ≥15 and <30 was required. A severe OSA patient had an AHI ≥30 (Epstein et al. 2009).

An ANCOVA was performed controlling for hypertension. Participants were categorised into either hypertensive or not hypertensive using the following criteria: not hypertensive = category 1: systolic blood pressure <130 mm Hg and diastolic < 85 mm Hg and hypertensive = category 2: systolic blood pressure >130 mm Hg or on antihypertensive medications. Partial

correlations were performed to control for the effect of hypertension. Variables which did not meet the assumption of normality were data transformed using log transformation prior to being used in the partial correlations.

The frequency of baroreflex sensitivity measurements was also compared between the two groups to assess the feasibility of the baroreflex sensitivity measurements using the spontaneous technique during wakefulness in the patients with OSA. As a result participants were placed into two groups based on the median number of detected baroreflex sensitivity measurements. Correlational analysis and partial correlation controlling for hypertension was then performed on the high number of baroreflex sensitivity measurements group.

**Results:**

The physical characteristics between the two groups were tested for significant differences using a one-way ANOVA which revealed the results shown in Table 4-a. As found in the previous studies, the mild/moderate patients with OSA had significantly lower body mass and associated anthropometric measures. Furthermore, the findings revealed mild/moderate patients with OSA were more likely to have normotensive blood pressure ($p<0.01$). Three patients were excluded from data analysis. Two patients because of their baroreflex sensitivity was not regarded as feasible (not having more than three detectable baroreflex sensitivity measures based on the procedures of Kardos et al. (2001) and one patient because of having ectopic heart beats throughout the ECG trace.

**Table 4-a**. Physical characteristics of the two groups where significantly different, ** = $p<0.01$. Where data was normally distributed values represent mean ± SD, median is used where data is non-normally distributed.

| Parameter | Mild/Moderate OSA | Severe OSA |
|---|---|---|
| N | 17 | 16 |
| AHI | 13.1** | 56.6 |
| ODI | 16.7** | 51.6 |
| Age (yr) | 53.2 ± 9.4 | 49.5 ± 13.3 |
| Height (cm) | 174.4 ± 6.5 | 175.4 ± 8.2 |
| Mass (kg) | 100.0** | 117.5 |
| Neck (cm) | 43.0** | 47.0 |
| Waist (cm) | 107.0** | 125.0 |
| Hip (cm) | 108.0 | 116.5 |
| BMI ($kg/m^2$) | 32.3** | 39.6 |
| Baroreflex sensitivity (ms/mm Hg) | 6.0 | 8.1 |
| Hypertension category | 1.0** | 2.0 |
| $25\%O_2$/ $6\%CO_2$ $\Delta$VE (l/min/BSA) | 11.9** (n = 15) | 6.8 (n = 13) |
| 13% $O_2$ $\Delta$VE (l/min/BSA) | 1.8 (n =15) | 1.9 (n = 13) |
| $13\%O_2/6\%CO_2$ $\Delta$VE (l/min/BSA) | 14.9 (n = 15) | 10.7 (n=13) |

In order to investigate whether the baroreflex sensitivity was significantly different between the two groups, an ANCOVA was performed using hypertension as a covariant. No significant difference could be found. No correlations were also found between baroreflex sensitivity, the ventilatory response to the gas mixtures data from the previous chapter, AHI and any of the other parameters investigated.

In order to conduct a partial correlation to control for the effects of hypertension on the correlation between AHI, body characteristics, ventilatory response to the hypercapnia and/or hypoxia normalised by BSA and baroreflex sensitivity, the variables: AHI and baroreflex sensitivity were first normalised using a log transformation. This was performed as the variables AHI and baroreflex sensitivity did not meet the assumption of normality using Kolmogorov-Smirnov (*D*) (AHI *D* (33), $p < 0.05$; baroreflex sensitivity *D* (33) $p < 0.01$; and Shapiro-wilk (*W*) (AHI *W* (33), $p < 0.01$; baroreflex sensitivity *W* (33), $p < 0.01$). A partial correlation was then conducted with the transformed variables.

The partial correlation controlling for hypertension on baroreflex sensitivity also found no significant relationships existed between baroreflex sensitivity with any of the parameters. The results of Spearman's rho and the partial correlation on the parameters of interest are displayed in Table 4-b and Table 4-c respectively. Figure 4-b displays two scatterplots; the first scatterplot panel a. displays logtransformed baroreflex sensitivity against log transformed AHI. Scatterplot panel b. is the result of the partial correlation between baroreflex sensitivity and AHI controlling for hypertension conducted on log transformed AHI and baroreflex sensitivity.

**Table 4-b.** Spearman's rho Correlation matrix of measured variables where * =$p$<0.05 and ** = $p$<0.01. BRS = Baroreflex sensitivity.

| | AHI | BRS | Neck | Mass | BMI | 25%$O_2$/6%$CO_2$ | 13% $O_2$ | 13%$O_2$/6%$CO_2$ |
|---|---|---|---|---|---|---|---|---|
| AHI | 1.00 | | | | | | | |
| BRS (ms/mmHg) | 0.20 | 1.00 | | | | | | |
| Neck (cm) | 0.56** | -0.09 | 1.00 | | | | | |
| Mass (kg) | 0.51** | -0.04 | 0.67** | 1.00 | | | | |
| BMI (kg/$m^2$) | 0.48** | -0.12 | 0.81** | 0.86** | 1.00 | | | |
| 25%$O_2$/6%$CO_2$ ΔVE (l/min/BSA) | -0.53** | -0.13 | -0.39* | -0.50** | -0.39* | 1.00 | | |
| 13% $O_2$ ΔVE (l/min/BSA) | -0.03 | -0.25 | 0.27 | 0.01 | 0.29 | 0.06 | 1.00 | |
| 13%$O_2$/6%$CO_2$ ΔVE (l/min/BSA) | -0.21 | -0.23 | -0.02 | -0.42* | -0.18 | 0.53** | 0.27 | 1.00 |

**Table 4-c.** Partial correlation matrix controlling for hypertension on the outcome variables AHI, body characteristics and variables representing the potential interaction between the baroreceptors and chemoreceptors. Where logBRS = log transformed baroreflex sensitivity whereas logAHI = log transformed AHI. Where signficiant * = $p<0.05$ and ** = $p<0.01$.

| | logAHI | logBRS | Neck | BMI | 25%$O_2$/6%$CO_2$ | 13% $O_2$ | 13%$O_2$/6%$CO_2$ |
|---|---|---|---|---|---|---|---|
| logAHI | 1.00 | | | | | | |
| logBRS | 0.37 | 1.00 | | | | | |
| Neck (cm) | 0.45* | 0.06 | 1.00 | | | | |
| BMI (kg/$m^2$) | 0.37 | -0.30 | 0.78** | 1.00 | | | |
| 25%$O_2$/6%$CO_2$ ΔVE (l/min/BSA) | -0.48* | -0.19 | -0.33 | -0.42* | 1.00 | | |
| 13% $O_2$ ΔVE (l/min/BSA) | -0.19 | -0.34 | 0.16 | 0.18 | 0.10 | 1.00 | |
| 13%$O_2$/6%$CO_2$ ΔVE (l/min/BSA) | -0.26 | -0.25 | 0.02 | -0.16 | 0.59** | 0.41* | 1.00 |

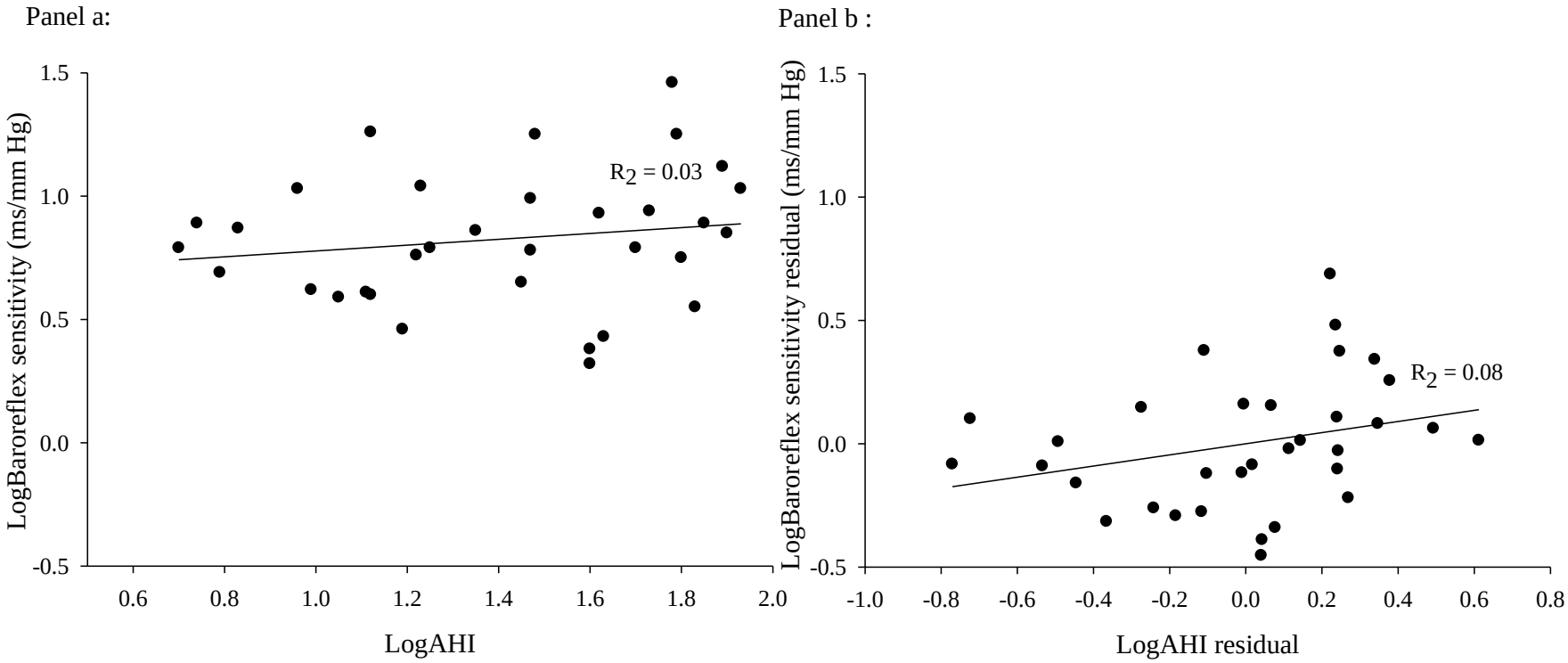


**Figure 4-b.** Panel a; Scatterplot of log transformed baroreflex sensitivity against log transformed AHI. Panel b; Scatterplot of partial correlation of baroreflex sensitivity residuals against AHI residuals showing the effect of hypertension as a control variable.

There was variability amongst the participants in the number of detected baroreflex measurement sequences (range = 8 to 185 measures). The number of detected measures of baroreflex sensitivity was not significantly different between the mild/moderate patients with OSA and the severe patients with OSA. No correlations between the study parameters and the number of detected baroreflex measures were found. Figure 4-c displays the variability between the number of detected baroreflex sensitivity measures and the baroreflex sensitivity.

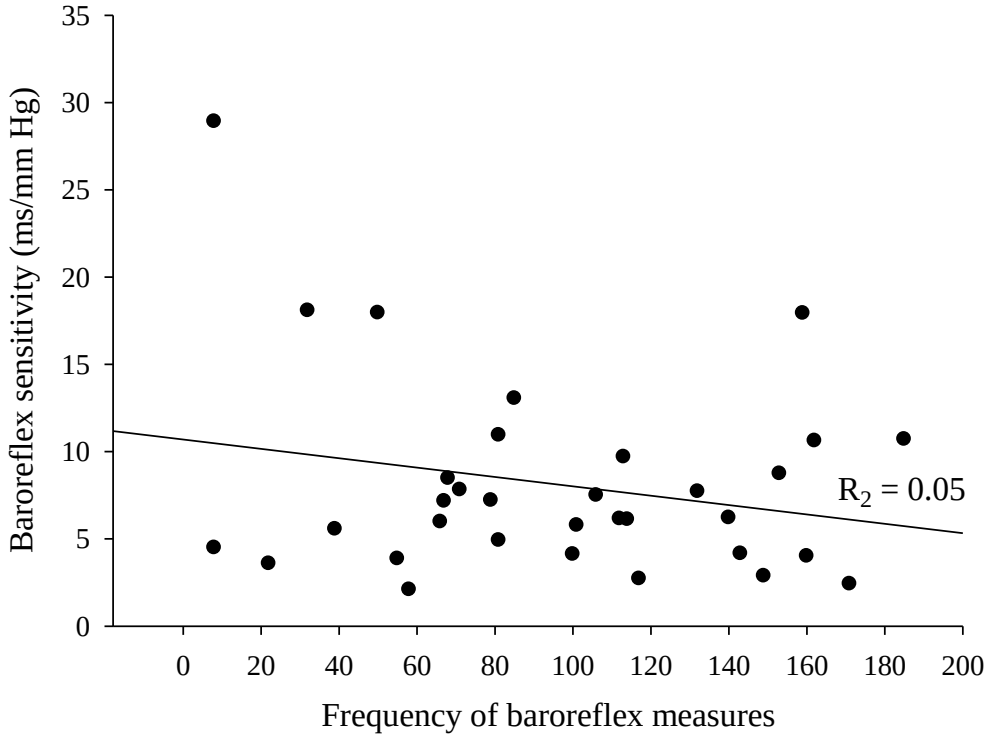


**Figure 4-c.** Number of detected baroreflex sequences compared to baroreflex sensitivity measurement.

To further investigate whether the variability in the number of baroreflex sensitivity measurements had affected the results, stratification was also applied based on the median number of baroreflex measurements. The median number of baroreflex sensitivity measures was 100 measures. Therefore, participants with less than 100 detected baroreflex sensitivity measurements were placed into a low measures group whereas participants with 100 or more measures were placed into a high measures group. A one-way ANOVA then compared all parameters between the two groups revealing the group with the high number of baroreflex measurements had a significantly ($p$ = 0.049) lower AHI (AHI = 26.12 ± 22.09), compared to the low number of baroreflex measurements group (AHI 43.11 ± 25.49) This provides some

evidence to suggest that the spontaneous method of assessing baroreflex sensitivity during wakefulness may not be appropriate amongst patients with severe OSA as further explained in the discussion section.

We excluded partcipants with fewer than 100 measures and reassessed correlations in the remaining 17. Significant correlations were found as displayed in the correlation matrix in Table 4-d. The mean baroreflex sensitivity when the 17 subjects were only considered was 6.9 ± 3.9 ms/mm Hg compared to 8.1 ± 5.7 ms/mm Hg when the 33 subjects were analysed. Furthermore, when a partial correlation was performed controlling for hypertension with the high number of baroreflex measurements group, the correlations were still significant as displayed in the correlation matrix in Table 4-e. Log transformed AHI and BRS were used as these variables did not meet the assumption of normality.

**Table 4-d.** Spearman's rho correlation matrix of measured variables where * =$p<0.05$ and ** = $p<0.01$. BRS: baroreflex sensitivity.

| | AHI | BRS | Age | 13% $O_2$ |
|---|---|---|---|---|
| AHI | 1.00 | | | |
| BRS (ms/mm Hg) | 0.06 | 1.00 | | |
| Age (years) | -0.51* | -0.61** | 1.00 | |
| 13% $O_2$ $\Delta$VE (l/min/BSA) | -0.09 | -0.79** | 0.62** | 1.00 |

**Table 4-e.** Results of partial correlation controlling for hypertension showing significant correlations. Where * = $p<0.05$ and ** = $p<0.01$. logBRS = log transformed baroreflex sensitivity and logAHI = log transformed AHI.

| | logAHI | logBRS | Age (years) | 13% $O_2$ |
|---|---|---|---|---|
| logAHI | 1.00 | | | |
| logBRS | 0.26 | 1.00 | | |
| Age (years) | -0.64** | -0.54* | 1.00 | |
| 13% $O_2$ $\Delta$VE (l/min/BSA) | -0.17 | -0.72** | 0.45 | 1.00 |

**Discussion:**

Our results suggest OSA does not alter the baroreflex sensitivity during wakefulness. We were unable to find a significant correlation between the severity of OSA, the measured baroreflex sensitivity and the ventilatory response to $CO_2$. After studying the data in detail however, it was recognised that individuals with a low number of baroreflex sensitivity measurements (<100 measures) have a significantly higher AHI (AHI 43.11 ± 25.49) suggesting that possibly the spontaneous methodology during wakefulness in the patients with severe OSA is not appropriate. When the participants with under 100 measures of baroreflex sensitivity were excluded and the effects of hypertension controlled, significant partial correlations were found between baroreflex sensitivity and age ($r$ = -0.54, $p$ <0.05) and the ventilatory response to the hypoxic gas mixture (13% $O_2$) ($r$ = -0.72, $p$ <0.01). The mean baroreflex sensitivity with the exclusion of the low number of measurements group was 6.9 ± 3.9 ms/mm Hg lower than the mean baroreflex sensitivity of 9.9 ms/mm Hg which was reportedly found using the spontaneous approach amongst 575 male healthy subjects with an age range of 18-60 years in Kardos et al. (2001) study.

*Alteration of baroreflex sensitivity in OSA:*

Previous research conducted with patients with OSA has observed a reduced baroreflex sensitivity in children (McConnell et al. 2009, Coverdale et al. 2012, Walter et al. 2013) and adults (Ryan et al. 2007, Carlson et al. 1996). However, previous studies though have employed different methods for investigating baroreflex sensitivity with most investigating baroreflex sensitivity during sleep (Ryan et al. 2007, McConnell et al. 2009, Walter et al. 2013) as opposed to wakefulness. Those studies which have investigated baroreflex sensitivity during wakefulness have either reported an unaltered sensitivity (Narkiewicz et al. 1998) or depressed baroreflex sensitivity (Carlson et al. 1996, Cortelli et al. 1994). To our knowledge, only two studies have used the spontaneous approach to assess baroreflex sensitivity amongst patients with OSA during different grades of wakefulness. Ryan et al. (2007) during nocturnal wakefulness (prior to falling asleep) and Crisalli et al. (2012) amongst children with OSA during wakefulness, finding an improvement in baroreflex sensitivity following adenotonsillectomy.

It has been suggested the sequence of events in OSA which include breathing cessation, nocturnal hypoxia, continuous brief arousals and sleep fragmentation enhances oxidative stress (Buckley & Schatzberg 2005) and elevated sympathetic activity (Vatansever et al. 2011). Previous research has also shown baroreflex sensitivity is significantly affected by obesity (Kardos et al. 2001, Grassi et al. 1995) and this is correctable through weight loss (Grassi et al. 1998). As we know, obesity is highly prevalent amongst the OSA population and our study population was predominately obese (BMI: 35.9 ± 6.0 kg/m$^2$). Obesity is associated with diabetes, cardiovascular diseases including hypertension, dyslipidemia and atherosclerosis (Hubert et al. 1983, Rahmouni, Haynes & Mark 2002). Moreover even in the absence of hypertension, obesity has been shown to be correlated to increased sympathetic overdrive resulting in subclinical organ damage to the heart, blood vessels and kidneys in young subjects (Lambert et al. 2010, Smith & Minson 2012). More specifically, abdominal visceral fat has been found to be significantly related to muscle sympathetic nerve activity with men displaying subcutaneous obesity being found to have similar muscle sympathetic nerve activity to non-obese men with similar abdominal visceral fat (Alvarez et al. 2004). Despite our study population being predominately obese however, no significant correlation was found between baroreflex sensitivity and any of the outcome variables related to body mass. This was the case with and without the use of a partial correlation to control for hypertension. Some of these findings may be partly attributed to study limitations which are discussed later.

In our study when only participants with higher than 100 baroreflex sensitivity measures were considered, a negative relationship was found between age and baroreflex sensitivity ($r_s$ = -0.61, $p$<0.01) which was significant also when hypertension was controlled with the use of a partial correlation ($r$ = -0.54, $p$ <0.05). A negative correlation between baroreflex sensitivity and age is in agreement with Kardos et al. (2001) study investigating baroreflex sensitivity using also the spontaneous methodology in a healthy working population (n = 575 males) with a mean age of 36.5 ± 10.4 years compared to our study population with mean age of 49.29 ± 8.7 years (n = 17).

*Interaction between baroreflex sensitivity and ventilatory response to $CO_2$:*

Despite little doubt that considerable interaction exists between the chemoreceptors and the baroreceptor reflexes (Cooper et al. 2005, Somers, Mark & Abboud 1991), the only correlation which was found was when the participants with less than 100 baroreflex

sensitivity measurements were excluded. As a result of this stratified exclusion of data, a strong negative correlation was found between the ventilatory response to the hypoxic gas mixture (13% $O_2$) and the baroreflex sensitivity with the participant's hypertension controlled for using a partial correlation ($r$ = -0.72, $p$ <0.01). It is known that activation of the arterial baroreceptors have an inhibitory influence on the chemoreflex which can be impaired in conditions such as heart failure, leading to a counterproductive cycle of increased sympathetic activity and this resulting in augmented peripheral chemoreflex sensitivity and increased sympathetic outflow (Olson & Somers 2013, Heistad et al. 1972). It is possible the more severe patients with OSA who tended to have the lower number of baroreflex sensitivity measures and so were subsequently excluded, were more likely to have the comorbidities associated with the impairment of the interaction between the baroreceptors and the chemoreceptors. However these findings may be the result of the spontaneous approach to assessing baroreflex sensitivity during wakefulness being inappropriate amongst patients with severe OSA as discussed in the limitations section.

Our previous study (Chapter 3) predominantly focused on the ventilatory response to $CO_2$. It was also found that the ventilatory response with the hypoxic gas mixture (13% $O_2$) between the mild/moderate and the severe OSA patient groups were not significantly different with no correlation found between the severity of OSA and the ventilatory response. Previously in humans, the interaction between the baroreceptors and the chemoreceptors has been reported as being specific to hypoxia which activates primarily the peripheral chemoreceptors (Somers, Mark & Abboud 1991). Furthermore, Cooper et al. (2005) published a study investigating healthy subjects assessing the effects of breathing hypoxia (12% $O_2$ in $N_2$) and hyperoxic hypercapnia (5% $CO_2$ in 95% $O_2$) independently. It was found hypoxia but not hypercapnia depresses baroreflex sensitivity, supporting the interaction occurs in both directions. The baroreflex activation inhibits peripheral chemoreflex responses and peripheral chemoreflex activation has an inhibitory effect on arterial baroreflex responses (Cooper et al. 2005). In this current study when participants of the high baroreflex measurement group were assessed a significant correlation between baroreflex sensitivity and ventilatory response to the hypoxic gas mixture (13% $O_2$) ($r$ = -0.72, $p$ <0.01) was found. It is possible if we investigated the effects of breathing a lower $pO_2$ gas mixture to the previously tested 13% $O_2$, significant findings may have been found suggesting an alteration in the ventilatory response providing some evidence towards a potential interaction between the alteration of the baroreflex and the peripheral chemoreflex being a mechanism in the

development of OSA. However caution is required, as a significant difference in baroreflex sensitivity between the OSA patient groups or significant relationship between baroreflex sensitivity and AHI was not found in this study.

Tamisier et al. (2011) also investigated healthy subjects using 24 hour ambulatory monitoring of blood pressure before and after 13 nights of intermittent hypoxia exposure similar to the stimulus experienced by patients with severe OSA (85-95% $O_2$ desaturation-resaturation). It was shown for the first time that arterial pressure rise is sustained throughout the waking hours following hypoxic exposure; this suggesting that sympathoactivation induced by intermittent hypoxia likely contributes to blood pressure elevation which may be derived from reduced baroreflex sensitivity (Tamisier et al. 2011).

It is well known that apnoeas produce both hypoxia and hypercapnia whereas studies such as Cooper et al. (2005) and Tamisier et al. (2011) have used a model which assesses the influence of hypoxic hypocapnia. Such an approach may underestimate the effect, since increased $CO_2$ enhances the cardiovascular responses to hypoxia in both healthy individuals (Tamisier et al. 2011, Morgan et al. 1995, Tamisier et al. 2004) and patients with sleep apnoea (Tamisier et al. 2011, Kara, Narkiewicz & Somers 2003). Similar to the gas concentration of 13% $O_2$ with 6% $CO_2$ we used in the previous study, Cooper et al. (2004) investigated healthy subjects breathing 12% $O_2$ with 5% $CO_2$ with baroreflex sensitivity also being measured. It was found although the gas mixture did cause significant increases in mean blood pressure; it did not alter baroreflex sensitivity. Cooper et al. (2004) did find however that breathing against inspiratory resistance decreased baroreflex sensitivity and this effect was amplified when combined with breathing the gas mixture. Apnoeic events are characterised by inspiratory flow obstruction (Cooper et al. 2004). It has been suggested that negative pressure breathing influences the cardiovascular system through a direct effect of increased respiratory drive, with the baroreceptor reflex effectively being gated by the activity in the central inspiratory neurones (Eckberg 2003).

*Study limitations:*

There is no "gold standard" to how baroreflex sensitivity should be measured. Traditionally, it was assessed by injection of vasoactive substances (Ryan et al. 2007). The method we used was non-invasive, it assesses the spontaneous cross-correlation and regression between systolic blood pressure and R-R interval computed over 10 second sliding windows. It has

been shown to yield more values per minute, have a lower within-patient variance and baroreflex measurement delay to sequential spontaneous measures (Westerhof et al. 2004). Despite this, there was large variability amongst participants in terms of the number of baroreflex sensitivity measurements (range: 8-185 measures). Currently there is no objective measure to indicate that a test is satisfactory so we therefore chose to exclude participants based on the median number of measures. After stratification based on the median number of measures (100 measurements), it was found that the group with the low number of measurements had a significantly higher AHI (AHI 43.11 ± 25.49, $p$ <0.05) causing us to question the usefulness of the spontaneous approach during wakefulness when investigating patients with severe OSA. This subsequent exclusion of so many patients may have skewed the analysis and caution is therefore required in the interpretation of the results. Furthermore the sample size used in our study is relatively small compared to other existing studies investigating BRS and our study does not include a control group for comparison.

Problems with assessing baroreflex sensitivity during wakefulness may be related to the potential stimulus such as noise surrounding the patient having a greater influence than during sleep. This occurrence of noise though was minimalized through performing the study in a quieter part of the hospital. Indeed some patients with severe OSA did struggle to not fall asleep during the test and so unfortunately had to be lightly stimulated on the shoulder by the researcher. A further limitation is that all non-invasive methods involve not only the arterial baroreflex but also other cardiovascular and thoracic stretch reflexes. Invasive methods such as phenylephrine bolus technique may be influenced by this, but possibly to a lower extent, although will be affected by other unquantified pharmacological effects (Davies et al. 1999). The influence of respiration on heart rate cannot be excluded. However, it is believed this influence is relatively small and not of major significance (Ryan et al. 2007). Moreover, when available, participant's breathing rate was measured via thoracic and abdominal belts (Embletta® Gold, Embla Systems, USA) and analysed afterwards using RemLogic software. In all cases, measured breathing rate was 15 ± 1 breaths per minute which corresponds with the recommendations stated in Bernardi et al. (2011) review on methods for assessing cardiac autonomic function in human research studies.

Finally, although participants were placed in a corresponding hypertensive group in an attempt to control for this using a partial correlation and ANCOVA as appropriate, the wide range of other conditions which are known as potential comorbidities of OSA such as diabetes

(Frattola et al. 1997) and other cardiovascular disorders (Katsube et al. 1996, Mortara et al. 1997) have also been shown to influence baroreflex sensitivity (Ryan et al. 2007).

**Conclusion:**

No association was found between baroreflex sensitivity and OSA severity. However, there were difficulties in achieving satisfactory measurements to the extent that 50% of the study population were excluded from further data analysis. Based upon this work, future application of this method in this patient group would appear to be challenging.

## Chapter 5 Association of metabolic and inflammatory markers with the severity of OSA

**Abstract**:

*Purpose:* In this current study we predominately focus on investigating the relationship between the apnoea hypopnoea index (AHI) and markers of inflammation. We investigate markers representing low level inflammation including C-reactive protein (CRP) and certain adipokines consisting of adiponectin and leptin which have been suggested to play a role in the pathogenesis of OSA. We also assess plasma concentrations of two endocannabinoids: 2-arachidonoylglycerol (2-AG) and arachidonoylethanolamide (AEA). Furthermore, the data from the previous studies with patients with OSA are also assessed for correlations with the investigated blood markers.

*Methods:* Fasted blood plasma samples were collected and analysed at the School of Sport, Health and Exercise Sciences, Bangor University Biochemistry Laboratory for the analysis of leptin, adiponectin and CRP via enzyme-linked immunosorbent assays. Further plasma samples were shipped to the Institute of Clinical Pharmacology, Hannover Medical School, Germany for the measurement of endocannabinoids 2-AG and AEA via liquid chromatography / in-line mass spectrometry.

*Results:* No significant correlations were found between any of the investigated blood markers and AHI. Significant correlations were however found between body characteristics, CRP, leptin and the two endocannabinoids. Furthermore, it was also found that patients with mild OSA had a significantly ($p$<0.05) lower plasma AEA concentration (n= 8, 0.77 ± 0.21 nM) compared to the moderate and severe patients with OSA combined (n = 42, 1.06 ± 0.32 nM). Positive correlations were also found between the ventilatory response to hypoxia with CRP and 2-AG.

*Conclusions:* The findings of this study provide evidence suggestive of a degree of inflammation being present amongst patients with OSA . Assessment of correlations suggests that the severity of OSA and the degree of inflammation is strongly associated with obesity.

**Introduction:**

It has been recognised the effects of intermittent hypoxia may contribute to comorbidities associated with OSA including hypertension, obesity, dyslipidemia, insulin resistance (Lavie 2009), diabetes (Punjabi et al. 2004) and metabolic syndrome (Trombetta et al. 2013). Evidence particularly, from animal models designed to simulate OSA supports intermittent hypoxia resulting from recurrent episodes of upper airway obstruction is an important pathophysiological pathway (He et al. 2014). It has been shown *in vivo* and cell culture studies that hypoxia activates transcriptional factors related to inflammation via different signalling pathways dependent upon whether the exposure to hypoxia is intermittent or sustained (Nanduri et al. 2008).

Intermittent hypoxia as occurs as a result of apnoeic events has been shown in a cell culture model to lead to a selective and preferential activation of inflammatory pathways mediated by the transcription factor nuclear factor kappa B (NF-κB) over adaptive, hypoxia inducible factor 1 dependent pathways, which prefers sustained hypoxia where activation of adaptive and protective pathways predominates (Ryan, Taylor & McNicholas 2005). Furthermore, the intermittent changes in blood oxygen saturation levels in OSA can be considered similar to the hypoxia and reoxygenation demonstrated in conditions characterised by ischemia and reperfusion associated with increased production of reactive oxygen species (ROS) (Lavie 2009).

ROS are normal by-products of cellular metabolism, which when overproduced, overwhelms antioxidant capabilities relating to pathogenic oxidative stress and inhibition of cellular mechanisms and cellular injury (Valko et al. 2007). The issue of increased ROS production in OSA though remains controversial with some studies failing to demonstrate increased oxidative stress with OSA (Oztürk et al. 2003, Wali et al. 1998). Questions have also arisen as to whether any oxidative stress would represent a consequence rather than a cause of tissue damage (Juránek & Bezek 2005, Grossman 2008). It has however been speculated that an increased production of ROS may trigger expression of multiple proinflammatory genes via activation of the oxidant-sensitive transcription factor NF-κB (Htoo et al. 2006).

Hypoxia-sensitive pathways comprising of hypoxia inducible factor (HIF) and NF-κB increase proinflammatory responses in macrophages, T cells, dendritic cells and neutrophils

(Scholz & Taylor 2013, Colgan & Taylor 2010, Scholz et al. 2013). It is now recognised that hydroxylases which have been shown to be inhibited in hypoxic conditions play a central role for the link between hypoxia and inflammation. Specifically a set of 4 different hydroxylases-prolyl-hydroxylases (PHD)-1, PHD-2 and PHD-3 and the asparagine-hydroxylase factor-inhibiting HIF (FIH) have been implicated in the posttranscriptional regulation of hypoxic and inflammatory signalling pathways (Eltzschig & Carmeliet 2011, Bartels, Grenz & Eltzschig 2013). Scholz et al. (2013) provide compelling evidence that hydrolases modulate inflammation via key posttranslational modifications in the IL-1β pathway. IL-1β is secreted from multiple cell types and is associated with a range of inflammatory, metabolic and infectious diseases (Scholz et al. 2013, Dinarello 2011). Upon binding IL-1β to its cognate receptor, a signalling cascade is initiated, in which signals via tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) result in the activation of NF-κB (Scholz et al. 2013).

NF-κB is a generic name for a family of dimeric transcription factors which serves as a key component in the regulation of inflammatory cytokines involved in the development of various conditions such as atherosclerosis and insulin resistance (Lurie 2011b). Furthermore NF-κB has been reported to play a dual role in the modulation of cell apoptosis (Abe 2007, Han et al. 2013). In patients with OSA Htoo et al. (2006) compared NF-κB activity compared to controls without OSA, the neutrophils in the patients with OSA demonstrated a several fold increase in NF-κB binding activity and a positive correlation was found between the severity of OSA and NF-κB activation. Furthermore recently, Han et al. (2013) has demonstrated that even in the early stage of exposure to intermittent hypoxia there is increased oxidative and inflammatory stress leading to acceleration of cell apoptosis via pathways which includes NF-κB in the human endothelial (EA.hy926) cells. These observations provide some support for intermittent hypoxia inducing vascular pathogenesis in patients with OSA (Abe 2007, Han et al. 2013). The endothelium is a major site for initiation of atherosclerosis (Han et al. 2013). Atherosclerosis is a chronic inflammatory disease triggered by endothelial dysfunction that is characterised by a pro-inflammatory and prothrombic state of the endothelium (Han et al. 2013, Endemann & Schiffrin 2004).

*Role of adipose tissue in inflammation and metabolic dysfunction:*

Almost all cross-sectional clinical and population-based studies have found significant associations between OSA and measures of excess body weight (Young, Peppard & Gottlieb 2002). Furthermore, OSA is heavily associated with the development of numerous co-morbidities with 60% of patients with metabolic syndrome also experiencing OSA (Drager et al. 2010, Trombetta et al. 2013, Trombetta et al. 2010). Although it is believed systemic inflammation plays a key role in the development of metabolic dysfunction the precise mechanisms are poorly understood (Drager, Jun & Polotsky 2010). The potential mechanisms which may contribute include hypoxia per se, sympathetic activation and activation of the NF-κB pathway causing disruption of hypothalamic-pituitary-adrenal axis. This may lead to systemic catecholamine-mediated lipolysis and lipotoxicity with hepatic transcriptional upregulation of lipid synthesis, impaired lipid clearance and out-of balance control of glucose, insulin regulating hormones and cytokines produced by adipose tissue (adipokines) such as IL-6, TNF-α, leptin and adiponectin (He et al. 2014, Lesser et al. 2012, Li et al. 2005, Coughlin et al. 2004).

Our understanding of adipose tissue has changed dramatically from a lipid storage organ to an endocrine and immunologically active one which secretes bioactive substances called adipokines that play an important role in the complex cross-talk between organs, regulating homeostasis (El-Kadre & Tinoco 2013). Furthermore adipose tissue is now regarded as one of the main sources of inflammatory mediators. In particular circulating levels of IL-6 secreted from adipose tissue is the most strongly correlated to adiposity and type 2 diabetes of all the cytokines (El-Kadre & Tinoco 2013). IL-6 along with IL-1 regulates at the post – transcriptional level CRP production (Artemiou et al. 2012).

CRP is an acute-phase reactant synthesised by the liver (Lurie 2011b) which is associated with an increased risk of atherosclerosis and cardiovascular disease though its relative importance has been questioned in a large study (Danesh et al. 2004). Furthermore, low-grade systemic inflammation as observed by elevated CRP levels has been suggested as one potential mediator of insulin resistance in OSA (Kokturk et al. 2005, Hargens et al. 2013, Kelly et al. 2010). Insulin resistance has been reported to be independently associated with OSA severity after the adjustment of obesity (Kelly et al. 2010).

Studies assessing the relationship between OSA severity and CRP have found conflicting results. Whilst some research have revealed an independent association from body mass parameters between the severity of OSA and CRP (Yokoe et al. 2003, Lui et al. 2009, Guven et al. 2012) others studies have found CRP production is more related to obesity than OSA (Akashiba et al. 2005, Ryan et al. 2007). Arnardottir et al. (2012) studied moderate to severe patients with OSA (n = 454) from five sites in Iceland (the Icelandic Sleep Apnoea Cohort) and found the association of OSA and the inflammatory biomarker CRP depends on obesity. A correlation between OSA severity, CRP and IL-6 levels was only found in obese males with a BMI $\geq$ 30 kg/m$^2$.

The effects of CPAP treatment on CRP is also unclear, CPAP has been shown to cause a reduction in CRP levels independent of BMI (Yokoe et al. 2003) however these findings have been challenged by studies reporting no reduction in CRP levels (Akashiba et al. 2005, Ryan et al. 2007). The effects of CPAP cessation has also been investigated with Phillips et al. (2007) studying the effects of short term (1 week) withdrawal of CPAP finding a marked increase in sympathetic activity without concomitant elevation of CRP and other vascular inflammatory markers.

Secreted also by the adipose tissue, adiponectin is an insulin sensitising hormone which decreases hepatic glucose output and increases fatty acid oxidation by the muscle (Kelly et al. 2010). In contrast to CRP, adiponectin protects against chronic inflammation with reduced adiponectin levels being related to increased endothelial inflammatory responses, the presence of coronary heart disease, dyslipidemia, insulin resistance and type 2 diabetes in humans (Wolk et al. 2005). Although adiponectin is specifically expressed in adipose tissue, plasma adiponectin levels have been found to be decreased with obesity (Arita et al. 1999, Yang et al. 2001). The mechanisms of this paradoxical finding are unknown but because there is a high prevalence of obesity in sleep apnoea it has been theorised OSA may influence adiponectin levels and it has been reported adiponectin levels in patients with sleep apnoea are lower than in normal subjects (Wolk et al. 2005). Furthermore Kelly et al. (2010) has revealed in obese pubertal children OSA severity is negatively associated with adiponectin levels even after adjustment for BMI. Many studies have used different durations of CPAP treatment to demonstrate an increased adiponectin concentration following CPAP intervention (Nakagawa et al. 2008, de Lima et al. 2010, Carneiro et al. 2009). Furthermore, a randomised controlled trial revealed no change in adiponectin levels following 3 months of CPAP compared to sham

CPAP treatment (West et al. 2007) and similar results were found in another randomised controlled study which used 4 weeks follow up from either therapeutic or sub-therapeutic levels of CPAP (Kohler et al. 2009). It has though been acknowledged that many studies with negative findings have had participants with very poor CPAP compliance (a use of less than 4 hours a night) or have not monitored or reported CPAP compliance information (Lurie 2011a).

Also produced in the white adipose tissue, leptin is an adipokine which was initially considered just to reduce food intake and increase energy expenditure (Friedman & Halaas 1998, Koch et al. 2014). The establishment of leptin resistance is regarded as a leading cause in the onset of obesity (Koch et al. 2014). Although leptin is mostly recognised for its anorexigenic and catabolic properties, leptin is also essential for maintenance of glucose homeostasis and is a potent insulin sensitizer (Yu et al. 2008, Kamohara et al. 1997), though the molecular mechanism underlying the glucose lowering properties and insulin sensitising effects of leptin are not well understood (Koch et al. 2014). Elevated leptin levels have been associated with atherosclerosis (Konstantinides et al. 2001), thrombosis (Bodary et al. 2002), neointimal hyperplasia (Bodary et al. 2007) and hypertension (Rahmouni et al. 2005). Furthermore, Wang et al. (2013) reports leptin causes endothelial dysfunction and enhances the pressor response to angiotensin II on blood pressure through increased sympathetic nervous system activation.

Several authors have attributed some pro-inflammatory properties to leptin (Loffreda et al. 1998, Fantuzzi & Faggioni 2000). It has been suggested that a relationship exists between TNFα and leptin production (Kirchgessner et al. 1997, Fawcett et al. 2000) which may provide a mechanism by which TNFα can modulate inflammation (Pickup, Chusney & Mattock 2000). Moreover, the current evidence which has indicated that leptin acts as a proinflammatory cytokine in the immune response has led to suggestion that leptin has potential roles in the development of autoimmune diseases (Tian et al. 2014) such as systemic lupus erythematosus (Xu et al. 2014, Wisłowska et al. 2008), rheumatoid arthritis (Bokarewa et al. 2003), multiple sclerosis (Matarese et al. 2010) and psoriasis (Zhu et al. 2013).

As with CRP and adiponectin the relationship between leptin concentration and OSA remains to be clarified (Lurie 2011b). In patients with OSA some studies have reported higher circulating leptin levels compared to BMI-matched control subjects (Ip et al. 2000, Phillips et al. 2000, Kapsimalis et al. 2008) with the severity of nocturnal hypoxemia associated with

leptin levels independent of obesity (Kapsimalis et al. 2008). Some authors however still consider the higher levels of leptin to be mostly related to obesity (Barceló et al. 2005) with some studies finding no significant association between OSA and leptin levels after controlling for body fat and/or BMI (Schäfer et al. 2002, Patel et al. 2004). CPAP treatment has been shown to reduce circulating leptin levels in patients with OSA despite unchanged BMI during the study period (Ip et al. 2000, Harsch et al. 2003). Although decreased leptin levels with CPAP have only been reported in non-obese patients with OSA (Barceló et al. 2005) or to be more pronounced in patients with OSA and a BMI<30 kg/m$^2$ (Lurie 2011b, Harsch et al. 2003).

*Involvement of the endocannabinoid system*

A key discovery enhancing our understanding of the control of adipose tissue and in particular the regulation of energy metabolism within adipose tissue, was finding the expression of functional cannabinoid receptors in the adipocytes which were up-regulated during adipogenesis (Matias et al. 2006, Roche et al. 2006, André & Gonthier 2010).

The endocannabinoids are endogenous molecules capable of binding and activating the same cannabinoid receptors activated by Delta (9)-tetrahydrocannabinol (THC), the major psychoactive principle of the hemp plant *Cannabis sativa* despite being chemically different. The endocannabinoid system consists of cannabinoid receptors, their ligands the endocannabinoids and endocannabinoid anabolic and catabolic enzymes (André & Gonthier 2010). Strictly speaking the term cannabinoid refers to compounds that can activate either cannabinoid receptor 1 (CB1) or cannabinoid receptor 2 (CB2), or both. However other molecules with similar structures to THC which do not activate the receptors; have often been included in this term (Burstein & Zurier 2009).

The adipocytes have been shown to possess all the enzymes involved in the biosynthesis and degradation of the endocannabinoids 2-arachidonoylglycerol (2-AG) and arachidonoylethanolamide (AEA) as well as oleylethanolamide (OEA) and palmitoylethanolamide (PEA) (Engeli et al. 2005, Gasperi et al. 2007, Matias et al. 2006) OEA and PEA are two cannabinoid receptor-inactive and biosynthetically related congeners of AEA (Côté et al. 2007). The endocannabinoid system is believed to be usually silent and to become transiently activated after stressful conditions (André & Gonthier 2010).

CB1 blockade in the adipocytes has been found to inhibit preadipocyte proliferation and increase expression of adipocyte maturation markers such as adiponectin, whereas its activation increases adipocyte differentiation, insulin sensitivity, glucose uptake and lipogenesis (André & Gonthier 2010, Gasperi et al. 2007, Motaghedi & McGraw 2008) . Stimulation of the endocannabinoid system though is not just limited to adipose tissue, in fact locations of the cannabinoid receptors is diverse with CB1 found ubiquitously but preferentially in locations of the brain and spinal cord (Howlett 2002) whereas CB2 is expressed in high levels in leukocytes and the spleen and in a lower extent in muscle, liver, intestine, testis (Liu et al. 2009) as well as adipose tissue (Roche et al. 2006, André & Gonthier 2010). Additionally, a second isoform of cannabinoid receptor appears to be present in additional tissues, especially the brain and kidney (Liu et al. 2009). The general effects of stimulation of the endocannabinoid system depend upon its location as highlighted in Table 5-a.

**Table 5-a.** Summarising the response to stimulation of the endocannabinoid system with the different sites of action (André & Gonthier 2010).

| Sites of action | Mechanisms | Over-activation Consequences |
|---|---|---|
| Brain | Increased food intake | Increased body weight/waist circumference |
| Gastrointestinal tract | Reduced satiety<br>Increased food assimilation | Increased body weight/waist circumference |
| Liver | Increased lipogenesis | Increased dyslipidemia<br>Increased steatosis |
| Pancreas | Increased insulin | Increased insulin resistance |
| Muscle | Decreased glucose uptake<br>Decreased fatty acid oxidation | Increased insulin resistance<br>Increased energy expenditure |
| Adipose tissue | Reduced adiponectin<br>Reduced leptin<br>Increased lipogenesis | Increased visceral fat<br>Increased dyslipidemia<br>Increased insulin resistance |

The role of THC in lymphocyte biology and immune/inflammatory response has been extensively reviewed (Burstein & Zurier 2009, Klein 2005, Klein & Newton 2007, Klein & Cabral 2006). Cannabidiol is typically the most abundant nonpsychoactive cannabinoid in the hemp plant (Burstein & Zurier 2009). Analogs of cannabidiol have been found to suppress the production of the inflammatory cytokine TNFα and appear to exert anti-inflammatory activity by suppressing fatty acid amidohydrolase (FAAH) activity, thereby increasing concentrations of the anti-inflammatory endocannabinoid AEA (Burstein & Zurier 2009).

AEA and 2-AG are the most studied endocannabinoids which act as mediators in the brain and in the peripheral tissues mainly through the stimulation of the brain (CB1) and peripheral (CB2) cannabinoid receptors (Sancho et al. 2003). AEA however can also interact with vanilloid receptor type 1(VR1) which is expressed primarily in afferent nociceptive neurons (Sancho et al. 2003, Zygmunt et al. 1999, Smart et al. 2000, Caterina et al. 1997). It is generally considered that in response to proinflammatory simulation, endocannabinoids such as AEA and 2-AG are rapidly produced, resulting in the stimulation of cannabinoid receptors in adjacent cells and subsequent down-regulation of the inflammatory response (Sancho et al. 2003, Berdyshev et al. 2001). Sancho et al. (2003) however observed that AEA exhibits NF-κB inhibitory activity via effects that are not mediated by the interaction of AEA with either cannabinoid or vanilloid receptors. Furthermore it is possible that newly synthesised AEA may limit the proinflammatory response by direct inhibition of proinflammatory cytokine release or by down-regulation of inducible nitric-oxide synthase in the cardiovascular endothelium. (Sancho et al. 2003, Stefano, Salzet & Bilfinger 1998, Berdyshev et al. 1997).

It has been demonstrated that 2-AG also inhibits cytokine production, however the role of CB1 and CB2 in these effects is also unclear (Ouyang et al. 1998, Gallily, Breuer & Mechoulam 2000, Facchinetti et al. 2003, Chang, Lee & Lin 2001, Rockwell et al. 2006) Rockwell et al. (2006) reports evidence that Interleukin 2 secretion is reduced with the presence of 2-AG independently from the activation of CB1 and/or CB2 but involving activation of a peroxisome proliferator-activated receptor (PPARγ). It has however also been found 2-AG reduces lipopolysaccharide cell death and other proinflammatory cytokine productions and increases anti-inflammatory cytokine concentration in a CB1 and/or CB2 activation dependent manner (Krishnan & Chatterjee 2012, Alhouayek, Masquelier & Muccioli 2014).

*In vivo c*hanges in endocannabinoid concentrations have been observed in inflammation related pathologies. Higher 2-AG and AEA concentrations have been found in animal models of several neuropathology's that include an inflammatory component such as multiple sclerosis (mice model (Baker et al. 2001)) and Parkinson's disease (non-human primate model (Stelt et al. 2005)). Targeting the endocannabinoid system in models of Alzheimer's disease has emerged as a potential approach to slow disease progression (Tanveer et al. 2012). Patients with coronary diseases have also been found to display higher serum levels of AEA and 2-AG compared to unaffected subjects (Sugamura et al. 2009). Little Research though has been conducted on investigating the influence of OSA on the circulating endocannabinoids with a systematic review of PubMed's database revealing only two studies were found using the search terms: sleep apnoea, endocannabinoids. These studies were Jumpertz et al. (2010) and Engeli et al. (2012). Note in Jumpertz et al. (2010) study, whether the patients were predominately diagnosed with obstructive or central/mixed sleep apnoea was not reported.

In this current study we predominately focus on investigating the relationship between the severity of OSA (using AHI), associated markers of inflammation and adipokines suggested to be involved in the pathogenesis. We focus attention on five markers consisting of CRP, adiponectin and leptin and the two endocannabinoids: AEA and 2-AG. The study of the endocannabinoids was only made possible through collaboration with Hannover Medical School, Germany. The previous ventilatory response data and the baroreflex sensitivity data are also assessed with the blood markers for correlations and it was originally thought the design of a regression model would be possible to understand which parameters best predict the severity of OSA and ventilatory response to $CO_2$.

**Method:**

*Participants*

Male newly diagnosed patients with OSA were recruited after they had received consultation regarding their sleep study results in the clinic at Ysbyty Gwynedd. All participants were diagnosed with OSA using an unattended home sleep study, respiratory polygraphy(Embletta® Gold, Embla Systems, USA). Measures of pulse oximetry, nasal airflow, thoracic and abdominal movements were analysed using RemLogic software. Diagnosis of OSA was performed by either an experienced RCCP (Registration Council for Clinical Physiologists) registered Clinical Physiologist or an experienced Sleep Technologist.

Patients performed the study within the two weeks prior to their treatment of CPAP. Written informed consent was obtained from all subjects prior to testing. Patients were excluded if they had a BMI≥50 kg/$m^2$ or they were on medications known to change their respiratory drive (i.e. opiate-based painkillers). The outcome data from all the patients with OSA from all the previous studies was also incorporated into this correlational study.

*General procedures*

This study was approved by the local North West Wales NHS Ethics Committee (Ref: Earing 11/WNo01/2) (Gwynedd, Wales) and carried out in accordance with the Declaration of Helsinki for research on human subjects. The study measurements were performed in a quiet room within the Pulmonary Function Department of Ysbyty Gwynedd. Participants arrived fasted having being instructed not to consume any food or drink (except water) within 12 hours of their visit. This was stated on their study information sheet and also verbally during a phone conversation on the day prior to the visit.

*Fasted blood samples:*

Participants reported between 08:00 and 12:00 to have 10 ml of fasted blood drawn via a cubital venipuncture. The venous blood was collected into two 6 ml Vacutainer® EDTA-plasma tubes. These blood filled vacutainers were then centrifuged within five minutes after blood drawing to avoid contamination of the blood plasma with endocannabinoids which are produced after drawing blood (Engeli et al. 2012, Vogeser et al. 2006). The vacutainers were centrifuged at 4000 rpm in a precooled centrifuge at 4°C (Universal 320R, Hettich Centrifuge, Germany) for ten minutes prior to pipetting the resulting plasma into two CryoTubes (ThermoFisher scientific). The samples were then immediately snap frozen in liquid nitrogen and transported in a portable dewar container. The frozen plasma samples were transported to the School of Sport, Health and Exercise Sciences, Biochemistry Laboratory where they were stored at -80°C for batch analysis after the completion of the data collection. In batches of approximately twenty, one CryoTube of frozen plasma per each participant was shipped approximately every three months in dry ice to Hannover Medical School, Germany for the quantification of the endocannabinoids: AEA and 2-AG. The remaining plasma tubes were analysed by me at the Biochemistry laboratory in the School of Sports, Health and Exercise Sciences, Bangor University, for the quantification of plasma adiponectin, plasma CRP and plasma leptin using the appropriate enzyme-linked immunosorbent assay (ELISA) kit.

Measurement of adiponectin was achieved using a high sensitivity (sandwich) human ELISA (Biovendor, Czech Republic) with a detection limit of 0.47 ng/ml and intra- and interassay coefficient of variation of 5.4% and 19.7% respectively. Leptin was quantified using a clinical range human ELISA (Biovendor, Czech Republic) with a detection limit of 0.2 ng/ml and intra- and interassay coefficient of variation of 6.4 % and 4.2% respectively. CRP was measured via CRP Human ELISA (Biovendor, Czech Republic) with a detection limit of 1 µg/ml and intra- and interassay coefficient of variation of 10.0% and 16.8% respectively. 20% CV was used as an appropriate quality control in all ELISAS performed based on the application of 20% CV in Reed et al. (2002) article.

*Endocannabinoid measurements:*

Unfortunately to investigate the endocannabinoids involves expensive facilities and expertise which was not available at Bangor University. Thankfully we were able to collaborate with a research team at the Institute of Clinical Pharmacology, Hannover Medical School, Germany. The general procedures, for the quantification of AEA and 2-AG involved using liquid chromatography / in-line mass spectrometry. Liquid chromatography–mass spectroscopy (LC–MS) gives a definitive identification and the quantitative determination of compounds, it provides a highly sensitive and selective measurement for all analytes of interest (Naik et al. 2005). Briefly, liquid chromatography is a technique which separates a mixture of compounds; the samples are placed on a tray for automatic injection into a column. Solvent is continuously pumped through the column, and the separated compounds are continuously sensed by a detector as they leave the column. This resulting detector signal is then plotted against time to form a chromatogram (Snyder, Kirkland & Dolan 2010). Mass spectrometry operates by converting the analyte molecules to a charged (ionised) state for subsequent analysis of the ions and any fragment ions that are produced during the ionisation process (Pitt 2009).

The plasma sample was combined with methanol/Tris buffer (50mM, pH 8.0), 1:1, containing 7 ng of synthesised d4-anandamid. To each sample, ice-cold chloroform/methanol (1:1) and 0.5 ml of 50 mM Tris buffer, pH 8.0 is then added. The solution was then centrifuged at 4°C (500 x g for 2 minutes), the chloroform phase was recovered and transferred to a borosilicate tube, and the water phase was extracted two more times with ice-cold chloroform. The combined extract was then evaporated to dryness at 32 °C under a stream of nitrogen. The dried residue was reconstituted in 110 µl of chloroform, and 2 ml of

ice-cold acetone was added. The precipitated proteins are then removed by centrifugation (1,800 x g, 10 minutes), and the clear supernatant was removed and evaporated to dryness. The dry residues are then reconstituted in 50 µl of ice-cold methanol, of which 35 µl was used for analysis by liquid chromatography/ in line spectrometry, by using an Agilent 1100 series LC-MSD, equipped with a thermostated autosampler and column compartment. Separation of the endocannabinoids was achieved using liquid chromatography with a guard column (Discovery HS C18, 2cm x 4.0 mm, 3 µm, 120A) and analytical column (Discovery HS C18, 7.5 cm x 4.6 mm, 3 µm) at 32 °C with a mobile phase of methanol/water/acetic acid (85:15:0.1, vol/vol/vol) at a flow of 1 ml/min for 12 min followed by 8 min of methanol acetic acid (100:0.1, vol/vol). The MSD (model LS) was set for atmospheric pressure chemical ionization, positive polarity and selected ion monitoring to monitor ions *m/z* 348 for AEA, 352 for d4-AEA, and 379 for 2-AG. The spray chamber settings were as follows: vaporizer, 400 °C; gas temperature, 350 °C; drying gas, 5.0 liters/min; and nitrogen was used as the nebulizing gas with a pressure of 60 psig. The calibration curves were produced using synthetic AEA and 2-AG (Cayman Chemical, Ann Arbor, MI). The amounts of AEA and 2-AG in the samples were determined by using linear regression of standard curves. (Engeli et al. 2012, Wang et al. 2003).

*Data analysis*

All data are presented mean ± SD and $p<0.05$ are considered statistically significant, respiratory measurements are expressed in BTPS. Where applicable the participants' previous data regarding their ventilatory response to hypercapnia/hypoxia and/or baroreflex sensitivity data were used in this study for correlational analysis. Spearman's rho was used to test if any correlations existed between the participant's AHI (the marker of the severity of OSA), ventilatory response to the gas mixtures, body characteristics, the measured blood parameters and baroreflex sensitivity. Spearman's correlation coefficient ($r_s$) was used as AHI was not normally distributed (Field 2009).

As with the previous study, the patients with OSA were split into two groups depending on their AHI. Group 1: consisted of patients with mild and moderate OSA and group 2: patients with severe OSA. Additionally ANOVAs was performed on patients with mild OSA vs. patients with moderate and severe OSA grouped together and on mild, moderate and severe OSA patient groups separately. ANCOVAs were also performed to control for BMI. To be classified as a patient with mild OSA an AHI of ≥5 and <15 with reported symptoms related

to sleepiness was present. To be classified as a patient with moderate OSA, a recording of AHI ≥15 and <30 was required. A patient with severe OSA had an AHI ≥30 (Epstein et al. 2009). A one-way ANOVA was performed on blood parameters between the two groups

**Results:**

In total 61 patients diagnosed with OSA were recruited from the sleep clinic, Ysbyty Gwynedd. Ideally all the patients with OSA would have had equal opportunity to participate in all the studies involved in this thesis, as was originally designed. Unfortunately though issues outside the control of the researchers led to some delays in some of the studies being able to start as previously intended.

A one-way ANOVA revealed no significant difference in any of the blood parameters (leptin, adiponectin, CRP, AEA and 2-AG) between the three groups of patients with mild, moderate or severe OSA. This was also the case when patients with mild/moderate OSA were compared with the patients with severe OSA. Further examination of the data though revealed the patients with moderate OSA grouped with the patients with severe OSA had a significantly higher plasma AEA concentration (n = 42, 1.06 ± 0.32 nM) compared to the patients with mild OSA group (n = 8, 0.77 ± 0.21 nM) ($p$ = 0.018). When an ANCOVA was performed with BMI as a covariant $p$ = 0.055. No significant difference in AEA was found when groups were analysed separately. Furthermore no significant differences in blood parameters were found between the groups when ANCOVA was used with BMI as a covariant. The results of the significant AEA concentration findings are displayed in Figure 5-a.

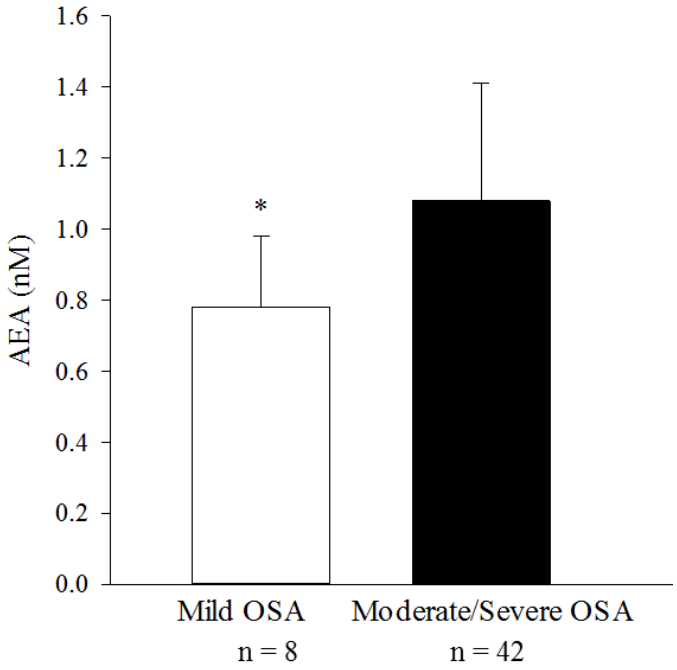


**Figure 5-a**. Bar chart displaying AEA concentration of mild group compared to moderate/severe OSA patient group. □ = mild OSA group and ■ = moderate/severe OSA patient group. Where significantly different * = $p<0.05$.

Spearman's rho also revealed AEA was significantly correlated to neck, waist, hip circumference and CRP as shown in the correlation matrix amongst the other significant correlations found (Table 5-b). Additionally, leptin was found to be significantly associated with ventilatory response to $CO_2$ when using Pearson's *r* test however Kolmogorov-Smirnov and Shapiro-Wilk test reveals that leptin is not normally distributed ($p = 0.004$ and $p = 0.003$ respectively) and so Spearman's rho interpretation is more appropriate. As with the findings of the previous study no relationships were found between baroreflex sensitivity, AHI and all the additional outcomes studied.

Table 5-b and Table 5-c display the correlation matrixes for the outcomes of most interest in this study. As with the findings of previous study all measurements of body characteristics were found to be highly related to the severity of sleep apnoea (AHI). Hip circumference was removed from the correlation matrix to conserve space, but was correlated with AHI (n = 61; $r_s = 0.31$; $p = 0.015$)

**Table 5-b.** Spearman's rho correlation matrix of measured variables where * = $p<0.05$ and ** = $p<0.01$.

| | n | AHI | Age | Neck | Mass | Waist | BMI | Leptin | Adiponectin | CRP | AEA | 2-AG |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AHI | 61 | 1.00 | | | | | | | | | | |
| Age (years) | 61 | -0.22 | 1.00 | | | | | | | | | |
| Neck (cm) | 61 | 0.50** | 0.10 | 1.00 | | | | | | | | |
| Mass (Kg) | 61 | 0.45** | -0.16 | 0.75** | 1.00 | | | | | | | |
| Waist (cm) | 61 | 0.42** | 0.09 | 0.80** | 0.88** | 1.00 | | | | | | |
| BMI ($kg/m^2$) | 61 | 0.40** | 0.04 | 0.80** | 0.87** | 0.92** | 1.00 | | | | | |
| Leptin (ng/ml) | 48 | 0.16 | 0.18 | 0.57** | 0.69** | 0.79** | .77** | 1.00 | | | | |
| Adiponectin (ng/ml) | 49 | -0.16 | 0.38** | 0.03 | -0.05 | 0.00 | -.10 | .03 | 1.00 | | | |
| CRP (ug/ml) | 40 | -0.03 | 0.18 | 0.58** | 0.35* | 0.49** | 0.49** | 0.58** | -0.09 | 1.00 | | |
| AEA (nM) | 50 | 0.02 | 0.10 | 0.41** | 0.23 | 0.31* | 0.25 | 0.21 | 0.16 | 0.45** | 1.00 | |
| 2-AG (nM) | 50 | -0.03 | 0.15 | 0.19 | 0.06 | 0.17 | 0.22 | 0.08 | -0.23 | 0.39* | 0.11 | 1.00 |

**Table 5-c.** Spearman's rho correlation matrix of measured variables where * = $p<0.05$ and ** = $p<0.01$.

| | n | AHI | Neck | BMI | Leptin | CRP | 2-AG | 25%$O_2$/6%$CO_2$ | 13% $O_2$ |
|---|---|---|---|---|---|---|---|---|---|
| AHI | 61 | 1.00 | | | | | | | |
| Neck (cm) | 61 | 0.50** | 1.00 | | | | | | |
| BMI (kg/$m^2$) | 61 | 0.40** | 0.80** | 1.00 | | | | | |
| Leptin (ng/ml) | 48 | 0.16 | 0.57** | 0.77** | 1.00 | | | | |
| CRP (ug/ml) | 40 | -0.03 | 0.58** | 0.49** | 0.58** | 1.00 | | | |
| 2-AG (nM) | 50 | -0.03 | 0.19 | 0.22 | 0.08 | 0.39* | 1.00 | | |
| 25% $O_2$/6%$CO_2$ ΔVE (l/min/BSA) | 32 | -0.51** | -0.42* | -0.44* | -0.32 | -0.27 | 0.03 | 1.00 | |
| 13%$O_2$ ΔVE (l/min/BSA) | 32 | -0.03 | 0.32 | 0.34 | 0.25 | 0.61** | 0.50** | 0.02 | 1.00 |

**Discussion:**

The main aim of this current study was to investigate the association of inflammatory and metabolic markers with the severity of OSA. It was hoped that a regression model could then be designed to allow prediction of the severity of OSA based on the blood markers which were found to correlate with AHI. However, no significant correlations were found between AHI and any of the blood markers. Furthermore, no significant correlations were found between the ventilatory response to $CO_2$ data and any of the blood markers analysed.

It was found that the patients with moderate and severe OSA grouped together (n = 42, 1.06 ± 0.32 nM) had a significantly higher AEA concentration compared to the patients with mild OSA group (n = 8, 0.77 ± 0.21 nM) ($p$ = 0.018). This was almost significant when BMI was used as a covariant ($p$ = 0.055). It is possible the lowered AEA concentration displayed amongst the patients with mild OSA reflects a higher degree of inflammation occurring in the patients with more severe OSA. The endocannabinoids are generally regarded as having anti-inflammatory properties (Crowe et al. 2014) and higher 2-AG and AEA concentrations have been found in animal models of several neuropathology's that include an inflammatory component such as multiple sclerosis (mice model (Baker et al. 2001)) and Parkinson's disease (non-human primate model (van der Stelt et al. 2005)). Furthermore a significant positive correlation with CRP was found between both of the analysed endocannabinoids in our study (AEA: $r_s$ = 0.45, $p$ = 0.015 and 2-AG: $r_s$ = 0.40, $p$ = 0.016). CRP is marker of low-grade systemic inflammation (Danesh et al. 2004, Kokturk et al. 2005, Hargens et al. 2013, Kelly et al. 2010). Unfortunately it is possible that the results are because the moderate/severe OSA patient group provides a better representation of the general OSA patient population with a sample size (n= 42) five times larger than the mild (n = 8) OSA patient group. The study aimed to recruit patients with OSA from the full spectrum of severity however did not achieve this in practice.

Engeli et al. (2012) is one of the few studies which has also investigated the endocannabinoids amongst patients with OSA. Two groups of patients with OSA were compared based on whether they had a normal glucose tolerance or type 2 diabetes mellitus. Groups were matched for age, sleep apnoea severity, BMI, body fat mass and blood pressure. Circulating AEA, 1-/2-AG (sum of 1- and 2-AG) and OEA was also compared to a healthy previously studied non-OSA control group. The control group displayed significantly ($p$ < 0.001) lower endocannabinoids (AEA, 1-/2-AG and OEA) and AEA was found to positively

correlate with the respiratory distress index. Additionally Engeli et al. (2012) reported the nightly decrease in mean oxygen saturation in the patients with OSA correlated with all three endocannabinoids. After adjustments for BMI, waist circumference, body mass, fasting insulin and glucose, and glucose infusion rate, though all three correlations with the endocannabinoids were diminished. Engeli et al. (2012) main finding was that blood pressure in patients with OSA was correlated with peripheral AEA concentrations. In our study however unfortunately blood pressure was not measured though no correlations were found between the endocannabinoids and the baroreflex sensitivity measures of the previous study.

Jumpertz et al. (2010) also investigated endocannabinoids in patients with sleep apnoea (no specification of type of sleep apnoea) and reported no significant difference between sleep apnoea patients and controls after adjustment for confounders including BMI, fasting insulin, HDL and LDL cholesterol. Jumpertz et al. (2010) did however find a significantly higher OEA concentration in patients with sleep apnoea which remained significant after adjustment for BMI, fasting insulin, LDL and HDL cholesterol and after direct comparison with BMI matched groups. OEA in cerebrospinal fluid has been shown to be elevated in volunteers following 24 hours of sleep deprivation (Koethe et al. 2009). Originally measurement of OEA was planned to be included in this thesis unfortunately though the analysis of the blood plasma for OEA was not able to take place.

In our current study, no correlations were found between AHI and the tested endocannabinoids, although AEA was correlated with waist ($r_s$ =0.31, $p$ = 0.027) and neck circumference ($r_s$ = 0.42, $p$ = 0.003). These positive correlations with increased adipose tissue further highlight the potential increase in activation of the endocannabinoid system in adipose tissue associated with reduced adiponectin and leptin and increased lipogenesis and in conditions of over activation will lead to increased visceral fat, dyslipidemia and insulin resistance (André & Gonthier 2010). It is likely then the increased AEA found in our study as with Engeli et al. (2012) study is related to obesity or potentially other comorbidities associated with OSA such as diabetes or hypertension. Increased concentrations of endocannabinoids have been found in obesity, associated with decreased FAAH gene expression in adipose tissue, (Engeli et al. 2012, Engeli et al. 2005, Blüher et al. 2006). Interestingly though 2-AG was not found to correlate with any of the body composition parameters in our study. It is also considered that the endocannabinoid system plays an important role in linking obesity to diabetes (Scherer & Buettner 2009). A close relationship

between insulin resistance and endocannabinoids in blood and tissues has been reported in the literature (Matias et al. 2006, Engeli et al. 2012, Blüher et al. 2006, Côté et al. 2007). Furthermore, insulin has been recognised as a negative regulator of AEA with the regulatory effect diminished by insulin resistance (Di Marzo et al. 2009). Engeli et al. (2012) observed plasma AEA, 1-/2-AG and OEA were significantly increased in diabetic patients with OSA compared to non-diabetic patients with OSA along with increased CRP and decreased adiponectin levels. A gradual loss of the inhibitory effect of insulin may represent a mechanism that explains increased circulating AEA concentrations in patients with OSA (Engeli et al. 2012). The patients in our study were not excluded or screened for the presence of diabetes.

Adiponectin has been shown to be reduced in obesity (Arita et al. 1999) and is thought to have potential antidiabetic (reduce insulin resistance), anti-atherosclerotic and anti-inflammatory properties (Trujillo & Scherer 2005). Reduced adiponectin levels are related to increased endothelial inflammatory responses, the presence of coronary heart disease, dyslipidemia, insulin resistance and type 2 diabetes in humans (Wolk et al. 2005). Kelly et al. (2010) has revealed in obese pubertal children OSA severity is negatively associated with adiponectin levels even after adjustment of BMI. Furthermore Vatansever et al. (2011) reported serum adiponectin concentrations were significantly decreased in patients with OSA compared to non-OSA controls with a negative correlation reported with AHI ($r$ = -0.34, $p$<0.05). In our study age was the only parameter which was found to correlate with adiponectin ($r_s$ = 0.38, $p$<0.007). The exclusion criteria employed in Vatansever et al. (2011) was however much stricter than in our study, it included not being prescribed any medications and no history of cardiovascular disease. More similar to our study, Makino et al. (2006) found plasma adiponectin levels were not different between OSA groups and were not correlated with AHI. Makino et al (2006) study included 213 Japanese patients with OSA with an age range of 27-80 years of age and like our current study recruited patients who have other various systemic and metabolic diseases which are known comorbidities of OSA.

As mentioned earlier, the analysed endocannabinoids in our study were both found to be significantly related to CRP. CRP is marker of low-grade systemic inflammation and is also suggested to potentially mediate insulin resistance in OSA in addition to being associated with an increased risk of atherosclerosis and cardiovascular disease (Danesh et al. 2004, Kokturk et al. 2005, Hargens et al. 2013, Kelly et al. 2010). Other previous research

investigating CRP in patients with OSA have found mixed results with some suggesting CRP is more related to obesity than OSA (Akashiba et al. 2005, Ryan et al. 2007). Further supporting this theory, no significant difference in CRP concentrations was found in our study between the groups of patients with OSA and no correlation existed between AHI and CRP. CRP though was found to be positively correlated ($p<0.01$) to body mass, BMI and neck, waist and hip circumference and leptin which was also positively correlated to body characteristics ($p<0.01$). It is debatable whether leptin levels are increased independently from obesity with the development of OSA (Ip et al. 2000, Phillips et al. 2000, Kapsimalis et al. 2008, Schäfer et al. 2002, Patel et al. 2004), current evidence does though indicate that leptin acts also as a proinflammatory cytokine which would support the correlation between leptin and CRP (Loffreda et al. 1998, Fantuzzi & Faggioni 2000, Xu et al. 2014, Wisłowska et al. 2008, Bokarewa et al. 2003, Matarese et al. 2010, Zhu et al. 2013). These correlations then further imply that systemic inflammation occurring in the participants of our study is more likely to be the result of obesity rather than sleep apnoea.

Exposure to either intermittent or continuous hypoxia has been shown to induce inflammatory stress (Han et al. 2013, Burki & Tetenta 2014). Patients with OSA are frequently exposed to intermittent hypoxia along with hypercapnia during sleep (He et al. 2014, Cooper et al. 2005). No correlation was present between AHI and the ventilatory response to the mild hypoxic gas mixture (13% $O_2$) as revealed in chapter 3. Using the previous ventilatory response data, in this study though we did find a positive correlation existed between the ventilatory response to the hypoxic gas mixture with CRP ($r_s = 0.61$ $p = 0.003$) an inflammatory biomarker and 2-AG ($r_s = 0.50$ $p = 0.007$) which has anti-inflammatory properties. Potentially, then the presence of inflammation, as a result of recurrent apnoea related events may explain the positive correlations found between the ventilatory response to hypoxia and correlation with CRP and 2-AG. Age was also found to be significantly correlated with the ventilatory response to hypoxia ($r_s = 0.42$ $p = 0.15$). This relationship though maybe explained as a result of the increased ventilatory response required to maintain arterial $O_2$ saturation with aging (Lhuissier, Canouï-Poitrine & Richalet 2012). Age though was also positively correlated with adiponectin ($r_s = 0.38$ $p = 0.007$) which is also known for having anti-inflammatory effects (Wolk et al. 2005).

As discussed previously in chapter 3 the ventilatory response to the hypercapnic hyperoxic gas mixture was found to be negatively related to AHI ($r_s = -0.51$, $p = 0.003$) and this was still

the case when a partial correlation was performed controlling for body mass ($r$ = -0.36, $p$ =0.047). Several studies have suggested that leptin may be involved in the control of breathing (O'Donnell et al. 2000, O'donnell et al. 1999, Tankersley et al. 1998). However this current study found it was only if Pearson $r$ test was applied, leptin was significantly correlated with the ventilatory response to $CO_2$ ($r$ = -0.38 $p$ = 0.04). Unfortunately Pearson r should not be used with this data set as leptin was found not to be normally distributed and so Spearmans rho's interpretation is more appropriate (Field 2009) this yields a value of $r_s$ = -0.33 p= 0.09. It's possible with the recruitment of more participants a significant correlation would be present although this is without controlling for obesity related parameters all of which leptin has been found to be significantly correlated to ($p$<0.01). Again many authors do consider leptin levels being mostly related to obesity rather than AHI so these results are not surprising especially as most of the study population are morbidly obese. Additionally we cannot rule out the presence of Obesity Hypoventilation Syndrome in our study population as early diagnosis is rare with Mokhlesi et al. (2007) for example identifying that 30% of the patients with OSA were later found to be diagnosed with Obesity Hypoventilation Syndrome.

Limitations:

In comparison to other studies investigating blood markers amongst patients with OSA our study sample size was relatively small and many other studies also include a control group for comparsion. The recruitment of patients with OSA was not stratified according to disease severity and this therefore also led to uneven group sizes which may skew our results. Additionally our findings may also be influenced by the lack of control over comorbidities such as obesity, diabetes and arthritis which are also associated with the presence of inflammation.

**Conclusion:**

No correlation was found between AHI and markers of inflammation, unfortunately inadequate recruitment may have been a contributing factor. The findings of this study provide further evidence to suggest that the severity of OSA is strongly influenced by markers of obesity which is associated with inflammation. Our study findings clearly highlight the influence of body mass on the ventilatory response and blood markers of inflammation in patients with OSA. Possible limitations of our study include the lack of participants for certain parameters. Future studies with a recruitment of many more participants are required

in order to allow the creation of a valid regression model and improve our understanding of the pathogenesis of OSA. We believe completion of this study though will facilitate future studies taking place between Bangor University and Ysbyty Gwynedd. It has been reported that inflammation may contribute to abnormalities in muscle function. Skeletal muscle (both respiratory and limb) abnormalities are common and profound in patients with chronic inflammatory disorders including chronic obstructive pulmonary disease (COPD). The next study chapter aims to develop and test a method of inducing inspiratory muscle fatigue to recognize if the function of the inspiratory muscles plays a role in the pathogenesis of OSA.

## Chapter 6 Development of a protocol to measure inspiratory muscle fatigue in patients with OSA:


### Abstract:

*Purpose:* Upper airway obstruction in OSA may lead to increased inspiratory efforts and consequently, periodical overload of the inspiratory muscles. This overloading along with the presence of nocturnal intermittent hypercapnia/hypoxia and systemic inflammation may lead to an increased risk of inspiratory muscle fatigue. Currently however, there is a need for a protocol which is relatively non-invasive and easy to perform to induce fatigue of the inspiratory muscles within the clinical environment.

*Methods:* This study comprises of two studies. The first tests and evaluates a novel protocol with healthy male participants (n = 63) from a range of physical activity backgrounds as well as assessing test-retest reproducibility. The second applied the protocol to male patients with OSA (n = 24) in Ysbyty Gwynedd to identify whether patients with OSA were prone to developing inspiratory muscle fatigue and whether associations exist between the results and their ventilatory response to hypercapnia and hypoxia.

*Results:* The first study found the protocol to be repeatable with an intraclass correlation coefficient of 0.97 using average measures and 0.75 with singular measures. The protocol found after eighty inspiratory resistive breaths the inspiratory pressure at RPE 14 was significantly correlated with neck ($r_s$ = -0.271; $p$ < 0.05) waist ($r_s$ = -0.296; $p$ < 0.05) and BMI ($r_s$ = -0.275; $p$ < 0.05). Despite this only 9% of variation ($R^2$ = 0.090) in pressure could be predicted based on these parameters using multiple regression. The participants were also split into two groups according to BMI. The overweight BMI group had a significantly lower inspiratory pressure at RPE 14 after 20, 40 and 60 resistive breaths ($p$<0.05).

The second study found in the patients with OSA the apnoea- hypopnoea index (AHI) was negatively correlated with RPE 14 after 80 resistive breaths ($r_s$ = -0.461; $p$ <0.05). Additionally PiMax was found to be negatively correlated with BMI ($r_s$ =-0.564; $p$<0.01) and neck circumference ($r_s$ = -0.496; $p$ = <0.05). There was no significant difference in PiMax between all groups. Using a partial correlation to control for BMI, AHI was still negatively correlated with inspiratory pressure at RPE 14 after 80 inspiratory resistive breaths ($r$ = -0.451; $p$<0.05). Furthermore, the patients with OSA had a significantly higher inspiratory pressure after 60 resistive breaths when BMI was used as a covariant compared to the

overweight participants of the first experiment ($p$<0.05). These outcomes suggest that with a higher AHI, patients with OSA tend to produce less force for a perceived effort while their total force production is elevated possibility due to recruitment of accessory muscles.

*Conclusions:* We have evaluated a novel, repeatable protocol to induce inspiratory muscle fatigue. BMI was found to have a significant effect on the results and markers suggestive of inspiratory muscle fatigue were identified amongst the patients with OSA. Furthermore, a negative correlation was found between AHI and inspiratory pressure at RPE 14 after 80 resistive breaths when BMI was controlled for. No correlation was found between the ventilatory response to hypercapnia or hypoxia. The results of this study therefore support the suggestion that the development of OSA may be related to separate mechanisms.

**Introduction:**

The ability to sustain ventilation is dependent on the central nervous system, the strength of the respiratory muscles, and the load applied to them (Goldstone, Green & Moxham 1994). The presence of an occlusive airway and subsequent apnoeas associated with OSA may lead to increased inspiratory efforts and consequently, periodical overload of the inspiratory muscles (Chien et al. 2013, Wilcox et al. 1990). Moreover, this overloading may cause an increased risk of fatigue (Chien et al. 2013) that may be accompanied with prolonged periods of apnoeas.

Skeletal muscle fatigue can be defined as a loss in the capacity for developing force and/or velocity of a muscle, resulting from muscle activity under load which is reversible with rest (ATS/ERS 2002, Janssens et al. 2013). Similarly respiratory muscle fatigue can be defined as an inability to continue to generate sufficient pressure to maintain alveolar ventilation (Roussos & Zakynthinos 1996, Zakynthinos & Roussos 2005). If the load placed upon the respiratory muscle pump exceeds its capacity, ventilatory failure may occur and consequently it has been hypothesised respiratory muscle fatigue contributes to task failure (Goldstone, Green & Moxham 1994, Guleria et al. 2002) and is alleviated by rest with mechanical ventilation (Brochard et al. 1989).

The term respiratory muscle fatigue is generally considered synonymous with inspiratory muscle fatigue since expiration is generally viewed as a passive process taking advantage of passive recoil of the respiratory system (Suzuki, Suzuki & Okubo 1991). Therefore, in this current chapter we focus predominately on the inspiratory muscles only. Although both inspiratory and expiratory muscle fatigue has been observed following expiratory resistive loading (Suzuki, Suzuki & Okubo 1991). Theoretically, fatigue could arise from a lack of effort or potentially a weak link in the chain of events from the central nervous system through to the peripheral contractile apparatus (Roussos & Zakynthinos 1996, Moxham 1990).

The inspiratory muscles consist of the intercostal muscles, the diaphragm which acts as the principal inspiratory pump muscle especially during sleep (Poole et al. 1997, McKenzie, Butler & Gandevia 2009) and the accessory muscles (scalene, sternocleidomastoid and platysma). Accessory muscles become more involved when greater inspiratory force is required such as in some obstructive respiratory diseases or when additional resistances are

applied (De Troyer et al. 1994). In theory the accessory muscles could support any weaknesses in the diaphragm during apnoea periods. The control of the inspiratory muscles can be considered on three levels. The first level is the autonomous level which is responsible for maintenance of basic pulmonary ventilation. The second is the adaptive level which coordinates the contraction of the inspiratory muscles with an increased respiratory load or other motor acts. Finally, the third level is the voluntary level, which allows the inspiratory muscles to be consciously controlled (Aleksandrova & Breslav 2009).

The diaphragm has been demonstrated to be more resistant to developing fatigue compared to limb muscles both *in vivo* and *vitro* (McKenzie, Butler & Gandevia 2009, Gandevia, McKenzie & Neering 1983). Furthermore, it has also been demonstrated that the inspiratory muscles recover from fatigue ten times faster than the elbow flexors performing a similar task (McKenzie & Gandevia 1991). One prominent characteristic of the neuromuscular system is its adaptability when subjected to a chronic stimulus (Enoka & Stuart 1992). Adaptations of the diaphragm often referred to as diaphragm remodelling, has been shown to occur as a result of the increased work of breathing in COPD which leads towards a more fatigue-resistant phenotype and hyperinflation which induces structural adaptations to cope with an unfavourable strength-length relationship (Orozco-Levi et al. 1999, Bachasson et al. 2013).

The current definition of muscle fatigue is ambiguous as muscle fatigue has been described irrespective of whether it is due to peripheral contractile fatigue (Janssens et al. 2013, Johnson et al. 1993, Laghi, Topeli & Tobin 1998) or inefficiency of the neural drive termed as central fatigue (Janssens et al. 2013, Gandevia 2001). Furthermore the amount of loss, the intensity of the muscle activity and the applied load is not further defined (Janssens et al. 2013).

Central fatigue refers to the condition in which muscle force generation during sustained or repetitive contraction decreases because of a reduced central motor output (Zakynthinos & Roussos 2005) (decreased motoneuronal output (ATS/ERS 2002). It is judged to be present when a truly maximum voluntary effort produces less force than one generated by direct electrical stimulation (ATS/ERS 2002). Bellemare and Bigland-Richie (1987) measured transdiaphragmatic pressure generation before, during and after inspiratory resistive loading and employed superimposed electrical phrenic nerve stimulation at various time points to determine if the participants were capable of "fully activating" the diaphragm (ATS/ERS 2002). It was reported task failure of the diaphragm occurs in part, due to failure of the voluntary drive to completely activate the fatiguing muscle. Guleria et al. (2002) also

demonstrated that participants were unable to fully activate the diaphragm at task failure and in comparison to Bellemare and Bigland-Richie (1987) study participants were also free to control minute ventilation, breathing frequency and duty cycle allowing participants to optimise their breathing pattern to increase endurance. Compared to the limb muscles it has been reported that the diaphragm is less susceptible to central fatigue over the same exercise period but substantial central fatigue has been demonstrated during prolonged series of expulsive contractions which markedly elevate abdominal pressure (McKenzie et al. 1992).

Peripheral fatigue can result because of alterations in the neuromuscular junction, changes in the propagations of the action potentials along the sarcolemmal membrane or into the t-tubules, changes in excitation-contraction coupling, or because of other alterations within the muscle cell such as alterations in metabolism or changes in contractile proteins (ATS/ERS 2002, Zakynthinos & Roussos 2005). High-frequency peripheral fatigue is present when there is a depression of forces generated by a muscle in response to high-frequency electrical stimulation whereas low frequency peripheral fatigue is a depression in force with low-frequency stimulation (ATS/ERS 2002). Low-frequency fatigue can occur in isolation, but high frequency fatigue is invariably associated with some alterations in muscle force generation at lower frequencies (Zakynthinos & Roussos 2005).

High frequency peripheral fatigue has been demonstrated in the diaphragms of healthy participants following a trial of high-intensity inspiratory resistive loading (ATS/ERS 2002, Zakynthinos & Roussos 2005, Aubier et al. 1981). This has been thought to reflect neuromuscular junction failure which has been theorised as a protective mechanism against excessive depletion of ATP stores, which would result in rigor mortis (Roussos & Zakynthinos 1996). In low frequency peripheral fatigue, the force generation in response to high-frequency stimulation is unimpaired indicating that the contractile proteins are capable of generating maximal force provided that sufficient calcium is released by the sarcoplasmic reticulum. As a result, impaired force generation occurs at submaximal frequencies of stimulation (ATS/ERS 2002). This type of fatigue is not related to depletion of ATP or phosphocreatine. The mechanisms of this type of fatigue are not well known (Roussos & Zakynthinos 1996). The most recent understanding in skeletal muscle is that low-frequency fatigue seems to be due to structural changes in proteins involved in intracellular calcium handling (Westerblad & Allen 2002). Guleria et al. (2002) has shown inducing low frequency peripheral fatigue is more difficult to achieve in the diaphragm than non-respiratory muscles

in humans. This is likely because the respiratory system chooses to respond to a large external load by preferentially recruiting extradiaphragmatic rib cage muscles (Guleria et al. 2002, Hershenson et al. 1989).

The onset of fatigue is often accompanied by an increased perception of effort (Enoka & Stuart 1992). Perception of effort is the sensation of how vigorous or heavy a physical task is (Borg 1982). It is a major characteristic of fatigue felt in disease and exercise (Enoka & Stuart 1992, Marcora, Bosio & de Morree 2008). Perception of effort has been theorised as a "sensation of innervation", referring to the conscious awareness of the central motor command which is sent to the active muscles (Lafargue & Franck 2009, de Morree, Klein & Marcora 2012). This considers a function of the sense of effort being to estimate the intensity of voluntary muscular force (Lafargue & Franck 2009, Carson, Riek & Shahbazpour 2002) Carson et al. (2002) provides support for fatigue altering the relationship between sense of effort and the motor command. Carson et al. (2002) demonstrated that in participants who carried out eccentric contractions of the triceps brachii in one arm, in the same experimental arm overestimated the level of force required to obtain target force levels of 25%, 50% or 75% of maximal voluntary muscle contraction defined by the unfatigued control arm. The participants believed that they were generating more force than they were achieving. This perception of increased effort as a result of fatigue is likely the result of the increased central motor command required to exercise at the same workload with the weaker muscles (de Morree, Klein & Marcora 2012). Appling this knowledge to the diaphragm implies that with the onset of fatigue there will be an increased activation of central motor command. This may increase the likelihood of activation of additional accessory muscles such as the extradiaphragmatic rib cage muscles as reported in Guleria et al. (2002) study which compared the presence of low frequency fatigue in the diaphragm to the quadriceps. This activation of accessory muscles could potentially relate to an increased force production for the same sense of effort.

Respiratory muscle fatigue is associated with acute hypercapnia due to a combination of the increased mechanical load of the lung, reduced muscle strength, decreased efficiency, and reduced energy supplies to the inspiratory muscles (Roussos & Zakynthinos 1996). In addition, patients with OSA are frequently exposed to nocturnal bouts of hypoxia and hypercapnia during sleep (Cooper et al. 2005). Whether hypercapnia impairs respiratory muscle function is debatable (Jonville, Delpech & Denjean 2002). Mador et al. (1997)

assessed the effects of 8% $CO_2$ being breathed for 20 minutes on two separate occasions. It was found acute hypercapnia mildly depressed limb contractility however did not produce significant changes in contractility of the diaphragm. Juan et al. (1984) on the other hand found acute respiratory acidosis equivalent to an arterial $CO_2$ tension of around 54 mm Hg decreases the contractility and endurance time of the diaphragm. Jonville et al. (2002) for the first time used magnetic stimulation of the phrenic nerves to evaluate the contribution of acidosis to diaphragmatic fatigue during exercise (Similowski et al. 1989). A significant decrease in twitch mouth pressure response was observed suggestive of diaphragmatic fatigue ten minutes following exercise with pronounced hypercapnia induced by hypoventilation. If hypercapnia does negatively influence respiratory muscle function, then this may be of particular concern in our patients with OSA as we found in chapter 3, patients with severe OSA have a diminished ventilatory response compared to patients with mild/moderate OSA breathing the hyperoxic hypercapnic gas mixture (25%$O_2$/6% $CO_2$) with a negative correlation found between AHI and ventilatory response to $CO_2$ ($r_s$ = -0.51; $p$ <0.01) which is present even when BMI is taken into account ($r$ = -0.36, $p$ = 0.047).

Obesity is highly prevalent amongst patients with OSA (Young, Peppard & Gottlieb 2002) and is regarded as a chronic inflammatory disease where the physiological resolution of inflammation is attenuated, leading to low-grade inflammation throughout the body (Newsholme & de Bittencourt 2014). Furthermore alterations in muscle structure due to fatty deposits within the muscles lining the upper airways has been reported (Whittle et al. 1999, Carrera et al. 2004, Pillar & Shehadeh 2008). Complications of obesity on the respiratory system include heightened demand for ventilation, elevated work of breathing and decreased functional residual volume and expiratory reserve volume (Parameswaran, Todd & Soth 2006). The reduction in lung volume has also been shown to increase the collapsibility of the upper airway (Tagaito et al. 2007). In addition to the prevalence of obesity, intermittent hypoxia occurring as a result of apnoeic events has been shown in cell culture models to lead to a selective and preferential activation of inflammatory pathways (Ryan, Taylor & McNicholas 2005).

It has been proposed that inflammation may further contribute to abnormalities in muscle function. Skeletal muscle (both respiratory and limb) abnormalities are common and profound in patients with chronic inflammatory disorders including chronic obstructive pulmonary

disease (COPD) (Kim, Mofarrahi & Hussain 2008, MacIntyre 2006) and congestive heart failure (Chien et al. 2013, Strassburg, Springer & Anker 2005).

A loss of muscle mass and the clinical appearance of "muscle wasting" can be caused by systemic inflammatory mediators accelerating muscle protein turnover through ubiquitins (MacIntyre 2006). Ubiquitins are small proteins present in all eukaryotic cells which play an important role in tagging proteins for destruction (Stryer 1995). Additionally, it has been speculated that overloading the muscles can lead to increased oxidative stress (Vollaard, Cooper & Shearman 2006). This would then cause increased production of free-radicals leading to further muscle damage (Chien et al. 2013, Jackson & O'Farrell 1993).

The intermittent changes in blood oxygen saturation levels in OSA have been considered similar to the hypoxia and reoxygenation demonstrated in conditions characterised by ischemia and reperfusion associated with increased production of reactive oxygen species (ROS) (Lavie 2009). Reports of increased ROS production as a result of apnoeic related events though does remain controversial (Oztürk et al. 2003, Wali et al. 1998). It has however been demonstrated that physical training can have a positive or negative effect on oxidative stress dependent upon the training load prescribed (Finaud, Lac & Filaire 2006). Moreover, age dependent increases in markers of oxidative damage to DNA, lipids and proteins determined biochemically in mammalian skeletal muscle have been widely reported (Starnes et al. 1989, Ji, Dillon & Wu 1990, Lawler et al. 1993, Leeuwenburgh et al. 1994, Sohal et al. 1994, Mecocci et al. 1999, Pansarasa et al. 1999, Zainal et al. 2000). Some researchers theorise that the accumulation of oxidative damage in skeletal muscle also contributes to the development of sarcopenia (loss of muscle mass with age) (Weindruch 1995).

It is plausible that inspiratory muscle fatigue may develop over the course of the night in patients with OSA. It has been reported the inspiratory efforts generated at the end of apnoeas are often very large, such that when transdiaphragmatic pressure and the tension-time index of the diaphragm are determined, end-apnoeic values in some patients approach or surpass the threshold of fatigue described in normal subjects (Vincken et al. 1987, Kimoff et al. 1994, Montserrat et al. 1997). Furthermore, these efforts occur under conditions of hypoxemia, hypercapnia and declining cardiac output (Garpestad et al. 1992). Additionally, impaired inspiratory muscle contractility has been observed in patients with OSA by means of the pleural pressure relaxation rate during voluntary sniff manoeuvres being prolonged in the morning compared to preceding night of sleep (Griggs et al. 1989). Montserrat et al. (1997)

however investigated patients with severe OSA to identify if diaphragmatic fatigue occurred during the large inspiratory efforts at the end of apnoeas during stage two of sleep at the beginning and at the end of the night. Montserrat et al. (1997) documented no support for the development of diaphragmatic fatigue however a relatively small number of participants were studied (n = 7).

More recently, Chien et al. (2010) investigated muscle strength, endurance and the fatigability of the inspiratory muscles and the knee extensors in patients with OSA compared to age and BMI matched controls. Chien et al. (2010) applied maximal voluntary ventilation to induce inspiratory muscle fatigue. Simultaneous surface electromyography was used to identify a significantly lower function of performance in both the inspiratory muscles and knee extensors in the OSA group during magnetic stimulation via the phrenic nerve and femoral nerve respectively. A higher fatigability was seen only in the inspiratory muscles of patients with severe OSA during both voluntary contractions and magnetic stimulations. Chien et al. (2010) used peripheral muscle (knee extensors) as a control because they are not considered to be overloaded during sleep. It was however reported that the patients with OSA had a lower physical activity level which may explain for the lower baseline muscular strength of both examined muscles in the OSA group and furthermore this may have meant the level of fatiguing task might have been relatively higher for the OSA group.

In healthy individuals, respiratory muscle fatigue has been reported to occur during voluntary hyperpnoea (Martin, Heintzelman & Chen 1982), high-intensity cycling (Johnson et al. 1993), treadmill exercise (Babcock et al. 1995), inspiratory resistive breathing (Fiz et al. 1998, Rohrbach et al. 2003, Gonzales & Scheuermann 2006) and repeated generation of transdiaphragmatic pressures (Bellemare & Bigland-Ritchie 1984). Gonzales and Scheuermann (2006) found in a healthy population that females demonstrated a slower rate of fatigue and less muscle fatigue at task failure during resistive breathing than males independent of muscle strength. In healthy participants, two minutes of maximal voluntary hyperventilation has been reported to result in failure of tension generation and low-frequency fatigue of the diaphragm (Polkey et al. 1997)

A potential contributor for the lack of research assessing whether inspiratory muscle fatigue occurs in clinical populations such as in OSA, may be attributed to the challenges researchers face in devising a method which can adequately assess respiratory muscle fatigue within the clinical environment. Although a variety of methods exist aimed at inducing

inspiratory muscle fatigue, they have mainly only been successfully implemented with healthy individuals. There is currently no consensus regarding an optimal protocol to induce and assess the fatigability of the inspiratory muscles (Janssens et al. 2013). Difficulties particularly arise in the measurement of inspiratory muscle fatigue as variables within the respiratory system including muscle interaction, lung volume and thoracoabdominal configuration need to be accounted for. Furthermore, laboratory techniques such as twitch-interpolation and the use of balloon catheters to measure oesophageal, gastric and transdiaphragmatic pressures are often unsuited for routine testing as they are invasive and use specialist equipment (ATS/ERS 2002).

Janssens et al. (2013) conducted a systematic review of the literature assessing inspiratory muscle fatigue within the healthy population. Many studies were reported to use inspiratory resistive loading by breathing against an inspiratory threshold load (32 studies) which in most of these studies (27 studies) involved participants' being instructed to breathe against a predefined percentage of their maximal inspiratory mouth pressure or transdiaphragmatic pressure ($Pdi_{max}$). 64% (n=49) of the studies included in Janssens et al. (2013) review used phrenic nerve stimulation to detect possible diaphragmatic fatigue. Supramaximal phrenic nerve stimulation is considered the most objective measurement of diaphragm fatigue (Perret et al. 1999). This method requires specialist invasive equipment (involves bilateral anterolateral or cervical stimulation of the phrenic nerves via electrical or magnetic stimulation) and is therefore not suitable to be performed routinely outside the laboratory environment with clinical populations (Janssens et al. 2013).

In this current study we aim to validate and test the design of a novel protocol designed to investigate the presence of inspiratory muscle fatigue within healthy subjects and patients with OSA. This protocol is designed to be used within environments where specialist invasive equipment which is often dedicated to the research setting, is not usually available such as in a clinical service. The methodology involves breathing against an inspiratory threshold load at a predefined percentage of maximal inspiratory mouth pressure and identifies the presence of fatigue based on changes in the inspiratory force production related to ratings of perceived exertion (RPE) (de Morree, Klein & Marcora 2012). The protocol is a further development from a test protocol previously designed and tested by myself with the help of a Master's student (James Magee) to induce inspiratory muscle fatigue in a healthy, relatively fit population (Magee 2012).

This chapter comprises of two related studies, the first study is to test and evaluate the novel protocol with healthy participants from a range of physical activity backgrounds and assess test-retest reproducibility. This experiment is also designed to help understand whether inspiratory muscle fatigue relates to the previous level of physical activity of the participants. The study took place in the laboratories of the School of Sport, Health and Exercise Sciences, Bangor University. The second study applied the novel protocol to patients with OSA in Ysbyty Gwynedd, Bangor to identify whether the patients were prone to developing inspiratory muscle fatigue. For further data analysis and interpretation, results of the inspiratory muscle fatigue protocol and the ventilatory response to the hypercapnia and/or hypoxia investigated in chapter 3 were assessed together for correlations. This was to identify if a potential interaction exists between the alteration in ventilatory response identified in chapter 3 and a change in inspiratory muscle function.

**Method:**

*Study 1: Testing of protocol to induce inspiratory muscle fatigue and test-retest reliability:*

*Participants:*

Following ethical approval from the School of Sport, Health and Exercise Sciences, Bangor University ethics committee, sixty three healthy male participants were recruited. Recruitment occurred using the Bangor University's email network as well as talks I gave in university lectures and laboratory practical sessions. Additionally, advertisements were placed in different locations such as in the university, sport centres, libraries and around the community. In order to match the gender of the patients recruited for the second experiment, all participants were male.

The study was performed in the School of Sport, Health and Exercise Sciences by myself and a MSc student, Alan Beg. As maximal inspiratory pressure efforts produce large changes in thoracic, upper airway, middle ear and sinus pressures, participants were excluded if they had suffered from a perforated ear drum (or other middle ear pathology), had a history of spontaneous pneumothorax or a recent trauma to the rib cage or were suffering from acute sinusitis (until condition resolved) (McConnell 2007).

*General procedures*

*Assessment of physical activity and physical characteristics:*

All participants completed the self-reported long version of the International Physical Activity Questionnaire (IPAQ). The long form was used as recommended for research requiring a more detailed assessment (Craig et al. 2003). The IPAQ is an instrument designed to measure habitual physical activity of individuals ranging from young to middle-aged adults (i.e. 15-69 years old). IPAQ was developed by the International Consensus Group in 1998-1999 to establish a standardised and culturally adaptable measurement tool across various populations in the world (Craig et al. 2003, Kim, Park & Kang 2013). IPAQ was used to assess the level of physical activity over the past seven days assessing the number of days and the periods of moderate or vigorous exercise recorded over four domains including: transportation, work, gardening/housework and leisure activities. IPAQ also considers the duration of time participants spend sitting.

The participants' height, weight, neck, waist and hip circumference were measured prior to the participants' lung function being assessed using spirometry performed on a portable hand held MicroLoop spirometer (Micro Medical Ltd., Basingstoke, UK), in accordance with the ATS/ERS guidelines (Miller et al. 2005).

*Measuring maximal inspiratory pressure:*

For the inspiratory muscle fatigue protocol an initial assessment of the participants' maximal inspiratory pressure (PiMax) is essential. Prior to performing maximal inspiratory pressures the participants were introduced to the Borg's 15-graded rating scale (RPE scale). The Borg's 15-graded rating scale has been validated for use with resistance exercise in numerous studies (Row, Knutzen & Skogsberg 2012, Gearhart et al. 2001, Eston & Evans 2009, Lagally, Amorose 2007, Lagally et al. 2002) including with sedentary adults (Tiggemann et al. 2010). The guidelines of Noble and Robinson (1996) were used to guide in explaining the RPE scale to the participant. The participants were instructed to specifically relate their RPE to their efforts of their breathing muscles only and to anchor their perceptual range a RPE of 6 was defined as a light bulb being completely off and 20 was defined as the brightest possible light. Patients were told there are no right or wrong answers and were encouraged to ask any questions. The 15-graded rating scale was used as this previously was used in the protocol

with healthy participants (Magee 2012). We subjectively perceived that the tested participants related better to an RPE value of 14 than value of 6 on the Borg's category-ratio (CR-10) scale. Participant rated their PiMax measures as a familiarisation of using the scale.

PiMax measures were performed using a MicroRPM respiratory pressure meter (MicroMedical Ltd., Basingstoke, UK) in accordance with British Association of Sport and Exercise Sciences (BASES) recommendations (McConnell 2007). Although not in line with those recommendations, a total of seven PiMax manoeuvres were performed rather than 10. This was because the pilot study with MSc student Magee showed no differences between scores and to avoid the presence of any fatigue prior to performing the protocol designed to induce inspiratory muscle fatigue.

The test procedure was explained to the participant and demonstrated as required. Each participant wore a nose clip and was instructed to expire to residual volume prior to inspiring whilst connected to the device with a maximal effort for at least two seconds. A disadvantage of this volitional test of PiMax is the mechanical linkage of each individual respiratory muscle within the chest wall and with other inspiratory or expiratory muscles, that influences the net pressure produced (ATS/ERS 2002). To minimise this effect, the participants were seated on a chair with their waist curved in a forward position to avoid recruitment of the accessory muscles throughout the protocol, as shown in Figure 6-a. Participants were instructed to try to breathe from the diaphragm also to avoid other accessory muscles becoming functional, such as the sternocleidomastoid and scalene muscles in the neck, muscles in the shoulder region and muscles in the pectoral girdle.

**Figure 6-a.** Drawing illustrating participants posture during the tests to minimise recruitment of accessory muscles.

The MicroMedical RPM was used in conjunction with PUMA respiratory pressure database and analysis software (MicroMedical Ltd., Basingstoke, UK). Participants were allowed to see the generated pressure trace and were given verbal encouragement to achieve a true maximal effort on each repetition. The mean value was taken from the largest two readings if within 10% of each other, if not the highest reading was used. At least 30 seconds of rest were given between each manoeuvre to avoid any presence of fatigue. An example pressure trace like that participants would have been able to see during this part of the test is shown in Figure 6-b.

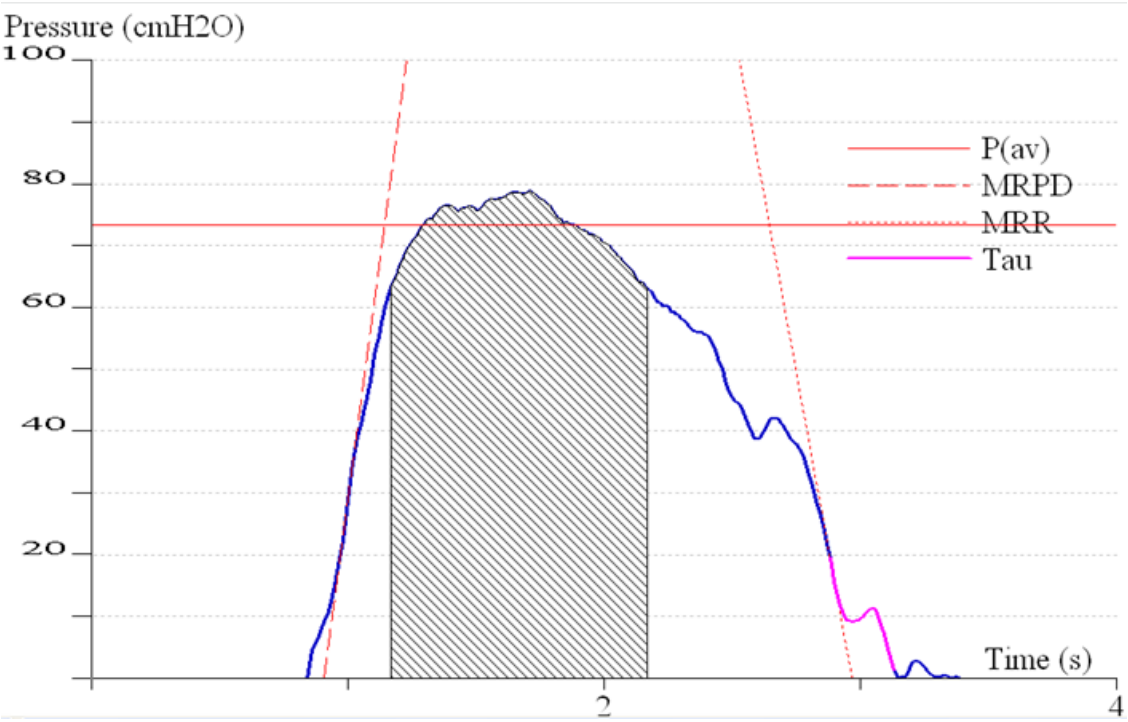


**Figure 6-b.** Screenshot of pressure trace participants were able to see during the maximal inspiratory pressure trial measurements.

*The inspiratory muscle fatigue protocol:*

*Rationale:*

Respiratory muscle fatigue is difficult to measure non-invasively using serial measurements of PiMax. This is particularly because during increased effort a large number of accessory muscles interact to aid the process of inspiration (De Troyer, Kirkwood & Wilson 2005). Therefore we developed a protocol whereby participants use their sense of effort to match force with the presence of fatigue being demonstrated by impaired force generation at submaximal efforts, revealing possible evidence of either central fatigue, low frequency peripheral fatigue or an interaction of both.

We measured inspiratory pressure achieved by participants when they were asked to attempt to inspire at an effort they perceive as relating to an RPE of 14 on the Borg's RPE scale (described as between somewhat hard and heavy). This judgement of effort is subjective and when the muscles become fatigued the participant may perceive they are generating more force than they actually are. For example, if a subject lifted a weight with an effort of 14 on the RPE scale and then closed their eyes and carried a number of loads causing their muscles to become fatigued, they may then perceive the same weight as heavier than they previously

had. In this study, the inspiratory muscles were designed to become fatigued through breathing through a Powerbreathe device (Powerbreathe Plus Fitness, H.A.B International Ltd) which caused inspiratory resistance. The effort used during inspiration was designed so that participants would gradually become fatigued and would generate less inspiratory force for a similar effort rating of 14 on the Borg scale. For hygiene reasons, Powerbreathe filters were used. If the filter became obstructed by saliva, the participant was instructed to shake the device into the washing up bowl which they were leaned over in order to maintain the same posture in all tests. The Powerbreathe device was also taken apart, sterilised and dried between participants. Resistance of the Powerbreathe device was set to 50% of the obtained PiMax as per the levels of resistance shown in Table 6-a.

**Table 6-a.** Showing the related Powerbreathe levels used to obtain resistance at 50% of PiMax. Where participant fell between levels half measures were used. Information was kindly acquired from H.A.B International Ltd. technical support.

| Powerbreathe Plus Fitness | | | | | | |
|---|---|---|---|---|---|---|
| Level | **0** | **1** | **2** | **3** | **4** | **5** |
| $cmH_20$ | 23 | 39 | 55 | 72 | 88 | 104 |
| mmHg | 17 | 29 | 40 | 53 | 65 | 76 |

The Powerbreathe device was used to apply a resistive load to the inspiratory muscles. The MicroMedical RPM device was used to measure PiMax prior to the protocol and to measure what the participant perceived as an inspiratory effort related to RPE of 14 on the Borg's RPE scale between inspiratory resistance breathing sets. From this point forward in the thesis, the inspiratory effort related to RPE 14 is referred to as $IP_{14}$.

*Protocol:*

Firstly after the completion of the maximal inspiratory pressure the RPE scale was again explained to the participant. Understanding of the RPE scale was then checked during the following familiarisation task for $IP_{14}$ measurements. Additionally, participants were introduced to a modified Borg scale which was designed to assess level of dizziness and dyspnoea at the end of the study protocol.

The familiarisation task was used to familiarise the participant to performing $IP_{14}$ through the MicroMedical RPM device prior to fatiguing the inspiratory muscles. Participants completed five inspiratory efforts at $IP_{14}$ with thirty seconds of rest between measures. After the familiarisation, participants competed five sets of twenty breaths (inspiration and expiration) totalling hundred breaths whilst connected to the Powerbreathe device. When connected to the Powerbreathe device, participants were instructed to fully inspire to maximal inspiratory capacity and expire to residual volume. It was stressed that participants should not perform the breath cycles quickly in order to avoid hyperventilation.

Between each set of twenty breaths, participants performed one $IP_{14}$ measure through the MicroMedical RPM device. After the completion of the entire trial, participants were then required to report their level of dizziness and dyspnoea using a modified Borg scale. This was to ensure participants were not hyperventilating during the test. Figure 6-c displays a flow chart illustrating the complete test protocol.

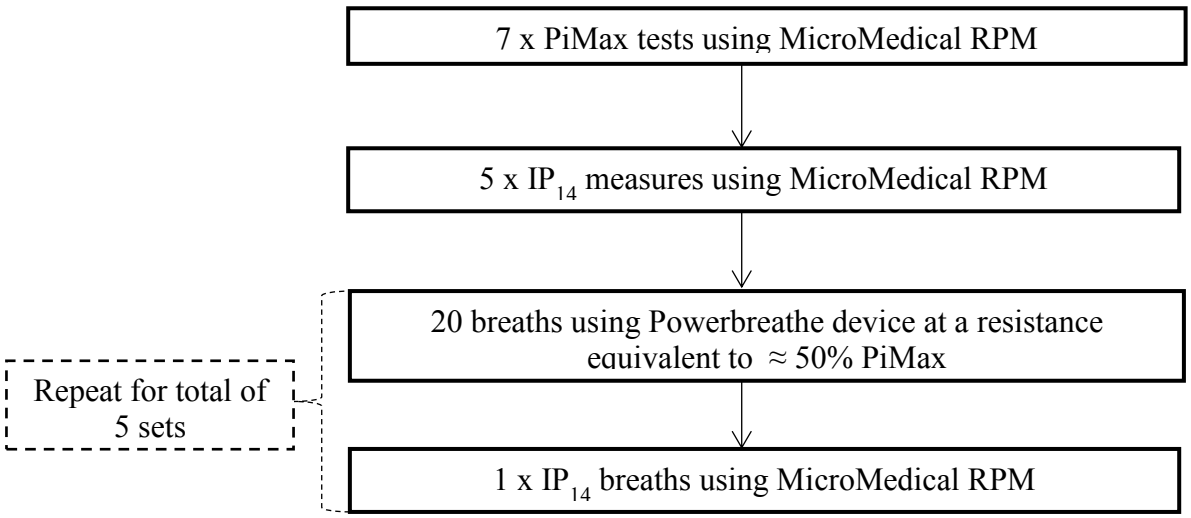


**Figure 6-c.** Flowchart describing the inspiratory muscle fatigue protocol

*Test-retest reliability:*

In order to assess test-retest reliability participants performed the same experimental protocol at least three days later to ensure recovery from the test. Participants performed the second test session at the same time of day as the first.

*Study two: Assessment of inspiratory muscle fatigue in the patients with OSA:*

*Participants:*

Male, newly diagnosed patients with OSA were recruited after they had received consultation regarding their sleep study results in the clinic at Ysbyty Gwynedd. All participants were diagnosed with OSA using an unattended home sleep study Respiratory Polygraphy (Embletta® Gold, Embla Systems, USA). Measures of pulse oximetry, nasal airflow, thoracic and abdominal movements were analysed using RemLogic software. Diagnosis of OSA was performed by either an experienced RCCP (Registration Council for Clinical Physiologists) registered Clinical Physiologist or an experienced Sleep Technologist. Patients performed the study within the two weeks prior to starting CPAP. Written informed consent was obtained from all subjects prior to testing. Patients were excluded if they had a BMI≥50 kg/m$^2$ or they were on medications known to alter their respiratory drive (i.e. opiate-based painkillers).

Patients who were found to have a $FEV_1$/FVC less than 60% were excluded from the dataset (n = 1). This was to reduce the possible presence of COPD in the study population as research literature has demonstrated a greater resistance to fatigue in patients with COPD (MacIntyre 2006). Furthermore, 1 patient's results were excluded due to incomplete test data as a result of technical error. The outcome data from all the patients with OSA from all the previous studies was also incorporated into this correlational study.

*General procedures*

This study was approved by the local North West Wales NHS Ethics Committee (Ref: Earing 11/WNo01/2) (Gwynedd, Wales) and carried out in accordance with the Declaration of Helsinki for research on human subjects. The study was performed in a quiet room within the Pulmonary Function Department of Ysbyty Gwynedd. Participants arrived fasted having been instructed not to consume any food or drink (except water) 12 hours prior to their visit. This was stated on their study information sheet and also verbally during a phone conversation on the day prior to the visit. Participants followed the same protocol as described in the flow chart (Figure 6-c). Unlike study one however, participants only performed the protocol once and did not complete the IPAQ prior to performing the study.

*Data analysis:*

All data were analysed using the Statistical Package for Social Sciences version 20 for Windows (SPSS Inc., Chicago IL, USA). All data are presented mean ± SD and $p<0.05$ are considered statistically significant.

In the first study, participants were placed into groups dependent on their level of physical activity reported by the IPAQ (group 1: low; group 2: moderate; group 3: high activity) and groups dependent on their BMI (group 1: BMI<25; group 2: BMI ≥25). In the second study, patients with OSA were split into two groups according to their AHI. Group 1: consisted of mild and moderate OSA and group 2: severe OSA. Additionally, tests were performed on patients with mild OSA vs. patients with moderate and severe OSA grouped together and tests were performed on mild, moderate and severe OSA patient groups separately. To be classified as a patient with mild OSA, an AHI of ≥5 and <15 with reported symptoms related to sleepiness was present. To be classified as a patient with moderate OSA, a recording of AHI ≥15 and <30 was required. A patient with severe OSA had an AHI ≥30 (Epstein et al. 2009).

*Test-retest reliability:*

In the first study, test-retest intraclass correlation coefficients were assessed using the $IP_{14}$ measures taken after every set of 20 inspiratory resistive breaths through the Powerbreathe device. The coefficient of variation (standard deviation/mean) was also calculated between each test of PiMax and absolute $IP_{14}$ measurements and a Bland and Altman plot illustrated the mean $IP_{14}$ between tests.

*Relative differences between groups:*

To allow assessment of fatigue amongst participants in both studies, each $IP_{14}$ measurement was converted to percentage of PiMax for each participant. This was to allow direct comparison between participants as PiMax varied considerably amongst participants. Mixed modal ANOVAS were performed on relative $IP_{14}$ measurements with each 20 breath Powerbreathe set between the groups.

Linear regression was also used to calculate the slope of the relative $IP_{14}$ measurements between the $1^{st}$ and $4^{th}$ set of 20 breaths through the Powerbreathe device. Slopes were tested for significant difference between groups. All outcome measures of this study and where applicable, the ventilatory response to hypercapnia/hypoxia data were assessed for correlations. Partial correlations were performed as appropriate and multiple regression was used to create a model to predict $IP_{14}$ as a result of resistive breathing with correlated parameters. Where data did not meet the assumption of normality, data transformations were used or Spearman rho test as appropriate.

**Results:**

*Body Characteristics of participants:*

The body characteristics and pulmonary function data of the participants in both experiments are displayed in Table 6-b. The patients with OSA were significantly heavier ($p<0.01$) and older ($p<0.01$) than the healthy participants recruited in the first experiment. There was however no significant difference in PiMax between the patients with OSA and the healthy participants of the first experiment ($p = 0.279$).

**Table 6-b.** Body characteristics, spirometry and PiMax values of each group. Where * = $p<0.05$ and ** = $p<0.01$ between the groups within the same experiment.

| | Study one | | Study two: Patients with OSA | | |
|---|---|---|---|---|---|
| | Normal | Overweight | Mild | Moderate | Severe |
| N | 37 | 26 | 9 | 5 | 11 |
| Age (yrs) | 29.1 ± 8.1 | 32.2 ± 7.2 | 51.2 ± 11.0 | 49.0 ± 12.2 | 51.6 ± 11.9 |
| Height (cm) | 174.9 ± 6.3 | 174.5 ±6.0 | 173.5 ± 8.9 | 176.7 ± 9.8 | 174.9 ± 8.5 |
| Weight (kg) | 70.6 ± 7.0 | 86.0 ± 10.0 | 93.1 ± 10.9 | 116.8 ± 24.3 | 114.5 ± 21.2* |
| Neck (cm) | 37.0 ± 1.7 | 39.8 ± 1.6** | 40.8 ± 2.2 | 45.5 ± 3.5* | 45.5 ± 3.2** |
| Waist (cm) | 85.5 ± 7.4 | 98.6 ± 8.2** | 101.2 ± 8.9 | 118.4 ± 13.9* | 121.1 ± 13.8** |
| Hip (cm) | 89.3 ± 6.2 | 99.5 ±7.0** | 104 ± 9.7 | 115.6 ± 14.0 | 115.8 ± 13.2 |
| BMI ($kg/m^2$) | 23.1 ± 1.5 | 28.2 ± 2.5** | 31.0 ± 3.1 | 37.3 ± 6.3 | 37.5 ± 6.7* |
| $FEV_1/FVC$ (%) | 88.7 ± 10.6 | 90.0 ± 6.3 | 79.9 ± 9.3 | 81.0 ± 4.6 | 78.0 ± 6.5 |
| PiMax ($cmH_2O$) | 107.2 ± 29.1 | 113.3 ± 26.4 | 108.6 ± 29.4 | 102.3 ± 19.9 | 104.9 ± 25.1 |

*Study 1: Interaction between relative differences in $IP_{14}$ and level of physical activity:*

No significant differences were found between the low, moderate and high activity groups and this was also the case when low (n = 7) and moderate (n = 25) physical activity categories were combined together and compared to the high activity level group (n = 31). The same data analysis was also performed on normal (BMI<25 $kg/m^2$) vs. overweight group (BMI ≥25 $kg/m^2$).

A multi-model ANOVA was performed assessing the effects of each 20 breath set on $IP_{14}$ between the overweight (n=26) and normal BMI group (n=37). There was no significant main effect of the sets of twenty resistive breaths and no significant interaction was found between BMI category and sets of twenty resistive breaths through the Powerbreathe device. However, the main effect of BMI group was almost significant ($p$ = 0.052) and therefore a follow up one-way ANOVA was performed. The one-way ANOVA found no significant difference in slopes between the groups however it was found the overweight BMI group had a significantly lower $IP_{14}$ compared to the normal BMI group after 20 breaths ($p$ = 0.025), 40 breaths ($p$ = 0.043) and 60 breaths ($p$ = 0.044) with the Powerbreathe device. No significant difference was found after 80 ($p$ = 0.096) or 100 breaths ($p$ = 0.329). The mean $IP_{14}$ across the entire trial was also significantly different ($p$ = 0.034). Due to the significant differences in homogeneity of variance of the mean $IP_{14}$ after 20, 100 breaths and the mean $IP_{14}$ across the entire trial Brown-Forsythe test was performed instead of ANOVA on these conditions (Field 2009). Figure 6-d illustrates the percentage of PiMax measured during $IP_{14}$ across the two BMI category groups with the corresponding number of completed Powerbreathe breaths.

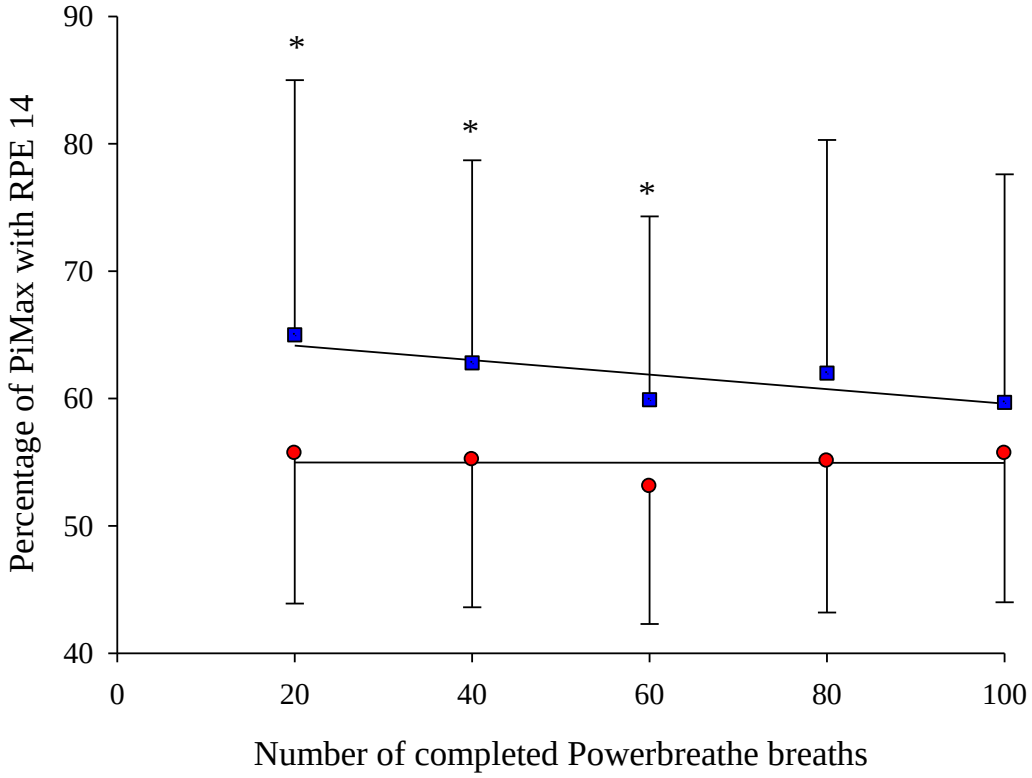


**Figure 6-d.** Percentage of PiMax with $IP_{14}$ measurements after each set of twenty Powerbreathe breaths. Where: ■ = normal BMI (<25 kg/m$^2$) and ● = overweight BMI (≥25 kg/m$^2$) and * = $p$<0.05.

*Assessing correlations and developing a regression model:*

Table 6-c shows the correlation matrix between body characteristics and $IP_{14}$ measurements. BMI was not found to be normally distributed according to Kolmogorov-Smirnov ($p$ =0.019 and the Shapiro-Wilk ($p$ = 0.020) tests of normality therefore Spearman's rho was used (Field 2009). Of particular interest, after eighty breaths with the Powerbreathe device, $IP_{14}$ was significantly correlated with neck ($r_s$ = -0.271; $p$ = 0.032) waist ($r_s$ = -0.296; $p$ = 0.018) and BMI ($r_s$ = -0.275; $p$ = 0.029). Multiple regression was performed to develop a prediction model for the value $IP_{14}$ after eighty breaths through the Powerbreathe device. The enter method was used first using all three variables as predictors. The model was insignificant and therefore backward model multiple regression was performed. The backward model excluded all variables except BMI which was able to predict 8.8% of the variation ($R^2$ = 0.088). The residuals of this model however did not meet the assumption homoscedasticity. Therefore log transformation was applied to the variable BMI. The assumption of both homoscedasticity and normality of residuals was then met. Using the enter method it was found that log transformed BMI can significantly explain 9% of the variance in $IP_{14}$ following eighty breaths using the Powerbreathe device ($F(1, 61) = 6.048$, $p < 0.05$, $R_2 = 0.090$.

**Table 6-c.** Results of Spearman's rho correlation analysis on body characteristics with $IP_{14}$ (Percentage of PiMax) after breathing through Powerbreathe device. ** = $p<0.01$ and * = $p<0.05$.

| | Neck | Waist | Hip | BMI | $IP_{14}$ – 20 breaths | $IP_{14}$ – 40 breaths | $IP_{14}$ – 60 breaths | $IP_{14}$ – 80 breaths | $IP_{14}$ – 100 breaths |
|---|---|---|---|---|---|---|---|---|---|
| Neck (cm) | 1.00 | | | | | | | | |
| Waist (cm) | 0.74** | 1.00 | | | | | | | |
| Hip (cm) | 0.58** | 0.76** | 1.00 | | | | | | |
| BMI ($Kg/m^2$) | 0.68** | 0.72** | 0.68** | 1.00 | | | | | |
| $IP_{14}$ – 20 breaths | -0.12 | -0.17 | -0.28* | -0.26* | 1.00 | | | | |
| $IP_{14}$ – 40 breaths | -0.23 | -0.17 | -0.23 | -0.27* | 0.71** | 1.00 | | | |
| $IP_{14}$ – 60 breaths | -0.26* | -0.21 | -0.24 | -0.24 | 0.67** | 0.82** | 1.00 | | |
| $IP_{14}$ – 80 breaths | -0.27* | -0.30* | -0.24 | -0.27* | 0.60** | 0.74** | 0.81** | 1.00 | |
| $IP_{14}$ – 100 breaths | -0.14 | -0.11 | -0.15 | -0.06 | 0.66** | 0.73** | 0.83** | 0.83** | 1.00 |

*Test-retest reliability:*

$IP_{14}$ after 20, 40, 60, 80 and 100 breaths through the Powerbreathe device was found to be highly reproducible across the 63 participants. Test and retest reliability was assessed using a two-way mixed model intra-class correlation coefficient which was found to have an absolute agreement with single measure of 0.754 with a 95% confidence interval from 0.671 – 0.816. The average measure of the intraclass correlation coefficient was 0.967 with a 95% confidence interval from 0.953 – 0.978. Figure 6-e shows a Bland-Altman analysis on the pooling of the 63 paired test and retest measurements of mean across all trials in which $IP_{14}$ was performed. The mean difference (bias) of the measurements between test and retest at $IP_{14}$ was 2.91. The SD of the difference was 13.78 and width of the 95% limits of agreements was 54.03. A small mean difference and apparent random distribution of points around the mean is observed on the Bland-Altman analysis deeming the methodology to be unbiased. Lastly the CV (standard deviation/mean x 100) for each $IP_{14}$ test measurement is shown in Table 6-d along with PiMax.

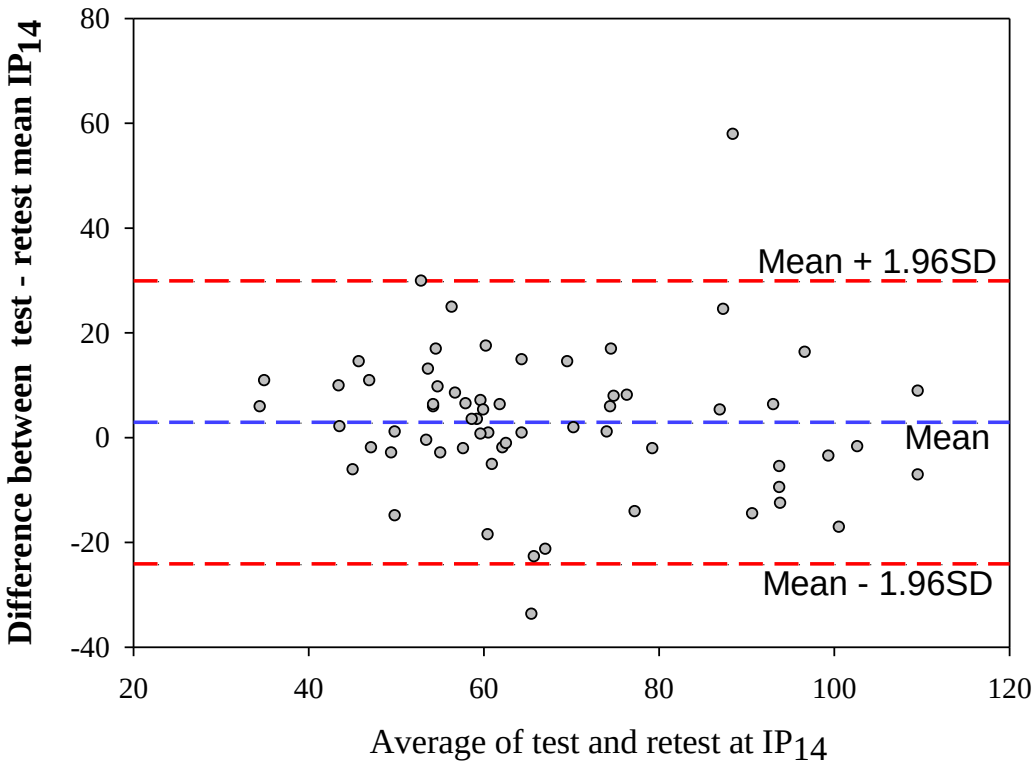


**Figure 6-e.** Bland and Altman plot showing the difference between the mean test and retest $IP_{14}$. Central blue line represents mean $IP_{14}$, red lines represent 95% limits of agreements [-24.10, 29.93].

**Table 6-d**. The CV of each measurement of $IP_{14}$ with each twenty breath set.

| Measurement (test-retest) | CV |
|---|---|
| PiMax – PiMax | 0.08 ± 0.74 |
| $20IP_{14}$ – $20IP_{14}$ | 0.14 ± 0.12 |
| $40IP_{14}$ – $40IP_{14}$ | 0.13 ± 0.13 |
| $60IP_{14}$ – $60IP_{14}$ | 0.13 ± 0.11 |
| $80IP_{14}$ – $80IP_{14}$ | 0.15 ± 0.12 |
| $100IP_{14}$ – $100IP_{14}$ | 0.16 ± 0.11 |

*Study two: Effects of OSA on $IP_{14}$:*

A mixed-model ANOVA revealed no significant differences in the patients with OSA $IP_{14}$ or ventilatory response slope. This was the case when mild, moderate and severe patients with OSA were compared independently, or grouped together as mild and moderate vs severe or severe and moderate vs. mild OSApatient groups. Additionally, due to the findings of the first study, multi-model ANCOVA's were performed using BMI as a covariate still though no significant differences were found in $IP_{14}$ between the OSA patient groups.

*Assessing correlations:*

As with the first study, Spearman's rho was used to assess for correlations as data was not normally distributed (Field 2009). A significant negative correlation was found between AHI and $IP_{14}$ after 80 breaths through the Powerbreathe device ($r_s$ = -0.461; $p$ <0.05). Additionally BMI ($r_s$ =-0.564; $p$ <0.01) and neck circumference ($r_s$ = -0.496; $p$ <0.05) were negatively correlated with PiMax. As found in other studies of this thesis BMI ($r_s$ = 0.423; $p$ <0.05) and neck circumference ($r_s$ = 0.493; $p$ < 0.05) was positively correlated with AHI. No correlations however were found between any of the inspiratory pressures with the ventilatory response data of the previous study (Chapter 3).

Due to the significant difference between $IP_{14}$ of normal BMI and overweight BMI group in the previous study and the significant correlation between BMI and AHI, partial correlations were also assessed controlling for BMI. The outcome variable AHI was not normally distributed (Kolmogorov-Smirnov (*D*) AHI (*D* (24), Shapiro-wilk (*W*) test $p$<0.05; *W* (24) $p$<0.05) and therefore was transformed using square root transformation. Using partial correlation analysis controlling for BMI, square transformed AHI was negatively correlated with $IP_{14}$ after 80 breaths through the Powerbreathe device ($r$ = -0.451; $p$<0.05) and almost significantly negatively correlated with $IP_{14}$ after 40 breathes through the Powerbreathe device ($r$ = -0.364; $p$ = 0.08). No other significant correlations were found.

*Comparing the patients with OSA to the overweight individuals (BMI≥25) from first study:*

When comparing patients with OSA (n = 24) as one group to the overweight group (BMI≥25; $n$ = 26) of experiment two, a multi-model ANOVA revealed no significant effect for the completed Powerbreathe breaths and no significant interaction between number of completed

breaths through Powerbreathe device and group. However, a significant difference between groups was found ($p$ = 0.029). A follow up one-way ANOVA revealed the patients with OSA have a significantly higher $IP_{14}$ after 40 ($p$ <0.05), 60 ($p$ < 0.01) and 80 breaths ($p$ < 0.05) through the Powerbreathe device using Brown-Forsythe test for 40 and 60 breaths as homogeneity of variance proved significant (Field 2009). These results are displayed in Figure 6-f. ANCOVA controlling for BMI between group was not performed as the assumption of homogeneity of regression slopes was not met ($p$ <0.05) (Field 2009).

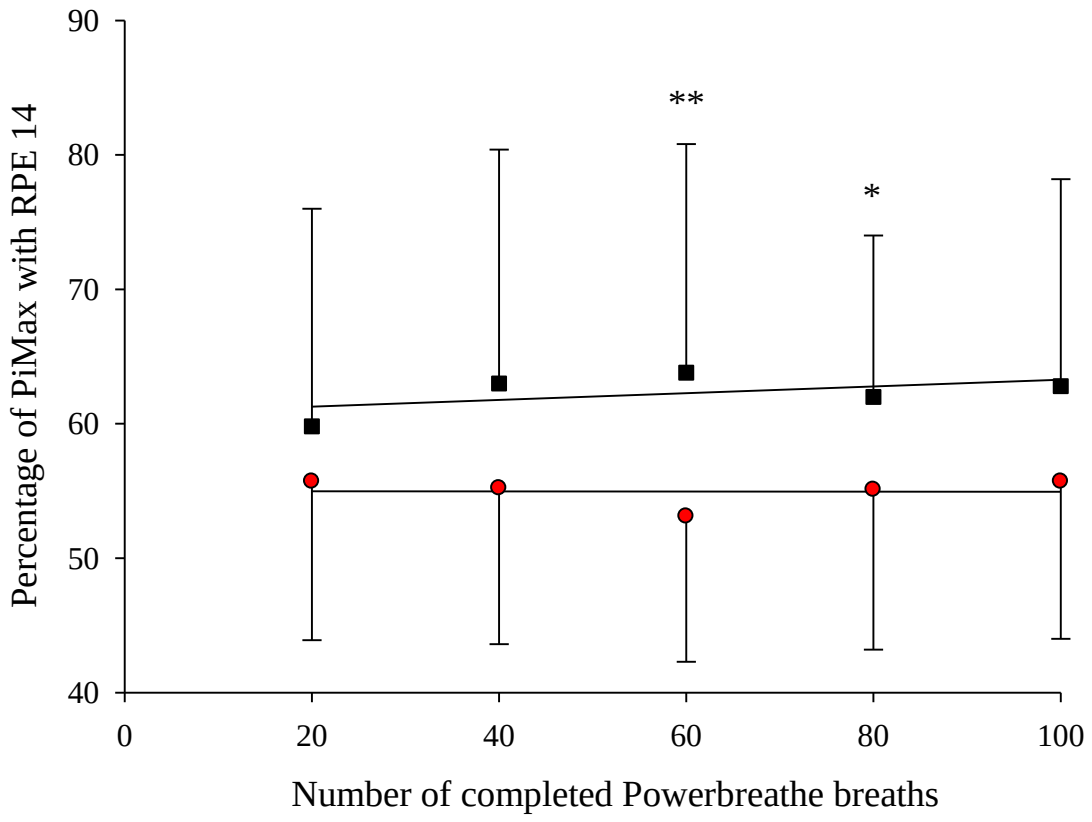


**Figure 6-f.** Comparing $IP_{14}$ between overweight participants group and patients with OSA. Where ■ = patients with OSA and ● = Overweight non-OSA group. Values represent mean ± SD, * = $p$ <0.05, ** = $p$ <0.01.

A mixed model ANOVA comparing the overweight no OSA group (n = 26) to the mild (n=8), moderate (n= 5) and severe (n=11) OSA group separately, also found no significant main effect for Powerbreathe breaths or interaction between group and breaths. Furthermore no significant main effect was found for group ($p$ = 0.06).

**Discussion:**

One of the main purposes of this current study was to develop a novel, easy to perform protocol which could be used to test the fatigability of the inspiratory muscles non-invasively with minimal cost. The first study confirms that the protocol has an acceptable degree of repeatability and BMI has a significant effect on the results. The second study then applies the protocol to a population of patients with OSA with varying degrees of severity. It provides some support for its use, with potential evidence for fatigue amongst the patients with OSA being demonstrated by an increase in AHI being associated with an alteration in $IP_{14}$ after the inspiratory resistive breathing through the Powerbreathe device. More specifically after 80 breaths through the Powerbreathe device a negative correlation with AHI was observed ($r_s$ = -0.461; $p$ <0.05) and this was still the case when the effects of BMI were taken into account ($r$ = -0.451; $p$<0.05). Furthermore neck circumference and BMI were found to be negatively associated with PiMax. It is well understood that increased neck circumference and BMI are predictive of OSA (Carter & Watenpaugh 2008).

*Development of a protocol with the healthy participants:*

The identified test-retest intraclass correlation coefficient obtained with the healthy participants in the first experiment yielded a value of 0.97 using average measures and 0.75 with the singular measures. These results are comparable to Dimitriadis et al. (2011) findings which assessed the test-retest reliability of PiMax using the MicroRPM the same equipment which was used in our current study for the measures of both PiMax and $IP_{14}$. Dimitriadis et al. (2011) reported an intraclass correlation coefficient of 0.78. An intraclass correlation coefficient value greater than 0.6 is considered an acceptable measure of reliability and values greater than 0.8 are considered high reliability (Dimitriadis et al. 2011). Although invasive techniques, such as esophageal and gastric balloons for recording esophageal, gastric and transdiaphragmatic pressure are considered to be more reliable, they require difficult, long and unpleasant procedures (Dimitriadis et al. 2011, Syabbalo 1998).

We were unable to find a significant difference in fatigue amongst the low, moderate and high activity groups categorised by the IPAQ. It is possible this may be because a large proportion of the study population consisted of University students. Many students struggled to relate to the questions of the IPAQ. For example, some of the questions regarding paid work, travelling in a motor vehicle, gardening and yard work were seen as irrelevant to many

of the students recruited. Using BMI however to differentiate participants into groups did reveal significant findings. It could be that the participants' BMI in our study was a better indication of the participants' level of physical activity. The overweight group (BMI≥25 kg/m$^2$) had a significantly lower $IP_{14}$ after 20, 40 and 60 breaths ($p$<0.05) and BMI was significantly negatively correlated with $IP_{14}$ after 20, 40 and 80 breaths through the Powerbreathe device. These findings suggest an alteration in perception of effort with increased body mass.

It has been previously suggested that obese children may perceive various intensities of physical exertion differently from their normal weight counterparts (Belanger et al. 2013). Marinov et al. (2002) compared obese to non-obese children performing a standardised workload, both groups had similar ventilatory efficiency but an increased awareness of fatigue that further limits physical capacity was present in the obese group. It is therefore possible that the overweight individuals' unnecessary limit their activity based on perceptions of exertion and therefore in this study overestimated the amount of effort they apply during the protocol. Moreover, the correlation matrix is particularly important as the significant negative correlation ($p$< 0.05) found when all the participants are considered between BMI and $IP_{14}$ provide further support for using BMI as a covariate in the second experiment with the patients with OSA.

*Effects of OSA on inspiratory muscle fatigue:*

The patients with OSA demonstrated a negative correlation between AHI and $IP_{14}$ after 80 breaths through the Powerbreathe device ($r_s$ = -0.461; $p$ <0.05). This was present even after BMI was taken into account using a partial correlation ($r$ = -0.451; $p$<0.05). This finding may provide some evidence for inspiratory muscle fatigue, although no significant differences were found between OSA patient groups in terms of $IP_{14}$ values and slope. The negative correlations found between PiMax with BMI ($r_s$ =-0.564; $p$<0.01) and neck circumference ($r_s$ = -0.496; $p$ <0.05) may potentially mean that some of the presence of fatigue is concealed as the $IP_{14}$ measures represent percentage of PiMax. There was however no significant difference found in PiMax between groups.

The patients with OSA were compared to the overweight group of the first experiment. Unfortunately, due to a lack of resources we were unable to perform respiratory polygraphy to rule out the presence of OSA in the overweight group, however all participants were asked

if they were aware of having sleep apnoea and all stated no. The OSA group had a significantly higher $IP_{14}$ at 40 ($p<0.05$), 60 ($p<0.01$) and 80 breaths ($p<0.05$) through the Powerbreathe device. These results may be seen in conflict with the findings of the first experiment where the overweight BMI group had a lower $IP_{14}$ compared to the normal BMI group. These findings however may be related to the difficulties which arise in assessing inspiratory muscle fatigue. The increased $IP_{14}$ in the patients with OSA may be related to greater recruitment of the accessory muscles (De Troyer et al. 1994). These muscles are likely to include the extradiaphragmatic rib cage muscles as observed in Guleria et al. (2002) study. These may become activated as a result of an alteration in the sense of effort and the motor command in order to maintain the same $IP_{14}$ during the presence of inspiratory muscle dysfunction or fatigue (Carson, Riek & Shahbazpour 2002). The onset of fatigue is often accompanied by an increased perception of effort (Enoka & Stuart 1992). The higher $IP_{14}$ may be because the patients with OSA perceive a RPE value of 14 (between somewhat hard and heavy) as higher effort potentially related to a dysfunction in the inspiratory muscles. The absence though of a reduction in $IP_{14}$ over the breathing sets with the Powerbreathe device in both groups does raise the possibility of either the inspiratory muscles being fatigued prior to the first 20 breathes through the Powerbreathe device or a fatigue not being demonstrated in the test.

The difficulty in clearly identifying inspiratory muscle fatigue within the patients with OSA is not surprising. Inducing inspiratory muscle fatigue in another clinical population; namely COPD is particularly reported as difficult (MacIntyre 2006). As a result of COPD, adaptations have been shown to occur such as the diaphragmatic sarcomeres becoming shorter and more oxidative or type I sarcomeres develop to increase endurance capabilities. Additionally, the density of the capillaries is also known to increase leading to the respiratory muscles "stealing" blood flow (MacIntyre 2006). To our knowledge, no studies have investigated whether such adaptations to the diaphragm may occur as result of the obstructions which occur during sleep in OSA. However, adaptations to the genioglossus muscle (upper airway dilatory muscle) have been reported (BuSha, Strobel & England 2002).

The most important finding in our study is the patients with OSA have a significant negative correlation between AHI and $IP_{14}$ after 80 resistive breaths through the Powerbreathe device ($r_s$ = -0.461; $p$ <0.05) and this is still the case when the effects of BMI were taken into account via partial correlation ($r$ = -0.451; $p$ <0.05). The finding of BMI affecting the results

in the first experiment supports the use of BMI as a covariant. No correlations were found between the ventilatory response to the gas mixtures of the previous study and $IP_{14}$. It is worth noting however only 19 of the 24 participants who completed the inspiratory muscle fatigue protocol also took part in the breathing of the gas mixtures. Nevertheless, these findings do suggest that the development of OSA may be related to two entirely separate mechanisms with some individuals more prone to OSA as a result of inspiratory muscle fatigue whilst others develop OSA as a consequence of a change in their ventilatory response to hypercapnia as observed with the hyperoxic hypercapnic gas mixture investigated in chapter 3. The findings of significant correlations between AHI and $IP_{14}$ after 80 breaths through Powerbreathe device supports $IP_{14}$ after 80 breaths being included in our regression model used to predict the severity of OSA in addition to the ventilatory response to $CO_2$. This final regression model will be discussed in the next chapter which concludes this thesis.

Study limitations:

Inducing inspiratory muscle fatigue is difficult, presently there is no easy to perform non-invasive technique available. In our study it has proven difficult to obtain results which clearly demonstrate fatigue as the change in performance is limited and so some degree of theoretical interpretation is required to explain our findings. No significant differences were identified between patient groups with mild, moderate or severe OSA. This finding may reflect the relatively small sample size present in each group (mild = 9, moderate = 5, severe= 11) in comparison to the overweight BMI ($\geq$25 kg/m$^2$) vs. normal BMI groups (<25 kg/m$^2$) (normal BMI =37, overweight =26) investigated in this chapter.

*Further study:*

Our study allowed the development of a cost-effective protocol which is non-invasive, repeatable, relatively easy to administer and allows the indirect assessment of fatigability of the inspiratory muscles amongst a healthy and a clinical population of patients with OSA. Measuring mouth pressure is relatively non-invasive and provides the sum pressure of the inspiratory muscles involved. The disadvantage is that specific muscles cannot be assessed independently. Recruitment of accessory muscles was minimised in our study by enforcing a leaning posture (Segizbaeva, Pogodin & Aleksandrova 2013), encouraging participants to concentrate on their breathing muscles and performing submaximal manoeuvres by using $IP_{14}$ as opposed to PiMax. Future studies assessing the prevalence of muscle fatigue in the

pathogenesis of OSA may benefit by focussing attention on specific muscles such as the major upper airway dilator muscle, the genioglossus.

### *Conclusion*

This present study successfully designed and evaluated a novel repeatable non-invasive protocol to induce inspiratory muscle fatigue using submaximal measures ($IP_{14}$) in healthy participants (normal BMI and overweight BMI). The use of the protocol with patients with OSA though was less clear with the possibility of fatigue occuring prior to the completion of the first set of Powerbreathe breaths BMI was significantly negatively associated with $IP_{14}$ in the healthy participants and the overweight participants had a significantly lower $IP_{14}$ during the protocol. In the patients with OSA, fatigue was identified by the $IP_{14}$ being significantly higher than the overweight group which was attributed to recruitment of additional accessory muscles. Furthermore, a negative correlation was found between AHI and $IP_{14}$ after 80 resistive breaths with and without BMI taken into account. No correlation was found between the data of the ventilatory response to $CO_2$ study therefore the results support the development of OSA may be related to two entirely separate mechanisms with some individuals more prone to OSA as a result of inspiratory muscle fatigue, whilst others develop OSA as a consequence of a change in their ventilatory response to hypercapnia.

## Chapter 7 General Discussion:

The objective of this thesis was to identify which physiological factors contribute most strongly to OSA, as assessed by the apnoea-hypopnoea index (AHI). This chapter discusses and integrates all the main findings presented in this thesis. The thesis first investigated the potential effects of the exposure to intermittent hypercapnia and hypoxia during sleep using a novel technique to assess the contribution of the central and/or peripheral chemoreceptors on the ventilatory response to $CO_2$ during wakefulness. This methodology was first developed with scuba divers and non-diving controls before being implemented with the patients with OSA. Following this, the implications of the baroreflex-chemoreflex interactions were assessed before investigating inflammatory blood markers present within the patients with OSA. Finally, with the understanding that the occlusive airway and subsequent apnoea associated with OSA may lead to increased inspiratory efforts whereby the inspiratory muscles are overloaded during sleep, the prevalence of inspiratory muscle fatigue in the patients with OSA was assessed. The main findings of these research studies and their potential limitations will now be discussed. After this a novel regression model will be put forward which is designed to allow the prediction of the severity of OSA based on the parameters investigated in this thesis and then finally based on this prediction model, the supported direction of potential future investigations will be considered.

*Investigation of the ventilatory response to $CO_2$ during wakefulness:*

The first aim was to assess whether the concomitant exposure to hypoxic and hypercapnic episodes related to the cessation of ventilation (Carter & Watenpaugh 2008, Punjabi 2008) in patients with OSA altered the ventilatory response to $CO_2$ during wakefulness. Previous studies investigating the ventilatory response in patients with OSA with hypercapnia and/or hypoxia have been conflicting with some studies reporting an increase, decrease or no change in the ventilatory response (Sin, Jones & Man 2000, Radwan et al. 2000). As discussed previously, some of these results could be attributed to different inclusion criteria and methodology implemented. Furthermore, the interaction between the peripheral and central chemoreceptors had often been overlooked. Therefore there was a requirement for a methodology which would allow us to isolate the response of the central and peripheral chemoreceptors to measure the contribution of each component in the measured ventilatory response to $CO_2$.

Incorporating the recommendations of Duffin (2007), we devised an experimental system which allowed us to assess the ventilatory response with every breath using a metabolic cart. We measured the ventilatory response to 25% $O_2$ with 6% $CO_2$ to measure the contribution of the central chemoreceptors and we measured the ventilatory response to 13% $O_2$ with 6 % $CO_2$ to measure the sum of the peripheral and central chemoreceptors combined. The difference between the two responses was then regarded as the contribution of the peripheral chemoreceptors. Furthermore, we also assessed the ventilatory response to hypoxia only, using 13% $O_2$.

First we assessed the experimental system with a healthy population known to have a reduced ventilatory response to $CO_2$. Experienced scuba divers were chosen as previously in the laboratory we had demonstrated a reduced ventilatory response to $CO_2$ during $CO_2$ rebreathing using 100% oxygen. Furthermore, no other research with scuba divers has investigated whether these previous findings can be attributed to an alteration involving the peripheral chemoreceptors. The findings of the previous $CO_2$ rebreathing study, led to the support of 6% $CO_2$ being used to identify a $CO_2$ retainer from non-retainer.

In this current study it was found that the experienced scuba divers had a lower ventilatory response with both the hypercapnic conditions compared to the non-diving matched controls. No significant difference was found between groups in terms of change in minute ventilation with the hypoxic hypercapnic condition from the hyperoxic hypercapnic condition. These findings suggest that any adaptation predominately involves the central chemoreceptors. Additionally, no associations were found between the ventilatory response to $CO_2$ and the number of dives performed possibly indicating that the changes in $CO_2$ sensitivity are achieved in a comparably short time or that sensitivity is inherited, with individuals who are sensitive to $CO_2$ leaving the diving population.

Assessing the ventilatory response amongst the patients with OSA using the same gas mixtures found that patients with severe OSA had a significantly lower ventilatory response to $CO_2$ in the presence of hyperoxia and hypoxia compared to the patients with mild/moderate OSA. As with the scuba diving study, no significant difference was found between groups in the change in the ventilatory response with the addition of 13% $O_2$ with the 6% $CO_2$ compared to the 25% $O_2$ with 6% $CO_2$ suggesting the altered ventilatory response involves predominately the central chemoreceptors. Furthermore, a negative correlation was found between AHI and the ventilatory response to the hyperoxic

hypercapnic gas mixture ($r_s$ = -0.51, $p$ <0.01) and this was present even when the effects of BMI were taken into account ($r$ = -0.36, $p$ <0.05). It was however also found the correlation between AHI and the ventilatory response was diminished with the addition of hypoxia with the concentration of 6% $CO_2$. This may provide some evidence for a role of the peripheral chemoreceptors perhaps compensating for a change in the central chemoreceptor response. The results with patients with OSA were compared to those found with the scuba divers and non-diving matched controls. The non-OSA patients had their minute ventilation normalised by their body surface area estimated using the Dubois and Dubois equation (Dubois & Dubois 1989) to allow comparison of ventilatory response to the patients with OSA. The scuba divers ventilatory response was similar to that observed in the patients with mild/moderate OSA and the non-diving controls were the most responsive. These results may give an estimation of the ventilatory response we would expect if non-OSA patients were recruited.

There are some limitations to these findings; firstly our study only tested the ventilatory response using 13% $O_2$ to achieve moderate hypoxia and a mean end-tidal $pO_2$ of 56.5 ± 3.99 mm Hg (Goodall, Ross & Romer 2010). In order to rule out any adaptation acting upon the peripheral chemoreceptors, a series of different hypoxic isoxic $CO_2$ responses at different $pO_2$ tensions should be measured to fully characterise the peripheral chemoreflex (Duffin 2007). Secondly, there is now some evidence which does observe that when carotid body denervation is performed the hypoxic ventilatory response is eliminated (as expected) but the central hyperoxic $CO_2$ response is also markedly depressed (Dahan, Nieuwenhuijs & Teppema 2007, Rodman et al. 2001, Dempsey & Smith 2014). This therefore suggests that although the role of the peripheral chemoreceptors is minimised in hyperoxia, it is possible it is not entirely diminished. Finally, other comorbidities associated with OSA such as metabolic syndrome and cardiovascular conditions have been previously shown to also alter ventilatory control (Trombetta et al. 2013).

*Investigation of the baroreflex-chemoreflex interaction in patients with OSA:*

There is little doubt that considerable interaction exists between the chemoreceptors and the baroreceptor reflexes (Cooper et al. 2005, Somers, Mark & Abboud 1991). We therefore hypothesised that individuals may display an altered baroreflex sensitivity associated with their previous ventilatory response findings. The baroreflex sensitivity of patients with OSA was investigated during wakefulness using the spontaneous method. Our findings suggest that

AHI is more influenced by body mass and an alteration in the ventilatory response to $CO_2$ than an alteration in baroreflex sensitivity.

Initially no significant association was found between baroreflex sensitivity and all other measured variables such as AHI, body mass and the ventilatory response data. Further analysis of the data revealed a substantial variation in the frequency of baroreflex measurements across individuals (range: 8-185 measures). After stratification based on the median number of measurements (100 measurements) the low number of measurements group were found to have a significantly higher AHI (AHI 43.11 ± 25.49, $p$ <0.05) causing us to question the usefulness of the spontaneous approach during wakefulness when investigating patients with severe OSA. Potentially this subsequent exclusion of so many patients though may have skewed the analysis and caution is therefore required in the interpretation of the results. Furthermore the sample size used in our study is relatively small compared to other existing studies investigating BRS and our study does not include a control group for comparison. Unfortunately, we were limited by available clinical space to perform this study. The effect of noise surrounding the patient was minimised through performing the study in a quieter part of the hospital. Furthermore, some patients with severe OSA did struggle to not fall asleep during the test and so unfortunately had to be lightly stimulated on the shoulder by the researcher in order to maintain wakefulness. A further limitation is the influence of respiration on heart rate however it is believed this influence is relatively small and not of major significance (Ryan et al. 2007). Finally in this study, most patients were not normotensive and were subsequently on medications. Although participants were placed in a corresponding hypertensive group in an attempt to control for this using a partial correlation and ANCOVA as appropriate, the wide range of other conditions which are known as potential comorbidities of OSA such as diabetes (Frattola et al. 1997) and other cardiovascular disorders (Katsube et al. 1996, Mortara et al. 1997) have also been shown to influence baroreflex sensitivity (Ryan et al. 2007).

*The presence of inflammation in patients with OSA:*

In this study we assessed blood plasma markers which represent the occurrence of low level inflammation including C-reactive protein (CRP) and certain adipokines consisting of adiponectin and leptin which have been suggested to play a role in the pathogenesis of OSA. We also assessed blood plasma concentrations of two endocannabinoids believed to have

mainly anti-inflammatory properties: 2-arachidonoylglycerol (2-AG) and arachidonoylethanolamide (AEA).

It was found that patients with mild OSA had a significantly ($p$<0.05) lower plasma AEA concentration (n = 8, 0.77 ± 0.21 nM) compared to the patients with moderate and severe OSA combined (n = 42, 1.06 ± 0.32 nM). This provides some potential evidence to imply a higher degree of inflammation amongst the patients with moderate/severe OSA. Unfortunately the results are confounded by the group sizes, The reason for the higher recruitment of moderate/severe patients with OSA is just a reflection of the majority of the patients coming into Ysbyty Gwynedd's sleep clinic. With the experience gained in this study, we would by audit or database generation ensure that future studies are both adequately powered and that there is sufficient opportunity to recruit a satisfactory range of patients.

Intermittent hypoxia designed to simulate the occurrence of apnoeic events have been shown to lead to a selective and preferential activation of inflammatory pathways (Ryan, Taylor & McNicholas 2005). In our study positive correlations were found between the ventilatory response to hypoxia with CRP ($r_s$ = 0.61 $p$ <0.01) and 2-AG ($r_s$ = 0.50 $p$ <0.01). No significant correlations were found between any of the investigated blood markers and AHI. This may in part be due to our relatively lenient inclusion criteria recruiting patients with a BMI up to 50 kg/m$^2$ and with the presence of other comorbidities associated with OSA. Similar to our study, Makino et al. (2006) applied similar inclusion criteria recruiting individuals who have other various systemic and metabolic diseases which are known comorbidities of OSA and found no correlation with adiponectin and severity of OSA. Vatansever et al. (2011) on the other hand applied relatively strict inclusion criteria such as having no prescribed medications and no history of cardiovascular disease, finding adiponectin was negatively correlated with AHI. Furthermore, studies have also supported CRP (Akashiba et al. 2005, Ryan et al. 2007) and leptin (Barceló et al. 2005, Schäfer et al. 2002, Patel et al. 2004) are more associated with obesity than OSA. In our study, significant correlations were found between AHI, body characteristics, CRP, leptin and the two endocannabinoids.

*Prevalence of inspiratory muscle fatigue:*

In order to investigate the occurrence of inspiratory muscle fatigue amongst patients with OSA, firstly a novel relatively easy to perform protocol was developed with a healthy population who had not been previously diagnosed with OSA. The protocol was found to be reproducible with an intraclass correlation coefficient of 0.97 using average measures and 0.75 with singular measures. In brief, the protocol consisted of inducing inspiratory muscle fatigue through 5 sets of 20 breaths through an inspiratory resistive breathing device with a resistance equivalent to 50% of the participant's maximal inspiratory pressure (PiMax). After each breathing set participants inspired through an inspiratory pressure meter at an effort equivalent to 14 on the Borg's rating of perceived exertion (RPE) scale. This measurement was termed $IP_{14}$.

With the healthy participants it was found BMI had a significant effect on $IP_{14}$ suggestive of fatigue. The overweight BMI group (BMI≥25 kg/m$^2$) had a significantly lower $IP_{14}$ after 20, 40 and 60 resistive breaths through the Powerbreathe device ($p$<0.05). These results support BMI being used as a covariate with the patients with OSA. PiMax was not significantly different between the patients with OSA and the controls. No significant difference in $IP_{14}$ was found between OSA patient groups with and without BMI used as a covariate.

A significant negative correlation was found between AHI and $IP_{14}$ after 80 resistive breaths using the Powerbreathe device ($r_s$ = -0.46; $p$ <0.05). Furthermore, BMI ($r_s$ =-0.56; $p$<0.01) and neck circumference ($r_s$ = -0.50; $p$ <0.05) were found to be negatively correlated with PiMax. Using a partial correlation controlling for BMI, square transformed AHI was negatively correlated with $IP_{14}$ after 80 breaths through the Powerbreathe device ($r$ = -0.45; $p$<0.05). These findings may provide some evidence for inspiratory muscle fatigue. However no significant differences were found between OSA patient groups in terms of $IP_{14}$ values and slope. Furthermore although it is possible that fatigue may have occurred prior to the completion of the first set of Powerbreathe breaths there is the possibility fatigue did not occur in the patients in the absence of no observed reduction in $IP_{14}$.

When comparing patients with OSA (n = 24) as one group to the overweight group (BMI≥25; $n$ = 26), OSA patients were found to have a significantly higher $IP_{14}$ after 40 ($p$ <0.05), 60 ($p$ < 0.01) and 80 breaths ($p$ < 0.05) through the Powerbreathe device. The

increased $IP_{14}$ in the patients with OSA may be related to greater recruitment of the accessory muscles (De Troyer et al. 1994) activated as a result of an alteration in the sense of effort and the motor command in order to maintain the same $IP_{14}$ during the presence of inspiratory muscle dysfunction or fatigue (Carson, Riek & Shahbazpour 2002).

There are various limitations in our approach; measuring mouth pressure provides the sum pressure of the inspiratory muscles involved. The disadvantage is specific muscles cannot be assessed independently. Recruitment of accessory muscles was minimised in our study by enforcing a leaning posture (Segizbaeva, Pogodin & Aleksandrova 2013), encouraging participants to concentrate on their breathing muscles and performing submaximal manoeuvres by using $IP_{14}$ as opposed to PiMax. Additionally, due to a lack of resources we were unable to perform respiratory polygraphy to rule out the presence of OSA in the overweight group. This aside, we have developed a relatively non-invasive method of assessing inspiratory muscle fatigue which is reproducible and relatively easy to perform.

*The final regression model:*

In order to create a multiple regression model designed to predict the severity of OSA (AHI) using three predictors comprising of the most significant variables which correlate with AHI (Neck: $r_s = 0.53$; $p<0.01$; $IP_{14}$ after 80 breaths through the Powerbreathe device: $r_s = -0.46$; $p <0.05$ and ventilatory response to 25% $O_2$ with 6% $CO_2$: $r_s = -0.51$; $p <0.01$) identified in this thesis, multiple imputation was performed. Multiple imputation aims to allow for the uncertainty about missing data by creating several different plausible imputed data sets and appropriately combining results obtained from each of them (Sterne 2009).This was performed to allow 37 cases to be used in the regression model as opposed to just 19. Both variables requiring data imputation (ventilatory response to $CO_2$ (n = 32) and $IP_{14}$ after 80 breaths (n = 24)) were normally distributed. If data are non-normally distributed then this can introduce bias in the imputations (Sterne 2009).

In agreement with Sterne (2009) recommendations of reporting multiple imputation. Data imputation was performed because of data sets were incomplete for the development of a regression model with three predictors. This was due to lower recruitment in the ventilatory response to $CO_2$ study and the inspiratory muscle fatigue study. Data imputation was therefore needed to allow equal number of cases to the neck variable (n = 37) to be compared with multiple regression. There are no known important differences between the individuals

who completed each study to bias the use of multiple imputations. For the imputation modelling Mersenne twister random number generator was used with SPSS version 20 and 5 imputation datasets were created (Sterne 2009). Unless replicating results from SPSS version 12 or earlier, the Mersenne twister algorithm is considered more reliable (Garson 2012). Data constraints were placed on $IP_{14}$ after 80 breaths through the Powerbreathe device based on original minimum and maximum data values obtained.

The multiple regression model meets the recommendations of requiring at least 10 subjects per predictor (Field 2009). Multicollinearity was not a concern and the residuals of the model met the assumption of normality and homoscedasticity. The results of the multiple regression using the pooled imputation data (n = 37) is shown in Table 7-a. Log transformed AHI and neck circumference was used as both AHI and neck circumference was not considered normally distributed. The prediction model was statistically significant, $F$ (3, 33) = 8.319, $p$<0.01 and accounted for approximately 43% of the variance of AHI ($R^2$= 0.43). AHI was primarily predicted by neck circumference (β = 0.42) and to a lesser extent by ventilatory response to $CO_2$ (β = -0.30) and $IP_{14}$ after 80 resistive breaths through the Powerbreathe device (β= -0.23).

**Table 7-a.** Multiple regression to predict log transformed AHI using the predictors: Neck circumference, ventilatory response to 25%$O_2$ with 6% $CO_2$ and $IP_{14}$ after 80 breaths through the Powerbreathe device.

| | | | | Correlations | | |
|---|---|---|---|---|---|---|
| | B | Standard Error (SE) B | β | Zero-order | Partial | Part |
| Constant | -4.92 | 2.58 | | | | |
| $IP_{14}$ after 80 breaths | -0.01 | 0.01 | -0.23 | -0.26 | -0.29 | -0.22 |
| logNeck | 4.27 | 1.50 | 0.42** | 0.56 | 0.46 | 0.39 |
| 25%$O_2$/6% $CO_2$ ΔVE (l/min/BSA) | -0.06 | 0.03 | -0.30* | -0.45 | -0.34 | -0.28 |

$R^2$ = 0.43, (p<0.01), * = $p$<0.05; ** = $p$<0.01.

*Implications of the model on future studies:*

The results of the multiple regression analysis can be viewed as further support for future interventions which are specifically designed to reduce neck circumference through weight loss. Based on the Starling model presented in the literature review, reducing neck circumference would mean less inspiratory effort will be required to maintain airway patency. This would reduce the risk of periodical overload of the inspiratory muscles which has been hypothesised to lead to potential fatigue (Chien et al. 2013, Wilcox et al. 1990).This presence of fatigue has been associated with acute hypercapnia due to the combination of the increased mechanical load of the lung, reduced muscle strength, decreased efficiency, and reduced energy supplies to the inspiratory muscles (Roussos & Zakynthinos 1996). It is this exposure to intermittent hypercapnia which we suggest to play a key role in causing the adaptation in the ventilatory response to $CO_2$ based on our reported findings in healthy populations of experienced scuba divers .

Neck circumference is a particular concern during the night because of the role of nocturnal rostral fluid shift. During the day, fluid accumulates in the intravascular and interstitial spaces of the legs due to gravity, upon lying down at night the fluid redistributes rostrally (upwards) and potentially accumulates in the neck causing the upper airway to narrow further (White & Bradley 2013). Interventions which have been shown to reduce nocturnal fluid shift include wearing compression stockings in the daytime (Redolfi et al. 2011) and intensive diuretic therapy with patients with diastolic heart failure (Bucca et al. 2007). Recently, Tremea et al., (2014) has published plans for a randomised controlled trial which aims to test the use of diuretics to treat both hypertension and OSA through reducing the extravascular fluid shift from the legs in sleep (Cichelero et al. 2014, Esler et al. 2010). CPAP was originally thought to solely act as a "pneumatic splint" of the upper airway (White & Bradley 2013, Sullivan et al. 1981). CPAP has also been shown using magnetic resonance imaging (MRI) to reduce fluid retention in the neck after 4-6 weeks of use measured during wakefulness (Ryan et al. 1991).

Weight loss is likely to play a key role in any future treatment intervention. With the high prevalence of obesity hypoventilation syndrome in patients with OSA (Mokhlesi, Kryger & Grunstein 2008, Powers 2008) and the significant correlations found in our study between all anthropometric measurements and ventilatory response to $CO_2$. In addition to the identified correlations between body characteristics and AHI found in every study of this thesis.

Furthermore, surgically induced weight loss has been shown to significantly improve pulmonary function. Weight loss surgery in patients with obesity hypoventilation syndrome has also demonstrated an improvement in diurnal hypoventilation, increasing their ventilatory response and therefore reducing the prevalence of hypercapnia (Dávila-Cervantes et al. 2004, Borel et al. 2012, Boone et al. 1996).

The findings of this thesis also provide evidence to support potential application of inspiratory muscle training. High intensity interval based supervised inspiratory muscle training has been shown in patients with moderate-to-severe COPD to achieve very high training loads, yielding large improvements in inspiratory muscle strength and endurance over a relatively brief total training period (Hill et al. 2006). Furthermore this thesis has devised a novel way of potentially assessing the effectiveness of such interventions on reducing the risk of inspiratory muscle fatigue. Further studies may validate such a protocol with other clinical populations where assessing inspiratory muscle fatigue has proved difficult particularly without invasive procedures (ATS/ERS 2002).

*Conclusions:*

This PhD thesis represents the first collaborative research between Ysbyty Gwynedd's Pulmonary Function Department, The School of Sport, Health and Exercise Sciences, Bangor University and Hannover Medical School, Germany. The findings of the studies act a stimulus for further investigation. By assessing the major physiological mechanisms behind the development of the severity of OSA, we have devised support for future potential interventions which will focus on reducing neck circumference, increasing ventilatory response to $CO_2$ and increasing inspiratory muscle endurance. We have also provided support for further validation and development of the entirely novel protocol of inducing inspiratory muscle fatigue amongst other clinical and healthy populations.

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