Micro- and Macro-vascular function in Obstructive Sleep Apnoea

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ABSTRACT

Obstructive sleep apnoea (OSA) is common worldwide. Cardiovascular diseases such as hypertension, heart failure and coronary artery diseases are highly prevalent in OSA population. Although the precise mechanisms for disease development in OSA are ambiguous, endothelial dysfunction (ED) is regarded as a crucial step. Indeed endothelial dysfunction has been demonstrated in peripheral vasculature in OSA population, however the effects of ventilatory dysfunction on coronary microcirculation and myocardial perfusion is unknown.

I performed comprehensive assessment of endothelial function in moderate to severe OSA subjects compared with healthy and hypertensive controls, using flow mediated dilatation (FMD), laser Doppler flowmetry (LDF), flow cytometry in addition to studying vascular elastic properties. In order to investigate coronary endothelial dysfunction, I studied myocardial perfusion in sub-endocardial region using myocardial contrast echocardiography (MCE). Additionally, as OSA is related with left ventricular diastolic dysfunction and high risk of atrial fibrillation, I also investigated cardiac remodeling using real time three-dimensional echocardiography (RT3DE). It is noteworthy that RT3DE and MCE have not been used in moderate to severe OSA subjects previously. Finally, the effects of continuous positive airway pressure (CPAP) therapy on these central and peripheral markers of macro- and micro-vascular dysfunction were also studied after 6 months.

I demonstrated endothelial dysfunction in the peripheral circulation, when compared with healthy subjects. Using ‘state of the art’ techniques, I showed endothelial dysfunction in the sub-endocardial region and cardiac remodeling in OSA compared with healthy subjects. However, contrary to available data, I did not note a significant change in the indices of arterial stiffness and in the flow cytometric analysis of endothelial progenitor cells and circulating endothelial cells

amongst the three groups. Most importantly, the peripheral and coronary endothelial dysfunction improved dramatically in OSA subjects, after they were treated with CPAP therapy for 6 months.

In summary, micro- and macro-vascular dysfunction exists in otherwise healthy moderate to severe OSA subjects compared with healthy subjects and can be improved with effective CPAP therapy. These findings may have strong clinical and prognostic implications, which ought to be tested in future studies.

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I dedicate this thesis to my beloved family.

DECLARATION OF WORK

I performed all the tests/techniques mentioned in this thesis and I am fully responsible for the collection and analysis of the data.

No portion of the work referred to in the thesis has been submitted in support of any application for another degree or qualification of this or any other university or other institute of learning.

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GLOSSARY

2DE	2-Dimensional Echocardiography
AA	Aortic Augmentation
ACE	Angiotensin Converting Enzyme
ACH	Acetylcystine
ADMA	Asymmetric Dimethyl Arginine
AHI	Apnoea-Hypopnoea Index
AIx	Aortic Augmentation Index
AIx@75	Aortic Augmentation Index at heart rate of 75/minute
ANOVA	One Way Analysis of Variance
ARB	Angiotensin Receptor Blockers
ASE	American Society of Echocardiography
ATP	Adenosine Triphosphate
BMI	Body Mass Index
BP	Blood Pressure
CAD	Coronary Artery Disease
CEC	Circulating Endothelial Cell
cGMP	Cyclic Guanosine Monophosphate
CFR	Coronary Flow Reserve
CPAP	Continuous Positive Airway Pressure
CVD	Cardiovascular diseases
DM	Diabetes Mellitus
ECG	Electrocardiogram
ED	Endothelial Dysfunction
EDTA	Ethylene Diamine Tetraacetic Acid

EDV	Endothelial Dependent Dilatation
EIV	Endothelial Independent Dilatation
EPC	Endothelial Progenitor Cell
FR	Blood Flow at Rest
FS	Blood Flow after Stress
FMD	Flow Mediated Dilatation
GTN	Glycerin Trinitrate
HDL	High density lipprotein
HTN	Hypertension
ICAM	Inter-cellular Adhesion Molecule
IDDM	Insulin Dependent Diabetes Mellitus
IP3	Inositol Triphosphate
IQR	Inter-quartile Range
IVSD	Inter-ventricular Septal diameter in Diastole
IVRT	Iso-volumetric Relaxation Time
LA	Left Atrium
LAVI	Left Atrial Volume Index
LDF	Laser Doppler Flowmetry
LDL	Low Density Lipoprotein
LV	Left Ventricle
LVEDV	Left Ventricular End Systolic Volume
LVEF	Left Ventricular Ejection Fraction
LVESV	Left Ventricular End Systolic Volume
MBFR	Myocardial Blood Flow Reserve
MCE	Myocardial Contrast Echocardiography
MRI	Magnetic Resonant Imaging

NIDDM	Non-insulin Dependent Diabetes Mellitus
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
OSA	Obstructive Sleep Apnoea
PET	Positron Emission Tomography
PU	Perfusion Unit
PWA	Pulse Wave Analysis
PWV	Pulse Wave Velocity
ROI	Region of Interest
ROS	Reactive Oxygen Species
RT3DE	Real Time 3-Dimensional Echocardiography
SD	Standard Deviation
SEVR	Sub-endocardial Viability Ratio
SNP	Sodium Nitroprusside
SPECT	Single Photon Emission Computed Tomography
SV	Stroke Volume
TDI	Tissue Doppler Imaging
TIA	Transient Ischemic Attack
VCAM	Vascular cell Adhesion Molecule
VEGF	Vascular endothelial growth factor
WHO	World Healthy Organization

Section 1 Introduction

1.1 Obstructive Sleep Apnoea and Cardiovascular Diseases

1.1.1 Introduction

Obstructive sleep apnea (OSA) is characterized by episodic partial/complete collapse of the upper airways alternating with normal breathing, leading to chronic intermittent hypoxia, oxygen desaturation, sleep fragmentation and cortical arousal. OSA is common worldwide and a higher than normal risk/prevalence of cardiovascular diseases has been observed in OSA population [Bradley et al 2009][Phillipson et al 1993]. Several landmark OSA studies observed epidemiological patterns of coronary artery disease, heart failure, cardiac arrhythmias and stroke in this population [Table 1-1]. OSA also strongly coexists with cardiac risk factors such as hypertension and impaired glucose tolerance. In fact, screening for hypertension has been recommended amongst newly diagnosed OSA patients [Chobanian et al 2003]. Hence unsurprisingly, OSA has been implicated in the initiation and progression of a myriad of cardiovascular disorders such as coronary artery disease (CAD), heart failure, systemic hypertension (HTN), pulmonary hypertension, cardiac arrhythmias and stroke [Table 1-1]. On this note, evidence from several cohort studies indicated that even mild form of OSA is independently associated with increased likelihood of cardiovascular morbidity.

Table 1-1: Epidemiological studies on the relationship between obstructive sleep apnea and cardiovascular disease

Reference	n	Dominant gender	Associated disease	Study design and findings
Shahar et al 2001	6424	Male	Stroke, Heart failure, Coronary artery disease	Large study, questionnaire based assessment, overnight polysomnography used to detect apnea hypopnea index, supported
Nieto et al 2000	6132	Female	Hypertension	Multi centre study, interview based assessment, manual blood pressure, demonstrated dose dependent rise in systolic and diastolic blood pressure with worsening sleep related breathing measures across all ages and ethnic groups
Peppard et al 2000	709	Male	Hypertension	Four year follow up study, Manual blood pressure monitoring, independent dose dependent response in hypertension was seen in association with sleep disordered breathing
Lavie et al 2000	2677	Male	Hypertension	OSA is independently and profoundly associated with hypertension

Hoffstein et al 1994	458	Male	Cardiac arrhythmias	Arrhythmia assessment by isolated ECG lead analysis, prospective study, prevalence of arrhythmia was comparable to nocturnal hypoxemia but not snoring. Rules out snoring as a cause of cardiac arrhythmias
Laaban et al 2002	169	Male	Heart failure	Left ventricular assessment by MUGA scan, prospective study of patients with polysomnographically diagnosed OSA, suggested impact of OSA on day time left ventricular systolic function
Peker et al 2000	62	Male	Coronary artery disease	Questionnaire based assessment, OSA and non-OSA patients were identified by full overnight sleep study, small study but statistically significant higher risk of CAD development in OSA group
Arzt et al 2005	1475	Male	Stroke	4 year follow up, apnea/hypopnea index of 20 or more was associated with increased risk of stroke (P=0.02)

OSA= obstructive sleep apnea; CAD= coronary artery disease; MUGA scan= multi gated acquisition scan; ECG= electrocardiogram

As screening tools, Epworth's score, Berlin's score and overnight oximetry are commonly used but neither of these is sensitive or specific to accurately diagnose OSA [Abrishami et al 2010] [Chervin et al 1999]. Multi-channel sleep study (MCSS) is regarded as a gold standard tool to diagnose and classify OSA subjects according to the disease severity. During a standard MCSS, heart rate, blood pressure, respiratory rate, oxygen saturations, airflow through the nasal apertures and chest wall movements are monitored. Based on the number of hypopnoeas and apnoeas per hour, Apnoea-Hypopnoea Index (AHI) is estimated. The OSA with the AHI of 5-15 is classed as mild, whilst 15-30 and more than 30 is regarded as moderate and severe respectively [Shamsuzzaman et al 2003]. Notably, the latter two groups are the primary target for treatment with continuous positive airway pressure (CPAP).

Convincing data from randomized controlled trials in OSA patients with hypertension or heart failure have demonstrated that the CPAP treatment improves clinical outlook and may improve OSA associated cardiac morbidity [Table 1-2,1-3]. Hence, significant links between cardiovascular morbidity and OSA and CPAP led benefits, warrant a better understanding of the syndrome and its related disease developing mechanisms.

In this chapter, I shall provide a comprehensive overview of OSA, including its epidemiological patterns, previously proposed mechanisms/hypotheses of OSA related disease developing mechanisms and its historical relatrion with various cardiovascular disorders.

1.1.2 Epidemiology

OSA affects up to 14% of the middle age Western population, with even higher prevalence in the men, elderly and obese population [Ferini-strambi et al 2004][Redline et al 1994] [Young et al 2002] [Duran et al 2001a][Duran et al 2001b]. It is therefore not surprising that the majority of epidemiological studies have male preponderance although some large studies have

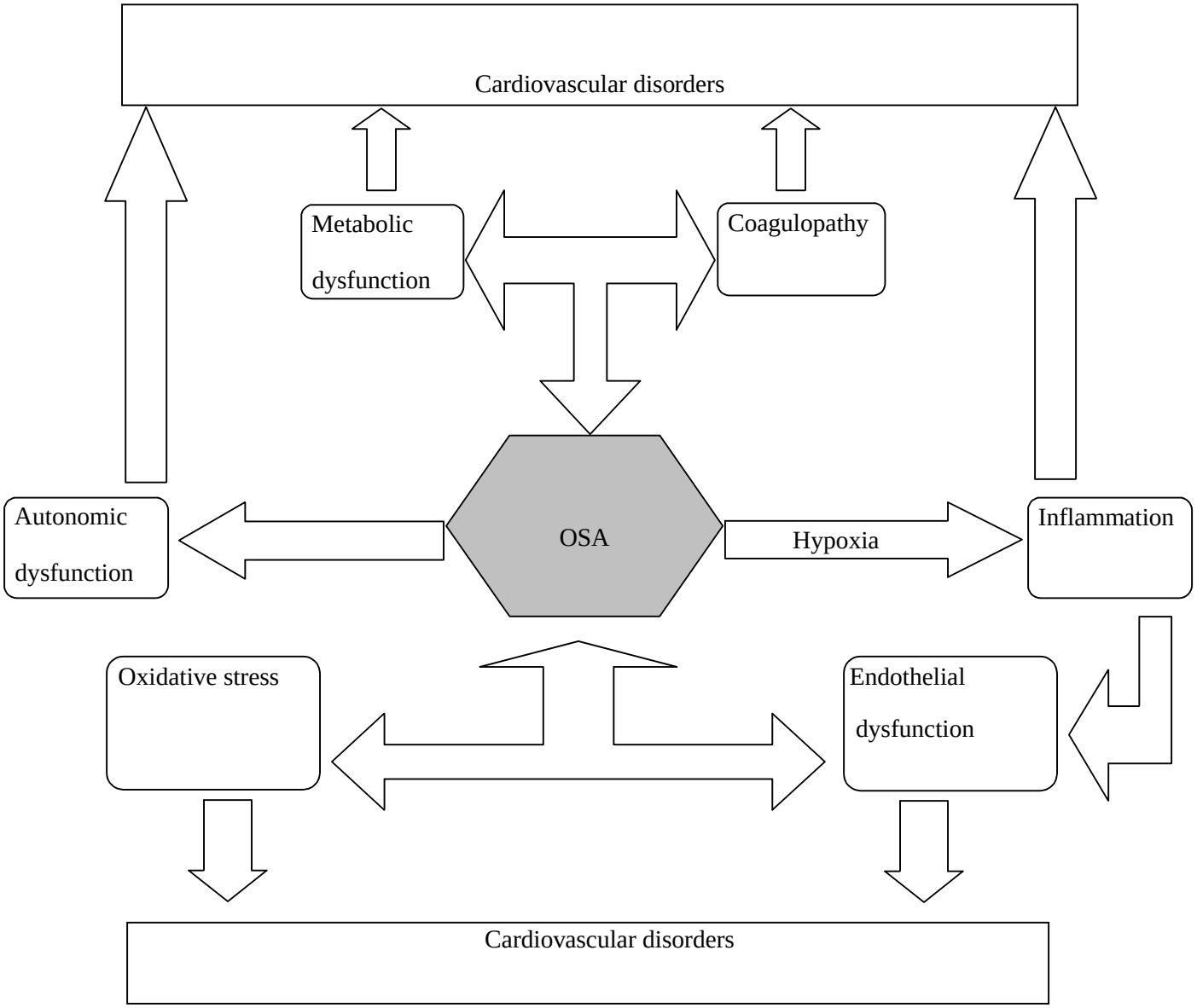
predominantly recruited females [Table 1-1]. Given that the risk of cardiovascular morbidity increases with age, it is sometimes difficult to appreciate if OSA causes cardiovascular disease or whether they are merely epiphenomenon.

Although a number of studies demonstrated the effect of age on the *development* of OSA independent of other confounding factors, data addressing the age related absolute change in disease *severity* are deficient. Some authors claim limited whilst others suggest marked deterioration in disease severity - as much as doubling after 10 years (calculated using AHI) in a given OSA population [Young et al 1993][Bixler et al 1998]. Like the increasing age, weight gain has also been shown to be a strong predictor of worsening OSA [Peppard et al 2000]. Of note, increased prevalence of OSA is seen in patients with congestive cardiac failure (CCF) [40%], stroke [60%] and end stage renal failure [50%] [Sin et al 1999][Yaggi et al 2005][Hanly et al 2001].

1.1.3 Pathophysiology

Although the association between OSA and CVD has been well known for decades, the underlying pathophysiological mechanisms have begun to unfold only recently. Endothelial dysfunction, autonomic overdrive, subclinical systemic inflammation, metabolic dysregulation and coagulopathy are some of the potential mechanisms which have been proposed to explain this complex link. Whilst vascular, metabolic, humoral and autonomic factors have their own relative impact; it is yet unclear which one plays the central role [Figure 1-1].

Figure 1-1: Pathophysiological mechanisms linking Obstructive Sleep Apnoea to Cardiovascular Disorders



1.1.3.1 Endothelial dysfunction (ED)

A large body of evidence suggests that impaired endothelial function accompanies OSA [Grebe et al 2006][Kato et al 2000][Budhiraja et al 2007]. The relationship between endothelial dysfunction and CVD is also well established and endothelial damage/dysfunction has a key role in the development of CVD [Napoli et al 2001]. The existence of endothelial dysfunction in OSA and vascular disorders is probably not a mere coincidence and in fact may indicate close underlying pathological links; therefore it is imperative to understand the cellular biology and physiology of the endothelium in OSA.

The endothelium is a *live* tissue which is more than merely a separation between the blood and the vessel wall. The role of endothelium in the maintenance of vascular tone, cellular proliferation, coagulation cascade and the modulation of the activity various blood constituents such as platelets and monocytes by secreting various vasoactive substances, is well recognized [Harrison et al 2003][Budhiraja et al 2004][Chin et al 1998][Sanner et al 2000][El-solh et al 2002].

How and what starts the process of endothelial dysfunction in OSA? Although the precise mechanisms of endothelial perturbation remain ambiguous, it has been postulated that OSA related hypoxia, inflammation or oxidative stress initiates the process [Venugopal et al 2002][Valgimigli et al 2003]. Also the inter-relationships amongst these factors are probably multifactorial. For example, NO is a vasodilator secreted by endothelium, and a reduction in NO is associated with endothelial dysfunction [Kato et al 2000], with an impairment of quantifiable endothelial mediated vasodilatory response [Celermajer et al 1996][Panza et al 1990][Linder et al 1990]. OSA patients also have reduced NO levels as well as impaired endothelial mediated vasodilatation [Ohike et al 2005]. Nonetheless, it is uncertain what

precisely leads to the reduced NO bioavailability in OSA. Inflammation and other cytokines may potentially play a role. Since the endothelium keeps a balance between several mediators and hormones, any imbalance *per se* – perhaps caused by inflammation – would result in adverse effects.

C-reactive protein (CRP) is robust marker of inflammation and is an important predictor of cardiovascular events. Reports of significant elevation in OSA population led to discover that CRP reduces NO synthase levels, supporting the role of inflammation in development of endothelial dysfunction [Shamsuzzaman et al 2002][Venugopal et al 2002]. Indeed there are other operating mechanisms too such as asymmetric dimethylarginine (ADMA), an endogenous NO antagonist is greater in OSA patients [Ohike et al 2005], suggesting yet another route to the development of endothelial dysfunction.

Whether endothelial dysfunction is the cause or the effect of cardiovascular problems (such as hypertension) remains ambiguous in OSA. Nevertheless, a strong link has been demonstrated between OSA and markers of endothelial dysfunction, even in normotensive population [Ip et al 2004]. Furthermore randomized CPAP trials have reported a healthy impact of endothelial status in OSA [Lattimore et al 2006].

1.1.3.2 Autonomic dysregulation

A dysfunctional autonomic system has been reported in OSA [Balachandran et al 2012]. Several components of autonomic nervous system mediate cardiovascular changes in OSA; however role of sleep related parasympathetic activity is yet to be proved. Regardless of causative mechanisms, deranged autonomic cardiovascular regulation is predictive of worse prognosis in high risk patients, ie with diabetes, heart failure, and myocardial infarction [Kataoka et al 2004][Hadase et al 2010]. Increasingly, dysfunctional autonomic system is

being regarded as an independent risk factor in normotensives and hypertensive subjects [solin et al 2009]. This may be the case also for patients with OSA, who are characterized by diurnally increased plasma levels of norepinephrine, elevated muscle sympathetic nerve activity, and altered heart rate variability [Singh et al 2008]. Arguably, dysfunctional autonomic system could be secondary to obesity, although robust data are limited [Narkiewicz et al 1998(a)]. Furthermore, repetitive hypoxia in OSA patients may also cause overactivation of sympathetic system [Somers et al 1995]. Similarly, central and peripheral chemorecepters have a close yet complex relationship with ventilation/breathing [Duffin et al 2010] and the overactivation of such receptors has been found in OSA patients nocturnally as well as during the day hours [Narkiewicz et al 1998(b)] [Tilkian et al 1976]. On the same note, hypertension and blood pressure oscillations in OSA are regarded as poor prognostic signs and predictors of target organ damage [Frattola et al 1993].

Available evidence suggests that long-term treatment with continuous positive airway pressure (CPAP) significantly improves autonomic indices in OSA patients [Narkiewicz et al 1998 (b)]. Notably, treatment of OSA with 100% oxygen (not necessarily with CPAP) corrects the hypoxia results in chemoreceptor deactivation, thus reducing sympathetic activity and normalizing hemodynamic parameters. Larger studies are required to assess the effects of interventions for OSA on autonomic function.

1.1.3.3 Haemostasis

Coagulopathy and abnormal platelet aggregability play important roles in the pathogenesis of atherothrombotic disease [Lurie et al 2011][Kannel et al 1987]. The impact of OSA on platelets per se is controversial; such effect may be secondary to endothelial dysfunction, raised

nocturnal catecholamine levels, or be simply a response to apnoeic episodes [Sanner et al 2000][Pearson et al 2010]. Regardless of the aetiology of platelet dysfunction, studies have noted improvements in platelet status with the correction of hypoxia using CPAP [Bokinsky et al 1995].

The effects of sleep disturbed breathing on red blood cells are less well studied. In an observational study of 624 OSA patients, small yet significantly higher hematocrit levels were reported in patients with lower nocturnal oxygen saturations [Hoffstein et al 1994 (a)]. Finally, other coagulation factors, such as plasma fibrinogen, are elevated in OSA and long term CPAP appears to reduce coagulation factor VII activity [Wessendorf et al 2010][Chin et al 2007]. Indeed this evidence is available but this does not overwhelmingly support the notion of disturbed coagulation in OSA. Therefore in summary, the effect of OSA on haemostasis and thrombosis remains a controversial territory due to inadequate evidence [Robinson et al 2004].

1.1.3.4 Oxidative stress

OSA is a state of oxidative 'stress' and the latter has a diverse role in the development of cardiovascular disorders [Lurie A et al 2011][Lavie et al 2003]. Repetitive hypoxic episodes lead to intra-cellular structures to adapt to lower oxygen levels. Therefore, the availability of normal oxygen concentration to these cells during the normoxic phase leads to the production of reactive oxygen species (ROS), which have the ability to oxidize cellular products, lipids and proteins. ROS from neutrophils and monocytes is augmented in OSA patients and has a close relationship with classical inflammatory mediators (such as bradykinin), which also suggests that systemic inflammation is one of the contributory factors in endothelial dysfunction. ROS upregulate adhesion molecules and diminish NO synthase activity, thus augmenting NO

breakdown and ultimately, diminished NO levels [Deanfield et al 2005]. If not corrected, the resultant endothelial damage/dysfunction may lead to atherosclerosis [Harrison et al 2003]. Case control studies have shown the tendency of neutrophils to produce more ROS in OSA, a phenomenon which is reversible with CPAP therapy [Schulz et al 2000]. Similarly, an enhanced expression of adhesion molecules in monocytes and an increased ROS production in OSA patients also responds to CPAP [Dyugovskaya et al 2002]. Both experimental and human studies are consistent with the concept that intermittent hypoxia in OSA represents a form of oxidative stress and may lead to dysfunctional endothelium.

1.1.3.5 Inflammation

Observational studies have shown that various vascular disorders as well as OSA are associated with sub-clinical inflammatory processes [Garvey et al 2009] [Ridker et al 2003][Danesh et al 2000]. It is believed that some of the inflammatory mediators/markers may induce vasculopathy through endothelial dysfunction. For example, raised CRP levels result in the direct as well as indirect (by monocytes activation) reduction of NO synthase [Venugopal et al 2002][Woollard et al 2002]. OSA increases levels of inflammatory mediators such as inter-cellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) [Ohga et al 1999][Ohga et al 2003], in endothelial cells as well as in neutrophils, thus promoting endothelial cell adhesiveness and oxidative stress. The underlying mechanisms is believed to be OSA associated hypoxia and sleep deprivation which modulates the expression of inflammatory mediators, particularly interleukins and tumor necrosis factor alpha [Vgontzas et al 1997]. CPAP therapy reduces various circulating inflammatory mediators such as ICAM-1, Interleukin-8 (IL-8) [Ohga et al 2003] and C-reactive protein [Minoguchi et al 2006].

1.1.3.6 Metabolic dysfunction

There is a well-recognized association of insulin resistance with OSA as well as with atherosclerosis [Grebe et al 2006][Punjabi et al 2002], suggesting that metabolic disturbances may well be a significant link between OSA and CVD. The severity of sleep apnea appears to correlate with the degree of insulin resistance. Unsurprisingly, severe OSA is accompanied by a 5-fold increase in the risk of overt diabetes mellitus [Spiegel et al 1999]. Conversely, high insulin levels in non-obese OSA patients have been reported and worsen with increasing AHI and oxygen desaturation levels [Punjabi et al 2002][Elmasry et al 2001 (a)]. Thus, obesity per se may not the sole determinant of insulin resistance, as previously believed [Vgontzas et al 2000].

Leptin is a hormone involved in satiety, body weight control and fat distribution is also regarded as an independent risk factor for CAD [Caro et al 1996][Wallace et al 2001]. Although leptin is also elevated in obese individuals it increases platelet aggregability regardless of the body fat distribution [Considine et al 1996][Davi et al 2002]. Correction of OSA reduces leptin levels, as well as intra abdominal fat levels [Chin et al 1999]. However, treatment with CPAP does not show any consistent improvement in glucose tolerance [Elmasry et al 2001 (b)].

Despite regional variations in the prevalence of coronary disease, men are consistently at a greater risk of developing and dying from CAD than women, and the gender-specific effects of sex hormones are implicated in this inequality. Serum testosterone levels have been observed to be negatively correlated with the severity of OSA [Canguven et al 2010]. Some studies observed an inverse relationship between testosterone levels and not only coronary disease and coronary risk factors but also with mortality [Park et al 2012]. There is no evidence to suggest that CPAP treatment improves testosterone levels in OSA. Perhaps testosterone level may be an

additional indicator in determining severity and in follow-up of OSA, although this hormone is strongly affected by a number of physical and physiological factors.

1.1.4 Obstructive sleep apnea and cardiovascular diseases

The most serious complications of OSA include heart failure, coronary artery disease (CAD), arrhythmias, stroke, systemic hypertension and pulmonary hypertension. Animal as well as human studies have revealed that OSA induced intermittent intra-thoracic pressure changes are associated with acute hemodynamic disturbances in physiological parameters and adverse effects on left ventricular pressures and dimensions [Bradley et al 1992][Sajkov et al 1994][Virolainen et al 1995][White et al 1995].

1.1.4.1 Hypertension

Large epidemiological studies have demonstrated a strong and consistent link between OSA and hypertension [Table 1-1]. A vast proportion of OSA population (upto 95%) has been reported to have some degree of high blood pressure [Marin et al 2012][Lavie et al 2000]. Though the causative pathways remain debatable; cross sectional analyses of several studies suggest that OSA is an independent risk factor for high mean, systolic and diastolic blood pressures [Table 1-1]. Therefore, hypertension management guidelines acknowledge OSA as an independent cause of hypertension and recommends blood pressure screening amongst OSA patients [Chobanian et al 2003].

There are several theories on the underlying mechanisms of raised blood pressure in OSA. Increases sympathetic activity secondary to aponeaic/hypopnoeic hypoxia and cortical micro-arousal [Solin et al 2003][Narkiewicz et al 1998 (a)][Singh et al 1998] and nocturnal fluctuations in catecholamines [Fletcher et al 1987][Baruzzi et al 1991] are some of the more

prominent factors in the causation of hypertension. Carlson et al reported augmented sympathetic activity in non-hypoxic OSA patients even during day hours, which were later supported by other studies [Carlson et al 1993][Somers et al 1995]. Finally, endothelial dysfunction - leading to failure of endothelial dependent and independent vasodilatation of resistant vessels – results in raised peripheral vascular resistance and consequently, hypertension.

Increasing evidence suggests that CPAP therapy in OSA could improve blood pressure in part by improving endothelial dysfunction as well as the correction of triggering factors, such as sympathetic system overactivation and hypoxia [Table 1-2][Bazzano et al 2007][Mills et al 2006][Faccenda et al 2001]. For example improved nocturnal catecholamine with concomitant improvement in nocturnal and daytime blood pressures has been demonstrated with the use of CPAP [Mills et al 2006]. Others CPAP studies have also shown to improve autonomic balance in small OSA cohorts [Imadojemu et al 2007]. Whilst antihypertensive effect of CPAP has been questioned by some [Robinson et al 2006], others suggest that beneficial effect of CPAP on lowering blood pressure extends beyond pharmacological therapy [Table 1-2].

Table 1-2: Randomized clinical trials of use of continuous positive airway pressure in obstructive sleep apnea patients with hypertension

Reference	Study design	Findings	Comment
Mills et al 2006	50 patients were randomised to therapeutic or sub-therapeutic CPAP for 2 week, to detect any effect on catecholamine clearance	Significant reduction in systolic as well as diastolic blood pressure and concurrent increased nor epinephrine clearance in CPAP arm suggest role of latter in hypertension in OSA	Single blinded study, short duration, manual blood pressure recordings
Faccenda et al 2001	Placebo controlled cross-over study of the effects of 4 wk of CPAP or oral placebo on 24-h blood pressure in 68 patients (predominantly male)	Improvement in diastolic blood pressure (p=0.04) was noted especially in patients with nocturnal desaturation or with prolonged CPAP use. Daytime sleepiness also improved	Normotensive study population, Deduction of mild OSA from study, lack of washout period and short study duration
Robinson et al 2006	Double blind trial randomising 35 patients to either therapeutic or sub-therapeutic CPAP for 2 months with ambulatory blood pressure monitoring	CPAP does not reduce mean blood pressure in non sleepy hypertensive OSA patients	Small study, does not individually comment on systolic and/or diastolic blood pressue. Does not involve mild OSA
Becker et al	32 patients were randomized to therapeutic	Interestingly reduction in AHI	First study to suggest drop in blood pressure with

2003	or sub therapeutic CPAP. For 9 weeks.	was noted in both groups however significant drop in mean, systolic and diastolic blood pressure was only noted in therapeutic CPAP arm	CPAP, good compliance in groups, small study size, however ambulatory Blood pressure monitoring may give spuriously low readings
Dorkova et al 2008	32 OSA patients were given CPAP for 8 weeks with pre and post CPAP assessment of various inflammatory and endothelial dysfunction markers with blood pressure changes	Reduction in systolic as well as diastolic blood pressures was noted	Small study, investigated the role of metabolic syndrome, oxidative stress and inflammation in the causation of CVD
Vgontzas et al 2008	In this study of 16 obese OSA patients several plasma markers and blood pressure were assessed with its response to CPAP	Improvement in overall blood pressure was recorded after 3 months on CPAP	Small study, emphasis of the link between OSA and inflammation
Barnes et al 2004	Comparison of the effects of CPAP and oral placebo or mandibular device (MAS) among 110 OSA patients, for 3 months. Objectives were to observe any improvement in sleepiness and quality of life	Small nocturnal reduction of blood pressure noted with MAS compared to CPAP besides symptomatic improvement	Considerable female population, this is one of the first studies to show significant improvement in OSA hypertensive patients with MAS, though the correct method to use was questioned in some. Included mild OSA as well.
Campos - Rodriguez et al 2006	In this double blind trial 4 weeks randomisation of OSA patients with treated hypertension, to therapeutic CPAP verse sub-therapeutic CPAP was done	Statistically insignificant drop in mean blood pressure in CPAP group but no change in systolic, diastolic or nocturnal	Reasonable size double blind study, short duration, inadequately powered to detect blood pressure variations

		blood pressure.	
Hla et al 2002	24 men with hypertension and with or without OSA were randomized to CPAP for 3 weeks.	CPAP arm showed reduction in nocturnal systolic and diastolic blood pressure.	Small study, only male investigated,
Barnes et al 2002	28 predominantly male patients with mild to severe OSA randomized to CPAP or oral placebo for 8 weeks to assess neurobehavioral improvement and reduction in blood pressure.	Neurobehavioral indices improved in both arms however subsidiary analysis for blood pressure did not show significant difference.	Single blinded study, high rate of patients withdrawal and doubts about CPAP compliance. only 7 patients were hypertensives and analysis failed to show significance of CPAP is such a small group.
Barbé et al 2001	To detect the effect of CPAP on blood pressure and quality of life of non sleepy OSA patients, 54 patients with severe OSA were randomised to nasal CPAP or sham CPAP for 6 weeks	Non-sleepy patients with severe OSA do not get worthwhile benefit from CPAP such as reduction in blood pressure and improvement in cognitive impairment	Multicenter placebo controlled blinded study, short CPAP duration,,
Campos - Rodriguez et al 2007	In this prospective long term trial (24 month follow up) ambulatory blood pressure monitoring was carried out to investigate any improvement in blood pressure parameters	CPAP has a dose dependent response with reduction in blood pressure	Small study, same group published opposite findings in a double blinded but similar trial in short term (4 weeks of CPAP)

CPAP= continuous positive airway pressure; MAS= mandibular advancement splint; OSA= Obstructive sleep apnea**;** CVD**=** cardiovascular disease

Indeed, owing to the link between severity of OSA and incidence of hypertension [Table 1-1], CPAP therapy has been extensively studied in patients with *moderate to severe* OSA [Table 1-2]. Although *mild* OSA is also linked with elevated blood pressure, limited evidence reveals no significant decline in the blood pressure with of CPAP treatment [Monasterio et al 2001].

1.1.4.2 Coronary artery disease (CAD)

Many epidemiological studies consistently report a close and strong presence of CAD in OSA population regardless of other risk factors [Martinez et al 2012][Table 1-1]. Circulatory physiology is greatly affected by change in sleep pattern, OSA related hypoxia, hypercapnia, blood pressure surges, sympathetic overactivation, and the acute imbalance of vasoactive hormones [Peker et al 1999]. These changes can not only provoke acute coronary syndromes but also, their persistence ultimately leads to chronic consequences such as heart failure [Gami et al 2004 (a)]. Interestingly, reversible electrocardiogram (ECG) changes can be noted in OSA patients without significant CAD [Hanly et al 1993]. The use of CPAP in OSA patients reduces incidence of new coronary events compared to controls, as observed by a large prospective trial [Milleron et al 2004]. Moreover, in a randomised study, CPAP has been found to reduce mortality [Mooe et al 2000], though the mechanism largely remains unknown.

1.1.4.3 Heart failure

Many observational studies have linked OSA with systolic as well as diastolic impairment of the left ventricle [Table 1-1] [Carr et al 2012] [Artz et al 2006]. Moreover patients with heart failure *per se* have a higher prevalence of sleep disordered breathing [Javaheri et al 1998]. The

precise reasons are uncertain but odema of neck soft tissues makes pharyngeal tissue prone to collapses thereby leading to further tightening of airways [Shepard et al 1996].

In health, during the inspiration the right ventricular output increases whilst the left ventricular (LV) output slightly decreases, leading to respective changes in their stroke volumes [Kasai et al 2011]. These subtle respiratory variations in ventricular volumes become more pronounced with apnoeas/hypopnoeas, due to accentuated negative intra-thoracic pressure induced by marked and forceful inspiratory efforts. Increased venous return, augmented right sided preload shifts the septum to the left (during diastole) impairs LV filling and diminishes LV preload and ejection pressure. Furthermore, transient and intermittent airway obstructions and changes in intra-thoracic pressure significantly increase LV transmural systolic pressures. Ultimately these haemodynamic stresses translate into cardiac remodelling, LVH and ultimately arrhythmias and heart failure [Kasai et al 2011]. In addition, OSA associated hypertension, exaggerated adrenergic responses and hormonal imbalance may all predispose to hypertensive heart failure [Tanriverdi et al 2006 (a)]. Normalization of left ventricular (LV) function with CPAP treatment has been reported in OSA patients [Mansfield et al 2004].

Mechanisms leading to diastolic dysfunction in OSA are less clear. Myocardial hypertrophy and persistently high blood pressure [Kasai et al 2011][Cloward et al 2003] and adverse changes in LV dynamics (a rise in after-load and a reduction in fractional shortening) in response to intermittent airway collapse, can also lead to impaired relaxation [Parker et al 1999]. Some studies based on the echocardiographic findings have concluded that OSA independently does not show any adverse impact on ventricular function in OSA [Hanly et al 1992].

Right ventricular hypertrophy, reduced contractility and ejection fraction (EF) [Sanner et al 1997] have all been reported in OSA patients. These findings may be a direct consequence of OSA related disturbed haemodynamics or secondary to pulmonary hypertension, which itself is prevalent in OSA [Chaouat et al 1996][Alchanatis et al 2001]. Successful treatment of OSA also improves right ventricular function [Nahmias et al 1996], and improvement in LV dimensions and contractility have been noted after CPAP [Table 1-3].

Table 1-3: Studies using Continuous Positive Airway Pressure in Obstructive Sleep Apnea patients with Heart Failure

Reference	Study design	Conclusion	Comments
Mansfield et al 2004	Open trial using CPAP for 12 weeks in OSA patients in middle aged population to detect improvement in heart failure	Substantial improvement in cardiac failure and quality of life	predominantly male, large number of drop outs, manual blood pressure recording, diastolic dysfunction not assessed
Kasai et al 2008	Aim of the study was to detect any prognostic benefits of CPAP in heart failure with OSA. 88 OSA patients with moderate to severe disease were randomized to CPAP or observations	CPAP treatment group significantly reduces morbidity and mortality depending upon strict compliance	Good comparative study for assessing the prognostic significance of CPAP in this particular group
Kaneko et al 2003	24 OSA patients were randomized to either CPAP with medical therapy or medical therapy alone for one month	CPAP significantly reduced heart rate, blood pressure and left ventricular dimensions and ejection fraction	Well conducted open study, inclusion of ischaemic and non ischaemic patients, small study
Wang et al 2007	To see any effect of CPAP on mortality in heart failure patients with OSA, 164 patients were recruited and control group involved untreated OSA patients	Untreated OSA with heart failure is high risk for mortality	A good prospective study with median follow up of over 7 years

Alchanatis et al 2000	CPAP was randomized again observation in fifteen of twenty six patients for three months with regular transthoracic echocardiogram	CPAP significantly improves ventricular function especially diastolic, also drop systolic blood pressure	Small study. Echocardiography induced possible observer bias, control group comprised of non OSA individuals, mild disease excluded from study
Arias et al 2005	In this study of 42 patients sham CPAP against effective CPAP in moderate OSA patients for 12 weeks	Impaired relaxation is a prominent finding in OSA patients. Effective CPAP treatment improves E/A ratio	Double blind yet small study, only male population included, echocardiography related observer bias may be another factor

OSA= obstructive sleep apnea; CPAP= continuous positive airway pressure

The effect of CPAP on exercise capacity in OSA patients with heart failure has not been studied extensively. However in some small studies CPAP seems to increase exercise capacity in heart failure patients regardless of coexistence of OSA [Schlosser et al 2006].

In most studies transthoracic echocardiography has been used as diagnostic tool, however in OSA patients where obesity is commonly prevalent, the efficacy of echocardiography has been questioned. Nevertheless, Laaban et al [Laaban et al 2002] studied a large cohort of OSA patients without known coronary artery disease and investigated cardiac functions using multiple-gated equilibrium cardiac imaging (MUGA) reporting poor LV systolic function.

1.1.4.4 Cardiac Arrhythmias

A wide spectrum of conduction disturbances ranging from premature ventricular contractions (PVCs) to complex arrhythmia such as complete heart block and ventricular tachycardia, have been reported in OSA [Harbison et al 2000]. In one small study (n=71), there was a higher incidence of life threatening ventricular arrhythmias amongst patients with sleep disordered breathing [Harbison et al 2000]. In heart failure patients with implantable cardiac defibrillators, device discharges occurred more frequently in OSA patients than those without, particularly nocturnally, suggesting a link between OSA and nocturnal malignant arrhythmias [Serizawa et al 2008]. Epidemiological studies demonstrating the arrhythmia burden of OSA are summarized in Table 1-4.

Table 1-4: Obstructive Sleep Apnea and association with Cardiac Arrhythmias

Reference	n	Tachyarrhythmias %	Bradyarrythmias %	Ectopics %
Guilleminault et al 1983	400	3	19	0
Becker et al 1995	239	0	10	0
Flemons et al 1993	76	0	6	1
Miller et al 1982	23	0	13	9
Bolm-audorff et al 1984	20	15	0	36
Tilkian et al 1977	15	13	46	67

Intermittent hypoxia can cause HR to decrease, increase, or remain constant depending upon status of autonomic system [Leung et al 2009]. In canine models, right atrial pacing augments the autonomic activity of the right pulmonary arterial sympathetic nerve endings during apnoea, and may induce atrial fibrillation (AF) [Cagirci et al 2011][Gami et al 2004][Ghias et al 2009]. Therefore, stimulation of such ganglionated plexi during obstructive apnoeas may be one of the mechanisms of AF in OSA. Myocardial stretch caused by the mechanical effects of OSA, myocardial ischaemia and activation of cardiac inflammatory pathways may also precipitate atrial and ventricular arrhythmias [Kasai et al 2011]. The mechanisms described above operate in parallel in OSA where a marked negative intrathoracic pressure impacts heavily on the LV leading to cardiac remodelling (LVH and LA dilatation) ultimately increasing risk of atrial arrhythmias and thromboembolism.

.Studies in heart failure patients suggest an increased prevalence of AF in patients with sleep apnea [Javaheri et al 1998]. Additionally the presence of OSA predicts pre-discharge recurrence

of AF after cardiac surgery [Mooe et al 1996] and post ablation [Jongnarangsin et al 2008]. Although CPAP treated OSA patients have better outcomes with regards to reduced risk of recurrent AF after electrical cardioversion [Kanagala et al 2003], the role of CPAP in primary prevention of AF in OSA patients is unknown.

It is clear now that OSA has a profound impact of LV function, promotes stasis and carries a high risk of AF. There is no direct study investigating threshold to prescribe oral anticoagulation in OSA subjects. Recently, a joint consensus document from the European Society of Cardiology (ESC) Heart Failure Association and the ESC Working Group on Thrombosis has been published [Lip et al 2012]. This report suggests that, in patients (regardless of the OSA status) with AF, oral anticoagulation is recommended, and the CHA(2)DS(2)-VASc and HAS-BLED scores should be used to determine the likely risk-benefit ratio. However, in heart failure patients with impaired LV and sinus rhythm, there is no evidence of an overall benefit of vitamin K antagonists (e.g. warfarin) on mortality, with excessive risk of major bleeding. Therefore, there is currently no compelling reason to use warfarin routinely for OSA patient with heart failure but in sinus rhythm.

Bradycardia is also highly prevalent in OSA patients [Guilleminault et al 1983][Flemons et al 1993]. Although pauses up to 2 seconds can be observed in apnea episodes in otherwise healthy individuals [Becker et al 1995], the prolongation of pause duration has been noted in OSA patients [Tilkian et al 1977]. Some degree of conduction block has been described in up to 10% of OSA patients, especially during REM sleep [Koehler et al 1998]. Recently a small study using insertable loop recorder in 23 moderate OSA patients questioned the usefulness of 24-hour holter monitor. The results seem to suggest bradyarrythmias are more frequent in OSA and loop recorder is superior to 24-hours holter in detecting the significant pauses (longer than 3 seconds) ($p=0.03$) [Simantirakis et al 2004]. Further more consistent with the most available data, CPAP significantly ameliorates these arrhythmias [Simantirakis et al 2004].

Hypoxia probably plays an important role in the genesis of bradycardia with improvement in heart rate on oxygen administration [Zwillich et al 1982]. CPAP - even in the absence of sinus or AV nodal disturbances - reduces apnea associated bradyarrythmias [Harbison et al 2000]. Atrial overdrive pacing [Garrigue et al 2002], perhaps by reducing the number of arrhythmias, significantly reduces these episodes in OSA as well as CSA, further lowering the risk of sleep associated rhythm disturbances.

1.1.4.5 Cerebrovascular disease

Epidemiological studies link OSA with escalated risk of stroke [Table 1-1] [Das et al 2012][Partinen et al 1995]. Unsurprisingly, OSA is also regarded as a poor prognostic marker in stroke patients [Good et al 1996] partly due to poor ventilation secondary to posture related problems amongst stroke patients. A small study of 24 stroke presence of OSA was significantly associated with adverse prognosis [Sahlin et al 2008]. Nevertheless further data is needed to determine whether OSA is related to cerebrovascular morbidity independent of other vascular risk factors. On a different note, sleep disordered breathing is also highly prevalent amongst stroke patients [Good et al 1996] due to associated sub-total/total paralysis of facial and pharyngeal muscles.

Hypertension, atherogenesis, hypercoagulability, impaired endothelial dysfunction, strong association with AF and changes in cerebral haemodynamics may all contribute [Ficker et al 1997]. In some instances the dramatic changes in haemodynamics may cause up to 80% fall in cerebral blood flow [Netzer el al 1998] contributing to cerebrovascular events in the OSA population. Currently there are little data on the effect of CPAP in OSA in post stroke settings to establish any symptomatic and/or prognostic benefits. Limited data indicates poor outcome

with CPAP use in OSA [Scala et al 2003]. Larger studies targeting primary risk reduction of stroke with the use of CPAP in OSA patients are much required.

1.1.5 Management strategies

OSA is an emerging health challenge partially because of its strong association with many cardiovascular disorders but more importantly due to its' individual recognition as an established cardiac risk factor. Close involvement of OSA in the development of heart failure and hypertension might suggest that treatment of OSA might ameliorate these secondary conditions. The current general consensus is that OSA should be treated with CPAP for ventilatory support as well as a tool for the secondary prevention of cardiac problems [Table 1-2; 1-3]. Whilst limited data suggest reduction in the incidence of cardiac manifestations with early use of CPAP [Dursunoglu et al 2007]; existing and/or secondarily induced cardiovascular diseases should still be treated on their own merit [Eastwood et al 2010].

OSA is a relatively under-diagnosed condition and hence, the majority of OSA patients would have developed a cardiovascular condition before a formal diagnosis of OSA is made. Indeed CPAP emerged as a novel therapeutic tool; its prognostic advantages have also been demonstrated in recent trials [Kasai et al 2008]. From the cardiac perspective, most of the studies on OSA have been done in population with preexisting hypertension or other cardiovascular disorders. The efficacy of CPAP in hypertension in some open, single and double blind studies has been challenged by some, whilst investigating a range of blood pressure parameters such as systolic, diastolic, mean 24-hours, nocturnal and day time blood pressure [Robinson et al 2006][Becker et al 2003]. Indeed most of the current evidence supports the use of CPAP in reduction of blood pressure with concomitant improvement in

patients' cognition and daytime sleepiness [Mills et al 2006][Dorkova et al 2008][Vgontzas et al 2008][Dursunoglu et al 2007][Good et al 1996].

In the last decade, various studies have determined the effect of nasal CPAP on heart failure associated with OSA [Mansfield et al 2004][Kaneko et al 2003]. Although most were of a small size, they have shown improvements in symptoms, LV dimensions and systolic (as well as diastolic) cardiac function. However, assessments were based on TTE which is not infallible; also, there were significant gender differences and in some, mild OSA was excluded. Few studies have focused on the prognostic significance of nasal CPAP but the results are promising, with a reduced mortality and hospitalization in patients treated with nasal CPAP [Kasai et al 2008][Wang et al 2007].

Compliance with CPAP is a well recognized issue [Weaver et al 2006] which necessitates an acceptable alternative with at least similar or more advantages. Barnes et al [Barnes et al 2004] have used a mandibular assisted splint (MAS) with improvements shown in clinical outcomes, although this was not superior to nasal CPAP, and the impact on prognosis has not been determined. The oral appliances have also been studied in 28 OSA patients to determine the impact on brain natriuretic peptide (BNP) and left ventricular dimensions [Hoekema et al 2008]. Though the author concluded significant reduction in elevated BNP levels, these benefits do not seem to translate into echocardiographic ventricular improvement in this small study. Surgical treatment of OSA [Won et al 2008] has not gained much popularity, due to potential operative risks and no prognostic value.

Antihypertensive drugs have shown to have beneficial effects on endothelium, whether directly or indirectly [Koh et al 1999][Li-Saw et al 2001]. As inflammation and coagulopathy caused by OSA induced hypoxia leads to atherogenesis, the use of aspirin and statins may help [Kennon et al 2001][Albert et al 2001].

1.1.6 Conclusion

Observational studies have demonstrated that OSA is a highly prevalent condition across all age and gender groups, carrying a serious risk of cardiovascular morbidity and mortality. Now regarded as an independent cardiac risk factor, OSA also leads to the development of other cardiovascular risk factors, such as hypertension. Although hypoxia, oxidative stress and autonomic dysfunction have all been implicated in the development of CVD in OSA population, the dysfunctional endothelium plays a pivotal role, by hormonal imbalance and impairment of vasomotion which lays the foundations for vascular disoders. Moreover, the coexistence of OSA with metabolic syndrome, obesity and diabetes mellitus makes cardiovascular complications a logical consequence.

OSA related symptoms such as insomnia, day time somnolence and poor exercise capacity could profoundly affect individual daily life. Therefore early recognition and treatment of OSA, supplemented by educational, behavioral and supportive interventions, may improve clinical status as well as the long term outcome. Further understanding of basic pathophysiology behind the generation of cardiovascular disorders in OSA patients may identify possible new targets for adjunct therapies such as use of anti-inflammatory drugs.

1.2 Endothelial Dysfunction, its assessment and Prognostic implications

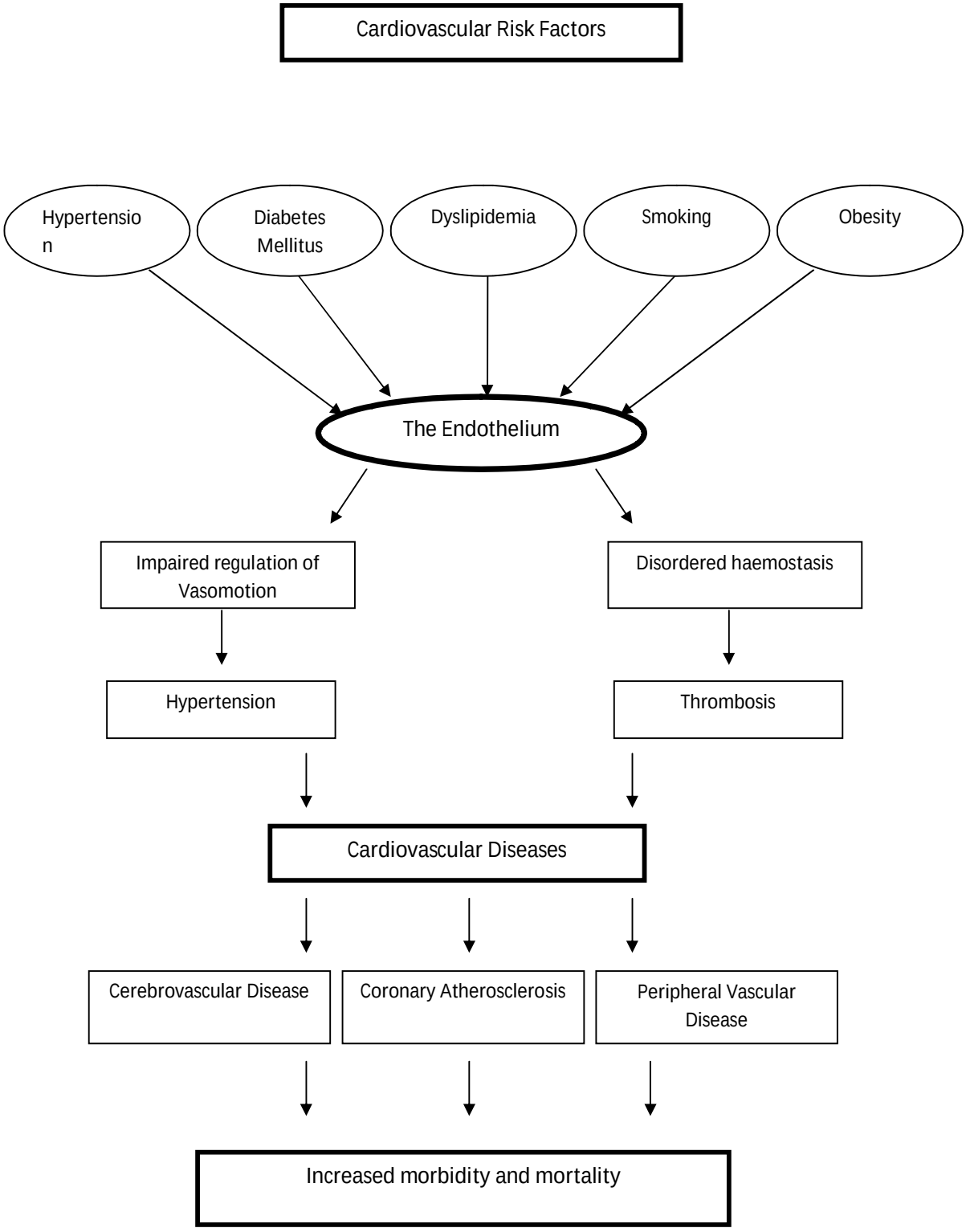
1.2.1 Introduction

The endothelium is a thin mono-cellular layer lining the entire vascular system, separating blood from the interstitium. It plays a core role in vascular tone by releasing various types of vasoactive substances, such as nitric oxide (NO). In addition to regulating vasomotion, the healthy endothelium also has anti-thrombotic (through prostacyclins), anti-inflammatory (through developmental endothelial locus-1{Del-1}) and anti-proliferative (through NO and prostaglandin I2) properties. All such mechanisms are regulated by a strict balance amongst several agonist and antagonist biochemical substances secreted by the endothelium [Choi et al 2009][Butt et al 2010][Stamler et al 1994].

Endothelial dysfunction (ED) is a systemic process wherein the endothelium loses the ability to maintain the vascular equilibrium, and is characterized by abnormal vascular homeostasis, a prothrombotic coagulation profile and vasoconstriction. Given the close association between ED and cardiovascular risk factors or overt cardiovascular disease per se, the presence of ED is, unsurprisingly, a crucial step in the development of atherosclerosis [Figure 1-2] [Hamasaki et al 2000][Butt et al 2010].

Over the last few years, the use of several invasive and non-invasive methods of assessment of ED has led to a better understanding of endothelial pathophysiology and mechanisms of disease development. Also a number of studies have shown that persistent ED is associated with adverse prognosis [Halcox et al 2002][Heitzer et al 2001]. More importantly, it has been proposed that strict physiological and/or pharmacological management of cardiovascular risk factors improves the functional status of the endothelium and reduces the risk of future cardiac events [Modena et al 2002].

Figure 1-2: The interaction between the Endothelium and Cardiovascular risk factors



In this chapter I have discussed modern perception of endothelial biology and provided an up-to-date overview of methods for the assessment of ED. I have also appraised the clinical and prognostic significance of the ED in relation to cardiovascular diseases and its major risk factors.

1.2.2 The Healthy Endothelium – A Brief Overview

The endothelium is the largest organ and gland of the human body and it produces a variety of vasoactive molecules under different stimuli [Table 1-5]. During health, these substances balance each other (such as pro- and anti-inflammatory substances) to maintain the vascular equilibrium.

Majority of endothelial functions are mediated thorough NO. It is derived from L-arginine and as a biologically active gas it is abundantly present in all tissues [Moncada et al 1993]. Its production requires involvement of the NOS enzyme family and several coenzymes [Moncada et al 1991]. Three different isoforms of NOS are known, NOS-I, NOS-II and NOS-III and although are present in endothelial cells. NOS-II that synthesizes NO for longer periods is important in maintaining the basal vascular tone whilst NOS-I and NOS-II produce short bursts of NO [Jones et al 1996]. Although, a variety of various physiological, chemical and pathological mechanisms can accentuate the production and release of NO, shear stress is probably the most notable stimulus for NO release [Cooke et al 2001]. Upon appropriate stimulation, special endothelial channels in the endothelial cell membrane open, causing an influx of calcium that activates nitric oxide synthase (NOS)-III leading to the production of NO [Miura et al 2001]. NO crosses the endothelial layer and diffuses into vascular smooth muscles and through a series of complex molecular reactions, produces cyclic guanyl monophosphate (cGMP). The latter subsequently modulates the influx of intracellular calcium causing smooth muscle relaxation and thus vasodilatation [Ohno et al 1993].

The vasodilatory reaction of endothelium (such as reactive hyperemia) is directly proportional to released NO concentration. Since the NO is directly released by the endothelial cells during reactive hyperemia, this response is known as endothelial dependent vasodilatation (EDV). Extrinsic nitrates such as Nitroglycerine bypasses the endothelium to act directly on smooth muscle cells to induce vasodilatation, and is referred to as endothelial independent vasodilatation (EIV). Both the EDV and to an extent EIV are attenuated in ED and the magnitude of this reduction correlates with the severity of the ED.

The leukocyte adhesion to endothelium is fundamental in leukocyte recruitment, which occurs during inflammatory, autoimmune and infectious settings. The interaction between leukocytes and endothelial cells requires numerous adhesion molecules expressed both on leukocytes and endothelial cells, thus accentuating leukocyte recruitment into sites of inflammation and tissue injury. However, accurate and functionally important endogenous/endothelial inhibitors of leukocyte adhesion have remained debatable, thereby questioning the anti inflammatory properties of the endothelium. Del-1 is a glycoprotein that is secreted by endothelial cells and is regulated upon hypoxia or vascular injury and has been implicated in vascular remodeling [Choi et al 2009]. Animal studies suggest that endothelial Del-1 acts as an anti-adhesive factor that interferes with the integrin LFA-1- receptor dependent leukocyte-endothelial adhesion. Endothelial Del-1deficiency increases LFA-1-receptor dependent leukocyte adhesion both *in vitro* and *in vivo*. This fact is supported by the findings that Del-1 (-/-) mice displayed significantly higher neutrophil accumulation in induced lung inflammation *in vivo* [Choi et al 2008]. Thus, it can be derived that endothelial Del-1 is an endogenous inhibitor of inflammatory cell recruitment and could provide a foundation for targeting leukocyte-endothelial interactions in disease. More importantly, since inflammatory processes are pivotal in the pathogenesis of atherosclerosis and mediate many stages of plaque formation and destabilization, the anti-inflammatory properties of the healthy endothelium could be crucial in the retardation of atherosclerosis [Crea et al 1997].

In addition to NO, prostaglandin I2 (PGI2) released by vascular endothelium has a profound negative impact on vascular smooth muscle proliferation [Stamler et al 1994][Sinzinger et al 1987]. A histological examination of vascular tissue revealed significantly lower number of activated smooth muscle cells in patients treated with PGI2 than in untreated ones, across all the age groups. A comparable suppression was demonstrated in the intima and the media of the vascular segments as well. PGI2 inhibits smooth muscle cell proliferation most probably by inhibiting platelet derived growth factor release from the platelets and stimulation of vascular smooth muscle cell Camp [Sinzinger et al 1986].

1.2.3 Assessment of Endothelial Dysfunction

Due to the intense focus on the endothelium, demand for a cost-effective, non invasive, reliable, reproducible, and easily available technique to assess endothelial functions has grown tremendously [Table 1-5].

Table 1-5: Examples of Studies investigating Endothelial Dysfunction in Populations with Cardiovascular Risk Factors

Reference	**Study design**				**Results and Comments**
	n	*ED marker*	*CV risk factor*	*Protocol*	
Al Suwaidi et al 2001	397	Coronary studies	Obesity	Obese (BMI>30) versus over weight (BMI 25-30) and normal weight subjects (BMI<25)	Obesity is associated with and is an independent predictor of poor ED. Patient has angiographically normal coronary arteries, DM and HTN excluded
Lip et al 1997	225	vWF, P-sel, Plasma Fibrinogen levels, PAI	Essential HTN	178 hypertensive compared with 47 controls	Larger study showing higher levels of ED markers in hypertensives and suggesting links between ED, blood pressure and cardiac remodeling
Celermajer et al 1993	200	Brachial artery FMD	Smoking	Healthy smokers versus healthy non-smokers	FMD was significantly lower in smoking group (4±3.9% versus 10±3.3%; p<0.0001). large group with stringent exclusion criteria
Sibal et al 2009	123	FMD, ADMA, ICAM, VCAM, E-sel	IDDM	61 IDDM patients versus 62 healthy subjects	FMD, ADMA were lower and adhesion molecules were higher significantly in IDDM patients (both p<0.001). coexisting confounding factors in study group
Taddei et al 1995	110	Plethysmography	Age	Middle aged hypertensive subjects were compared matched normotensives	An inverse correlation noted between age and EDV. Mean age (46 years) of study group may be considered ‘too young’
Meyer et al	107	FMD, capillary	NIDDM	63 NIDDM versus 44 healthy	Both FMD and capillary blood flow were

2008		blood flow		controls	impaired in DM (Both p<0.05) coexistence of confounders in study group where female gender was under-represented
Lip et al 2001	102	vWF, P-sel, Plasma Fibrinogen levels	Malignant HTN	Malignant HTN (n=18) compared with healthy and disease controls, 6 month follow up	Patients with blood pressure dependent elevated levels of VWF and plasma fibrinogen levels in small malignant HTN group
Hamburg et al 2004	62	Brachial artery FMD	Family history of premature CAD	34 healthy subjects with parental history of CAD compared with 28 healthy individuals with no FH.	Impaired FMD was demonstrated only in men with parental history of CAD in this non blinded study. Matched female representation.
Maas et al 2008	36	FMD, NO assays	Dyslipidemia	24 hypercholesterolemic subjects versus 12 healthy controls.	Diminished NO excretion (p=0.003) and 36% reduction in FMD ((p=0.027) noted in hypercholesterolemia. Use of NO as a validated ED marker is questionable.

CV: cardiovascular, ED: endothelial dysfunction, CAD: coronary artery disease, FH: family history, BMI: body mass index, FMD: flow mediated dilatation, EDV: endothelial dependent vasodilatation, DM: diabetes mellitus, IDDM: insulin dependent diabetes mellitus, NIDDM: Non-insulin dependent diabetes mellitus, HTN: hypertension, NO: nitric oxide, ADMA: Asymmetric dimethylarginine, VWF: von Willebrand factor, P-sel: P-selectin, E-sel: E-selectin, ICAM: intercellular adhesion molecule, VCAM: Vascular cell adhesion molecule,PAI: plasminogen activator inhibitor

Historically, invasive methods were amongst the first to be developed. For example, coronary angiography has been utilized extensively for endothelial assessment [Ludmer et al 1986]. Changes in the luminal diameter of a coronary artery in response to intra-coronary infusions of pharmacological agents such as acetylcholine (ACH) or adenosine were an important method for coronary endothelial assessment. Ludmer et al first reported coronary vasodilatation post-intracoronary ACH infusion in healthy coronary arteries. In contrast, ACH induced paradoxical vasoconstriction in diseased (atherosclerotic) coronaries, which is due to relative unavailability of NO [Ludmer et al 1986]. Indeed previous coronary studies do not provide a direct evidence of impaired NO release from coronary endothelium; however it must be noted that ACH induced endothelial responses are predominantly NO dependent. Therefore, the vasoconstrictor response in coronary arteries has been regarded as a useful indirect tool for identification and/or risk stratification of ED and early atherosclerosis [Anderson et al 1999].

Currently, ED can also be assessed by quantifying coronary blood flow and diameter through intra-coronary Doppler techniques in response to pharmacological stimuli. In this method, once a coronary artery is engaged with a guide catheter, a Doppler wire is introduced, usually in left anterior descending coronary artery. Once basal flow tracing is obtained, further signals are recorded after an intracoronary ACH or adenosine infusion. Coronary blood flow can be calculated by analyzing the peak flow velocity and coronary diameter. The invasive nature and potential complications of coronary angiography precludes this as routine method of ED assessment [Al Suwaidi et al 2001].

Flow Mediated Dilatation

Flow mediated dilatation (FMD) is amongst the most common techniques for endothelial assessment. In this method, the response of the endothelium in a large artery to changes in blood flow is to mediate the relaxation of smooth muscle cells, possibly via NO. This requires

specialized high resolution vascular ultrasound, in addition to a trained operator, to produce valid and reproducible results. Mean basal arterial diameter is measured using high frequency ultrasound. Inflation of a sphygmomanometer cuff (to a pressure level that will prevent blood flow) distal to the point of measurement for more than 4 minutes will, upon release of the cuff, induce shear stress, which will in turn releases NO, thus causing vasodilatation. EIV can also be determined by measuring mean arterial diameter post nitroglycerine administration sublingually [Celemajor et al 1992].

FMD is most commonly performed on brachial artery although femoral and radial arteries can also be used. One big problem with the use of a variety of vascular beds in various research laboratories is the different reference ranges for different beds. Reassuringly, most studies report FMD as an accurate and reproducible method of ED assessment [Hijmering et al 2001]. To ensure reproducible results the test should be performed in a controlled environment (such as quiet environment and within a controlled room temperature). Several factors such as food, drugs and temperature influence FMD, therefore the test should be performed after at least 8-12 hour fasting, abstaining from caffeine, smoking and drugs which could potentially influence the results. To standardize this technique the International Brachial Artery Reactivity Task Force has recommended guidelines to ensure reproducibility and accuracy [Corretti et al 2002].

1.2.3.2 Other Imaging Methods

Cardiac Positron Emission Tomography (PET) has the distinctive ability to assess coronary flow reserve and coronary endothelial function, based on response of blood flow to pharmacological stress (intravenous adenosine or dipyridamole) and the cold pressor test. Quantitative analysis of coronary vasomotor function is valuable for accurate estimation of function and treatment monitoring in the presence of a number of coronary risk factors [Rosas et al 2008]. Recent data supports predictive value of PET assessment of coronary vasomotor

imaging in subjects with coronary artery disease [Uren et al 1994]. In addition, this technique has shown to demonstrate vasomotor dysfunction in asymptomatic young hypertensives and/or dyslipidemic patients [Rosas et al 2008]. Hence, quantitative analysis using PET has a great prospect of wide application in recognizing microvascular dysfunction and "individualized" monitoring of the outcomes of primary or prophylactic medical interventions to optimize cardiovascular outlook.

Similar to PET, Myocardial Resonance Imaging (MRI) also estimates the coronary blood flow and coronary flow reserve non-invasively yet without any associated radiation exposure [Hirsch et al 2008][Wöhrle et al 2006]. With MRI coronary artery flow indices can be measured with either a breath-hold or a respiration-triggered acquisition; however in the breath-hold MRI measurements, cardiac output are significantly depressed during breath-holding phase after deep inspiration, but the advantage is that the breath-hold method requires lesser scan time. If coronary sinus blood flow and LV mass is estimated using phase-contrast cine MRI, both the blood flow per gram of myocardial tissue and coronary flow reserve can be precisely quantified. Coronary flow reserve, with quantitative MRI flow measurement, is measured to be between four to five folds. The MRI quantification of coronary flow reserve and other flow indices seems to be an ideal method for evaluating coronary hemodynamics and may be valuable in evaluating endothelial dysfunction of the coronary circulation [Hirsch et al 2008][Wöhrle et al 2006].

Nevertheless, whilst both the imaging techniques have their relative pros and cons, the expense and limited availability of these modalities render these techniques unsuitable for routine use to assess endothelial status.

Lately, myocardial contrast echocardiography (MCE) has been gaining popularity as an imaging tool to assess the integrity of the myocardial microcirculation [Dwivedi et al 2007][Di Bello et al 2004]. This approach provides an estimation of the two crucial components of capillary perfusion: Blood volume fraction and flow velocity, unlike other

experimental and clinical methods that only measure myocardial blood flow (MBF). The latter can be used to accurately calculate of CFR providing information about the functional consequences of ED. This technique utilizes inert microbubbles that possess similar rheological properties as red blood cells and remain entirely within the vascular space [Jayaweera et al 1994]. During the intravenous infusion of these microbubbles and following attainment of a steady state in the myocardial tissue, the microbubbles are destroyed using high energy ultrasound and the rate of bubble replenishment is measured (represents mean red blood cell velocity) [Wei et al 1998]. Under resting conditions and if resting flow is normal, this replenishment is completed within 5 seconds. However, it takes longer to fill if resting flow is reduced. The rate of filling is faster after stress which is usually induced by intravenous dipyridamole or dobutamine. After full replenishment, the ultrasound signals represent the relative blood volume within the beam, which correlate to the blood volume within the myocardium itself. Normalizing this value to the signals from the left ventricular cavity provides a value of blood volume fraction. The product of the blood volume fraction and flow velocity is equal to MBF which could be utilized for the measurement of CFR [Wei et al 1998]. In addition, not only is MCE a useful tool for the non invasive assessment of ED, but it can also be used in evaluating cardiovascular prognosis in selected cohorts [Dwivedi et al 2007].

Peripheral endothelial function can also be assessed by venous impedance plethysmography which quantifies local blood flow in response to intra-arterial drug administration [Taddei et al 1995]. However, due to the partially invasive nature of this (administration of intravascular infusions) precludes its common use. Similarly, laser Doppler flowmetry (LDF) coupled with iontophoresis, which measures endothelial function at capillary level by scanning red cell flux in a small tissue sector is another method of ED assessment [Yoshida et al 2010].

1.2.4 Plasma Markers

The endothelium secretes a number of substances and logically it raises the question if these substances can act as surrogate markers for ED [Table 1-5]. Lately, several endothelial products have been successfully utilized for this purpose; for example, plasma Von Willebrand factor (VWF), have been used as marker of ED in a number of studies [Yaqoob et al 1993]. More recently, quantitative and qualitative analyses of certain endothelial cell strains have also been reported to be useful as putative ED markers [Shantsila et al 2007 (a)]. The routine use of such substances has their individual pros and cons and is discussed in this section.

1.2.4.1 Von Willebrand Factor (VWF)

This glycoprotein is released from endothelial cells and, as demonstrated by severe von Willebrand's disease (which resembles haemophilia), has an integral role in thrombogenesis [Vischer et al 2006]. As a strong association has been reported between ED and VWF levels [Figure 1-3]; the latter has been meticulously investigated in recent years and can be regarded as a surrogate marker for the presence as well as progression of ED [Lip et al 1995][Jansson et al 1991]. However, plasma VWF levels are greatly affected by genetic (ABO blood group and VWF gene mutations) and non-genetic factors (ageing, diabetes and oxidative stress). Therefore, the future role of VWF as a 'gold standard' biomarker of ED greatly depends upon validation studies of such genetic and non-genetics determinants [Vischer et al 2006]. Finally, the acute phase response of VWF results in high levels of this protein in inflammatory disorders and malignancy, and in such disease groups VWF can not be utilized as a robust ED biomarker [Pottinger et al 1989]. Despite these limitations, the results from studies predicting stroke risk in the elderly are promising and similar studies might further refine the role of VWF in ED [Conway et al 2003].

1.2.4.2 Endothelin (ET)

Endothelin (ET) is a 21-amino acid vasoconstrictor peptide and was first described to have a vital role in vascular homeostasis in 1988 [Yanagisawa et al 1988]. Later, genetic analysis demonstrated three distinct genes encoding for three structurally and pharmacologically distinct ET isoforms named ET-1, ET-2, and ET-3 [Inoue et al 1989]. The vasoconstrictor effect of ET is mediated by ET-A and ET-B receptors located on vascular smooth muscles.

ET is a mitogenic, vasoconstrictor and pro-inflammatory molecule and is found elevated in diseases associated with ED such as hypertension (HTN), diabetes mellitus (DM) and coronary artery disease (CAD) [Kishimoto et al 1995]. The association of endothelins with ED is also supported by data demonstrating reversal of ET mediated actions by blocking ET-A receptors in patients with atherosclerosis [Bohm et al 2002]. In addition, ET correlates well with other markers of ED [Vancheeswaran et al 1994]; however, limited understanding of the subcellular interaction(s) between ET and endothelial functions makes its use as biomarker of ED ambiguous at present.

1.2.4.3 Adhesion Molecules

These are a family of molecules that are located on the surface of endothelial cells, as well as various other cells. The origin and function of these circulating and soluble adhesion molecules are largely unclear, but they most likely originate from shedding or proteolytic cleavage from the cell surface and promote leukocyte adhesion.

Vascular cell adhesion molecule-1 (VCAM-1], endothelial–leukocyte adhesion molecule-1 (E-selectin) and intercellular adhesion molecule-1 (ICAM-1] are some of the more extensively studied molecules [Blann et al 1998 & 1999]. E-Selectin is exclusively expressed on endothelial cells and due to this exclusivity, could be used as a surrogate marker of ED [Newman et al 1993]. High levels of soluble E-selectin have been observed in conditions associated with ED such as HTN, cancer, DM and sepsis [Newman et al 1993]. Nevertheless, soluble E-selectin levels often fail to correlate with VWF levels in atherosclerosis or

connective tissue diseases [Blann et al 1994 & 1995] and therefore its precise role as a plasma marker of ED remains unclear. Plasma adhesion molecules, ICAM and VCAM, are also found on other cells in addition to endothelial cells, such as lymphocytes and fibroblasts, and hence not specific for the endothelium [Gearing et al 1993].

1.2.4.4 Soluble Thrombomodulin

Thrombomodulin is an integral membrane protein expressed on the surface of endothelial cells [Maruyama et al 1985]. It functions as a cofactor in the 'thrombin-thrombomodulin complex', leading to inhibition of fibrin formation, as well as platelet activation and activation of circulating protein C [Mitchell et al 1986]. Like soluble adhesion molecules, a truncated form of membrane thrombomodulin can be found in plasma (i.e. soluble thrombomodulin). Given the lack of correlation between soluble thrombomodulin levels, cytokines and fibrinogen, this proteoglycan is believed to reflect 'endothelial damage' rather than 'endothelial activation' [Seigneur et al 1993]. Whilst soluble thrombomodulin have been reported in the plasma and urine of healthy subjects, levels are grossly deranged in conditions associated with ED, such as sepsis, chronic renal failure, cancer and connective tissue disease [Gearing et al 1993][Newman et al 1993].

1.2.4.5 Nitric Oxide (NO)

As described before, majority of vasomotive endothelial functions are predominantly mediated through NO. Additionally, owing to its intracellular production, quantitative and/or qualitative analysis of NO could make this molecule an accurate surrogate marker for ED. However, complex sub cellular biochemical NO synthetic reactions render accurate laboratory measurement extremely complex. Although by-products of endogenous NO, found in plasma and urine, can be measured, these methods are indirect, expensive and of poor

reproducibility. A major problem with a commonly used biochemical method is that increased consumption of nitrate rich foods can also result in spuriously high levels [Ignarro et al 1990][Saccani et al 2006]. These facts, whilst supporting easy availability of NO, indicate that the reliability of measuring NO as diagnostic plasma/serum/urine marker of ED is still uncertain.

1.2.4.6 Asymmetric Dimethylarginine (ADMA)

ADMA is a competitive NO antagonist. It is produced as a metabolic by-product of a complex proteolytic cleavage process in human cell cytoplasm and is detected in the plasma and urine. There is ample evidence supporting the presence of elevated ADMA levels in conjunction with ED [Abbasi et al 2001]. In addition, its close biochemical link with homocystiene indicates the potentially significant contribution of ADMA in the initiation of ED and manifestations of atherosclerosis [Abbasi et al 2001]. Of note, folic acid induced endothelial improvement also supports the same notion [Holven et al 2003].

Hence, when considering the available data, ADMA seems to be emerging as a novel marker of ED; however, rapid fluctuations in plasma ADMA levels in response various factors such as high-salt diet, fatty meal consumption, pregnancy and renal impairment [Fard et al 2000] Holden et al 1998][Vallance et al 1992] are the major limitations to its routine use.

1.2.5 Cell Markers

1.2.5.1 Circulating Endothelial Cells (CECs)

Circulating endothelial cells (CECs) represent mature endothelial cells driven from the vessel wall in response to endothelial injury, and rarely exist in healthy individuals in detectable quantities [Erdbruegger et al 2006]. As quantitative assays of CECs have been shown to have a linear correlation with endothelial integrity; these cells could be regarded as a novel and

reliable surrogate biomarker of endothelial integrity [Lee et al 2005][Erdbruegger et al 2006]. High CEC counts are found in a number of disease states associated with ED. Enumeration of elevated CECs levels in acute coronary syndrome, stroke, DM, and peripheral vascular disease could act as a diagnostic marker in addition to reliably indicating disease severity and prognosis [Lee et al 2005][Nadar et al 2005][Sinzinger et al 1986]. Although estimation and enumeration of CECs is complex, technologies such as immunobeads isolation and fluorescence-mediated flow cytometry have made this a plausible proposition.

1.2.5.2 Endothelial Progenitor Cells (EPCs)

Bone marrow-derived circulating endothelial progenitor cells (EPCs) help maintaining vascular integrity by restoring endothelial continuity and neo-vascularization of ischaemic tissues [Kawamoto et al 2007]. Although the exact mechanism resulting in the mobilization of EPCs largely remains unknown, a spectrum of biochemical substances such hypoxia and vascular endothelial growth factor (VEGF) have been described as mediators of this process [Hristov et al 2004]. EPCs not only mediate neovascularization having been demonstrated in transplanted limbs but have also been useful in predicting cardiovascular prognosis [Kawamoto et al 2007].

These concepts have led to further improvements in efficient isolation, mobilization, recruitment, and transplantation of EPCs as a therapeutic approach. Since these cells are related to endothelial repair and regeneration, depletion of EPCs in conditions associated with ED, leads to impairment of the repair process and thus promote endothelial and vascular damage. Of note, the extent of EPCs paucity can also be correlated with disease severity [Valgimigli et al 2004]. Therefore, depletion or extremely low EPCs levels in smoking, DM, and atherosclerosis suggests that EPCs may be a consistent and robust *in vivo* early marker of ED [Tepper et al 2002][Kondo et al 2004] [Vasa et al 2001]. However, since EPCs diminish with age, the correlation with ED may not be possible in the elderly population [Scheubel et al 2003].

1.2.6 Inter-relationships between different Methods of Endothelial Assessment

The availability of a wide variety of ED assessment tools introduces a possibility of wide variability among different methods. As yet, there are very few randomized and blinded data comparing invasive and non invasive methods to demonstrate agreement between these techniques.

Coronary vaso-reactivity and brachial artery FMD were compared in a study on subjects with angiographically normal arteries [Teragawa et al 2005]. The results suggested that brachial FMD significantly correlates with coronary diameter and flow changes indicating reasonable agreement between the methods. Similarly, FMD has also been compared with plasma markers such as VWF [Chong et al 2004][Figure 1-3]. Here again, authors reported a significant inverse correlation between FMD and VWF ($p=0.001$).

Figure 1-3: Correlation between von Willebrand factor and Flow Mediated Dilatation

Correlation line between FMD and vWf shown, with 95% confidence intervals [FMD= flow mediated dilatation; VWf=von Willebrand factor] Correlation between FMD and vWf: r=-0.517, p<0.001. Reproduced from Felmeden DC *et al*., Blood Coag Fibrinolys 2003; 14: 425-431, with permission

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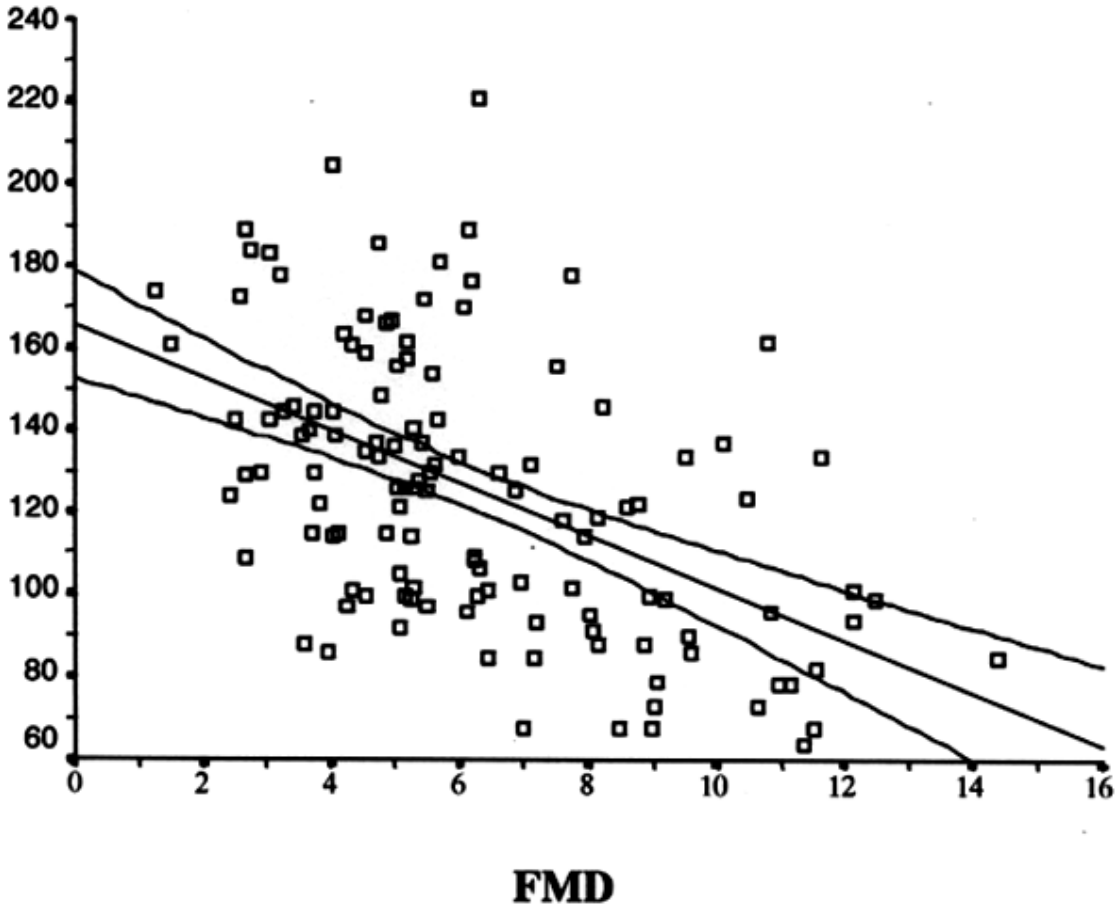


Further larger studies using newer methods (such as new biological and biochemical marker) are needed to test the correlation between the different methods of endothelial assessment.

1.2.7 Endothelial dysfunction in relation to cardiovascular risk factors

Endothelial dysfunction (ED) has been demonstrated in prominent prognostic cardiovascular risk factors such as DM, HTN) and smoking [Figure 1-2] [Table 1-5], leading to the hypothesis that ED could be the final common pathway for major cardiovascular risk factors. However as yet, it is largely unclear if the prognostic power of these risk factors is secondary to the presence of ED.

1.2.7.1 ED and Demographic variables

The effects of ageing on endothelial functions have been investigated in human models and generally the results seem to support that ageing is associated with attenuated NO production [Toda et al 2012][Taddei et al 1995]. For example, ACH induced changes in coronary artery diameter were investigated in an elderly cohort and the results seem to suggest that ageing *is* associated with attenuated EDV [Taddei et al 1995]. Age related attenuation of the NO levels is probably the consequence of the 'vascular senescence' phenomenon [Minamino et al 2004]. The latter is described as a stage when the vascular cell growth is arrested after completing their life span. These senescent cells are abundantly present in atherosclerotic lesions, express proinflammatory molecules reducing the in vivo NOS levels, and thus contribute to impaired NO production [Minamino et al 2004]. In addition, evidence from animal studies suggests that age related enhanced oxidative stress could also be another contributing factor [Blackwell et al 2004].

Obesity also has a profound impact on endothelial function as demonstrated in a large study (n=397) investigating the impact of body mass index (BMI) on coronary vasomotor response [Campia et al 2012][Al Suwaidi et al 2001]. In multivariate regression analysis, obesity was found an independent predictor of poor coronary blood flow and vasoreactivity [Al Suwaidi et al 2001]. Although pathophysiological pathways explaining this association largely remain obscure; intracellular accumulation of certain fatty acids could augment the oxidative stress

and cause ED [Bakker et al 2000]. Long chain fatty acyl co-enzymes-A esters may also inhibit endothelial mitochondrial function, resulting in impaired adenosine translocation leading to over production of ROS, ultimately resulting in manifestations of ED [Bakker et al 2000].

1.2.7.2 ED and Smoking

Active or passive, smoking has an independent and dose-dependent relationship with ED and atherosclerosis [Celermajer et al 1993 & 1996]. Nicotine is converted by the liver into various metabolites such as cotinine, cotinine-N-oxide, nicotine-1'-N-oxide and norcotinine. These metabolites increase the expression and the activity of angiotensin converting enzyme [Ljungberg et al 2008] which subsequently promotes the over production of angiotensin II. The latter result in the production of superoxide ions and enhances vasoconstriction [Rajagopalan et al 1996] and may be responsible for the development of ED. Interestingly, smoking causes raised VWF [Lip et al 1995], and also predisposes to the formation of white thrombi (platelet thrombus) which, though clinically quiescent [Hung et al 1995], are frequently reported on atherosclerotic plaques [Davies et al 1988]. In an environment of impaired coagulation (such as ED), these so called 'benign white thrombi' might proliferate leading to an acute coronary occlusion.

1.2.7.3 ED and Diabetes Mellitus

ED is dominantly reported in association with insulin dependent (IDDM) and independent diabetes (NIDDM), and indicates a high risk of developing micro- and macro-angiopathic complications [Johnstone et al 1993]. Although underlying mechanisms are broadly similar, ED is more frequently associated with angiopathic complications in NIDDM [Neri et al 1998]. In a large cohort (n=209) of IDDM patients, for example, those who developed

microalbuminuria after a 10 year follow up had significantly impaired endothelial functions compared to those patients who did not develop microalbuminuria [Myrup et al 1994]. Similarly, in a relatively smaller study of 70 IDDM patients, ICAM and VCAM levels were substantially raised in patients with retinopathy compared to those without diabetic eye disease [Fasching et al 1996].

It is believed that persistently elevated blood glucose levels cause glycosylation of the phospholipids and proteins inducing increased intra-cellular oxidative stress. These glycosylated products are initially reversible but after undergoing complex biochemical reactions, they transform into more stable and irreversible molecules which generate ROS and increase oxidative burden [Calles-Escandon et al 2001]. Additionally, the role of ED in intracellular depletion of nicotinamide adenine dinucleotide phosphate (NADPH) and over expression of growth factors in subject with DM could also provide insight into diabetes related neovascularization and atherogenesis [Calles-Escandon et al 2001].

1.2.7.4 ED and Hypertension

ED has been demonstrated in the peripheral as well as coronary circulation of hypertensive patients In a large study of 111 hypertensive patients, coronary Doppler recordings showed significantly diminished coronary vasoreactivity to both, ACH and adenosine infusions, indicating dysfunctional endothelium [Hamasaki et al 2000]. FMD of the brachial artery in hypertension may be less sensitive than in-vitro measurement in resistance arteries, whilst EDV in the human forearm and coronary vasculature are strongly correlated [Teragawa et al 2005]. Another indirect method of endothelial assessment is to visualize the intima and media thickness (IMT) of the carotid artery [Sramek et al 2000]. Strictly speaking, this is not a routine test of assessment of “endothelial function”, but IMT measurement is believed to reflect the global endothelial state and overall atherosclerotic burden on the large arteries.

Similar to the FMD, IMT measurement also has issues regarding observer dependency, but a good correlation has also been shown between IMT and FMD [Hashimoto et al 1999].

Other tests such as response of blood flow as measured by plethysmography to intra-arterial infusions such as, acetylcholine, methacholine or nitroprusside, are also frequently used to study the effect of drugs on vascular and endothelial function in hypertension. Unsurprisingly, the use of anti-hypertensive therapy is associated with an improvement of endothelial function [Nadar et al 2004].

ED per se could even lead to HTN and vice versa and there are several explanations for this [Figure 1-2]. Clearly, strong and well documented evidence supports the concept of endothelial disturbance in hypertension [Spencer et al 2002][Panza et al 1993] with relationships to the severity of carotid artery atherosclerosis and abnormalities in coagulation profile, which may contribute to an accentuated risk of stroke [Lip et al 1994]. However, it is unclear if these changes are of aetiological nature or simply the consequences of hypertension [Luscher et al 1994].

With the constant exposure to high blood pressures in HTN, certain changes occur in endothelium that 'activates' it. However, endothelial activation, regardless of different versions of its definitions by molecular biologists and immunologists, ultimately leads to the enhanced production of procoagulant and inflammatory agents [Blann et al 2000][Harrison et al 2003]. Hence, with persistent exposure to the high-pressure blood flow, accentuated inflammatory mediators and the effects of the neutrophils and platelets adhered to the endothelium; the latter becomes damaged [Pearson et al 1994]. This 'damaged' or 'dysfunctional' endothelium then participates in a vicious cycle whereby the blood pressure tends to remain elevated due to consistent changes in NO and endothelin bioavailability.

Conversely, ED related lack of NO, oxidative stress, impaired coagulation profile and exaggerated vascular smooth muscle proliferation could lead to increased peripheral vascular resistance [Buga et al 1991]. As the blood pressure is directly related to cardiac output and

peripheral vascular resistance, the hemodynamic effects of the latter could contribute to the development of HTN. It has been reported that cyclooxygenase dependent vasoconstrictor pathway contributes to the development of ED in essential hypertensive patients [Taddei et al 1993]. Others, whilst investigating the vascular effects of NG monomethyl L arginine (an arginine analog) in essential hypertension, report a defect in the endothelium-NO system as a root cause of ED development in HTN [Panza et al 1994].

1.2.7.5 ED and Dyslipidemia

There is a linear relationship between dyslipidemia and CAD risk [Multiple Risk Factor Intervention Trial Group 1982]. Studies carried out to determine statin's abilities demonstrated they have multiple pleiotropic effects (i.e. cholesterol-independent benefits). These properties are highly diverse and assume such effects such as the improvement of endothelial status-damaged by multiple pathologic conditions- with secondary increase in plasma NO bioavailability [Agewall et al 2006]. Enhanced production of reactive oxygen species (ROS) due to hypercholesterolemia, and in conditions with increased low density lipids (LDL) and/or reduced high density lipids (HDL) levels could impair endothelial functions [Casino et al 1993]. In addition, oxidized LDL also down- regulates the activity of endothelial NOS, and thereby induces ED [O'Driscoll et al 1997]. Antioxidant ability of the statins manifests in various ways, such as reduction in the ROS production, fact that makes them useful in the treatment of the conditions like dyslipidemia or cardiovascular diseases [Agewall et al 2006].

1.2.8 Role of Endothelial Dysfunction in Cardiovascular Prognosis

It is evident that a variety of cardiovascular risk factors significantly contribute to the development of ED [Table 1-6]. Based on such links, ED could be the *final common pathway* shared by most cardiac risk factors and could have a crucial role in the cardiovascular prognosis.

Table 1-6: Important Prognostic Studies on Endothelial Dysfunction

Reference	Study design					Conclusion
	n	*ED assessment Method*	*ED marker*	*Follow up*	*Primary end points*	
Modena et al 2002	400	Brachial FMD	EDV and EIV	67 months	Any cardiovascular events (non specified)	Highly significant event rate in patients with poor FMD ($p< 0.0001$), inclusion of only post menopausal women was a limitation
Halcox et al 2002	308	Intracoronary studies	Coronary response to ACH	46 months	Cardiovascular events	11% event rate, ACH induced coronary vasoconstriction is predictive of CV events
Heitzer et al 2001	281	Brachial FMD	EDV and EIV	4.5 years	Death, MI, stroke, PTCA, CABG	Worse outcome in patients with poor ED ($p<0.001$) and EIV ($p<0.05$). Significant improvement in vasomotor response was noted with vitamin C ($p=0.001$)
Perticone et al 2001	225	Brachial FMD	EDV and EIV	31.5 months	Any vascular event (MI, stroke, PVD)	8.17% event rate, observer bias can not be ignored in FMD
Gokce et al 2003	199	Brachial FMD	EDV and EIV	1.2 years	Death, MI, USA, stroke, PTCA, CABG	FMD markedly reduced in patients with events ($4.4 \pm 2.8\%$ compared with those without an event $7.0 \pm 4.9\%$, $p < 0.001$).
Suwaidi et al 2000	157	Intracoronary studies	Coronary response to	28 months	Cardiac death, MI, PCI	Presence of ED is significantly associated with future risk of adverse cardiac events

			ACH			($p<0.05$)
Schächinger et al 2000	147	Intracoronary studies	Coronary response to ACH, CPT	7.7 years	Cardiac death, MI, PCI, CABG	19% event rate, vasomotor responses are predictive of cardiac events

ED: endothelial dysfunction, ACH: acetylcholine, USA: unstable angina, MI: myocardial infarction, PCI: percutaneous coronary intervention, PTCA: percutaneous Transluminal coronary angioplasty, CABG: coronary artery bypass grafting, CPT: cold pressor test, CV: cardiovascular, PVD: peripheral vascular disease, FMD: flow mediated dilatation, EDV: endothelial dependent vasodilatation, EIV: endothelial independent vasodilatation

In order to test this concept, a number of invasive and noninvasive studies have investigated the relationship between ED and cardiovascular prognosis [Table 1-5, 1-6]. Halcox et al investigated the coronary endothelial function invasively and noted the occurrence of unpredictable cardiovascular events (cardiovascular death, myocardial infarction, stroke, and unstable angina) in healthy subjects and patients with CAD on follow up [Halcox et al 2002]. The authors reported that coronary EDV response to ACH was a strong and independent predictor of future cardiovascular events ($p=0.003$); although EIV was not associated with worse cardiovascular outcome. Similar results were also reported by Schächinger et al, who observed a 19% relative increase in the risk of cardiovascular events in patients with CAD and poorer endothelial dependent responses [Schächinger et al 2000].

Thus, the *existence* of ED in coronary arteries is associated with poor cardiovascular outcome. However, whether the *severity and extent* of ED would have an impact on clinical outcome, remained unclear. To address this issue, Suwaidi et al compared patients with different grades/severity of ED (on the basis of their response to ACH) and found significantly higher incidence of cardiac events in patients with severe ED compared to those with the milder form ($P<0.05$) [Suwaidi et al 2000]. These findings reinforce the general belief that the extent of ED has an additional impact on cardiovascular prognosis.

Similar to the invasive techniques, the non-invasive methods of ED assessment have also been used to predict adverse cardiovascular prognosis in a number of studies. In one study by Perticone et al, endothelial status was investigated in 225 hypertensive patients using brachial FMD [Perticone et al 2001]. The patients in the lowest FMD tertile had the worst prognosis, demonstrating a link between *occurrence* and *severity* of ED with cardiovascular prognosis. Similarly, in another study using FMD with prolonged follow up (4.5years), higher rates of cardiovascular events (32%) were noted in patients with poor ACH induced vasodilatory response [Heitzer et al 2001].

Similarly, plasma indices have also been utilized in a number of recent studies investigating cardiovascular prognosis. For example, VWF levels were found profoundly and significantly elevated in patients admitted with acute coronary syndromes and levels were independently predictive of major adverse cardiovascular events (MACE) [HR 1.02 (95% CI 1.005-1.040); P = 0.009][Boos et al 2008]. Earlier, Lip et al also concluded that hypertensive patients who experienced new cardiovascular events had more ED, as demonstrated by elevated D-dimer and VWF levels [Lip et al 2002]. Finally, these observations were also supported by findings from Anglo-Scandinavian Cardiac Outcome Trial (ASCOT) where among high risk patients with hypertension, raised levels of P-selectin were found predictive of myocardial infarction [Varughese et al 2007].

As a close agreement exists between invasive and no invasive methods as described above [Teragawa et al 2005][Chong et al 2004], a variety of non-invasive methods can be reliably utilized for the assessment of cardiovascular prognosis. Further larger studies using newer methods (such as new biological markers) are needed to test the prognostic value of ED.

1.2.9 Management of Endothelial Dysfunction

ED is potentially a reversible condition. Several physiological and pharmacological therapeutic strategies have proven to improve ED. These include, for example exercise, folic acid, ACE inhibitors, statins and antidiabetic drugs [Table 1-7]. It can be hypothesized that the prognostic benefits of some pharmacological agents (such as statin, ACE Inhibitors) may secondary to endothelial improvement with the use of these agents.

Table 1-7: Important Interventional (Physiological and Pharmacological) Studies on Endothelial Dysfunction

Reference	Study Design					Results and Comments
	n	*Intervention*	*Study groups*	*ED markers*	*Follow up*	
Mancini et al 1996	155	ACEI	51 patients were randomized to 40mg quinapril compared to placebo (n=54)	Intra coronary studies	6 months	In this double blinded study, Quinapril group showed significant improvement in coronary dynamics versus placebo. This was possibly due to better oxidative profile
Böger et al 1998	80	L-Arginine	49 hypercholesterolemic versus 31 healthy subjects	ADMA	Nil	In this double blinded trial, ADMA levels were found raised in disease group ($p<0.05$). Levels normalized after L-arginine administration ($p<0.01$).
De Souza et al 2000	68	Exercise	Healthy men underwent a home-based aerobic exercise	Plethysmography	3 months	30% increase in ACH-mediated vasodilatation after exercise ($p<0.01$) Suggested regular aerobic exercise can retard age-related attenuation in vascular responses EDV
Dupuis et al 1999	60	Statins	ACS patients (n=30) versus healthy volunteers (n=30)	FMD	6 months	Compared to placebo, EDV improved ($p=0.02$) with no concurrent changes in coagulation profile or endothelin levels, in statin group
Ziaccardi et al 2002	56	Weight Reduction	Obese females underwent strict weight reduction	TNF-α, IL-6, ICAM-1, VCAM-1 and P-sel	12 months	10% weight reduction significantly reduces cytokines ($p<0.01$) and adhesion molecules ($p<0.02$). However exclusively young

			programme			female included. Obese group have higher levels at baseline
Mather et al 2001	44	Metformin	1gram/day Metformin in 29 DM patients versus placebo (n=15)	Plethysmography	12 weeks	Subjects randomized to Metformin demonstrated marked improvement in ACH-stimulated flows compared to other group (p= 0.002). Insulin resistance was found to be the sole predictor of EDV
Quyyumi et al 1997	39	L-Arginine	32 CAD patients compared with 7 healthy volunteers	Intra coronary studies	Nil	Intracoronary infusion of L arginine enhanced EDV significantly (p<0.001). however, small and non-blinded study
Prasad et al 2000	31	ARB	Effects of Losartan were on a cohort of patients with atherosclerosis	FMD	8 weeks	Small study yet showed improvement in FMD after ARB therapy (1.4±0.9% to 3.2±0.8%, P=0.03)
Ting et al 1997	23	Vitamin C	Dyslipidemic (n=11) versus healthy subjects (n=12)	Plethysmography	Nil	Vitamin C improves EDV in dyslipidemic (p=0.001) supporting anti-oxidant actions of Ascorbic acid
Husain et al 1998	19	Aspirin	19 patients with documented CAD and/or coronary risk factors.	Doppler flow velocities	Nil	Intravenous Aspirin significantly improved ACH-mediated femoral vascular resistance index (19 ±5%, p=0.002). intravenous use is exceedingly rare in practice
Pistrosch et al 2004	12	Rosiglitazone	12 Patient received Rosiglitazone and	Plethysmography	12 weeks	Small double blind study demonstrating increased forearm blood flow post Rosiglitazone significantly (12.8 ± 1.3

			Nateglinide			versus. 8.8 ± 1.3 ml/100 ml; P < 0.05)

ED: endothelial dysfunction, ACH: acetylcholine, SNP: sodium nitroprusside, FMD: flow mediated dilatation, EDV: endothelial dependent vasodilatation, EIV: endothelial independent vasodilatation, DM: diabetes mellitus, ACS: acute coronary syndrome, ACEI: angiotensin converting enzyme inhibitor, ARB: angiotensin receptor blockers, CAD: coronary artery disease, ADMA: Asymmetric dimethylarginine, MDT: multidisciplinary team, TNFα: tumor necrosis factor α, IL-6: interleukin-6, ICAM-1:intercellular adhesion molecule-1, VCAM-1: vascular cell adhesion molecule-1, P-sel: P-selectin

Several possible mechanisms have been postulated to explain how such pharmacological interventions improve the endothelium. For example, therapeutic use of L-Arginine (precursor of NO) is believed to increase the concentration and activity of endothelial NO. In addition, reduction in plasma ET-1 levels, enhanced apoptosis of vascular lesion cells and normalization of elevated ADMA levels have been described as supplementary benefits of L-Arginine [Böger et al 1998]. Other agents such as ACE inhibitors and statins may to improve ED possibly by reduction in superoxide ions, attenuation of contractile effect of angiotensin and enhanced increase in NO release from endothelial cells [O'Driscoll et al 1997][Mancini et al 1996]. Such improvement in oxidative stress by statins is in addition to its cholesterol lowering effects [Dupuis et al 1999].

Furthermore, the evidence from the last two decades strongly support the prognostic benefits obtained in different populations with ACE inhibitors and statins [ONTARGET Investigators 2008][Yusuf et al 2000]. Whilst evidence as described above clearly demonstrates improvement in endothelial status with the use of these agents, how much of such endothelial improvement translates into the prognostic benefits remains largely unclear. Larger prognostic studies investigating pharmacological interventions with set primary and secondary end points and concomitant endothelial assessment are needed to settle this debate.

1.2.10 Conclusion

The endothelium is a unique vasoregulatory organ which secretes a number of molecules to achieve and maintain the vascular equilibrium. Any derangement in the endothelium has significant negative consequences. Ample evidence suggests that major cardiovascular risk factors impair endothelial function inducing a state of ED and this could predispose to the development of atherosclerotic plaques if left unchecked. Since ED holds such a critical

position in the development of various cardiovascular disorders, this demands its accurate assessment for risk stratification. Over the years, a large number of useful invasive and noninvasive techniques have been utilized to assess ED accurately. Endothelial functional status can be improved by using a number of physiological and/or pharmaceutical agents. Improved prognosis with primary prevention strategies could suggest that improved ED could lead to better outcome. However, data supporting this point are not only limited but also indirect. The answer probably lies in further large studies with cardiovascular primary and secondary end points and prolonged follow up.

Section 2 Hypotheses and Methodology

2.1 Hypotheses

The aims of the present study were to test the following three hypotheses:

1. Significant perfusion abnormalities are demonstrable in OSA using MCE, compared with disease and healthy controls.
2. Degree of perfusion abnormalities demonstrated in OSA with MCE correlates with established markers of endothelial damage/dysfunction.
3. Myocardial perfusion and endothelial abnormalities in OSA patients improve with prolonged CPAP therapy.

2.2 Subject Recruitment

2.2.1 Study Population

Forty five stable, newly diagnosed moderate/severe (apnoea hypopnoea index>15 diagnosed by multi channel polysomnography [FSI Grey Flash recorder, Stowood Scientific Instruments Ltd, Oxford UK]) OSA patients were selected from sleep laboratory at City Hospital Birmingham, UK. These patients were compared with 45 healthy controls and 45 stable, treated hypertensive controls (disease controls). OSA was excluded from both control groups using multi channel sleep study. The hypertensive subjects were recruited from hypertension clinic whilst healthy subjects were selected from patients' relatives, staff and from the hospital records of sleep study negative patients [Table 2-1].

Table 2-1: Clinical Characteristics of Obstructive Sleep Apnoea patients, Hypertensive and Healthy Controls

Variable	OSA	HTN	Healthy	p
n	45	45	45	
Male/Female	34/11	30/15	30/15	0.21
Age (years)	50(9)	49 (12)	46(7)	0.120
Aponea Hypopnea index	33 (21-50) *Ψ	4(1.2-5.8)Ψ	3(0.9-5)*	<0.01
Body mass index (Kg/m2)	34(8)	32(6)	32(6)	0.118
Systolic Blood pressure (mmHg)	142(16)	150(21)^	135(15)^	<0.01
Diastolic Blood pressure (mmHg)	83(11)	85(10)	82(9)	0.22
No. of current smokers	14	12	16	
Comorbidities				
Treated hypertension	0	45	0	
Treated dyslipidemia	0	0	0	
Known coronary artery disease	0	0	0	
Diabetes Mellitus	0	0	0	
Past stroke/TIA	0	0	0	
Current drug therapy				
ACE inhibitors	0	23	0	
ARB	0	20	0	
Calcium channel blockers	0	20	0	
Beta blockers	0	15	0	

Aspirin	0	34	0	
Diuretics	0	7	0	

Values are described as Mean (Standard Deviation) and Median (Inter-quartile Range). P <0.05 was considered statistically significant.

* Significance between OSA and Healthy subjects
^ Significance between Hypertensive and Healthy subjects
Ψ Significance between OSA and Hypertensive subjects

OSA= Obstructive Sleep Apnoea, HTN= Hypertension, TIA= Transient Ischemic Attack, ACE= Angiotensin Converting Enzyme, ARB= Angiotensin Receptor Blockers

All study participants were deemed otherwise healthy after careful interview, detailed clinical examination, baseline blood tests (Full blood count, renal function tests, liver function tests, inflammatory markers, blood glucose, thyroid and cholesterol profile) electrocardiogram (ECG) and transthoracic echocardiography.

All study subjects fasted for 12 hours and abstained from smoking, alcohol, tea and coffee for 24 hours prior to the study. Hypertensive subjects were advised to omit their medications on the study day, as prolonged treatment omission was deemed unethical. All scans were performed in a quiet, darkened, temperature controlled room after patient rested for 15-20 minutes. Blood pressure (BP) measurement and baseline venous blood sampling were performed prior to any scanning, from the left and right arm respectively whilst subjects were rested in supine position.

After baseline measurements, the OSA subjects were autotitrated with an automated CPAP device before being issued with a fixed pressure CPAP (REMstar Pro M Series with C-Flex - Phillips Respironics, Pennsylvania, USA). CPAP compliance was monitored/recorded throughout the study at regular intervals. Satisfactory CPAP compliance was defined as a

minimum usage of 4 hours per night for at least 75% of the week's nights (i.e. more than 5 nights per week). After a mean duration of 26 weeks on CPAP therapy, all available OSA subjects were followed up.

Ethical approval was granted by local research ethics committee and written informed consent was obtained from all participants.

2.2.2 Exclusion criteria

Patients with confounding effect on endothelial function such as those with pre-existing diabetes mellitus (defined according to WHO criteria; fasting plasma glucose more than 7.0 mmol/L or 2-hour post-prandial plasma glucose of more than 11.1mmol/L, and/or the patient taking on anti-diabetic treatment such as insulin or oral therapy or on dietary management), hyperlipidaemia (defined as patients taking antidyslipidemic drugs) and coronary artery disease (such as previous myocardial infarction or revascularisation procedure) were excluded from this study. Patients with known acute or chronic conditions such as with cardiovasucular disease disease, cerebrovascular disease, malignancy, connective tissue or inflammatory disease, hepatic/renal impairment and acute infections were excluded from the study.

2.2.3 Study Design

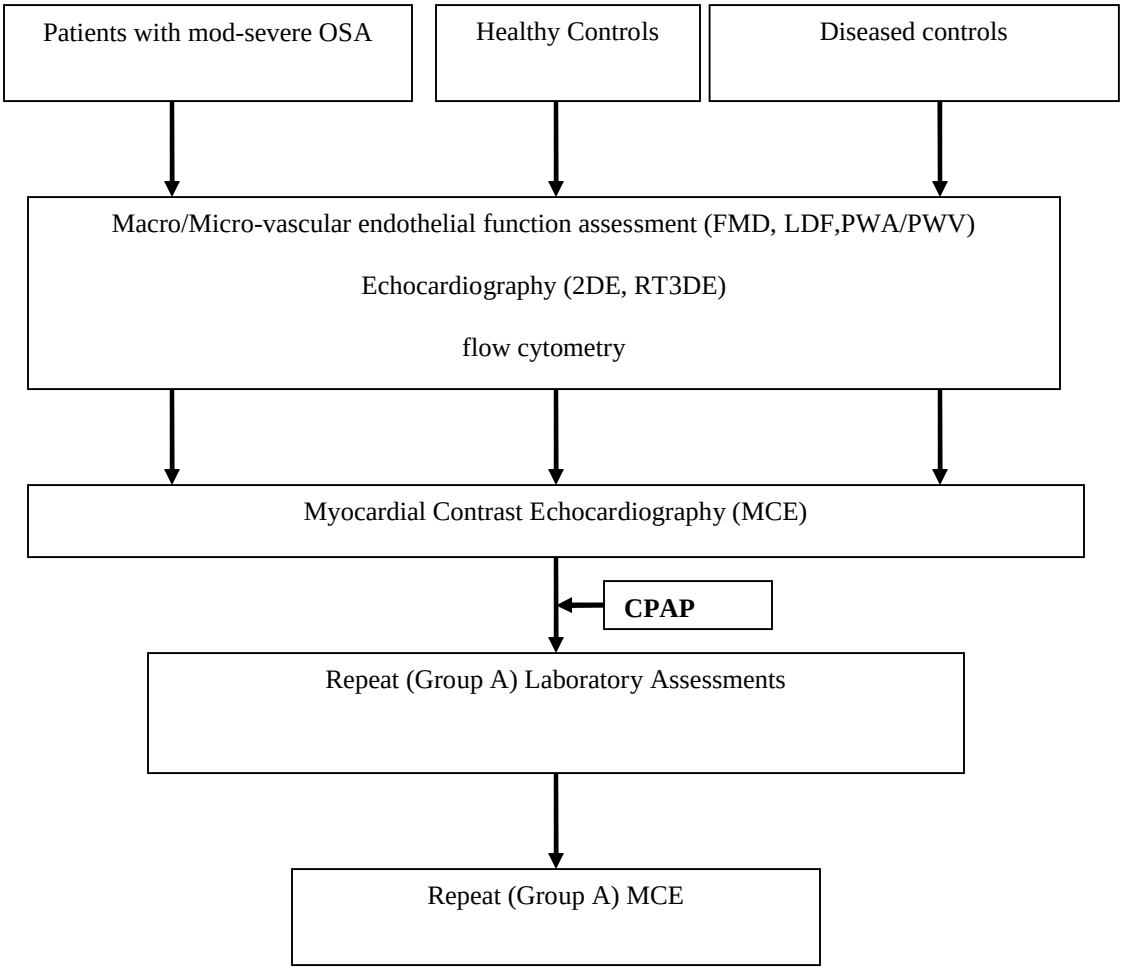
2.2.3.1 Cross-Sectional Study

I compared 'OSA patients', 'healthy controls' and 'disease controls' in the cross-sectional arm. The three groups were well-matched for age, sex and body mass index and underwent the tests as detailed in the next section [Figure 2-1].

2.2.3.2 Longitudinal Study:

All OSA patients were prescribed with CPAP therapy and were followed up after six months of treatment [Figure 2-1]. The pressures and compliance was ensured in first month after prescription and adequate ventilatory pressures were set. Poorly compliant subjects were excluded.

Figure 2-1: Study Design



OSA= Obstructive Sleep apnoea; 2DE=2-dimensional echocardiography;RT3DE=Real time three dimensional echocardiography;FMD= Flow mediated dilatation;LDF=Laser Doppler flowmtry;PWA=Pulse wave analysis;PWV= Pulse wave velocity;FC= Flow cytometry;CPAP= continuous positive airway pressure

2.3 Methods for assessment of Endothelial function

2.3.1 Flow Mediated Dilatation (FMD)

All images were acquired with a GE Vingmed System 5 ultrasound system using a hand-held 10 MHz vascular ultrasound probe in accordance with the international guideline [Corretti et al 2002]. High resolution ultrasound scanning of right brachial artery was performed 3-5 cm above the antecubital fossa whilst patients lying flat. Anterior to posterior wall diameters (leading edge to leading edge) were recorded synchronized by R-wave on the ECG. Endothelium-dependent dilation was assessed by response to flow-mediated hyperaemia. The sphygmomanometer cuff placed around the right upper arm was inflated above the systolic blood pressure for 4.5 minutes followed by prompt deflation and recording of brachial artery image for 5 minutes. Once the baseline brachial artery diameter and flow restored, endothelium-independent dilation was assessed 3 minutes after administration of 0.4mg glyceryl trinitrite (GTN, Nitrolingual® Hohenlockstedt, Germany). Endothelial responses were estimated as percentage of the brachial diameter changes compared to baseline levels.

2.3.2 Laser Doppler flowmetry (LDF)

Assessment of microvascular endothelial function has been performed using scanning laser Doppler flowmeter (Periscan system PIM II, Perimed AB, Stockholm, Sweden, 780 nm red laser) with iontophoresis (0.1mA for 60 sec.) of 2% acetylcholine (ACH- to evaluate endothelial-dependant response) and 1% sodium nitroprusside (SNP- to evaluate endothelium-independent response) [Yim-Yeh et al 2011]. The current was delivered through two drug delivery chambers (Model LI 611, Perimed, Sweden) placed on ventral aspect of

right upper forearm 5 cm apart, and were connected to current intensity-regulated generator (Perilont, Perimed AB).

Three baseline scans were taken to measure the mean baseline perfusion. 2% ACH (as an endothelium-dependent vasodilator) was iontophoresed for 60 sec at 0.1 mA followed by pulse scanning (each scan for 38 seconds) for minimum of 7 minutes. Similarly 1% SNP was delivered once for 60 seconds at 0.1 mA which was followed by seven minutes of pulse scanning. Mean baseline perfusion, mean maximum perfusion and maximum percentage change in a region of interest (ROI), were calculated using Perimed software.

2.3.3 Assessment of Arterial Stiffness using Pulse wave analysis/velocity

Pulse wave analysis and velocity was measured using Sphygmocor (Sphygmocor, Atcor medical, Sydney, Australia). Carotid arterial waveforms were recorded non-invasively over 10 seconds using a high-fidelity hand-held tonometer [Wilkinson et al 1998]. Aortic augmentation (AA), augmentation index (AIx), rate adjusted augmentation index (AIx@75) and sub-endocardial viability ratio (SEVR) were recorded using sphygmocor CVMS software system (Version 8).

After performing the baseline measurements, waveform was recorded every five minutes for 20 minutes after 2 puffs of Salbutamol (Salamol CFC-free, IVAX pharmaceutical, Ireland). Subjects were rested till the pulse wave data returned to baseline, and waveforms were recorded again every 5 minutes for 20 minutes after the sublingual GTN administration (0.4 mg).

Aortic pulse wave velocity was also recorded by making sequential ECG-gated tonometer recordings at the carotid and femoral arteries. The straight-line distances between the sternal notch and both waveform measurement sites were determined. Pulse wave velocity was also estimated using sphygmocor CVMS software system (Version 8).

2.3.4 Flow cytometry

A fasting venous blood sample was taken from the left antecubital vein prior to any imaging and was collected into EDTA vacutainers. The first 10 ml of drawn blood was not used for cellular analysis to avoid contamination of the sample by endothelial cells dislodged by venepuncture. All samples were analyzed for flow cytometry (FACSCalibur, Becton Dickinson, Oxford, UK) within three hours of sampling. A full blood count was obtained simultaneously using hematoanalyser (Advia, Bayer).

For EPC and CEC quantification, 200ul of whole EDTA blood was mixed with 500ul of phosphate buffer solution (PBS) followed by incubation with fluorochrome-labelled monoclonal antibodies anti-CD45-PerCP (Becton Dickinson, Oxford, UK), anti-CD34-PE (Becton Dickinson, Oxford, UK), anti-CD146-FITC (Biocytex, France) and KDR-PE (R&D, UK) for 20 minutes in the dark at room temperature. The sample was then lysed with BD lysing solution® and washed once in PBS. The resultant pellet was then resuspended with PBS before running for flowcytometeric analysis.

The samples were analyzed using a 2 laser 4-colour FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK). A minimum of 1,000,000 mononuclear cellular events was analyzed per sample. Cells were plotted according their forward scatter and side scatter characteristics and gated to include only mononuclear cell events, excluding cell doublets, platelets, dead cells/debris and high side scatter events. In our project EPCs were defined as cells strongly positive for CD34++/KDR++ antibodies and CECs as cells strongly positive for CD34++/CD146++ antibodies.

Figure 2-2 illustrates flow cytometry immunophenotyping of circulating EPCs and CECs. A population of cells with distinct forward-scatter and side-scatter characteristics (R1) was shown to be strongly positive for CD34 expression, yet bearing low levels of CD45 [Figure

2-2A]. CD45 negative and positive events are plotted along the X-axis [Figure 2-2B]. A subset of R1 cells labelled as R2, demonstrating high levels of CD146, yet negative for KDR represented CECs [Figure 2-2C]. Similarly, EPCs were highly positive for KDR with little or no expression of CD146 [Figure 2-2D].

2.3.5 Echocardiography

In all subjects, 2DE, RT3DE and MCE was performed using Phillips iE33 ultrasound machine (Bothel, WA, USA). A modern off-line QLAB software [Xcelera, Phillip (iE33) Ultrasound Quantification Module, USA] was used for RT3DE volumetric assessment and estimation of myocardial perfusion.

2.3.5.1 Two Dimensional Echocardiography (2DE)

Para-sternal long axis, para-sternal short axis (at aortic valve level, mitral leaflet level, papillary muscle level and at apex) and standard apical views (4-chamber, 5-chamber, 2-chamber and 3-chamber) were acquired. The aortic root and valvular assessment was performed according to the American Society of Echocardiography's (ASE) recommendations [Quiñones et al 2002]. The ASE 16-segment LV model was used for analysis. LV diastolic dimensions, LV mass index, Left ventricular ejection fraction (LVEF) and left ventricular systolic/diastolic volume (LVESV/LVEDV) were assessed by using M-mode and the modified Simpson's biplane method [Senior et al 1994]. All above parameters and mitral annular systolic velocity (S') surrogated LV systolic function.

Assessment of the markers of acute diastolic function was performed by evaluating trans-mitral flow and tissue Doppler imaging (TDI). For every parameter, at least three samples of flow direction were taken sequentially and then the average was calculated. Trans-mitral flow was obtained in the apical 4-chamber position, where the pulsed Doppler and the sample

volume cursor were in parallel with the flow direction. Mitral early inflow peak velocity (E), late inflow peak velocity (A), deceleration time of E wave (DT) and A wave duration were calculated with conventional Doppler measurement technique. Colour-mapped tissue Doppler images (TDI) were obtained on apical 4-chamber view with a frame rate exceeding 175 frames/seconds. After TDI preset was activated, sample volume of pulsed Doppler was located at the corner of mitral annulus and septal wall and then lateral wall in apical four-chamber position. Peak velocities of early (E'), late (A') diastolic, systolic (S') wave and iso-volumetric relaxation time (IVRT) were calculated [De Boeck et al 2003].

The left atrial (LA) volume surrogates chronic diastolic dysfunction and was calculated as below:

1- Ellipse formula – (πxD1xD2xD3)/6, where D1=longitudinal dimension of LA in the 4-chamber view, D2=horizontal dimension of LA in the 4-chamber view, D3=vertical dimension of LA in para-sternal long axis view.

2- Area-length formula – (0.85 x 4-chamber LA area x 2-chamber LA area)/common length

3- Using RT3DE planimetry

Left atrial volume index was obtained by dividing left atrial volume by body surface area (Mosteller formula).

2.3.5.2 Real time Three Dimensional Echocardiography (RT3DE)

RT3DE dataset was acquired in apical windows. LV and LA images were taken by wide-angled acquisition (full-volume method) during end expiration. Using QLAB software the LV/LA volume was measured using a semiautomatic tracing of endocardial border at each frame during one cardiac cycle [Anwar et al 2008]; however automatic tracings were

manually modified if necessary. Papillary muscles, LA appendage and pulmonary vein apertures were excluded during analysis. Stroke volume (SV), LVEF and LVESV/LVEDV were recorded.

2.3.5.3 Myocardial Contrast Echocardiography (MCE)

Advances over the past fifteen years in the development of contrast agents and ultrasound equipment technology have allowed myocardial contrast echocardiography (MCE) to evolve from the experimental laboratory into clinical practice for the evaluation of ischaemic heart disease.

MCE utilises acoustically active gas filled microspheres (microbubbles) which have rheology similar to that of red blood cells, and are small enough to traverse capillaries, these unique properties allow assessment of the myocardial microcirculation which provides information about the epicardial coronary arteries.

2.3.5.3.1 Contrast agents

Contrast agents are typically 2 to 4 microns in size and consist of a gas encapsulated by a stabilising outer shell [Grayburn et al 2002]. To ensure successful negotiation of the pulmonary microvasculature and adequate visualisation of the myocardium, microbubbles have undergone a number of modifications over recent years. The prolonged survival of microbubbles has been achieved primarily through the use of stable outer shells and the substitution of air with a high density; high molecular weight gas both of which confer improved stability. Table 2-2 summarises the currently available contrast agents and their physical properties.

Table 2-2 Currently available ultrasound contrast agents

Agent	Bubble size	Gas	Shell composition
Albunex	4.5 μm	Air	Albumin
Levovist	2-3 μm	Air	None – bubbles adhere to galactose microparticles
EchoGen	2-5 μm	Perfluoropentane	Stabilised surfactant
Sonogen	2-5 μm	Perfluoropentane	Anionically charged surfactant
Optison	4.7 μm	Perfluoropropane	Albumin
Definity	1.5 μm	Perfluoropropane	Phospholipid
Sonovue	2.5 μm	Sulphur hexafluoride	Phospholipid
AI-700	2 μm	Perfluorocarbon	Synthetic polymer

The significant acoustic difference created between microbubbles and blood allows easy detection by specific ultrasound imaging techniques. Importantly, microbubbles oscillate at a rate of several million times per second in response to ultrasound waves, the same frequency used for echocardiography. Therefore, microbubbles not only act as passive reflectors but also as strong sources of sound. Oscillations generated thereby, have characteristics in received waves which are not present in transmitted waves. These signal characteristics are critical for effective separation of microbubble signals from tissue signals. Microbubbles are haemodynamically inert and remain entirely within the intravascular space. These two properties differentiate it from tracers used in other forms of cardiac imaging (such as PET, SPECT and CMR).

2.3.5.3.2 Principles of Myocardial Contrast Echocardiography

When insonated, gas bubbles pulsate with compression and expansion at the peak and trough of the ultrasound wave respectively. In an ultrasound beam with a frequency of 3MHz, bubble oscillation will reach three million times per second which is significantly more than the oscillation produced by the red cells. With this movement sound is generated and, combined with that of thousands of other bubbles it results in the scattered echo from the contrast agent. Characterising this echo, such that it can be differentiated from that of tissue, improves the sensitivity of contrast ultrasound and is the basis for new contrast specific imaging modes. Unlike solid tissue, gas bubbles have acoustic properties that vary with the strength of the insonating signal. With increasing power, insonation of gas bubbles can result in linear oscillation, non-linear oscillation or bubble destruction [Stewart et al 2003].

Insonated gas bubbles display the physical property of resonance - a frequency of oscillation at which the absorption and scattering of ultrasound is particularly efficient. Insonation of gas bubbles at their resonant frequency results in non-linear oscillation of the bubble, whereby the alternate expansion and contraction of the bubble is not equal. This results in the generation of harmonics - ultrasound produced at a frequency which is a multiple of the insonating frequency [Stewart et al 2003]. Recognition of this property of contrast media led to the development of harmonic imaging [Burns et al 1996]. With the receiver tuned to receive double the transmit frequency, an image is generated predominantly from the first harmonic signal, greatly improving the signal-to-noise ratio. Although initially developed as an aid to contrast echo, tissue also generates harmonics and the ability to enhance conventional grey scale imaging was rapidly appreciated. Harmonic imaging is now a standard feature on most echocardiography machines.

2.3.5.3.3 Assessment of Myocardial perfusion

For MCE studies, acoustic power and compression were maximized, and gain settings were optimized at the onset of each study and held constant throughout. The focus was initially set

at two-thirds of the depth of the image and then moved at the level of myocardial segment to be examined. Mechanical index was set at 0.9 for flash images. The definitive setting of the ultrasound images was optimized after initial contrast infusion, kept constant throughout the study, and matched at follow-up MCE study. Fifteen consecutive heart beats after the flash were acquired in digital format for subsequent off-line analysis. The intravenous contrast used in this study was Sonovue® (Bracco Research SA, Geneva, Switzerland), a second-generation ultrasound contrast agent that consists of microbubbles containing sulfur hexafluoride surrounded by a phospholipid shell. It is reconstituted by the addition of normal saline to the final solution of 5 ml. Sonovue was administered intravenously at the rate of 1 ml/min using a VueJect® (BR-INF 100, Bracco Research SA, Geneva, Switzerland) infusion pump. Contrast images were acquired in apical 4-chamber, 2-chamber, and 3-chamber views. As soon as myocardial video intensity had reached a plateau, a flash of ultrasound with a high mechanical index was given to destroy microbubbles in the sector and then the replenishment of bubbles was observed, digitally acquired and stored onto a magneto optical disk.

For stress, study subjects were then infused with dipyridamole (0.56 mg/kg over 4 min) followed by re-administration of intravenous contrast and acquisition of stress contrast images.

MCE was analysed using modern QLAB software. Basal segments, segments with poor image quality and frames showing wide variations in contrast intensity were excluded from analysis. On average, 4 segments per study were measured. After setting the background frame (frame immediately following microbubbles destruction) a region of interest (ROI) was mapped within a specific myocardial segment in background frame as well as frames showing replenishment of microbubbles. After full replenishment, the ultrasound signals represent the relative blood volume (α) within the beam, which correlate to the blood volume within the myocardium itself. Plateau myocardial contrast intensity (α) representing

myocardial blood flow volume, the rate of rise of signal intensity (β), representing mean myocardial blood flow velocity, and their product, myocardial blood flow (‘α’ x ‘β‘) at rest (FR) and after stress (FS) were measured, enabling calculation of mean MBFR (ratio of FS and FR) for each subject [Wei et al 1998].

2.4 Power Calculations

As my project is about vascular function, I decided to take a commonly used index of this function as my test statistic – the change is arterial diameter after reactive hyperaemia, known by many as flow mediated dilatation. This method is accepted and widely used in cardiovascular research [Cooke et al 2001] [Corretti et al 2002]. In addition, the department has a long history of using this method [Chong et al 2004][Felmeden et al 2003][Foster et al 2010]. Tanriverdi et al reported a significant difference in FMD in 40 OSA patients [mean (SD) FMD change 4.6 (1.3)%] compared to 24 controls [6.3 (0.8)%], a relative reduction of over 25% [Tanriverdi et al 2006].

Nevertheless, it is my hypothesis that that magnitude of the change in FMD in OSA is of the same order as the well-known abnormality in FMD in patients with hypertension, effectively a positive control group, and is inferior to that in healthy controls. Impaired FMD is hypertension is established [Muiesan et al 2001][Felmeden et al 2003]. Accordingly, my sample size calculation is for 40 subjects per group. This brings the 1-beta power of 0.8 for a difference of at least 0.05 between the groups, with an overall p value of <0.001. This is summarized in the following Minitab ANOVA printout of a possible model of changes in FMD in OSA and hypertension (HT) compared to controls.

This model is actually more conservative than that of Tanriverdi et al, who found FMD to be 73% lower in OSA compared to controls. My model is that FMD in OSA is only 83% of that of controls.

Minitab printout of my Model - One-way ANOVA: All groups versus C10

One-way ANOVA: All groups versus C10

```
Source   DF       SS      MS      F      P
C10       2   22.400  11.200  16.63  0.000
Error   117   78.799   0.673
Total   119  101.199

S = 0.8207   R-Sq = 22.13%   R-Sq(adj) = 20.80%

                                  Individual 95% CIs For Mean Based on
                                     Pooled StDev
   Level         N    Mean   StDev  ------+---------+---------+---------+---
   1: Controls  40  5.8120  0.8207                           (-----*------)
   2: HT        40  5.0120  0.8207       (-----*------)
   3: OSA       40  4.8120  0.8207  (-----*------)
                                     ------+---------+---------+---------+---
                                         4.80      5.20      5.60      6.00

   Pooled StDev = 0.8207
```

However, with three groups I require more power, and so aimed to collect data from 45 subjects per group, giving extra confidence, both scientifically and statistically. As it happened, in reality, FMD in OSA was marked 54% lower (when assessed as arterial

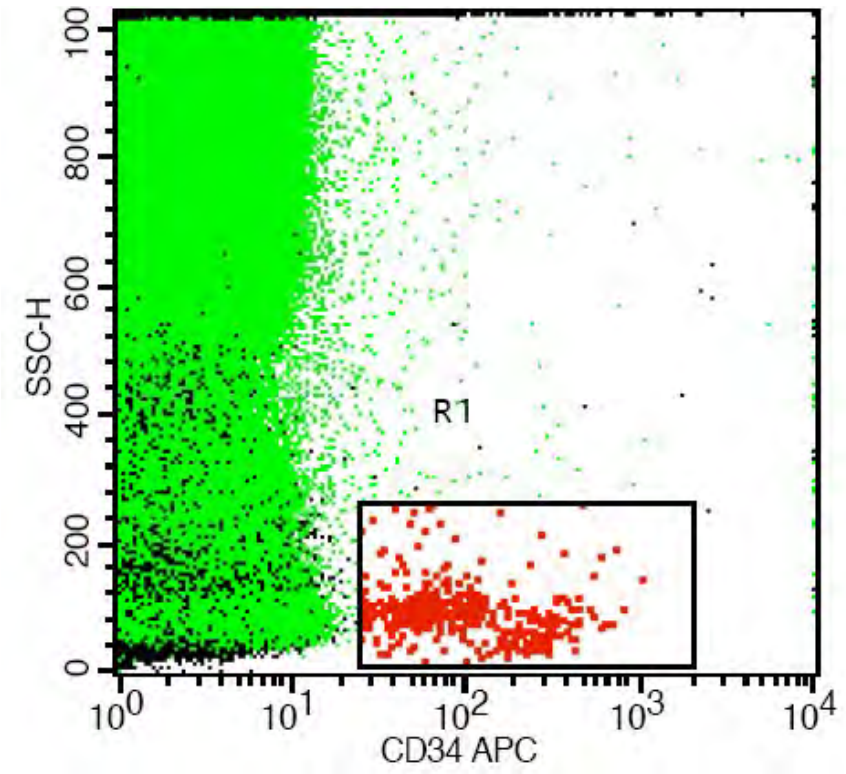
diameter in mm or as percentage) and compared to the healthy controls [Table 3-3], a much greater difference than that of Tanriverdi et al. This validates my sample size calculation.

2.5 Statistical Analyses

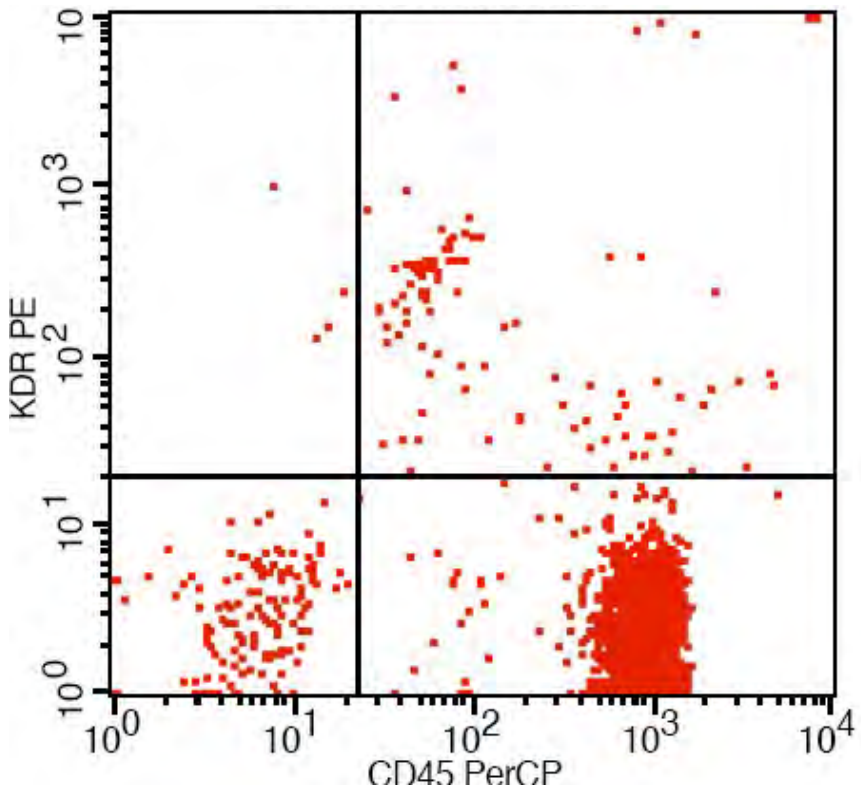
Minitab-15 (Minitab Ltd. Coventry, UK) statistical software was used to perform statistical analysis. Data are expressed as mean ± standard deviation (SD) for normally distributed data; or median and inter-quartile range (IQR) for descriptive and/or non-normally distributed data. For categorical data, chi-squared test was used. Data among three groups (OSA, hypertensive and healthy controls) were analyzed by one way analysis of variance (ANOVA) or Kruskall-Wallis test depending upon distribution, whilst Paired t test or Wilcoxon test was used for paired analysis. A post-hoc Tukey test was performed where to assess inter-group differences (with Log transformation where appropriate). Two-way ANOVA was used to investigate the longitudinal influence of time on the parameters of pulse wave analysis and pulse wave velocity. Pearson correlation was used for normally distributed data whilst non-normally distributed data was log transformed before the correlation analysis. A value of $p<0.05$ was considered statistically significant. The influence of independent variables (age, body mass index, blood pressure, total cholesterol, glucose, Epworth score, AHI) on a given parameter was explored using univariate analysis; only the statistically significant variables were then entered into a stepwise multivariate linear regression analysis.

Figure 2-2: A typical Fluorescence-activated cell-sorting (FACS) analysis for a subject. (A) 1% of events, plotting forward scatter (FSC) vs. side scatter (SSC). R1 selects all presumed cellular CD positive events; (B) A region of R1 showing CD45 negative (in bottom left box) and CD45 positive cells (bottom right box showing); (C) A region of R1 that expresses CD34 and CD146 highly and CD45 to a lesser degree, labelled as R2 (shaded box) is representing Circulating Endothelial Cells; (D) Sequential analysis of cells from R1 from (A) showing cells staining positively for both CD34 and KDR yet negative for CD45. These cells represent Endothelial Progenitor Cells and are labelled as R3. A representative FACS analysis is shown below:

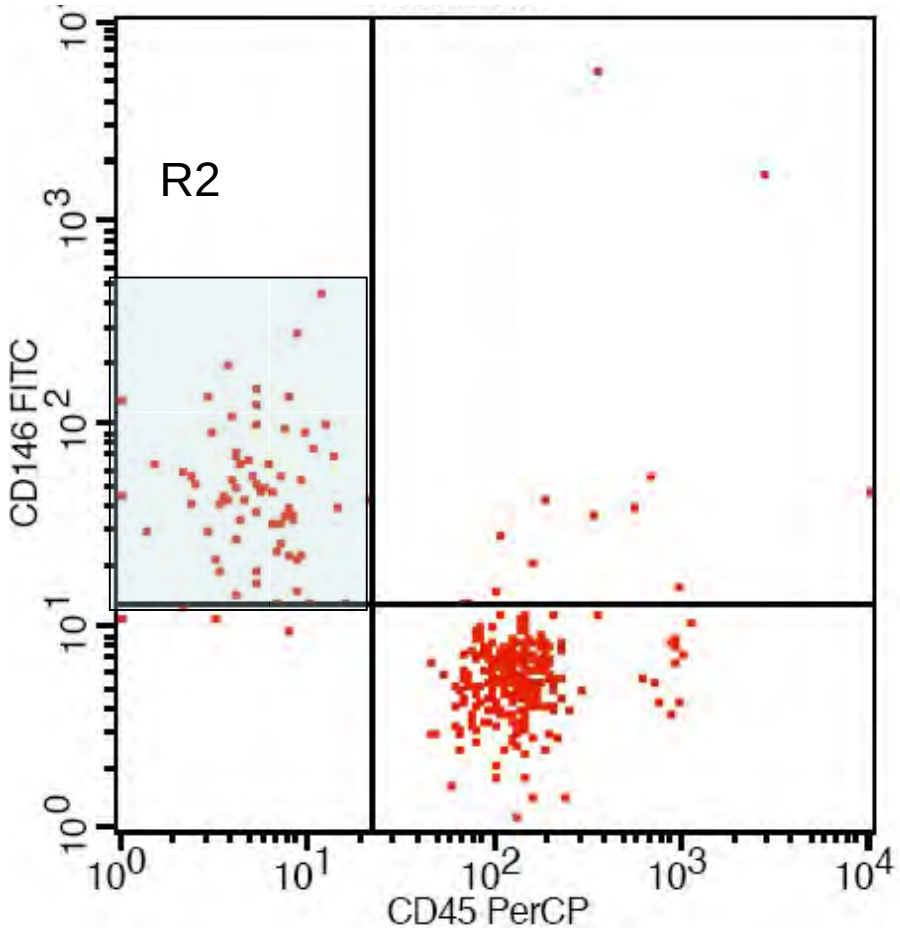
(A)



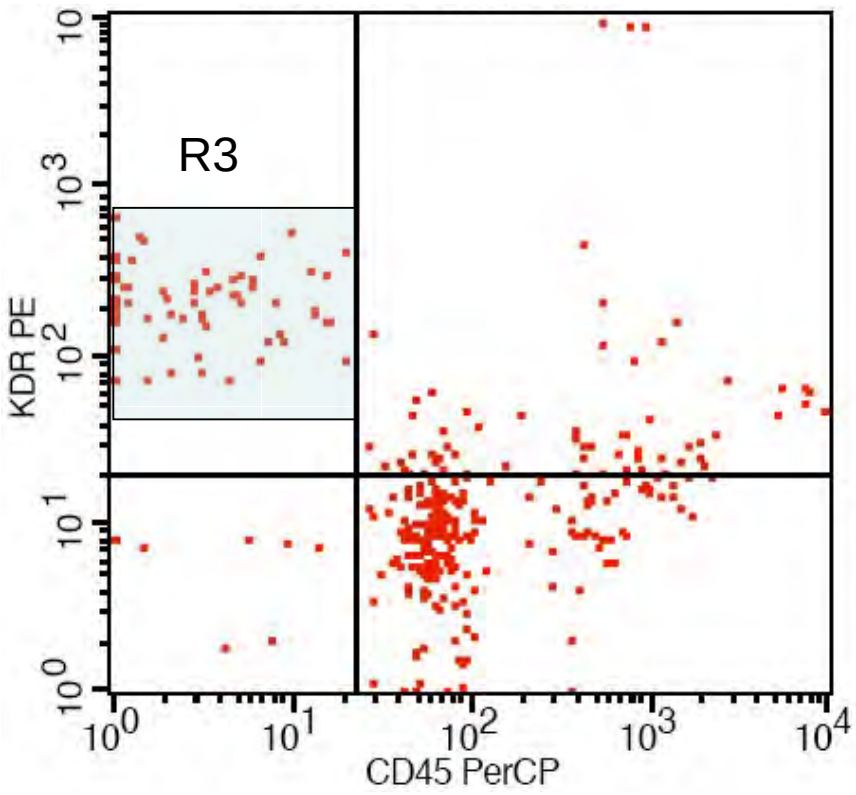
(B)



(C)



(D)


R3
KDR PE
CD45 PerCP
10^0
10^1
10^2
10^3
10^4

Section 3 Validation and Clinical Studies

3.1 Validation Studies

I assessed inter- and intra-observer variability, on 10 subjects for each of the techniques described in 'Hypotheses and Methodology''. These subject numbers are a quarter of the study group and hence 10 subjects were recruited only. For inter- and intra-observer variability study, I analysed the data for Coefficient of variation using Minitab. Using standard deviation of the difference between two groups and dividing this standard deviation by the mean of the study group, I obtained the percentage for the inter- and intra-observer variability.

These subjects were specifically recruited for this purpose and were not a part of the main study recruitment. They were recruited in the same manner as described in the section 'Hypotheses and Methodology' and provided informed consent. During intra-observer variability, I compared my method outcome when it was performed at two different times. During inter-observer variability my results were compared against an expert technician. Standard statistical tests were used as described in section 'Statistical analyses'. Following table represent the percentage difference in the variability for each technique.

Table 3-1: Percentage inter- and intra-observer variability

Technique	Inter-observer variability (n=10)	Intra-observer variability (n=10)
FMD	2.7%	1.9%
LDF	6.3%	8%
Flow Cytometry	1.9%	8.2%
Pulse wave analysis	9%	5.1%
2DE	1.4%	5.9%
RT3DE	11%	6.8%
MCE	6.1%	7.3%

FMD: Flow mediated dialatation, LDF: Laser Doppler flowmetry, 2DE: 2-dimensional echocardiography, RT3DE: Real time 3-dimensional echocardiography, MCE: Myocardial contrast echocardiography

3.2 Cross sectional studies

3.2.1 Assessment of Macro-vascular Function using Flow Mediated Dilatation in Moderate to Severe Obstructive Sleep Aponea compared with hypertensive and healthy controls

3.2.1.1 Abstract

Background: OSA is associated with a wide variety of cardiovascular diseases and increased cardiovascular morbidity and mortality. Impaired flow mediated dilatation (FMD) has been utilized to demonstrate endothelial dysfunction (ED) in several cardiovascular diseases. I hypothesized that ED could exist in conjunction with moderate to severe OSA contributing to its cardiovascular complications.

Methods: I compared FMD of the brachial artery in untreated moderate to severe OSA patients with hypertensive and healthy control subjects (n=45, in each group) without sleep-disordered breathing. Endothelial-dependent vasodilatation (EDV) was assessed by establishing reactive hyperaemia and endothelial-independent vasodilatation (EIV) was determined by using sublingual Glyceryl trinitrate (GTN).

Results: Baseline brachial artery diameter was comparable in all three groups. Both EDV and EIV were significantly impaired in OSA and hypertensive cohorts compared to healthy subjects.

Conclusion: Macro-vascular dysfunction, as demonstrated by impaired FMD, is present in otherwise healthy patients with moderate-severe OSA. This may indicate potential pathophysiological mechanisms leading to ED, impaired smooth muscle function and untimately increased cardiovascular risk in normotensive subjects with moderate to severe OSA.

3.2.1.2 Introduction

Over the past decade, FMD has evolved as a non-invasive and reproducible technique of endothelial assessment [Corretti et al 2002]. The stimulus in the form of reactive hyperaemia, induced by arterial occlusion, provokes the endothelium to release nitric oxide (NO), with subsequent vasodilatation which can be ultrasonographically acquired and quantified as a surrogate of vasomotor function [Dijkhorst-Oei et al 1999]. This technique has been validated and standardized according to the recommendations of the International Brachial Artery Reactivity Task Force [Corretti et al 2002].

Impaired endothelial dependent and -independent responses have been reported in OSA using FMD [Celermajer et al 1992][Corretti et al 2002][Tanriverdi et al 2006 (a)][Bayram et al 2009][Amra et al 2009]. However these studies have important limitations, such as inclusion of milder form of the disease. Since only moderate to severe OSA is the primary target for CPAP and presence of mild OSA could have diluted the results. Secondly, high frequency of confounding variables such as hypertension and diabetes mellitus in these studies may suggest that the reported ED may only be a reflection of coexisting diseases. Additionally, in some previous studies, presence of the OSA in the control (non-OSA) group has not been meticulously excluded. Finally, lack of adequate power to detect a statistically significant change has also been an issue previously. Given strong bond between OSA and cardiovascular disorders, it is imperative to determine the varcular functional viability in OSA, however in the presence of above mentioned limitations, the answer remains ambiguous.

The purpose of this study was to assess endothelial-dependent and -independent dilatation in otherwise healthy patients with moderate to severe OSA. I hypothesized that ED exists in otherwise healthy normotensive OSA individuals and can be demonstrated using FMD. I tested

this by perforimg FMD in otherwise healthy untreated moderate to severe OSA subjects who were compared against hypertensive and healthy subjects.

3.2.1.3 Methods

The methods have been described in details under section 'hypotheses and methodology'.

3.2.1.4 Results

Table 3-2 depicts the baseline demographic, anthropomorphic and polysomnographic data whilst Table 3-3 shows brachial artery measurement data including absolute and percentage changes in brachial artery diameters in response to reactive hyperaemia and sublingual nitrates.

FMD was performed on all of 135 study patients (OSA=45, hypertensive=45, healthy=45). All three groups were matched for age, sex and body mass index. On average, patients were middle aged (50±9 years) with comparable female gender representation (32%). OSA patients had greater median apnoea-hypopnoea index compared to both hypertensive and healthy subjects (both $p<0.0001$). In the hypertensive cohort, systolic blood pressure was higher compared to healthy group ($p<0.0001$) [Table 3-2].

Mean baseline brachial artery diameter was comparable amongst three groups ($p=0.138$) [Table 3-3]. Following forearm ischaemia, endothelium dependent FMD was significantly impaired both in OSA and hypertensive groups compared with healthy individuals (p for both comparisons<0.0001). However no difference was found between OSA and hypertensive subjects ($p=0.51$) [Table 3-3].

EIV, measured following the sublingual administration of GTN spray was also significantly attenuated in OSA cohort compared with healthy subjects (p=0.02). Again, no difference was found between OSA and hypertensive subjects (p=0.59) [Table 3-3].

On Univariate analysis, no relationship was found between arterial responses and age, BMI, AHI, Blood pressure, glucose and total cholesterol [Table 3-4]

3.2.1.5 Discussion

To the best of my knowledge, this is the first study reporting impaired endothelium-dependent and endothelial independent brachial artery responses in otherwise healthy patients with moderate to severe OSA compared with healthy and hypertensive subjects *without OSA.*

Endothelium-dependent dilatation results from the release of nitric oxide (NO) from endothelial cells, in response to stimuli such as shear stress or acetylcholine infusion. Arterial dilatation in response to NO donors such as GTN is dependent upon normal vascular smooth muscle function. These results provide first evidence that impaired FMD responses in OSA subjects cannot be attributed solely to decreased brachial artery NO bioactivity, but is a function, at least in part, of downstream vascular reactivity.

I noted impaired EDV in OSA and a smaller ratio of EDV to EIV compared with the healthy subjects. This suggest endothelial dysfunction and these findings of are consistent with most studies [Bayram et al 2009][Amra et al 2009][Tanriverdi et al 2006 (a)][Ip et al 2004][Kraiczi et al 2001]. Impaired FMD has traditionally been recognized as an indirect marker of NO bioactivity. The exact mechanisms responsible for determining the magnitude of the FMD response are complex and incompletely understood. FMD may directly reflect brachial artery NO

bioavailability, but it also depends on baseline brachial artery diameter [Lockhart et al 2011]. Endothelium-dependent FMD has been shown to be a homoeostatic response to short-term increases in local shear stress, which is known to be critically dependent on the magnitude of the imposed stimulus and status of the distal forearm microcirculation [Lockhart et al 2011]. Finally, recurrent exposure to hypoxia-reoxygenation during apnoea/hypopnoea is believed to reduce endothelial NO synthesis and/or accentuating its degradation by production of reactive oxidative species [Lavie et al 2003][Busse et al 1995]. It is worth mentioning that ED is being regarded as a final common pathway, mediated by several cellular (inflammation, NO release dysfunction, coagulopathy), metabolic (metabolic dysfunction) and nervous (autonomic dysregulation) mechanisms which may contribute to variable extent in different patient population. Indeed a range of coaguopathies, metabolic syndrome and over driven sympathic system have been reported, but this study does not clearly show if either of above mentioned mechanisms are responsible and to what extent.

I also noted that brachial artery dilatation measured in response to sublingually administered GTN was impaired in OSA patients compared with healthy control subjects. This endothelium independent response reflects arterial smooth muscle function and can be impaired in asymptomatic subjects with coronary risk factors [Zhang et al 2000].

Exogenous organic nitrates, such as GTN, produce vasodilation by entering smooth muscle cells and undergoing conversion to NO. Sulfhydryl groups, in antioxidant compounds such as glutathione and cysteine, are important in the formation of active intermediates [Torfgård et al 1994]. Inflammation and/or oxidative stress may result in reduced bioconversion of GTN to NO, within smooth muscle cells, producing impaired GTN mediated response [Mülsch et al 2001]. In a recent study, higher hs-CRP was associated with lower GTN induced vascular response [Ayer et al 2011]. CRP has direct pro-inflammatory effects and is associated with markers of oxidative stress [Abramson et al 2005]. Furthermore, there is evidence for impaired endothelium-

independent responses in patients with Type 2 diabetes, proposing that increased oxidative stress associated with the diabetic state may impair the biochemical conversion of organic nitrates into NO [Abramson et al 2005]. It is thus possible that inflammation, associated with obesity, results in a relative overabundance of reactive oxygen species in the vessel wall, which in turn reduces the bioconversion of GTN to NO, representing as smooth muscle dysfunction and impaired EIV. However, the design of the present study did not permit to directly determine the relative importance of impaired generation of endothelium-derived NO and the smooth muscle response to NO in contributing to the magnitude of vasodilatation. However, at the very least this can be postulated that, as previously believed, impaired brachial reactivity is not purely NO related, and vascular components may have strong contribution in impaired vasoreactivity.

Several other factors such as dysfunctional autonomic nervous system are also crucial in the endothelial responsiveness. Over-activation of sympathetic system has been well reported in obese patients with and without OSA [Narkiewicz et al 1998(b)]. A recent study tested vascular endothelial function whilst investigation sympathetic activity simultaneously [Kaplon et al 2001]. An inverse relationship has been demonstrated with between FMD and the sympathetic drive surrogated by plasma epinephrine levels. Considering previously reported sympathetic over drive and higher epinephrine levels in OSA population [Narkiewicz et al 1998(b)], it can be derived that my results are likely to be secondary to dysfunction autonomic system, instead of endothelial dysfunction. However I did not have the mandate to investigate autonomic system as per the mandated by the study design.

Limitations

The observational model is one important limitation of this study. However, these data are relevant to human health, risk stratification and disease development. In order to ensure

uniformity, the tests were performed and analyzed by single operator and was validated with excellent inter- and intra-observer variability. It must be noted that these observations are limited to healthy middle aged subjects with moderate to severe OSA and cannot be extrapolated to OSA patients with milder form disease or subjects with coexisting cardiovascular disease/risk factors.

The blood pressure was significantly higher in hypertensive group compared with healthy controls. Blood pressure was also modestly elevated in OSA subjects compared with healthy controls but it did not reach statistical significance. In deed it can be argued that the vascular changes noted in the present study could just be a reflection of underlying hypertension in OSA subjects. Although my OSA subjects were not known hypertensives and blood pressure was monitored on several occasions to ensure normotensive recruitment. However, I did not perform 24 hour blood pressure monitoring due to logistical limitations. An element of white coat response can not be ignored although the patients were rested prior to all blood pressure recordings. Due to well known over active sympathetic drive in OSA subjects, it is quite possible the morning blood pressure was temporarily yet mildly elevated during the study period. Finally as described in the results I did not find a statistical relationship between the blood pressure and the vascular reactivity. Regardless of these arguments, there is no doubt that more stringent screening is required to exclude undiagnosed hypertensive cohorts and for this purpose use of 24-hour blood pressure monitoring is crucial. This is one big limitation of my study and this aspect should be taken care of in future studies.

3.2.1.6 Conclusion

In conclusion, this study submits the findings of impaired macro-vascular endothelial dependent and independent vasoreactivity in OSA subjects compared with healthy and hypertensive controls and is due to a combination of endothelial and vascular smooth muscle dysfunction.

Table 3-2: Clinical Characteristics of Obstructive Sleep Apnoea patients, Hypertensive and Healthy Controls

Variable	OSA	Hypertensive controls	Healthy controls	p
n	45	45	45	
Male/Female	34/11	30/15	30/15	0.54
Age (years)	50 (9)	49 (12)	46 (7)	0.120
Aponea Hypopnea index	33 (21-50) *Ψ	4(1.2-5.8)Ψ	3(0.9-5)*	<0.0001
Body mass index (Kg/m2)	34(8)	32(6)	32(6)	0.118
Systolic Blood pressure (mmHg)	142(16)	150(21)^	135(15)^	<0.0001
Diastolic Blood pressure (mmHg)	83(11)	85(10)	82(9)	0.220
No. of current smokers	14	12	16	0.452

Values are described as Mean (Standard Deviation) and Median (Inter-quartile Range). P <0.05 was considered statistically significant.

* Significance between OSA and Healthy subjects
^ Significance between Hypertensive and Healthy subjects
Ψ Significance between OSA and Hypertensive subjects
OSA= Obstructive Sleep Apnoea

Table 3-3: Brachial Artery reactivity data in Obstructive sleep apnoea, Hypertensive and Healthy subjects

	OSA	HT	Healthy	p
Baseline Brachial artery diameter (mm)	4.5±0.6	4.5±0.6	4.2±0.7	0.138
Absolute change in diameter after reactive hyperaemia mm)	0.26 (0.19-0.41)*	0.33 (0.16-0.46)^	0.48 (0.34-0.63)*^	<0.0001
Percentage change after reactive hyperaemia	6 (4-9)*	7 (3.4-9.5)^	11 (9-15)*^	<0.0001
Absolute change in diameter after GTN	0.50 (0.40-0.63)	0.51 (0.36-0.58)	0.59 (0.45-0.67)	0.05
Percentage change after GTN (mm)	11 (7-15)*	11 (8-14)^	14 (10-17)*^	0.018
EDV/EIV ratio	0.54*	0.63^	0.78*^	0.02

*Significance between Obstructive Sleep Aponea and Healthy subjects

^Significance between Hypertensives and Healthy subjects

OSA= Obstructive Sleep Apnoea; GTN= Glycerol Trinitrate; HT= Hypertension

Table 3-4: Univariate analyses of Pre-CPAP brachial artery reactivity in Obstructive Sleep Apnoea

	FMD hyperaemia %change		GTN %change	
	r	**p**	**r**	**p**
Age	-0.168	0.270	-0.103	0.500
Height	0.205	0.176	0.047	0.759
Weight	0.133	0.386	0.075	0.685
BMI	-0.002	0.989	-0.096	0.531
AHI	0.102	0.506	-0.091	0.551
Epworth score	-0.278	0.096	-0.036	0.833
Systolic BP	0.082	0.594	0.103	0.499
Diastolic BP	0.162	0.288	-0.004	0.989
Glucose	-0.394	0.872	-0.325	0.657
Total cholesterol	0.239	0.318	0.735	0.159

r=correlation coefficient; AHI= Apnoea Hypopnoea Index; FMD= Flow mediated Dilatation; BP= Blood Pressure; CPAP= Continuous Positive airway pressure; GTN= Glycerol trinitrite; BMI= Body mass index

3.2.2 Microvascular Reactivity assessed by Laser Doppler Flowmetry in Moderate to Severe Obstructive Sleep Aponea compared to Hypertensive and Healthy controls

3.2.2.1 Abstract

Background: Micro-vascular dysfunction has been implicated in the pathogenesis of cardiovascular complications of obstructive sleep apnea (OSA). I investigated micro-vascular function in moderate to severe OSA using laser Doppler flowmetry (LDF). I hypothesized that LDF derived ED exists in OSA compared with matched healthy and hypertensive controls.

Methods: Otherwise healthy patients with moderate to severe OSA were compared with non-OSA hypertensive and healthy controls. Micro-vascular reactivity was assessed by LDF, which allowed the measurement of the response to iontophoretically applied acetylcholine (endothelium-dependent vasodilatation {EDV}) and sodium nitroprusside (endothelium-independent vasodilatation {EIV}).

Results: Both endothelial dependent and independent responses were blunted significantly in OSA and hypertensive patients compared to healthy subjects. Both EDV and EIV were comparable between OSA and hypertensives.

Conclusion: Impaired endothelial microvascular function exists in otherwise healthy normotensive subjects with moderate to severe OSA.

3.2.2.2 Introduction

There are a variety of non invasive methods to estimate endothelial function in macrovasculature such as flow-mediated vasodilatation. In microcirculation, ED can be noninvasively measured in the forearm cutaneous vasculature using laser Doppler flowmetry (LDF). This technique has been successfully utilized in a number of disease states and has proven to be a reproducible tool [Gomes et al 2008][Beer et al 2008][Kubli et al 2000]. This device allows monitoring of the changes in cutaneous perfusion precipitated by the iontophoresis of acetylcholine (ACH) and sodium nitroprusside (SNP).

Microvascular endothelial status has been rarely assessed in OSA using this technique. There are significant limitations even in the sparcely available data [Friberg et al 1998][Trzepizur et al 2009]. Also, no previous study has used this method in otherwise healthy subjects with moderate to severe OSA. It must be understood that the understanding of microvascular endothelial integrity is particularly important, as moderate to severe form of OSA is an emerging and widely acknowledged risk factor for cardiovascular health.

I aimed to assess endothelial-dependent and independent perfusion changes in the forearm skin of patients with moderate to severe OSA who were compared with matched non-OSA hypertensive and healthy subjects. I hypothesized that ED may exist in the microvasculature of otherwise healthy normotensive OSA patients. In order to test this hypothesis I performed LDF coupled with iontophoresis in moderate to severe OSA subjects, hypertensive and healthy cohorts.

3.2.2.3 Methods

The methods have been described in details under section 2 ‘Hypotheses and Methodology’.

3.2.2.4 Results

45 subjects with moderate to severe OSA were recruited after the diagnosis was established with polysomnography but only 31 patients were available for LDF scanning prior to the initiation of the CPAP treatment. In total, the LDF was performed on 98 patients (OSA=31, hypertensive=34, healthy=33). All three groups were matched for age, sex and BMI. On average, OSA patients were middle aged (50±10 years) with similar proportion of female gender representation (30%). OSA patients has greater median AHI compared to both, hypertensive and healthy subjects [$p<0.0001$] [Table 3-5].

Mean baseline perfusion was comparable amongst the three groups [Table 3-5]. ACH induced increase in cutaneous blood flow was significantly impaired in OSA and hypertensive groups compared to healthy individuals [$p<0.0001$] [Table 3-6]. However, the EDV was comparable between OSA and hypertensive cohorts ($p=0.22$).

Following iontophoresis of SNP, endothelium independent percentage perfusion was significantly impaired in OSA and hypertensive groups compared with healthy subjects, with median increase of 96% (IQR 58-151) and 103% (IQR 69-139) in OSA and hypertensive groups respectively, versus 180% (IQR 125-237) in healthy subjects {both $p<0.0001$} [Table 3-6]. Again there was no significant difference in EIV, between the OSA and hypertensive cohorts ($p=0.43$).

On Univariate analysis, no relationship was found between microvascular responses and age, BMI, AHI, Blood pressure, glucose and total cholesterol [Table 3-7]

3.2.2.5 Discussion

In this study, I demonstrated for the first time that microvascular endothelial activity, represented by cutaneous perfusion, is significantly impaired in patients with moderate to severe OSA who are otherwise healthy and free from any cardiovascular history and/or risk factors, compared to healthy subjects. Impaired EDV can be explained by impaired NO release from endothelial cells but EIV is predominantly controlled by smooth muscles in vascular wall. Hence impairment of EIV may represent smooth muscle dysfunction alongside the ED, which has never been described in moderate to severe OSA before.

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LDF in combination with iontophoresis is a validated and reproducible technique for the assessment of ED [Pellaton et al 2002][Rousseau et al 2008]. Although LDF has not been studied widely in OSA population, my results are consistent with the published data. Trzepizur et al has recently reported impaired endothelial dependent (ACH induced) micro-vascular function in OSA patients compared with healthy controls [Trzepizur et al 2009]. Additionally, these findings are consistent with previous OSA studies, where alternative techniques were used instead of LDF [Carlson et al 1996][Kato et al 2000][Kraiczi et al 2001].

Sleep deprivation, impaired sympathetic responses and hypoxia- all of which are widely prevalent in OSA- have been implicated in the impairment of endothelial dependent vasoreactivity [Gokce et al 2002][Morris et al 1996][Bonetti et al 2003][Amir et al 2004][Phillips et al 2000][Phillips et al 2004]. For example, Amir et al reported vascular malfunction in subjects with fewer sleeping hours [Amir et al 2004]. Indeed sleep deprivation induces surges in the systolic blood pressure due to autonomic disturbances releasing endothelial inflammatory mediators and causing ED [Phillips et al 2000][Pagani et al 2009] [Chae et al 2001]. Similarly, a close relationship has been demonstrated between serum nitrites/nitrates concentrations and oxygen desaturation may offer another explanation of severely blunted endothelial dependent responsiveness in OSA [Phillips et

al 2004]. In a study investigating cardiovascular morbidity, has reported autonomic dysfunction as a major causative factor [Okano et al 2010]. This study was carried out using LDF in high risk patients. There is also some evidence that improvement in metabolic profile translates into better vascular responsiveness [Forst et al 2008]. Therefore this can be concluded that some mechanisms over and above ED also exist and may operate in OSA.

I also demonstrated impaired endothelial *independent* functions in moderate to severe OSA compared to healthy subjects. Although these findings are in contrast to the data reported by Trzepizur et al, my data are consistent with LDF studies in smokers and diabetic subjects [Trzepizur et al 2009] [Morris et al 1995][Lim et al 1999][Caballero et al 1999][Pellaton et al 2002].

Impaired endothelial independent vasoreactivity at arteriolar/capillary level could be linked with smooth muscle dysfunction secondary to sub-clinical inflammatory processes in OSA subjects [Bravo et al 2007]. Up-regulation of certain cytokines such as Interleukin-1β, interleukin-6, Interleukin-10 and tumor necrosis factor-α, may modulate micro-vascular smooth muscle tone by reducing SNP induced cGMP levels [Ferrell et al 2002][Meier-Ewert et al 2004][Iversen et al 1999] [Papapetropoulos et al 1996]. The reduction in cGMP levels is also reported to be due to hypoxia driven attenuated nitric oxide synthase activity [Orshal et al 2004]. Nevertheless, this cumulative reduction in cGMP may limit the biological effect of extrinsic nitrates on smooth muscles expressed as impaired endothelial independent vasoreactivity [Papapetropoulos et al 1996].

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These finding of impaired endothelial dependent responses in hypertensive patients compared with healthy controls is consistent with the available data [Farkas et al 2005][Lindstedt et al

2006][Cupisti et al 2000]. However no previous study has demonstrated impaired endothelial independent response in healthy hypertensives as observed in the present study. This could be explained by the smaller study sizes and the co-existence of important confounding factors (such as renal failure) in the studied hypertensive patients which could restrict SNP mediated response markedly [Nakanishi et al 2002][Lindstedt et al 2006].

Hence it can be concluded that the findings of impaired EDV and EIV in OSA population, in fact, represent a more general vascular abnormality comprised of endothelial activation as well as smooth muscle dysfunction.

Limitations

Open study design and relatively smaller numbers are important limitations. I excluded milder form of OSA and our findings are limited to healthy middle aged subjects with moderate to severe OSA and cannot be extrapolated to patients with milder severity or OSA with coexisting disease causing altered vascular function (such as with atherosclerosis). A modestly elevated blood pressure was noted in OSA compared with healthy subjects, in spite of strict recruitment. As shown in the Table, no relation was found between the blood pressure and the LDF and hence it can be assume that such a modestly elevated BP is not the mediator of vascular responsiveness. This is one important limitation and should be focussed at in future larger studies.

3.2.2.6 Conclusion

In conclusion, ED exists at microciculatory level in moderate to severe OSA even in the absence of confounding cardiovascular factors. These changes may be subtle initially however I propose that repetitive transient ventilatory loss could lead to key changes in cellular physiology and thereby priming the development of future cardiovascular disease.

Table 3-5: Clinical characteristics of Obstructive Sleep Apnoea patients and the Controls

Variable	OSA	Hypertensive controls	Healthy controls	p
n	31	34	33	
Male/Female	24/7	23/11	22/9	0.364
Age (years)	50(10)	49(11)	46(9)	0.087
Aponea Hypopnea index	33 (20-48) *Ψ	3(2-4)Ψ	3(2-4)*	<0.0001
Body mass index	35(8)	31(6)	32(8)	0.084
Systolic Blood pressure (mmHg)	145(17)	149(20)^	137(14)^	<0.0001
Diastolic Blood pressure (mmHg)	83(12)	85(9)	83(10)	0.771
No. of current smokers	10	12	11	0.561

* Significance between OSA and healthy subjects
^ Significance between hypertensive and healthy subjects
Ψ Significance between OSA and hypertensive subjects
OSA= obstructive Sleep Aponea

Table 3-6: Changes in Micro-Vascular reactivity in response to Acetylcholine and Sodium Nitroprusside; values in Median (Inter-quartile Range)

	OSA	Hypertensive Controls	Healthy Controls	p
Mean Baseline perfusion (PU) pre-ACH	0.69 (0.62-0.78)	0.75 (0.6-0.87)	0.58 (0.44-0.8)	0.101
Mean maximum perfusion post-ACH (PU)	1.3 (1.1-1.7)	1.3 (1.1-1.7)	1.4 (1.1-2.1)	0.449
Mean percentage increase post-ACH (%)	101 (52-135)*	76 (51-109)^	234 (120-305)*^	<0.0001
Mean Baseline perfusion pre-SNP (PU)	0.74 (0.65-0.82)	0.72 (0.62-0.85)	0.62 (0.56-0.81)	0.061
Mean maximum perfusion post-SNP (PU)	1.3 (1.2-1.7)	1.5 (1.3-1.9)	1.5 (1.3-1.9)	0.350
Mean percentage change post-SNP (%)	96 (58-151)*	103 (69-139)^	180 (125-237)*^	<0.0001

* Significance between Obstructive Sleep Aponea and Healthy subjects
^ Significance between Hypertensive and Healthy subjects
OSA= Obstructive Sleep Aponea, ACH= Acetylcholine, SNP= Sodium Nitroprusside, PU= perfusion unit

Table 3-7: Univariate analyses of the Pre-CPAP micro-vascular reactivity in Obstructive Sleep Apnoea

	ACH %LDF change		SNP LDF %change	
	r	**p**	**r**	**p**
Age	-0.137	0.461	0.174	0.349
Height	-0.289	0.215	-0.343	0.06
Weight	-0.034	0.854	-0.116	0.535
BMI	0.107	0.567	0.109	0.559
AHI	0.133	0.477	-0.185	0.318
Epworth score	-0.269	0.193	-0.174	0.406
Systolic BP	-0.138	0.459	0.081	0.661
Diastolic BP	0.205	0.269	0.065	0.730
Glucose	-0.003	0.989	0.091	0.632
Total cholesterol	0.292	0.111	0.197	0.287

r=correlation coefficient; AHI= Apnoea Hypopnoea Index; FMD= Flow mediated Dilatation; BP= Blood Pressure, LDF= Laser Doppler flowmetry, CPAP= Continuous Positive airway pressure; ACH= Acetylcholine; SNP= Sodium nitroprusside; BMI= Body mass index

3.2.3 Endothelial Progenitor Cells and Circulating Endothelial Cells in Moderate to Severe Obstructive Sleep Aponea Compared to Hypertensive and Healthy Subjects

3.2.3.1 Abstract

Background: Obstructive sleep apnea (OSA) is associated with endothelial damage/dysfunction (ED) and enhanced cardiovascular risk. Increased circulating endothelial cells (CECs) have been reported in conditions with ED, such as coronary artery disease. Similarly lower endothelial progenitor cell (EPC) counts have been observed in patients with cardiovascular risk factors. I hypothesized that a similar distribution of CECs and EPCs might exist in moderate to severe OSA.

Methods: In the present study, CECs and EPCs were enumerated using flow cytometry in peripheral blood sample of 40 otherwise healthy untreated patients with moderate to severe OSA and compared with hypertensive and healthy subjects (n=40, in each control groups) without sleep-disordered breathing.

Results: Subjects in all three groups were age, sex and body mass index matched. OSA patients had significantly higher aponea-hypopnoea index (AHI) score compared with controls. There were no significant differences in CECs, EPCs count or CEC/EPC ratio amongst the three groups.

Conclusion: I found no differences in CECs or circulating EPCs in OSA compared with the control groups. Moderate to severe OSA may not be associated with cellular markers of vascular endothelial cell damage/repair in subjects with no concomitant vascular disease.

3.2.3.2 Introduction

Endothelial progenitor cells (EPCs) contribute significantly to vascular homeostasis and may represent reparative cells. It is postulated that these special cells replace dysfunctional endothelial cells [Khakoo et al 2005][Hristov et al 2003], and hence, a close association between EPCs, endothelial function and atherosclerotic risk factors has been reported [Hill et al 2003] [Chen et al 2004][Tepper et al 2002]. For example, patients at risk of atherosclerosis have strikingly reduced numbers of circulating EPCs. Additionally, decreased EPC counts have been reported to be predictive of future cardiovascular events in patients with stable as well as unstable coronary artery disease [Schmidt-Lucke et al 2005] [Werner et al 2006]. On the contrary, increased levels of circulating EPCs have been associated with better survival in patients with acute lung injury [Burnham et al 2005], indicating that EPCs may be predictive of favourable prognosis after vascular injury.

Circulating endothelial cells (CECs) have been described as the apoptotic cells which tend to coexist in conditions with vascular damage [Boos et al 2006][Jimenez et al 2003][Gonzalez-Quintero et al 2003]. Unsurprisingly, markedly elevated levels of these apoptotic CECs have been reported *in vitro* and *ex vivo* in patients with coronary artery disease [Kavurma et al 2005][Mayr et al 2001]. Additionally, CECs counts are increased in a range of conditions where vascular damage might be expected, such as acute coronary syndrome, congestive heart failure, both primary and secondary pulmonary hypertension and systemic sclerosis [Quilici et al 2004] [Chong et al 2004] [Bull et al 2003] [Del Papa et al 2004]. Thus, denuded CECs could be a marker for vascular endothelial cell damage, and thus might be increased in the untreated OSA population. Likewise, reduced EPC counts could also represent endothelial dysfunction (ED) in OSA.

I hypothesized that the CECs and EPCs distribution patterns could represent the burden of ED in subjects with moderate to severe OSA. I tested the hypothesis by performing flow cytometry on peripheral blood sample of modeare to severe OSA subjects and compared with hypertensive and healthy controls.

3.2.3.3 Methods

The methods have been described in details under section 2 'Hypotheses and Methodology'.

3.2.3.4 Results

45 subjects with moderate to severe OSA were recruited once the diagnosis was established however due to technical reasons only 40 patients were available for flowcytometric analysis prior to the initiation of the CPAP treatment. Therefore in total, the flow cytometric analysis was performed on 120 patients (OSA=40, hypertensive=40, healthy=40). All three groups were matched for age, sex and body mass index [Table 3-8]. On average, patients were middle aged (50±9 years) with similar proportion of female gender representation (20%). OSA patients has greater median AHI compared to both, hypertensive and healthy subjects (both $p<0.001$). In the hypertensive cohort, systolic blood pressure was higher compared to healthy group ($p<0.0001$).

As shown in Table 3-9, the number of circulating CD34++ cells, both in absolute count ($p=0.713$) and as percentage ($p=0.764$) of peripheral blood mononuclear cells, was not significantly different in OSA patients compared to that seen in both control groups [Table 3-9]. There were no statistically significant differences between the groups with respect to numbers of CECs ($p=0.083$) or circulating EPCs ($p=0.262$) and EPC/CEC ratio ($p=0.766$) [Figure 2-2] [Table 3-9].

Assessment of correlations between numbers of CECs and circulating EPCs in OSA and age, sex, body mass index, smoking, apnoea hypopnoea index, Epworth score and blood pressure yielded no statistically significant correlations [Table 3-10].

3.2.3.5 Discussion

In this study, I observed no significant differences in EPCs or CECs counts in the healthy OSA patients, compared with of age- and sex- and body mass index matched hypertensive and healthy controls. To the best of my knowledge this was the first and largest cross sectional comparison to date investigating EPCs/CECs using flow cytometry among patients with moderate to severe OSA, hypertension and normotensives.

My findings seem to suggest that OSA may not be associated with ED. Indeed EPCs have been proposed as a regenerative tool for treating human vascular disease, but are these cells indisputable markers of ED? Conflicting results have been reported in the identification, characterization, and precise role of EPCs in vascular biology and true significance of these cells in vascular repair and growth is still under scrutiny [Avogaro et al 2011]. Vascular homeostasis and specifically repair are complex multicellular processes that require several cellular phenotype-like endothelial cells, smooth muscle cells, monocytes, and bone marrow–derived cells. Hagensen et al. has recently questioned the role of EPCs in vascular repair by showing that bone marrow–derived cells rarely contribute to the endothelium of developing plaques or participate in endothelial regeneration after plaque rupture [Hagensen et al 2010]. The authors convincingly demonstrate that EPCs rarely contribute to plaque endothelium or regeneration of overlying endothelium after plaque disruption. This study indirectly suggests that the local vessel is the major source of plaque endothelium and predominantly contributes to regeneration of overlying endothelium after plaque rupture. All this is further complicated by the lack of a

specific marker and validated methods to unequivocally identify this circulating cell subset and by the presence of platelet microparticles in a standard assay for putative EPCs [Prokopi et al 2009]. Interestingly, a rare population of endothelial colony-forming cells has been identified in human umbilical cord blood and adult peripheral blood that embodies all of the properties of an EPC; however, there is no proof that the human endothelial colony-forming cells play a role in neoangiogenesis in vivo [Yoder et al 2007].

CECs are regarded as apoptotic cells and the apoptotic process takes place through a complex series of inter- and intra-cellular events [Avogaro et al 2011]. The consequence of the apoptotic process is detachment of endothelial cells, which are released into the bloodstream and can be recognized and measured as CECs. However, in some circumstances, endothelial cells do not detach as entire cells, but as apoptotic endothelial microparticles, which have strong prognostic value [Avogaro et al 2011]. Given the complexity of the ultimate so-called 'arterial denudation' and relative lack of data on this subjects, perhaps it is reasonable to assume that a certain threshold exists in the duration or the severity of the OSA, which needs to be reached before the apoptotic process could be triggered.

Nevertheless, my results are consistent with findings reported by Martin et al who investigated EPCs and CECs using immunomagnetic beads and flowcytometric techniques in a middle aged OSA population [Martin et al 2008].. The authors report no quantitative differences in CECs (7.0 ± 1.5 vs. 4.9 ± 0.9; $p=0.18$) or EPCs (1077 ± 318 vs. 853 ± 176; $p>0.51$) between OSA and the controls.

My findings can be justified by technical, logistical and individual factors. Firstly, the total numbers of identifiable EPCs and CECs in peripheral blood are extremely small, even in patients with proven ED [Rafii et al 2000]. Although our study design is one of the largest cross sectional studies, yet due to the rare event rate, it could have been under powered to detect the difference. Secondly, I had stringent exclusion criteria where subjects with conditions known to alter EPCs and/or CECs count, were excluded in order to assess the un-confounded effect of moderate to severe OSA on these cell strains. Hence, it is plausible that relative changes in EPCs and CECs count in OSA might exist distinctively, i.e. in OSA and coexisting cardiovascular diseases. Thirdly, depending upon a number of predisposing factors such as severity of OSA and fat distribution, individual vulnerability to develop derangement in the cell count could be highly discrepant in OSA [Barbe et al 2001] [Schafer et al 2002].

In previous OSA studies, exaggerated circadian variability has been indentified in certain hemodynamic variables such as blood pressure, the so-called ‘‘nondipping and dipping patterns’’ [Akashiba et al 1999]. These circadian fluctuations in endothelial status have been identified using more specific tests such as flow mediated dilatation [Maruo et al 2006]. However, the existence of circadian variability in EPCs and CECs numbers is not known in OSA and it can be hypothesized that these cells may alter transiently or/and intermittently during hypoxia. As I obtained blood samples in the morning (0900-1100 AM), I can not exclude the presence or the extent of circadian rhythm.

Limitations

I enumerated EPCs and CECs using modern flow cytometry alone. The choice between flow cytometry and immunobead technique is debatable as both have serious limitations [Shantsila et

al 2007 (a)(b)]. Furthermore, I did not perform qualitative analysis of the functional properties of these cells, which may have shown more profound impact of OSA compared to mere quantitative changes.

Mildly elevated BP, though statistically insignificant compared with healthy group, is another limitation of this study. On statistical analyses no correlation was found between the BP and the cellular counts. Although the cellular count was comparable amongst the group, it is reasonable to challenge the contribution of mildly elevated BP.

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3.2.3.6 Conclusion

This study did not demonstrate significant differences in EPCs and CECs distribution in otherwise healthy subjects with moderate to severe OSA free from confounding vascular disease or cardiovascular risk factors, compared with healthy or hypertensive controls. Therefore, I suggest that EPCs and CECs may not represent dysfunctional endothelium/endothelial damage, in a normotensive subset of moderate to severe OSA patients in the absence of coexisting cardiovascular disease or risk factors.

Table 3-8: Clinical Characteristics of Obstructive Sleep Apnoea patients, Hypertensives and Healthy Controls

Variable	OSA	Hypertensive controls	Healthy controls	p
n	40	40	40	
Male/Female	31/9	32/8	29/11	0.343
Age (years)	50(9)	49.1(12)	45.8(7)	0.120
Aponea Hypopnea index	33 (21-50) *Ψ	4(1.2-5.8)Ψ	3(0.9-5)*	<0.0001
Body mass index	34(8)	32(6)	32(6)	0.118
Systolic Blood pressure (mmHg)	142(16)	150(21)^	135(15)^	<0.0001
Diastolic Blood pressure (mmHg)	83(11)	85(10)	82(9)	0.22
No. of current smokers	14	12	16	0.21

Values are represented as Mean (Standard deviation) and Median (Inter-quartile Range). A $p<0.05$ was considered statistically significant.* Significance between OSA and Healthy subjects; ^ Significance between Hypertensive and Healthy subjects; Ψ Significance between OSA and Hypertensive subjects

OSA: obstructive sleep apnoea

Table 3-9: Endothelial Progenitor Cells and Circulating Endothelial Cell in Obstructive Sleep Apnoea patients, Hypertensive and Healthy groups

	OSA	Hypertensive controls	Healthy controls	p
n	40	40	40	
%CD34++ count	0.0004 (0.0003-0.0007)	0.0005 (0.0003-0.0007)	0.0004 (0.0003-0.0007)	0.764
CD34++ count (Per ml)	3092 (1756-3891)	2753 (1646-5409)	3144 (1948-5043)	0.713
Absolute EPC count (Per ml)	79 (43-149)	104 (64-182)	99 (48-404)	0.262
Absolute CEC count (Per ml)	81 (41-142)	107 (49-178)	133 (63-274)	0.083
EPC/CEC ratio	1 (0.79-1.60)	1 (0.67-1.36)	1.1 (0.54-1.71)	0.766

OSA= obstructive Sleep Apnoea; EPC= Endothelial Progenitor Cell; CEC= Circulating Endothelial Cell

Table 3-10: Univariate analyses of the Pre-CPAP Cellular count in Obstructive Sleep Apnoea

	Abs EPC		**Abs CEC**	
	r	**p**	**r**	**p**
Age	0.040	0.796	-0.024	0.873
Height	0.184	0.227	0.271	0.072
Weight	-0.135	0.377	-0.100	0.514
BMI	-0.224	0.140	-0.234	0.122
AHI	-0.016	0.917	-0.130	0.395
Epworth score	0.082	0.629	0.093	0.526
Systolic BP	0.025	0.869	0.019	0.902
Diastolic BP	0.160	0.294	0.004	0.979
Glucose	-0.108	0.497	-0.058	0.715
Total cholesterol	-0.248	0.101	-0.070	0.646

r=correlation coefficient; AHI= Apnoea Hypopnoea Index; FMD= Flow mediated Dilatation; BP= Blood Pressure, CEC= Circulating endothelial cells; EPC= endothelial progenitor cells; BMI= Body mass index

3.2.4 Assessment of Pulse Wave Analysis and Pulse Wave Velocity in Moderate to Severe Obstructive Sleep Aponea compared to Hypertensive and Healthy controls

3.2.4.1 Abstract

Background: Moderate–severe obstructive sleep apnea (OSA) is associated with endothelial dysfunction (ED) and hypertension. However it is uncertain if moderate-severe OSA in otherwise healthy subjects is also associated with increased arterial stiffness and impaired macro-vascular function.

Method: In 35 patients with moderate to severe OSA [Aponea-hypopnoea index (AHI) 36±20], and 35 matched diseased and healthy control subjects without OSA, arterial stiffness was assessed by applanation tonometry– derived pulse wave analysis (PWA) and carotid-femoral pulse wave velocity (PWV). Baseline and Glycerol trinitrate (GTN)/ Salbutamol induced changes in aortic augmentation (AA), aortic augmentation index (AIx), AIx corrected for heart rate of 75/minute (AIx@75), Sub-endocardial viability ratio (SEVR)] and PWV were recorded in all subjects.

Results: Systolic blood pressure (SBP) was higher in hypertensives compared to healthy subjects (p=0.001). Indices of PWA or the PWV were not statistically different in OSA patients at baseline or in response to GTN/salbutamol, compared with the controls.

Conclusion: Systemic arterial function and responses to endothelium-dependent (salbutamol induced) and -independent vasodilators (GTN induced) are preserved in patients with moderate to severe uncomplicated OSA, indicating preserved macro-vascular endothelial function.

3.2.4.2 Introduction

Multiple causal factors such as OSA related endothelial dysfunction (ED), modulations in sympathetic tone, high blood pressure and activation of inflammatory mediators have been implicated to cause vascular damage and in the development of atherosclerosis [Somers et al 1995][Shamsuzzaman et al 2002][Ip et al 2004]. Available evidence suggests that arterial stiffness and its interaction with dysfunctional endothelium significantly contribute in the accentuation of atherosclerosis and could be associated with cardiovascular risk factors [Koji et al 2007] [Ross et al 1999][Shimokawa et al 1999][Celermajer et al 1992][Schachinger et al 2000]. Although minimally symptomatic OSA has been shown to be associated increased arterial stiffness and hypertension [Kohler et al 2008 (a)], however only a few studies have investigated functional deformation in the arterial waveform in moderate to severe OSA [Kohler et al 2008 (b)][Drager et al 2007][Tomiyama et al 2009]. However, prominent presence of the cardiovascular diseases/risk factors in these available data may have heavily confounded the outcome [Wilkinson et al 2002] [Zhong et al 2005]. In summary, there are no uniform data reflecting early changes in vascular transfer function in otherwise healthy normotensive patients with moderate to severe OSA.

To investigate if arterial stiffness and hence vascular functions are impaired in patients with moderate to severe OSA, I conducted a cross-sectional study investigating validated indices of arterial stiffness and their responses to glycerol trinitrite (GTN) and Salbutamol to assess endothelial dependent- and independent vascular vasoreactivity. I hypothesized that subjects with

moderate to severe OSA have enhanced arterial stiffness compared with healthy and hypertensive controls.

3.2.4.3 Methods

The methods have been described in details under section 2 'Hypotheses and Methodology'.

3.2.4.4 Results

Although 45 subjects with moderate to severe OSA were recruited after the diagnosis was established with polysomnography but due to certain reasons only 35 OSA patients were available for assessment of arterial stiffness prior to the initiation of the continuous positive airway pressure (CPAP) treatment. These subjects were compared with equal number of age-, sex- and BMI-matched healthy and hypertensive controls [Table 3-11]. On average, OSA patients were middle aged (49±10 years) with similar proportion of female gender representation (25%). OSA patients has greater mean AHI compared to both, hypertensive and healthy subjects {both $p<0.0001$}; AHI was comparable between hypertensive and healthy individuals. In the hypertensive cohort, systolic blood pressure was higher compared to healthy subjects ($p=0.002$) partly due to omitting antihypertensive drugs for at least 12 hours before test.

Arterial stiffness assessed using AA, AIx, AIX@75, SEVR and PWV was comparable at baseline, amongst the three groups [Table 3-12; 3-13]. The endothelial-dependent (induced by systemic application of Salbutamol) and -independent changes (induced by sublingual application of GTN) in these parameters were not significantly different in OSA patients compared with control groups. During longitudinal analyses, only healthy subjects demonstrated normal endothelial dependent responses (Salbutamol use) whilst endothelial independent responses (GTN induced) were preserved in all three groups as shown below [Table 3-14; 3-15; 3-16].

On Univariate analysis, no relationship was found between vascular responses and age, BMI, AHI, Blood pressure, glucose and total cholesterol [Table 3-17].

3.2.4.5 Discussion

To the best of my knowledge, this is the first well-controlled, cross-sectional study on the assessment of vascular elastic properties in otherwise healthy patients with moderate to severe OSA. I demonstrated that patients with moderate to severe OSA had preserved vascular transfer function compared with matched control subjects without OSA.

I used a number of surrogate markers for arterial stiffness and pressure waveform reflection. For example, the AIx and SEVR are accurate surrogates for vascular elasticity and excellent predictor of adverse cardiovascular events [Wilkinson et al 2002] [Weber et al 2004][Noon et al 2008]. However, according to recent CAFÉ (Conduit Artery Function Evaluation) study, AIx@75 (AIx corrected for heart rate of 75 beats per minute) is a better and sensitive surrogate of central arterial stiffness [Williams et al 2009]. Finally, PWV is regarded as a superior marker of arterial stiffness in middle aged population [Zoungas et al 2007] [Drager et al 2007 (a)].

These data are in contrast with the findings from previous OSA studies; however, most of available data carry notable limitations. For example, considering dominant use of radial approach in most previous studies, physiological consequences of 'site-dependent heterogeneity' and 'amplification phenomenon' can not be ignored [Phillips et al 2005][Zhong et al 2005][Jelic et al 2002][Kohler et al 2008 (a)][Laurent et al 2006]. I used carotid approach based on the consensus from 'European Network of non-invasive investigations of large arteries' which concluded that the carotids artery may provide more accurate information of the transfer function

compared to the radial artery [Kelly et al 1989][O'Rourke et al 1992][O'Rourke et al 1999]. My PWV measuring technique (carotid-femoral) could also account for our findings of preserved PWV which is contrary to available data where ankle-brachial technique was used [Kitahara et al 2006] [Tomiyama et al 2009] [Drager et al 2010 (a)] [Drager et al 2007].

Hypertension has prominently coexisted in OSA subjects in previous investigation and could be an important confounder [Zhong et al 2005]. It must be noted that hypertension on its own merit increases arterial stiffness independent of OSA. Jelic et al, reported higher AIx in hypertensive OSA patients compared to normotensive OSA subjects ($p<0.001$) [Jelic et al 2002]. Therefore due to presence of hypertension and other cardiovascular disorders with concurrent use of antihypertensives, the reported vascular changes cannot be attributed entirely to the OSA [Kohler et al 2008][Noon et al 2008][Drager et al 2007][Zhong et al 2005].

I also did not demonstrate difference in GTN or Salbutamol induced changes in the indices of PWA or PWV, amongst the groups. This is consistent with the blinded data by Waring et al [Waring et al 2006]. In this investigation comparing hypertensive and healthy subjects, Waring et al reported comparable GTN-induced and salbutamol-induced responses in OSA patients and controls [Waring et al 2006]. Our study is novel as the effects of GTN and salbutamol on PWA indices have not been tested previously in the *moderate-severe form* of OSA.

This study was carried out at the time of diagnosis of OSA and it is unknown how long the disease had existed in these individuals. It can be assumed that certain threshold duration of OSA is necessary to observe any changes in pressure waveform.

Limitations

The existence of circadian variability in indices of arterial stiffness is not known in OSA and may be important factor. In order to reduce these variations, vascular scanning was performed at fixed times (0900AM-1100AM).

Hypertension has a close correlation with arterial stiffness and has been reported by several investigators as above. In my OSA population the systolic as well as diastolic blood pressure was higher than healthy subjects, but did not reach statistical significance. It can be argued that con existing hypertension may have a significant impact on the investigated parameters. I have described the recruitment process in details, under the METHODS section. There are several reasons why my OSA subjects still had modestly elevated BP, such as white coat hypertension, autonomic dysregulation and indeed under lying hypertension. I did not investigate the BP by 24 monitoring due to unfavourable logistics and neither the autonomic dysregulation was investigated. Due to a strong and close interaction between the two, I acknowledge slightly elevated BP as one of the most important limitations of the present study. Indeed, BP needs to be studied in OSA subjects with appropriate tools in order to rectify such bias. Having said that, I did not notice a statistical relation between markers of arterial stiffness and recorded BP. This is given in the Table below, showing univariate analyses [Table 3-17].

3.2.4.6 Conclusion

This cross-sectional study has demonstrated that in otherwise healthy patients with moderate to severe OSA, indices of arterial stiffness and hence macro-vascular functions are preserved, when compared with well-matched diseased and healthy control subjects without OSA. It can also be derived that only OSA patients with cardiovascular diseases/risk factors may have increased arterial stiffness and hence enhanced cardiovascular risk.

Table 3-11: Clinical Characteristics of Obstructive Sleep Apnoea Patients, Hypertensive and Healthy Controls

Variable	OSA	Hypertensive controls	Healthy controls	p
n	35	35	35	
Male/Female	25/10	26/9	23/12	0.128
Age (years)	49(10)	48(11)	47(9)	0.654
Body mass index	32(6)	30(5)	31(6)	0.364
Waist circumference (cm)	110(16)*	105(14)	98(14)*	0.003
Aponea Hypopnea index	36 (20) *Ψ	4(2)Ψ	3(1)*	<0.0001
Systolic Blood pressure (mmHg)	141(17)	150(20)^	135(16)^	0.002
Diastolic Blood pressure (mmHg)	83(12)	85(9)	80(8)	0.113
No. of current smokers	7	7	9	0.147

Values expressed as Mean (Standard Deviation) or Median (Inter quartile range). P<0.05 is considered statistically significant
* Significance between OSA and healthy subjects
^ Significance between hypertensive and healthy subjects
Ψ Significance between OSA and hypertensive subjects

OSA: obstructive sleep apnoe

Table 3-12: Indices of Pulse Wave Analysis and Pulse Wave Velocity before and after systemic administration of Salbutamol, as Median (Inter-quartile range)

Index	OSA	HT	Healthy	P
Baseline 1				
AA	15 (9-21)	15 (9-23)	13 (10-21)	0.800
AIx	26 (20-32)	29 (16-35)	27 (17-34)	0.893
AIx@75	18 (11-31)	22 (14-30)	21 (13-28)	0.777
SEVR	162 (138-194)	167 (147-188)	164 (145-191)	0.930
PWV	6 (5-7)	5 (5-7)	5 (5-6)	0.138
After 5 Minutes				
AA	13 (7-22)	14 (7-25)	14 (3-16)	0.170
AIx	25 (12-35)	28 (12-39)	21 (7-31)	0.154
AIx@75	21 (8-27)	19 (11-30)	17 (5-22)	0.141
SEVR	157 (146-183)	161 (142-182)	155 (142-187)	0.946
PWV	6 (5-7)	5 (4-6)	5 (5-6)	0.499
After 10 Minutes				
AA	13 (7-20)	14 (5-23)	12 (6-15)	0.554
AIx	25 (14-33)	26 (9-34)	22 (10-30)	0.642
AIx@75	18 (7-29)	21 (6-28)	16 (7-27)	0.522
SEVR	159 (143-176)	161 (144-179)	164 (139-192)	0.929
PWV	6 (5-7)	6 (5-7)	5 (5-6)	0.286
After 15 Minutes				
AA	12 (7-20)	13 (5-21)	14 (7-17)	0.844
AIx	22 (13-33)	26 (9-35)	23 (11-31)	0.890
AIx@75	17 (8-29)	21 (11-27)	17 (12-26)	0.834
SEVR	163 (143-181)	156 (146-181)	160 (141-185)	0.829
PWV	6 (5-7)	5 (5-6)	5 (5-6)	0.126
After 20 Minutes				
AA	12 (7-21)	11 (4-22)	12 (7-19)	0.942
AIx	23 (15-33)	21 (10-35)	21 (12-32)	0.872
AIx@75	18 (7-28)	19 (6-29)	15 (8-25)	0.868
SEVR	163 (144-181)	157 (144-174)	150 (140-190)	0.901

PWV	6 (5-7)	5 (5-6)	5 (5-6)	0.245

OSA= Obstructive Sleep Apnoea, HT=Hypertension, AA=Aortic Augmentation, AIx=Augmentation Index, AIx@75=Augmentation Index at heart rate of 75/minute, SEVR=Sub-Endocardial Viability Ratio, PWV= Pulse Wave Velocity

Table 3-13: Indices of Pulse Wave Analysis and Pulse Wave Velocity before and after systemic administration of Glyceryl trinitrate, as median (Inter-quartile range)

Index	OSA	HT	Healthy	P
Baseline 2				
AA	14 (9-21)	15 (8-23)	15 (9-18)	0.956
AIx	26 (19-33)	26 (13-38)	29 (17-33)	0.923
AIx@75	22 (13-31)	22 (9-35)	21 (17-28)	0.934
SEVR	163 (145-187)	156 (139-172)	162 (139-199)	0.451
PWV	5.8 (4.8-7)	5.3 (4.8-6.5)	5.4 (4.8-6)	0.612
After 5 Minutes				
AA	3 (-3 - 7)	4 (-2 - 11)	7 (1-11)	0.126
AIx	4 (-4 - 14)	7 (-2 - 16)	12 (3-24)	0.086
AIx@75	0 (-6 - 13)	10 (-3 - 16)	7 (0-17)	0.131
SEVR	160 (145-183)	156 (140-170)	158 (142-191)	0.846
PWV	5.6 (5-8)	5.4 (4.8-6)	5.3 (5-5.9)	0.362
After 10 Minutes				
AA	1 (-4 - 8)	6 (-5 - 12)	5 (-1 - 10)	0.322
AIx	2 (-7 - 13)	6 (-9 - 20)	10 (-2 - 17)	0.272
AIx@75	-1 (-9 - 8)	4 (-12 - 17)	5 (-3 - 10)	0.186
SEVR	164 (143-176)	160 (146-174)	158 (142-189)	0.986
PWV	6 (5-7)	5 (5-8)	5.5 (5-6)	0.467
After 15 Minutes				
AA	2 (-4 - 7)	5 (-5 - 12)	6 (-1 - 12)	0.320
AIx	2 (-6 - 13)	12 (-7 - 21)	11 (-1 - 20)	0.281
AIx@75	-2 (-12 - 11)	6 (-7 - 18)	7 (-3 - 15)	0.199
SEVR	163 (145-179)	161 (152-182)	150 (148-191)	0.904
PWV	6 (5-7)	5 (5-7)	5 (5-6)	0.486
After 20 Minutes				
AA	3 (-3 - 10)	7 (-3 - 15)	5 (0.3-12)	0.669
AIx	5 (-7 - 19)	15 (-5 - 25)	8 (-0.3 - 18)	0.597
AIx@75	-2 (-8 - 16)	8 (-10 - 22)	4 (-3 - 15)	0.510
SEVR	164 (144-178)	163 (152-185)	163 (147-198)	0.984
PWV	6 (5-7)	5 (5-7)	5 (5-6)	0.255

OSA= Obstructive Sleep Apnoea, HT=Hypertension, AA=Aortic Augmentation, AIx=Augmentation Index, AIx@75=Augmentation Index at heart rate of 75/minute, SEVR=Sub-Endocardial Viability Ratio, PWV= Pulse Wave Velocity

Table 3-14: Indices of Pulse Wave Analysis and Pulse Wave Velocity versus time after Salbutamol in Pre-CPAP Obstructive sleep apnoea, Hypertensive and Healthy Subjects

	Pre Sal	5 min	10 min	15 min	20 min	P
OSA						
AA	15 (9-21)	13 (7-22)	13 (7-20)	12 (7-20)	12 (7-21)	0.159
AIX	26 (20-32)	25 (12-35)	25 (14-33)	22 (13-33)	23 (15-33)	0.120
AIx@75	18 (11-31)	17 (8-27)	18 (7-29)	17 (8-29)	18 (7-28)	0.440
SERV	162 (138-194)	157 (146-183)	159 (143-176)	163 (143-181)	163 (144-181)	0.600
PWV	6 (5-7)	6 (5-7)	6 (5-7)	6 (5-7)	6 (5-7)	0.119
HT						
AA	15 (9-23)	14 (7-25)	14 (5-23)	13 (5-21)	14 (4-22)	0.372
AIX	29 (16-35)	28 (12-39)	26 (9-34)	26 (9-35)	25 (10-35)	0.147
AIx@75	22 (14-30)	19 (11-30)	21 (6-28)	21 (11-27)	19 (6-29)	0.380
SERV	167 (147-188)	161 (142-182)	161 (144-179)	156 (146-181)	157 (144-174)	0.119
PWV	5 (5-7)	5 (4-6)	6 (5-7)	5 (5-6)	5 (5-6)	0.092
Healthy						
AA	13 (10-21)*	10 (3-16)*	12 (6-15)	14 (7-17)	12 (7-19)	0.002
AIX	27 (17-34)*	21 (7-31)*	22 (10-30)	23 (11-31)	21 (12-32)	0.015

AIx@75	21 (13-28)*	17 (5-22)*	16 (7-27)	17 (12-26)	15 (8-25)	0.025
SERV	164 (145-191))*#∞	155 (142-187)*	164 (139-192)	160 (141-185))#	150 (140-190))∞	0.006
PWV	5 (5-6)	5 (5-6)	5 (5-6)	5 (5-6)	5 (5-6)	0.544

Values as Median (Inter-quartile range); $p<0.05$ is significant

* Significant difference between time 0 and 5 min

^ Significant difference between time 0 and 10 min

Significant difference between time 0 and 15 min

∞ Significant difference between time 0 and 20 min

OSA= Obstructive Sleep Apnoea, HT=Hypertension, Sal= Salbutamol, AA=Aortic Augmentation, AIx=Augmentation Index, AIx@75=Augmentation Index at heart rate of 75/minute, SEVR=Sub-Endocardial Viability Ratio, PWV= Pulse Wave Velocity

Table 3-15: Indices of Pulse Wave Analysis and Pulse Wave Velocity versus time after Glyceryl trinitrite in Pre-CPAP Obstructive sleep apnoea patients, Hypertensive and healthy controls

	Pre-GTN	5 min	10 min	15 min	20 min	P
OSA						
AA	14 (9-21)*^#∞	3 (-3 - 7)*	1 (-4 - 8)^	2 (-4 - 7)#	3 (-3 - 10)∞	<0.0001
AIX	26 (19-33))*^#∞	4 (-4 - 14)*	2 (-7 - 13)^	2 (-6 - 13)#	5 (-7 - 19)∞	<0.0001
AIx@75	22 (13-31)*^#∞	0 (-6 - 13)*	-1 (-9 - 8)^	-2 (-12 - 11)#	-2 (-8 - 16)∞	<0.0001
SERV	163 (145-187)	160 (145-183)	164 (143-176)	163 (145-179)	164 (144-178)	0.651
PWV	5.8 (4.8-7)	5.6 (5-8)	6 (5-7)	6 (5-7)	6 (5-7)	0.388
HT						
AA	15 (8-23)*^#∞	4 (-2 - 11)*	6 (-5 - 12)^	5 (-5 - 12)#	7 (-3 - 15)∞	<0.0001
AIX	26 (13-38)*^ #∞	7 (-2 - 16)*	6 (-9 - 20)^	12 (-7 - 21)#	15 (-5 - 25)∞	<0.0001
AIx@75	22 (9-35)*^#∞	10 (-3 - 16)*	4 (-12 - 17)^	6 (-7 - 18)#	8 (-10 - 22)∞	<0.0001
SERV	156 (139-172)#∞	156 (140-170)√	160 (146-174)	161 (152-182)#	163 (152-185)∞√	<0.0001
PWV	5.3 (4.8-6.5)	5.4 (4.8-6)	5 (5-8)	5 (5-7)	5 (5-7)	0.734
Healthy						
AA	15 (9-18)*^	7 (1-11)*	5 (-1 - 10)^	6 (-1 - 12)#	5 (0.3-12)∞	<0.0001

	#∞					
AIX	29 (17-33)*^ #∞	12 (3-24)*	10 (-2 - 17)^	11 (-1 - 20)#	8 (-0.3 - 18)∞	<0.0001
AIx@75	21 (17-28)*^ #∞	7 (0-17)*	5 (-3 - 10)^	7 (-3 - 15)#	4 (-3 - 15)∞	<0.0001
SERV	162 (139-199)	158 (142-191)	158 (142-189)	150 (148-191)	163 (147-198)	0.485
PWV	5.4 (4.8-6)	5.3 (5-5.9)	5.5 (5-6)	5 (5-6)	5 (5-6)	0.352

Values as Median (Inter-quartile range); $p<0.05$ is significant

* Significant difference between time 0 and 5 min

^ Significant difference between time 0 and 10 min

Significant difference between time 0 and 15 min

∞ Significant difference between time 0 and 20 min

√ Significant difference between time 5 and 20 min

OSA= Obstructive Sleep Apnoea, HT=Hypertension, GTN= Glycerol trinitrite, AA=Aortic Augmentation, AIx=Augmentation Index, AIx@75=Augmentation Index at heart rate of 75/minute, SEVR=Sub-Endocardial Viability Ratio, PWV= Pulse Wave Velocity

Table 3.16: Comparison of the Baseline values pre-Salbutamol and pre- Glyceryl trinitrate for three groups; as Median (Inter-quartile range)

	OSA		
	B1 (pre Sal)	**B2 (pre-GTN)**	**P**
AA	15 (9-21)	14 (9-21)	0.874
AIx	26 (20-32)	26 (19-33)	0.546
AIx@75	18 (11-31)	22 (13-31)	0.150
SEVR	162 (138-194)	163 (145-187)	0.654
PWV	6 (5-7)	5.8 (4.8-7)	0.798
	HT		
AA	15 (9-23)	15 (8-23)	0.691
AIx	29 (16-35)	26 (13-38)	0.421
AIx@75	22 (14-30)	22 (9-35)	0.741
SEVR	167 (147-188)	156 (139-172)	0.091
PWV	5 (5-7)	5.3 (4.8-6.5)	0.321
	Healthy		
AA	13 (10-21)	15 (9-18)	0.165
AIx	27 (17-34)	29 (17-33)	0.446
AIx@75	21 (13-28)	21 (17-28)	0.246
SEVR	164 (145-191)	162 (139-199)	0.812
PWV	5 (5-6)	5.4 (4.8-6)	0.311

OSA= Obstructive sleep Apnoea; HT= Hypertension; B1= Baseline 1 ; B2= Baseline 2; Sal= Salbutamol; GTN= Glycerol trinitrite; AA=Aortic Augmentation, AIx=Augmentation Index, AIx@75=Augmentation Index at heart rate of 75/minute, SEVR=Sub-Endocardial Viability Ratio, PWV= Pulse Wave Velocity

Table 3-17: Univariate analyses of the Pre-CPAP arterial stiffness markers in Obstructive Sleep Apnoea

	AIx75		**PWV**	
	r	**p**	**r**	**p**
Age	0.199	0.260	0.618	0.010
Height	-0.358	0.48	-0.478	0.062
Weight	-0.347	0.57	-0.216	0.228
BMI	0.181	0.306	0.048	0.790
AHI	0.109	0.539	-0.197	0.273
Epworth score	0.038	0.845	0.187	0.342
Systolic BP	0.163	0.358	0.552	0.111
Diastolic BP	0.439	0.110	0.311	0.08
Glucose	0.117	0.529	-0.102	0.592
Total cholesterol	0.043	0.809	-0.113	0.532

r=correlation coefficient; AHI= Apnoea Hypopnoea Index; FMD= Flow mediated Dilatation; BP= Blood Pressure; PWV= Pulse wave velocity; AIx@75= Augmentation index corrected for heart rate of 75/min; BMI= Body mass index

3.2.5 Left Ventricular Systolic and Diastolic function as assessed by Two- and Three-Dimensional Echocardiography in Moderate-Severe Obstructive Sleep Apnoea compared with healthy and hypertensive controls

3.2.5.1 Abstract

Background: Previous studies have yielded incongruent results regarding the effect of moderate to severe obstructive sleep aponea (OSA) on left ventricular (LV) function, in the absence of pulmonary or cardiac co-morbidity. In addition, there are limited data on the assessment of LV function using real time three-dimensional echocardiography (RT3DE).

Methods: I performed a prospective cross-sectional study investigating healthy subjects with moderate to severe OSA compared with stable hypertensive and healthy controls (n=40 in each group). Two-dimensional echocardiography (2DE) and RT3DE were used to measure LV systolic function (M-mode and modified Simpson's method) and acute and chronic indices of diastolic function (left atrial volume index, trans-mitral flow patterns and tissue Doppler imaging).

Results: OSA and hypertensive groups exhibited LV diastolic but not systolic dysfunction compared with healthy individuals. Left atrial (LA) volume and LA volume index was significantly greater in OSA compared to the control groups ($p<0.001$ for all comparisons).

Conclusion: Episodic changes in breathing patterns during sleep (repetitive apnoeas/ hypopnoeas) could be significant factors in the LV remodelling leading to LV diastolic dysfunction in non-hypertensive patients with moderate to severe OSA.

3.2.5.2 Introduction

Some obstructive sleep apnoea (OSA) studies have reported left ventricular (LV) systolic dysfunction [Krieger et al 1991][Malone et al 1991]. Although the underlying mechanisms are largely unclear however a reduced LV output is believed to be due to exaggerated negative intrathoracic pressure dynamics, and hypoxia driven augmentation in vascular resistance and rise in transmural pressure [Karam et al 1984][Parker et al 1999][Millman et al 1991] [Laks et al 1995]. Interestingly, some propose that OSA merely interacts with subclinically existing cardiac and respiratory diseases and enhances the appearance of heart and respiratory failure [Marrone et al 1998]. More importantly, only a few studies have specifically investigated LV function in moderate to severe OSA which is the primary target for continuous positive airway pressure (CPAP) treatment. Even in the available data, there are important limitation owing to loose inclusion criteria and mere use of 2DE techniques [Nahmias et al 1996]. It must be noted that RT3DE has been proven useful tool for accurate LV assessment in a number of conditions but there is no data on the use of RT3DE in the moderate to severe OSA population.

In summary, the LV systolic dysfunction is a debatable issue in moderate to severe OSA subjects. However, LV diastolic dysfunction has been reported by most OSA subjects with or without LV hypertrophy [kim et al 2008][Alchanatis et al 2002][Hedner et al 1990].

In this project, healthy OSA patients were compared with patients with stable and treated hypertension as well as healthy individuals, using both Two- and Three-dimensional echocardiographic techniques. I tested the hypothesis that ventilatory changes in moderate to severe OSA subjects causes LV remodelling manifested as impaired LV systolic and diastolic function, compared with healthy and hypertensive controls.

3.2.5.3 Methods

The methods have been described in details under section ‘Hypotheses and Methodology’.

3.2.5.4 Results

As shown in Table 3-18, the echocardiography was performed on total of 120 patients (OSA=40, hypertensive=40, healthy=40) prior to CPAP treatment. Subjects with suboptimal image quality were excluded. OSA patients has greater mean AHI compared to both hypertensive and healthy subjects (both $p<0.0001$).

Left Ventricular Systolic Function

There were no differences in LV volumes and other parameters including ejection fraction (EF), stroke volume (SV) and fractional shortening (FS) amongst the 3 groups using M-mode, modified Simpson’s and RT3DE methods [Table 3-19].

Left Ventricular Diastolic Function

Table 3-20 presents diastolic indices provided by conventional echocardiography and tissue Doppler (TDI). Early and late mitrial inflow velocities and their ratio (E, A and E/A), were significantly impaired in both, OSA and hypertensive groups, compared to healthy subjects ($p <0.001$ for both comparisons). OSA and hypertensive patients showed a significantly reduced TDI derived E’ velocity (<0.0001 for both) and prolonged iso-volumetric relation time (IVRT) than did normal control subjects ($p=0.02$ for both). Post-hoc analysis showed a significant difference in E/E’ between hypertension and healthy controls, but only a non-significant trend between OSA and healthy subject ($p=0.58$). All above diastolic indices were comparable between OSA and hypertension [Table 3-20].

Left atrial (LA) volume and LA volume index (LAVI) were significantly greater in OSA and hypertensives compared to healthy controls using 2DE and RT3DE [Table 3-21]. On Univariate analysis, no relationship was found between ventricular dimensions and age, BMI, AHI, Blood pressure, glucose and total cholesterol [Table 3-22]

3.2.5.1 Discussion

Important findings of this study are following: 1) systolic function is preserved in healthy OSA subjects; 2) OSA subjects exhibit LV diastolic impairment; 3) RT3DE derived LAVI is significantly greater in OSA compared with healthy controls. This is a novel study as the LV function, LA volume and LAVI have not previously been comprehensively explored using RT3DE in moderate to severe OSA. Due to high prevalance of atrial fibrillation and stroke in OSA subjects, our findings of greater LAVI may have important clinical and prognostic implications.

LV Systolic function

OSA has been described in conjunction with LV systolic dysfunction in a number of observational studies. In three large case series, 37%, 11% and 26% of the populations with impaired LV systolic function have been reported to have proven OSA [Sin et al 1999] [Javaheri et al 1998][Wang et al 2007]. Intesrestingly, LV systolic dysfunction tends to occur less in females (men 38% versus women 31%; $p<0.05$) and in subjects with excessive daytime somnolence [Javaheri et al 1998] [Arzt et al 2006][Sin et al 1999].

LV function has also been reported to be preserved in OSA patients in several studies and our findings are in line with these data [Alchanatis et al 2002] [Kraiczi et al 2001][Kim et al 2008]. For example, Kim et al studied 62 patients with mild, moderate and severe OSA and observed normal LV dimensions, mass index, LVEF and FS compared to health subjects ($p>0.05$) [Kim et

al 2008]. Similarly, whilst measuring FS (39.4±2.6% versus 39.9±4.1%; p>0.05), septal wall (9.2±0.8 mm versus 9.9±1.2 mm; p>0.05) and posterior wall thickness (8.6±1.0 mm versus 8.3±1.2mm; p>0.05) as a surrogate for LV systolic function, Alchanatis et al observed no difference between OSA and healthy subjects [Alchanatis et al 2000]. It is noteworthy that the present study is one of the largest to focus on healthy moderate to severe OSA and the first one to utilize RT3DE in this particular group.

Indeed, few have reported impaired LVEF in OSA compared with healthy subjects but the techniques used in these investigations was not validated for LV systolic assessment [Alchanatis et al 2002]. Repetitive nocturnal blood pressure surges have been reported in OSA patients and may be the most likely mechanism by which chronic OSA induce left ventricular systolic dysfunction [Portaluppi et al 1997][Levy et al 1996] [Verdecchia et al 1990]. My OSA patients were normotensive and although I did not measure nocturnal blood pressure fluctuation, it can be derived that only persistently elevated blood pressure would impair LV systolic function.

Traditional methods of LV systolic function assessment (M-mode and modified Simpson's method), may not be accurate due to sub-optimal image acquisition with 2DE, patient habitus and unusual LV geometry in OSA subjects. RT3DE has proven useful in accurate LV assessment compared with 2DE and older 3D reconstruction techniques [Von Ramm et al 1990][Smith et al 1991][Shiota et al 1998][Zwas et al 1999].

LV Diastolic Function using Conventional Indices

Diastolic dysfunction is regarded as a condition with increased resistance to LV filling, resulting in discernible transformations in the diastolic pressure-volume curve, causing symptoms of pulmonary congestion at rest and exertion [Brutsaert et al 2000]. The present study shows

deranged of markers of diastolic function in moderate to severe OSA and hypertension groups compared to healthy subjects.

Most studies on LV diastolic function in OSA patients have demonstrated impaired LV filling patterns [Arias et al 2005][Kraiczi et al 2001][Fung et al 2002] [Niroumand et al 2001] which are consistent with my reported findings. Few have reported preserved LV diastolic function [Niroumand et al 2001].

Evidence from animal studies suggest that surges in blood pressure, changes in LV hemodynamics, left ventricular hypertrophy and their interplay could be potential mechanisms leading to diastolic dysfunction in OSA [Flecher et al 1992][Gradman et al 2009]. Although same factors seem to induce diastolic impairment in *homo sapiens* too but it must be borne in mind that due to fundamental differences in artificially induced OSA in animals and naturally occurring OSA in humans, the presence of an additional perpetrator can not be completely ruled out [Stradling et al 1997]. For example, OSA is widely reported to cause 'endothelial dysfunction'; a condition associated with paucity of nitric oxide. Since nitric oxide is a key vasodilator, impairment of its secretion results in vasoconstriction and increased peripheral vascular resistance which may ultimately contribute to impaired LV filling [Schulz et al 2000][Ip et al 2000].

Left Atrial Volume/Left Atrial Volume Index

LA remodeling is viewed as a surrogate marker for chronic diastolic dysfunction. Notably, increased LA volume is an independently predictive of atrial fibrillation, heart failure and worse prognosis in asymptomatic populations with preserved LV systolic function [Pritchett et al 2005] [Takemoto et al 2005]. In present study of OSA patients without structural heart disease, greater LA volume might reflect chronicity of diastolic dysfunction. Due to age related altered flow dynamics and remodeling, I corrected LA volume for body surface area and reported as LAVI

[Lang et al 2005][Pritchett et al 2003]. My findings of larger LAVI in OSA are in accord with limited available data [Otto et al 2007] [Khan et al 2008]. Nevertheless previous studies were based on 2DE and were relatively smaller. This study is the first study to report greater LAVI in OSA population using RT3DE which may explain high preponderance of AF and stroke in OSA subjects. This may also elucidate high AF recurrence rate after cardiovarsion and ablation in OSA population [Ng et al 2011].

Limitations

Inter- and intra-observer variation in echocardiographic examinations is an important issue. However in order to ensure uniformity, all the measurements and recordings were performed and analyzed by the same operator. Besides, there was an excellent agreement between 2DE and RT3DE results.

High blood pressure is a well known cause of systolic and diastolic dysfunction. As I have demonstrated impaired diastolic function (with enlarged left atrium) with concomitantly high BP in OSA population, the effect of the latter on LA and LV dimensions can be questioned. The OSA subjects were non-hypertensive and compared to hypertensive cohort their average BP is much lower (150 versus 142). Besides OSA group's average BP is comparable with the healthy cohort. Such a modest rise is unlikely to cause a clear and marked diastolic dysfunction and subsequent LA dilatation. It's notable that no correlation existed between BP and the LV functional and structural parameters as given in the Table below.

3.2.5.6 Conclusion

In conclusion, this study reports preserved LV systolic yet impaired diastolic function in moderate to severe OSA subjects using both 2DE and RT3DE. LAVI have been demonstrated to

be significantly greater in OSA population which may have important predicting and managing atrial arrythmias.

Table 3-18: Clinical Characteristics of Obstructive Sleep Apnoea Patients, Hypertensives and Healthy controls

Variable	OSA	Hypertensive controls	Healthy controls	p
n	40	40	40	
Male/Female	33/7	31/9	30/10	0.647
Age (years)	50(10)	49(11)	46(9)	0.120
Aponea Hypopnea index	39 (22) *Ψ	4(2)Ψ	3(2)*	<0.0001
Body mass index	34(8)	32(6)	32(6)	0.118
Systolic Blood pressure (mmHg)	142(16)	150(20)^	135(15)^	<0.0001
Diastolic Blood pressure (mmHg)	83(11)	85(10)	82(9)	0.22
No. of current smokers	16	12	13	0.374

Values are described as Mean (Standard Deviation) and Median (Inter-quartile Range). P <0.05 was considered statistically significant.
* Significance between OSA and Healthy subjects
^ Significance between Hypertensive and Healthy subjects
Ψ Significance between OSA and Hypertensive subjects
OSA= Obstructive Sleep Apnoea

Table 3-19: Systolic Indices amongst the Obstructive Sleep Apnoea Patients, Hypertensives and Healthy controls using M-mode, Modified simpson's method and real time three dimensional echocardiography

	OSA	Hypertension	Healthy	p
n	40	40	40	
	M-Mode			
IVSd (cm)	1.2 ± 0.20	1.2±0.28	1.3±2.72	0.763
LVEDV (ml)	112.5±37.08	104.3±30.38	111.8±35.80	0.499
LVESV (ml)	42±20.22	34.8±16.08	36.7±17.14	0.175
LVEF (%)	64.1±7.84	67.4±8.52	67.4±9.8	0.152
FS (%)	35.3±5.78	37.8±6.64	38±8	0.152
	Modified Simpson's			
LVEDV (ml)	87.9±24.18	80.5±19.84	93.2±26.27	0.053
LVESV (ml)	30.6±13.04	28.4±12	34.4±14.13	0.113
LVEF (%)	65.9±9.61	65.8±8.92	64±7.77	0.552
	RT3DE			
LVEDV (ml)	84.2±25.05	87.7±23.29	84.5±24.26	0.779
LVESV (ml)	28.9±11.43	28.7±10.24	29.2±11.40	0.979
SV(ml)	54.8±17.27	58.1±16.19	55.2±15.30	0.461

LVEF (%)	66.3±7.6	67.4±6.59	65.8±6.68	0.574

Values are described as Mean (Standard Deviation). P <0.05 was considered statistically significant.
* Statistical significance lies between hypertensive and Healthy subjects
^ Statistical significance lies between OSA and Healthy subjects
OSA= Obstructive Sleep Apnoea, IVSd=Interventricular Septal thickness in Diastole, PWd= Posterior Wall thickness in Diastole, LVEDV= Left Ventricular End-Diastolic Volume, LVESV= Left Ventricular End-Systolic Volume, LVEF=Left Ventricular Ejection Fraction, FS= Fractional Shortening, SV=Stroke Volume

Table 3-20: Comparison of Diastolic Indices in Obstructive Sleep Apnoea, Hypertension and Healthy subjects

	OSA	Hypertension	Healthy	p
n	40	40	40	
E (m/sec)	51.8±13.48^	48.2±10.35*	59.5±15.03^*	0.001
A (m/sec)	53±13.37^	56.7±17.15*	41.5±10.32^*	<0.0001
E/A	1.01±0.35^	1.1±0.89*	1.5±0.45^*	<0.0001
E' (sep) (m/sec)	6.1±2.06^	5.1±1.35*	7.5±2.15^*	<0.0001
E/E'(sep)	9.5±3.93	10±3.52*	8.2±2.21*	0.05
IVRT (ms)	0.01±0.023^	0.01±0.028*	0.078±0.021^*	0.002

Values are described as Mean (Standard Deviation). P <0.05 was considered statistically significant.
* Statistical significance lies between Hypertensive and Healthy subjects
^ Statistical significance lies between OSA and Healthy subjects
OSA= Obstructive Sleep Apnoea, E= Mitral Early Inflow Peak Velocity, A= Late Inflow Peak Velocity, sep E'= Septal E prime, IVRT=Iso-Volumetric Relaxation Time

Table 3-21: Left Atrial Volume Index amongst Obstructive Sleep Apnoea Patients, Hypertensives and Healthy controls

	OSA	Hypertension	Healthy	p
n	40	40	40	
Ellipsoid Method	26.6±9.53^†	22.2±5.48*†	17.1±4.53 ^*	<0.0001
Area-length Method	28.1±9.50 ^†	23.7±5.74*†	18.6 ±4.93^*	<0.0001
RT3DE	26.3±8.04^	23.1±5.32*	18.2±4.33^*	<0.0001

Values are described as Mean (Standard Deviation). P <0.05 was considered statistically significant.
* Statistical significance lies between Hypertensive and Healthy subjects
^ Statistical significance lies between OSA and Healthy subjects
† Statistical significance lies between OSA and Hypertensive subjects
OSA= Obstructive Sleep Apnoea, RT3DE= Real time 3-Dimensional Echocardiography

Table 3-22: Univariate analyses of the Pre-CPAP Left ventricular function in Obstructive Sleep Apnoea

	3DLAA		E/A		3DLVEF	
	r	**p**	**r**	**p**	**r**	**p**
Age	0.141	0.420	-0.129	0.421	-0.230	0.183
Height	0.124	0.479	0.435	0.064	-0.257	0.137
Weight	0.233	0.179	0.202	0.206	-0.182	0.297
BMI	0.225	0.194	-0.046	0.774	-0.098	0.576
AHI	0.016	0.925	-0.090	0.578	0.299	0.081
Epworth score	-0.116	0.443	-0.062	0.727	-0.122	0.521
Systolic BP	-0.008	0.948	-0.269	0.089	-0.151	0.387
Diastolic BP	-0.140	0.423	-0.292	0.64	0.004	0.981
Glucose	0.199	0.274	0.84	0.616	-0.032	0.861
Total cholester	0.08	0.646	0.203	0.201	0.104	0.552

r=correlation coefficient; AHI= Apnoea Hypopnoea Index; FMD= Flow mediated Dilatation; BP= Blood Pressure; LVEF= Left ventricular ejection fraction; LAA= Left atrial area; BMI= Body mass index

3.2.6 Myocardial Blood Flow Reserve (using Myocardial Contrast Echocardiography) in Moderate to Severe Obstructive Sleep Apnoea compared with hypertensive and healthy controls

3.2.6.1 Abstract

Background: Obstructive sleep apnoea (OSA) is associated with a variety of cardiovascular diseases and increased cardiovascular morbidity and mortality. It is unknown whether the coronary blood flow responses are appropriate for myocardial metabolic requirements in otherwise healthy subjects with moderate to severe OSA. In this cross-sectional study, myocardial perfusion was assessed in moderate to severe OSA using quantitative myocardial contrast echocardiography (MCE).

Methods: Rest and stress MCE was performed in 120 patients (n=40 in moderate-severe OSA, hypertensive and healthy cohorts) with normal left ventricular systolic function and no known cardiovascular disorder. Real-time MCE dataset were acquired in three apical views at rest and after dipyridamole challenge. Myocardial blood flow was quantified in the mid- and apical cardiac segments, for the estimation of myocardial blood flow reserve (MBFR) {ratio of myocardial blood flow at rest (FR) to myocardial blood flow following stress (FS)}.

Results: All the groups were matched for age, sex and body mass index (BMI). Resting and post stress myocardial blood flow and MBFR were significantly attenuated in OSA patients and hypertensives compared with healthy subjects.

Conclusion: Impaired myocardial perfusion (owing to microvascular dysfunction) is attenuated in otherwise normal subjects with moderate to severe OSA and can be demonstrated using MCE. This may indicate altered flow-metabolic coupling, which may lead to nocturnal myocardial ischemia and other cardiovascular disorders particularly in susceptible individuals with moderate to severe OSA.

3.2.6.2 Introduction

Myocardial blood flow reserve (MBFR) is an established marker of coronary perfusion and has been reported to be subclinically impaired in hypertension, diabetes mellitus and heart failure [Hamasaki et al 2000][Houghton et al 1992][Moir et al 2006]. The major determinants of MBFR - blood flow at rest and at maximum stress- are largely dependent on the endothelial dependent coronary microvasculature [Chilian et al 1986]. Since endothelial dysfunction (ED) has been consistently reported in obstructive aleep apnoea (OSA), it can be postulated that paucity of nitric oxide may affect myocardial blood flow patterns due to adverse changes in microvascular vasomotion and coronary vascular resistance. Resultant subclinically impaired coronary flow dynamics may explain high development of cardiovascular diseases and poor clinical outlook in the OSA population [Vane et al 1998] [Britten et al 2004].

A variety of imaging tools such as magnetic resonant imaging (MRI), positron emission tomography (PET) and single photon emission computed tomography (SPECT) have been used for perfusion assessment [Orea-Tejeda et al 2003][Hamasaki et al 2000][Marini et al 2009] [Watkins et al 2009][Herzog et al 2009]. MCE has surfaced as a non invasive, cost effective, reproducible and bedside technique which has been validated against MRI and SPECT [Moir et al 2006] [Wei et al 1998] [Di Bello et al 2004]. Yet MCE has not been used to assess myocardial perfusion in subjects with moderate to severe OSA.

In this cross sectional study, I hypothesized that significant perfusion abnormalities exist in normotensive moderate-severe OSA patients compared with healthy individuals. In order to test this hypothesis, I performed MCE to estimate important myocardial blood flow indices in moderate to severe OSA with no confounding cardiovascular risk/disorder and compared them with healthy and hypertensive controls.

3.2.6.3 Methods

The methods have been described in details under section 2 'Hypotheses and Methodology'.

3.2.6.4 Results

45 subjects with moderate to severe OSA were recruited after the diagnosis was established with polysomnography. Perfusion analysis was performed in 120 patients (OSA=40, hypertensive=40, healthy=40), and the rest were excluded due to suboptimal image quality [Table 3-23].

On the test day, heart rate in OSA was 73 ±11 beats per minute which was comparable with other two groups (p=0.76). Hypertensive subjects had higher systolic blood pressure compared to healthy group (p=0.002).

At rest, the average segmental blood volume fraction (α) was significantly attenuated in subjects with moderate to severe OSA compared to healthy (OSA 21±6 versus healthy 24±4; p=0.005) and hypertensives (OSA 21±6 versus hypertensive 24±5; p=0.005). The resting blood flow velocity (ß) was significantly higher in healthy subjects compared with patients with OSA (healthy 1.7±0.5 versus OSA 1.1±0.5; p<0.0001) and hypertension (healthy 1.7±0.5 versus hypertensive 1.3±0.4; p<0.0001). The 'FR' (multiplication of α and ß at rest) was significantly

attenuated in OSA and hypertensive subjects compared with healthy group ($p<0.0001$) yet comparable between OSA and hypertensive cohorts ($p=0.104$) [Table 3-24].

Post-dipyridamole stress 'α' and 'ß' were significantly impaired in OSA and hypertensives (<0.001 for all comparisons) compared with healthy subjects yet both indices were comparable between OSA and hypertensive subjects ($p=0.06$ and 0.29 respectively). The post stress myocardial blood flow, 'FS', calculated as a product of post stress α and ß, was also significantly impaired in both OSA and hypertensive groups compared with healthy individuals and ($p<0.0001$ for both comparisons) [Table 3-24].

The MBFR, calculated as a ratio of the 'FS' and 'FR' was found to be impaired in OSA and hypertensive subjects compared to healthy controls ($P<0.0001$ for both comparisons). The MBFR was comparable between the OSA and hypertensive groups ($P<0.001$) [Table 3-24].

On Univariate analysis, no relationship was found between perfusion indices and age, BMI, AHI, Blood pressure, glucose and total cholesterol [Table 3-25]

3.2.6.5 Discussion

For the first time, I report sub-clinically yet seriously impaired myocardial perfusion estist in otherwise healthy patients with moderate to severe OSA. These findings might clarify the disease developing mechanisms and cardiovascular prognosis in OSA population.

Coronary perfusion is largely regulated by the endothelial nitric oxide secreted from sub-endocardial arteriolar network [Chilian et al 1986]. In addition to ED, hypoxia, dysregulation of β-adrenoreceptor mediated vasodilatation and α-adrenoreceptor mediated vasoconstriction in OSA is likely to induce microvascular dysfunction, increase vascular resistance consequently

impair myocardial perfusion [Kelm et al 1995][Kirby et al 1995][Pinto et al 1993]. It has been reported that, out of the above, ED is *the* most important underlying mechanism for development of cardiovascular disease, due to its consistent demonstration in OSA population [Kelm et al 1995] [Al Suwaidi et al 2001][Celermajer et al 1996][Johnstone et al 1993][Hamasaki et al 2000][Casino et al 1993] [Grebe et al 2006][Kato et al 2000].

Relationship between blood flow patterns and ED has only been studied at experimental level in OSA. Studies seem to suggest that induction of ED leads to significant changes in vascular tone at arteriolar level and beyond [Hamilton et al 2008][Hamilton et al 2009]. This would lead to uncoupling of the coronary blood flow-myocardial work relationship, which has been reported in a canine study [Hamilton et al 2008]. Indeed, there are fundamental physiological differences in canine and human models for example, highly variable severity of the OSA and its gradual evolution in the humans [Stradling et al 1997]. Hamilton et al experimented that induction of ED and OSA in *homo sapiens* was manifested by marked increase in coronary vascular resistance and profound attenuation of the blood flow [Hamilton et al 2009]. It must be noted that the myocardial energy demand is tightly coupled with the myocardial blood flow under physiological conditions [Kitamura et al 1972][Baller et al 1979]. In post-apnoeic phase of OSA, myocardial energy demand (oxygen consumption) increases due to oxygen desaturation, the rise in blood flow is vital to maintain adequate myocardial oxygenation [Somers et al 1995][Ringler et al 1990]. However, coronary arterial oxygen contents are at a minimum at this point of ventilation in the OSA subjects [Chen et al 1999]. This net reduction in myocardial blood flow in the face of increased oxygen requirement (due to augmented vascular resistance), could lead to nocturnal myocardial ischemia. Indeed initially, these changes in flow could be of brief duration, and in patients with milder forms of OSA without concomitant coronary disease may not have clinical sequelae; but may well become crucial in those with OSA of at least moderate severity especially with coexisting significant coronary stenoses [Peled et al 1999] [Ciaramita et al 2004].

These data are the first to report attenuated perfusion at rest, at stress and MBFR in moderate to severe OSA. Since OSA study subjects were normotensive, these perfusion abnormalities can not be attributed to commonly occuring hypertension in OSA population.

I also demonstrated impaired MBFR in otherwise healthy hypertensives compared with healthy subjects. These observations are in accordance with the available data. Di Bello et al demonstrated attenuated myocardial perfusion in a small group of patients with essential hypertension compared with healthy controls [Di Bello et al 2004]. However, due to notable prevalence of OSA in hypertensives, we only recruited proven OSA negative hypertensive individuals in order to study unconfounded effect of high blood pressure on myocardial perfusion.

Limitations

The observational study design is one limitation of this study; however all the measurements and recordings were performed and analyzed by the same operator. Furthermore, inter- and intra-observer variability was measured on 10 subjects and was found to be less than 10%.

Myocardial perfusion as described above depends largely on the microcirculation. High blood pressure in OSA is one of the effects of OSA and is well reported to cause micro circulatory dysfunction. I showed slightly high BP in OSA though comparable with healthy subjects. This raises the suspicion if the BP contributed to such finding. It’s difficult to answer but uni/multi-variate analysis did not reveal any such connection. This is a pilot project for MCE in OSA subjects and such aspects should be focussed at in future investigations.

3.2.6.6 Conclusion

I conclude that healthy subjects with moderate to severe OSA have impaired myocardial perfusion, which is likely to be owing to the ED. These observations provide insight into mechanisms leading to enhanced risk of cardiovascular disorders and cardiovascular prognosis in moderate to severe OSA patients.

Table 3-23: Clinical Characteristics of Obstructive Sleep Apnoea patients, Hypertensives and Healthy Controls

Variable	OSA (SD)	Hypertensive controls (SD)	Healthy controls (SD)	p
n	40	40	40	
Male/Female	32/8	28/12	39/11	0.486
Age (years)	50(10)	49(12)	46(8)	0.083
Aponea Hypopnea index	33 (21-48) *Ψ	4(3-4)Ψ	3(2-4)*	<0.0001
Body mass index	34(8)	31(5)	32(7)	0.084
Systolic Blood pressure (mmHg)	143(16)	149(21)^	135(15)^	0.003
Diastolic Blood pressure (mmHg)	83(12)	85(9)	82(10)	0.514
No. of current smokers	11	12	15	0.164

Values are given in Mean ± Standard deviation or Median ± Inter-quartile Range. $p<0.05$ was considered Significant.

* Significance between OSA and Healthy subjects; ^ Significance between Hypertensive and Healthy subjects; Ψ Significance between OSA and Hypertensive subjects

OSA=Obstructive Sleep Apnoea

Table 3-24: Comparison of Indices of Myocardial Perfusion amongst Obstructive sleep apnoea and controls

	OSA	HT	Healthy	p
n	40	40	40	
Segments	4	4	4	
FR	26±13*	32±13^	41±12*^	<0.0001
FS	45±26*	54±25^	141±39*^	<0.0001
MBFR	1.8±1*	1.7±0.8^	3.5±1*^	<0.0001

Values are given in Mean ± Standard deviation. $p<0.05$ was considered Significant.

OSA= Obstructive Sleep Aponea; HT= Hypertension; FR= Myocardial Blood Flow at Rest; FS= Myocardial Blood Flow at Stress; MBFR= Myocardial Blood Flow Reserve

* Significance between Obstructive Sleep Aponea and Healthy subjects; ^ Significance between Hypertensive and Healthy subjects

Table 3-25: Univariate analyses of the Pre-CPAP Myocardial perfusion in Obstructive Sleep Apnoea

	MBFR	
	r	**p**
Age	0.077	0.632
Height	0.266	0.093
Weight	0.067	0.675
BMI	-0.098	0.540
AHI	-0.146	0.361
Epworth score	0.241	0.164
Systolic BP	-0.063	0.195
Diastolic BP	-0.182	0.256
Glucose	-0.180	0.309
Total cholesterol	-0.257	0.105

r=correlation coefficient; AHI= Apnoea Hypopnoea Index; FMD= Flow mediated Dilatation; BP= Blood Pressure; MBFR= Myocardial blood flow reserve; BMI= Body mass index

3.3 Longitudinal studies

3.3.1 Impact of Continuous Positive Airway Pressure treatment on flow mediated dilatation in moderate to severe Obstructive Sleep Apnoea

3.3.1.1 Abstract

Background: Obstructive sleep apnoea (OSA) related endothelial dysfunction has been demonstrated using flow mediated dilatation. However the impact continuous positive airway pressure (CPAP) on macro-vascular function is not well established in moderate to severe OSA. I hypothesized that CPAP improves FMD in moderate to severe OSA.

Methods: 40 with moderate-severe OSA had forearm vascular reactivity studies before and after 26 weeks of CPAP treatment. Endothelial-dependent vasodilatation (EDV) was assessed by measuring brachial artery diameter changes in response to reactive hyperaemia and endothelial-independent vasodilatation (EIV) was determined after administration of sublingual glyceryl trinitrite (GTN).

Results: Pre-treatment, only modest changes in brachial artery diameter were noted. However after CPAP, EDV and EIV rose significantly ($p<0.0001$).

Conclusion: I conclude that treatment with CPAP improves endothelium dependent- and independent vasoreactivity in moderate to severe OSA. As ED is a systemic processm it can be

proposed that the CPAP treatment may improve cardiovascular outcome in selected OSA subjects.

3.3.1.2 Introduction

It is known that OSA related hypoxia, hypercapnia, sympathetic activation, and intrathoracic pressure swings can trigger complex cellular and biochemical processes, which may predispose to a pre-atherosclerotic condition, 'endothelial dysfunction' (ED) [Dean et al 1993][Zwillich et al 1995][Lavie et al 2003][Shimokawa et al 1999][Ross et al 1999]. Unsurprisingly, ED has been consistently demonstrated in OSA population and is considered responsible for chronic vascular diseases such as hypertension, coronary artery disease, heart failure, stroke and arrhythmias [Butt et al 2010][Peppard et al 2000].

A number of studies have validated FMD as an excellent non-invasive technique to evaluate vascular function in OSA before and after CPAP treatment [Celermajer et al 1992][Corretti et al 2002][Tanriverdi et al 2006 (a)]. Notably, treatment with the CPAP improves clinical outlook in OSA population. Some studies have reported improved nitric oxide bioavailability in OSA after the application of overnight nasal CPAP [Ip et al 2000]. Similarly improved FMD after CPAP in OSA has been reported proposing that CPAP may ameliorate ED and reduce the risk of development of cardiovascular diseases in OSA population [Lattimore et al 2006]. However these available OSA studies had loose exclusion criteria. Also the effects of long term CPAP therapy on the FMD in otherwise healthy and normotensive subjects with *moderate to severe* OSA.

I hypothesized that prolonged use of CPAP improves vascular responsiveness in normotensive moderate to OSA subjects. To test this hypothesis I perfomed FMD on moderate to severe OSA subjects before and after 6 months of CPAP therapy.

3.3.1.3 Methods

The methods have been described in details under section 2.

3.3.1.4 Results

Table 3-26 represents salient patient characteristics pre- and post-CPAP whilst Table 3-27 shows brachial artery reactivity data. This includes absolute and percentage changes in brachial artery diameters in response to reactive hyperaemia and sublingual nitrates, before and after CPAP therapy.

FMD was performed on 45 OSA patients at baseline however 40 patients were available after 6 month of CPAP therapy. The use of CPAP averaged 5.4 hours per night, 5 nights per week per patient. A significant post-CPAP drop was noted in systolic ($p<0.0001$) and diastolic blood pressure ($p=0.017$) and the Epworth score ($p<0.0001$) [Table 3-26].

Pre- and post-CPAP, mean baseline brachial artery diameter was comparable ($p=0.02$). Following hyperaemia, FMD improved significantly in post-CPAP subjects ($p<0.0001$) [Table 3-27]. Endothelial-independent vasodilatation (EIV), measured following the sublingual administration of GTN spray, also increased significantly ($p=0.01$) [Table 3-27].

3.3.1.5 Discussion

In line with the findinfs of this study, I report that 6 months of CPAP treatment improves macro-vascular function in the normotensive subjects with moderate to severe OSA.

One of the postulated mechanisms in the cardiovascular diseases development is that OSA can precipitate or accentuate atherogenesis by causing ED, although to date there is no direct evidence which supports this hypothesis [Dean et al 1993]. Indeed endothelial injury (paucity of NO) could be an important primary event in atherogenesis, followed by intimal thickening and formation of atherosclerotic plaques [Ross et al 1999][Shimokawa et al 1999]. CPAP is an effective treatment for OSA and enhances endothelial NO release in the macro-circulation [Ip et al 2000][Bayram et al 2009] [Lattimore et al 2006]. In addition to NO-dependent pathways, CPAP also enhances *non-NO pathways*, supporting my reported findings of improved EIV [Lattimore et al 2006]. Such favourable changes in vascular reactivity have been attributed to the amelioration of physiological effects of OSA (such as repetitive hypoxia, surges in systemic blood pressure and enhanced sympathetic activity) with the CPAP use. However these CPAP studies have notable limitation. For example, Ip et al exclusively studied men and hence the results can not be generalised [Ip et al 2000]. Similarly, under-powered cohorts, short follow up, inclusion of mild OSA and under-representation of females are notable limitations in others [Lattimore et al 2006][Imadojemu et al 2002]. In particular, the cardiovascular risk factor or diseases were not meticulously excluded in previous studies. Hence, independent impact of moderate to severe OSA on macro-vascular function remains debatable [Bayram et al 2009]. For the first time, the present data have shown that macro-vascular endothelial dysfunction in otherwise healthy and normotensive subjects with moderate to severe OSA is reversible with compliant long term use of CPAP. These findings are consistent with the available data [Lattimore et al 2006][Imadojemu et al 2002][Bayram et al 2009][Ip et al 2000].

Hypoxia, altered *oxygen dependent* biosynthesis of NO from L-arginine and cyclic surges in systemic blood pressure are by far the most commonly mechanism of impaired vasoreactivity in OSA [Tahawi et al 2001] [Remsburg et al 1999] [Dean et al 1993]. Relief of these physiological

derangements with the effective use of CPAP has been proposed as the mechanism leading to endothelial recovery after CPAP therapy.

It must be noted that a close correlation exists between ED in peripheral and coronary circulation [Sorensen et al 1995] [Anderson et al 1995]. Based on my findings of CPAP led improved FMD in brachial artery, I suggest that appropriate use of CPAP may also ameliorate coronary endothelial dysfunction.

Limitations

Modestly elevated blood pressure in the study group was one limitation of our study. I monitored blood pressure of the OSA subjects in clinics and community and ensured only normotensive blood pressure. I feel that this blood pressure level may reflect white coat response in study group. Due to unfavourable logistics, I could not perform 24 hour blood pressure which could have been useful in demonstrating the mean blood pressure more accurately. One could argue that changes in the FMD are secondary to improvement in blood pressure. Therefore, I performed univariate/multivariate analysis and found no relationship between changes in the blood pressure and the improvement in FMD in OSA subjects. Indeed, absence of the control group is another notabel important limitation of this study. I did not perform FMD at the end of the washout period which may also raise concerns about carryover effect from treatment. However, the tests were performed and analyzed by single operator to ensure uniformity. The technique was also validated with satisfactory inter- and intra-observer variability.

3.3.1.6 Conclusion

I propose that effective long term use of CPAP ameliorates OSA induced attenuated brachial vasoreactivity. Therefore I suggest that CPAP may provide the opportunity to lower vascular risk and may improve cardiovascular outlook and prognosis.

Table 3-26: Patient Characteristics

	Pre-CPAP	Post-CPAP	p
n	40	40	
Age (Years)	49±10	49±11	0.541
AHI	37 (21-55)	Not recorded	-
BMI (Kg/m2)	35±8	34±8	0.18
Epworth score	15 (8-18)	8 (3-22)	<0.0001
Systolic BP (mmHg)	143±16	133±13	<0.0001
Diastolic BP (mmHg)	83±11	79±8	0.017

Values expressed as Mean (Standard Deviation) or Median (Inter quartile range). AHI= Apnoea-Hypopnea Index; BMI= Body Mass Index; BP= Blood Pressure; CPAP= Continuous Positive Airway Pressure

Table 3-27: Brachial Artery reactivity before and after treatment with continuous positive airway pressure therapy

	Pre-CPAP	Post-CPAP	p
Baseline Brachial artery diameter (mm)	4.5±0.5	4.4±0.6	0.2
Absolute change in diameter after reactive hyperaemia (mm)	0.3±0.1	0.5±0.2	<0.0001
Change after reactive hyperaemia (%)	7±4	13±6	<0.0001
Absolute change in diameter after GTN (mm)	0.5±0.2	0.7±0.3	<0.0001
Post GTN change (%)	11±4	16±7	<0.0001

Values expressed as Mean (Standard Deviation); GTN= Glycerol Trinitrate; CPAP= Continuous Positive Airway Pressure

3.3.2 Impact of Continuous Positive Airway Pressure Therapy on Micro-Vascular Function in Moderate to Severe Obstructive Sleep Apnoea assessed by Laser Doppler Flowmetry

3.3.2.1 Abstract

Background: Obstructive sleep apnoea (OSA) is associated with endothelial dysfunction and carries an increased risk of hypertension and cardiovascular diseases due to acceleration of atherosclerosis. This study aimed to evaluate the impact of 6 month treatment with continuous positive airway pressure (CPAP) on the micro-vascular endothelial function in moderate to severe OSA using laser Doppler flowmetry (LDF).

Methods: Micro-vascular reactivity was assessed by LDF in 28 OSA patients at baseline and after 6 months treatment with CPAP. This technique (LDF) was coupled with iontophoresis which allowed the measurement of the response to topically applied acetylcholine (endothelium-dependent vasodilatation {EDV}) and sodium nitroprusside (endothelium-independent vasodilatation {EIV}).

Results: Both, EDV and EID were impaired in study patients at baseline and improved significantly after CPAP therapy ($p<0.0001$). A significant drop in systolic and diastolic blood pressure was also noted.

Conclusion: This study shows CPAP led improvement in attenuated micro-vascular endothelial function in normotensive moderate to severe OSA subjects. Improvement in endothelial status could have important implications on cardiovascular morbidity/mortality in OSA population.

3.3.2.2 Introduction

A meta-analysis of 16 randomized controlled trials demonstrated that regular and efficient use of CPAP reduces systolic, diastolic and mean arterial blood pressure in patients with OSA and may even help prevent hypertension [Bazzano et al 2007]. Also, the reversal of ED with continuous positive airway pressure (CPAP) use has been demonstrated in OSA subjects [Carlson et al 1996][Kato et al 2000][Kraiczi et al 2001]. Several techniques have been used to study the impact of CPAP on systemic vasculature; for example impact of CPAP on macro-vasculature has been studied on conduit arteries using flow mediated dilatation and it showed enhanced post-CPAP vasoreactivity [Imadojemu et al 2002][Ip et al 2000]. However micro-vasculature is a level of the systemic circulation which is most relevant in the development of hypertension and atherosclerotic process [Taddei et al 1995]. Microcirculatory function can be assessed using laser Doppler flowmetry (LDF) coupled with acetylcholine (ACH) and sodium nitroprusside (SNP) iontophoresis. Indeed, this non invasive technique has previously been successfully used in a number of cardiovascular conditions and is considered highly appropriate for repeated microcirculation studies [Debbabi et al 2010][Beer et al 2008][Trzepizur et al 2009]. However no previous study has used this to investigate the effects of long term CPAP treatment on microvascular dysfunction in otherwise patients with moderate to severe OSA.

In the present study I tested the hypothesis that 6 months use of CPAP improves microvascular endothelial function in patients with newly diagnosed moderate to severe OSA who have no

known cardiovascular disease or risk factor. I tested this hypothesis by performing LDF and iontophoresis in OSA subjects at baseline and after 6 months of CPAP therapy.

3.3.2.3 Methods

The methods have been described in details under section 2.

3.3.2.4 Results

Table 3-19 show important patients' characteristics whilst Table 3-22 shows micro-vascular data including peak rise in cutaneous perfusion and percentage changes in response to ACH and SNP, before and after CPAP.

LDF was performed on the right forearm of thirty one patients prior to the initiation of CPAP therapy. After 6 months of effective CPAP therapy, 28 patients with reasonable female gender representation (25%) were available for follow up. Recorded/Objective data on the use of CPAP during follow up averaged 5.1 hours per night 5 nights per week [Table 3-28].

A significant reduction is systolic ($p<0.0001$) and diastolic blood pressure ($p=0.017$) and the Epworth score ($p<0.0001$) was observed in post-CPAP group [Table 3-28].

Pre- and post-CPAP mean baseline perfusion was comparable ($p>0.05$). After CPAP treatment ACH induced endothelial dependent function improved significantly ($p<0.001$) [Table 3-29]. Endothelial-independent perfusion changes, measured following locally administered SNP, also increased significantly post-CPAP ($p=0.007$) [Table 3-29].

3.3.2.5 Discussion

In present study, I demonstrated that microvascular (endothelial) dysfunction in otherwise healthy subjects with moderate to severe OSA improves after 6 months of CPAP use. I used LDF as a

tool for endothelial assessment which is validated for this purpose but has not been used in moderate to severe OSA [Koitka et al 2004][Rousseau et al 2008].

These findings are consistent with the available data suggesting improved vascular function after CPAP [Carlson et al 1996][Kato et al 2000][Kraiczi et al 2001][Ip et al 2000][Lattimore et al 2006]. Although LDF has rarely been used in OSA subjects yet our data is consistent with the available data [Trzepizur et al 2009]. For example, Trzepizur et al reported improved microvascular function after 2 month of CPAP therapy in OSA patients compared with healthy controls [Trzepizur et al 2009]. EIV remained unchanged according to the authros but this study could have been under-powered (n=12) to detect significant difference in endothelial independent responses.

The exact mechanisms of CPAP led improvement of endothelial *dependent* responses in resistant vessels are largely unclear. Chronic sleep deprivation, autonomic dysregulation and perhaps most importantly chronic hypoxemia- all of which are widely prevalent in OSA- have been proposed in the development of ED in OSA [Amir et al 2004] [Takase et al 2004] [Phillips et al 2000] [Pagani et al 2009]. Based on available data, it can be proposed that CPAP therapy enhance endothelial dependent responses by amelioration of sleep fragmentation, improved oxygen saturation and regulation of autonomic function.

I also observed significant improvement in SNP-mediated responses after CPAP therapy. Our findings of improved EIV are consistent with recent double blind study where 27 OSA patients known to have marked oxygen desaturation were randomized to CPAP for 6 weeks [Cross et al 2008]. Both, endothelium-dependent and endothelium independent responses were impaired but improved markedly after CPAP use [Cross et al 2008]. Larger number of studied did not show any CPAP led improvement in SNP mediated responses [Kato et al 2000][Lattimore et al 2006]

but the coexisting presence of confounding factors in such investigations should not be ignored. Such confounder risk factors such as smoking, hypertension and concurrent use of medications, could have significant bearing on the endothelial responses and independent impact of CPAP on microvascular function. In contrast, I had stringent enrolment criteria where all OSA patients were carefully selected and exclusion of coexisting cardiovascular diseases/risk factors was ensured.

It has been reported that activated inflammatory cytokines in OSA subjects down regulate the endothelial receptors, limiting biological effect of the extrinsic nitrates resulting in impaired endothelial indepdent vasoreactivity [Bravo et al 2007] [Ferrell et al 2002][Meier-Ewert et al 2004] [Iversen et al 1999]. CPAP use inturrupts this inflammary cascade and thus may improve EIV.

Limitations

Absence of a control group and observational design are main limitations of our study. However, the tests were performed and analyzed by single operator to ensure uniformity and was validated in ten patients with excellent inter- and intra-observer variability. Furthermore, our observations are limited to *moderate to severe* OSA and cannot be extrapolated to other categories.

Again, the significant reduction in systolic and diastolic BP was noted after CPAP therapy. It can be argued that any improvement in LDF responses are secondary to such improvement in BP, however, I did not note any relationship between the reduction of BP and change in microvascular reactivity.

3.3.2.6 Conclusion

I report that long term CPAP therapy improves micro-vascular reactivity in moderate to severe OSA and therefore may have a significant impact on the cardiovascular morbidity and mortality in OSA population.

Table 3-28: Patient Characteristics

	Pre-CPAP	Post-CPAP	p
n	28	28	
Age (Years)	51±9	51±10	0.621
AHI	37 (20-54)	Not recorded	-
BMI (Kg/m2)	35±9	35±9	0.773
Epworth score	14 (8-18)	4 (0-8)	<0.0001
Systolic BP (mmHg)	145±17	133±12	<0.0001
Diastolic BP (mmHg)	84±13	79±8	0.005

Values expressed as Mean (Standard Deviation) or Median (Inter quartile range). AHI= Apnoea-Hypopnea Index; BMI= Body Mass Index; BP= Blood Pressure; CPAP= continuous positive airway pressure therapy; BP= blood pressure

Table 3-29: Changes in Micro-vascular Blood Flow in response to Acetylcholine and Sodium Nitroprusside before and after Continuous Positive Airway Pressure Therapy

	Pre-CPAP	Post-CPAP	p
Mean Baseline perfusion pre-ACH (PU)	0.69 (0.57-0.72)	0.61 (0.51-0.71)	0.127
Mean maximum perfusion post-ACH (PU)	1.4 (1.1-1.5)	1.3 (1-1.7)	0.811
Mean percentage increase post-ACH (%)	103 (55-133)	156 (83-238)	0.001
Mean Baseline perfusion pre-SNP (PU)	0.73 (0.64-0.8)	0.6 (0.53-0.74)	0.08
Mean maximum perfusion post-SNP (PU)	1.4 (1.2-1.7)	1.4 (1.2-1.6)	0.569
Mean percentage change post-SNP (%)	97 (58-146)	153 (92-223)	0.007

Values in Median (Inter-quartile Range). ACH= Acetylcholine; SNP= sodium nitroprusside, PU= Perfusion unit, CPAP= continuous positive airway pressure therapy

3.3.3 Impact of Continuous Positive Airway Pressure Therapy on Endothelial Progenitor Cells and Circulating Endothelial Cells in Moderate to Severe Obstructive Sleep Apnea

3.3.3.1 Abstract

Background: Decreased endothelial progenitor cells (EPCs) and increased circulating endothelial cells (CECs) have been described in obstructive sleep apnoea (OSA) and several cardiovascular diseases. I investigated the impact of continuous positive airway pressure (CPAP) therapy on endothelial repair capacity and apoptosis in otherwise healthy subjects with moderate to severe OSA.

Methods: 36 newly diagnosed patients with moderate to severe OSA with no coexisting cardiovascular comorbidities were enrolled. Circulating levels of EPCs, a marker of endothelial reparation, and CECs, a marker of endothelial apoptosis, were enumerated using flow cytometer before and after 6 months of nasal CPAP therapy.

Results: Compared with pre-CPAP, a trend of increasing numbers of EPC was observed post-CPAP, however it did not reach statistical significance (p=0.677). Additionally, the absolute CEC count and EPC/CEC ratio remained unchanged during the follow up (p= 0.61 and 0.238 respectively).

Conclusion: EPC and CEC counts were comparable at baseline and after 6 months of effective CPAP treatment in moderate to severe OSA. Treatment with CPAP may not be associated with improvement in these markers of endothelial repair capacity and vascular endothelial cell damage in OSA patients without concomitant vascular disease.

3.3.3.2 Introduction

The cumulative effect of the perturbed vascular milieu in OSA is believed to result in endothelial cell injury or dysfunction/damage, which has been recognized as crucial early event in the pathogenesis of cardiovascular disorders [Carlson et al 1996][Schultz et al 2005].

Endothelial progenitor cells (EPCs) represent a circulating pool of bone marrow derived cells to replace dysfunctional/damaged endothelium and hence contribute to vascular homeostasis [Shantsila et al 2007 (a) (b)]. Reduced levels of circulating EPCs are predictive of worse clinical outcome and several cardiovascular risk factors inversely correlate with the number and activity of EPCs [Tepper et al 2002][Chen et al 2004][Schmidt-Lucke et al 2005][Werner et al 2006]. Reduced EPCs count has been demonstrated in OSA subjects [Jelic et al 2009].

Several cardiovascular risk factors are closely associated with endothelial apoptosis [Asai et al 2000][Tricot et al 2000]. Increased numbers of apoptotic cells (CECs) have been reported in OSA and in a variety of vascular conditions such as acute coronary syndrome and heart failure [Solovey et al 1997][Quilici et al 2004][Chong et al 2004].

Small studies have reported favourable fluctuations in EPCs and/or CECs levels in OSA subjects, after CPAP therapy [Jelic et al 2009][Jelic et al 2008][El Solh et al 2007]. However, the effect

of long term CPAP therapy on EPCs and CECs count in healthy subjects with moderate to severe OSA remains undetermined.

In this chapter, I tested the hypothesis that these cells strains - acting as markers of ED in moderate to severe OSA - may improve after 6 months of CPAP use. I tested this hypothesis by performing flow cytometry on the venous blood sample of a carefully selected moderate to severe OSA individuals before and after 6 months of CPAP therapy.

3.3.3.3 Methods

The methods have been described in details under section 2.

3.3.3.4 Results

Table 3-30 depicts the baseline demographic, anthropomorphic and polysomnographic data whilst Table 3-31 shows distribution patterns of CECs and EPCs, pre- and post-CPAP.

Flow cytometric analysis was performed on the venous blood samples of 40 OSA patients prior to initiation of the treatment with CPAP. After 6 months of CPAP therapy, 36 patients (22% female) were available for follow up. Recorded/Objective data on the use of CPAP during follow up averaged 5.9 hours per night, 5 nights per week.

A significant post-CPAP reduction is systolic ($p<0.0001$) and diastolic blood pressure ($p=0.017$) and the Epworth score ($p<0.0001$) was observed [Table 3-30].

As shown in Table 3-31, the number of circulating CD34++ cells, both in absolute count and as percentage of peripheral blood mononuclear cells, was not significantly different in OSA patients before or after CPAP use ($p=0.206$ and 0.161 respectively).

The absolute numbers of CECs and circulating EPCs in subjects with OSA, pre- and post-CPAP are depicted in Table 3-31. There were no statistically significant differences between the groups with respect to numbers of CECs (p=0.610), circulating EPCs (p=0.677) and EPC/CEC ratio (p=0.238) [Table 3-31].

Assessment of correlations between numbers of CECs and circulating EPCs and age, sex, BMI, smoking, AHI, Epworth score and blood pressure yielded no statistically significant correlations.

3.3.3.5 Discussion

In the present study I observed no differences in EPCs or CECs count in the peripheral blood sample of subjects with moderate to severe OSA pre- and post-CPAP.

These findings are in contrast with the previous OSA studies where CPAP led increase in EPCs has been demonstrated after short term [Jelic et al 2009] [Jelic et al 2008]. However, smaller cohorts, under-representation of female gender, inclusion of subjects with cardiovascular disorders and concurrent use of antihypertensives are major limitations of these studies. It must be noted that all these factors have substantial and independent impact on the EPCs and CECs count and hence I meticulously excluded subjects with any confounding factor. Additionally the CPAP follow up was limited to 4 weeks in previous studies compared with present study where subjects were followed up after 6 months of adequate CPAP treatment. Finally reduction in CECs has also been reported after CPAP in limited available data [El Solh et al 2007] but most of these studies were small with gender exclusivity and hence the results can not be extrapolated to the whole population. However, I did not notice any significant changes in CECs numbers after CPAP therapy.

Considering a well reported association between ED and OSA as well as CPAP led improvement in ED our findings are somewhat surprising [Kato et al 2000][Ip et al 2004]. However, there are several possible reasons for our findings. Since the CPAP use results in dramatic and acute changes in thoracic pressures and plasma oxygen concentration it can be proposed that such physiological effects may not be so obvious and dramatic after prolonged CPAP therapy [Simons et al 2011][Milovanova et al 2009][Dempsey et al 2007]. Secondly, although our study is largest of its kind, due to the relatively rare event rate, our study sample may have been under-powered to detect a statistically significant difference. This is evident by the trend of increase in EPCs after CPAP, which did not reach statistical significance. Additionally, differential susceptibility to cardiovascular morbidity in OSA partially depends on factors such as hypoxia, sleepiness and obesity; it can be hypothesized that CPAP therapy may interact with these physiological factors resulting in different observations [Stanchina et al 2003][Findley et al 1983][White et al 2005][Barbe et al 2001] [Schafer et al 2002]. Although there are serious limitations associated with all EPCs/CECs enumeration techniques, the assay of endothelial colony-forming units may have been more likely to detect pre- and post-CPAP differences in the cell counts [Shantsila et al 2007 (a)] [Hill et al 2003]. Finally, it can also be derived that CPAP led improvement in CEC/EPCs distribution only occurs in OSA patients with coexisting cardiovascular diseases.

Limitations

I did not assess the oxygen desaturation, using nadir SaO2. It must be noted that hypoxia is an important determinant of endothelial damage in OSA [Dyugovskaya et al 2002] and it can be hypothesized that CPAP led changes are more prominent in conjunction with greater degree of hypoxia [Stanchina et al 2003][Findley et al 1983][White et al 2005]. More importantly, I did not assess the functional component of EPCs or CECs and it can be hypothesized that long term

CPAP therapy may predominantly alter EPC and/or CEC functional characteristics compared with the total circulating cell count.

Blood pressure is reported to have a strong influence on the EPC and CEC count. In parallel, a large body of evidence suggest CPAP led reduction of BP in OSA. Although, I showed BP reduction in this study, but no significant quantitative change in EPC and CEC and neither any relationship between the change in BP and the change in cellular count.

3.3.3.6 Conclusion

In conclusion, this study did not reveal any differences in EPCs and CECs, pre- and post-CPAP, in subjects with moderate to severe OSA who did not suffer from established cardiovascular disease or risk factors. However it is possible that CPAP dominanatly affects the functional components (as migratory capacity or EPCs and apoptotic properties of CECs) and that such change may be more prominent in subjects with coexisting cardiovascular disorders.

Table 3-30: Patient Characteristics

	Pre-CPAP	**Post-CPAP**	**p**
n	36	36	
Age (Years)	49 (10)	49 (10)	0.821
AHI	37 (21-55)	Not recorded	
BMI (Kg/m2)	35 (8)	34 (8)	0.180
Epworth score	13 (6)	5 (6)	<0.0001
Systolic BP (mmHg)	143 (16)	133 (13)	<0.0001
Diastolic BP (mmHg)	83 (11)	79 (8)	0.017

Values expressed as Mean (Standard Deviation) or Median (Inter quartile range). $P<0.05$ is considered statistically significant

CPAP: Continuous Positive Airway Pressure; AHI= Apnoea-Hypopnea Index; BMI= Body Mass Index; BP= Blood Pressure.

Table 3-31: Distribution of Endothelial Progenitor Cells and Circulating Endothelial Cells before and after continuous positive airway pressure therapy

	Pre-CPAP	Post-CPAP	p
N	36	36	
%CD34++ count	0.0004(0.0003-0.0007)	0.0005(0.0004-0.0006)	0.161
CD34++ count (Per ml)	3350(1749-4006)	3166(2346-4789)	0.206
Absolute EPC count (Per ml)	75 (38-151)	80 (45-204)	0.677
Absolute CEC count (Per ml)	82 (38-144)	83 (59-123)	0.610
EPC/CEC Ratio	1 (0.8-1.6)	1 (0.7-1.5)	0.238

EPC: Endothelial Progenitor Cell, CEC: Circulating Endothelial Cell, CPAP: Continuous Positive airway Pressure

3.3.4 Effect of Continuous Positive Airway Pressure treatment on Pulse Wave Analysis and Pulse Wave Velocity in Obstructive Sleep Aponea

3.3.4.1 Abstract

Background: Obstructive sleep apnea (OSA) is associated with arterial stiffness, hypertension and several cardiovascular disorders. The aim of the present study was to determine changes in arterial stiffness in healthy subjects with moderate to severe OSA following long term continuous positive airway pressure (CPAP) therapy.

Methods: In 26 patients (6 females) with moderate to severe OSA, arterial stiffness was assessed by applanation tonometry– derived pulse wave analysis (PWA) and pulse wave velocity (PWV). Baseline and Glycerol trinitrate (GTN)/Salbutamol induced changes in aortic augmentation (AA); aortic augmentation index (AIx); AIx corrected for heart rate of 75/minute (AIx@75); Sub-endocardial viability ratio (SEVR)] and PWV measurements were recorded at enrolment and after 6months of CPAP therapy.

Results: After CPAP, significant reductions were noted in the PWA indices (AIx@75 – pre-therapy 19 (IQR 10 - 28) to post therapy 4 (IQR -1.25 – 28); p=0.001). Salbutamol induced responses were significantly better post-CPAP compared to pre-CPAP ($p<0.01$) however only modest and statistically insignificant changes was noted in GTN induced responses. PWV measurements were also comparable before and after CPAP use and no changes were observed in GTN/Salbutamol induced responses.

Conclusion: These results suggest that clinically important changes in arterial stiffness may occur following long term treatment of moderate to severe OSA with CPAP.

3.3.4.2 Introduction

Whilst the independent effect of the OSA on cardiovascular health has widely been reported [Young et al 2005] [Arzt et al 2005][Peker et al 2006], several studies report a gradual decline in the cardiovascular event rate after CPAP use [Marin et al 2005][Peker et al 2002]. The exact explanation for such a reduction is unclear, but antihypertensive effect of CPAP treatment has been implicated [Chobanian et al 2003][Pepperell et al 2002][Gotsopoulos et al 2004]. Nevertheless, the size of anti-hypertensive effect of CPAP is controversial and appears to be modulated by several factors such as OSA severity, degree of sleepiness, concomitant use of antihypertensive medication, duration and efficacy of treatment and contentiously the arterial stiffness [Barnes et al 2004][Robinson et al 2006][Börgel et al 2004][Campos-Rodriguez et al 2006]. Due to a close correlation with ageing and various cardiovascular risk factors, arterial stiffness has also a strong predictive role beyond causing high blood pressure [Vaitkevicius et al 1993][Dart et al 1991]. Several investigators have probed if cardiovascular risk could be modified by reducing arterial stiffness [Oliver et al 2003][Weber et al 2004]. Indeed short term CPAP therapy has shown promising reduction in the arterial stifness in OSA population, however effects of prolonged use of CPAP in moderate to severe OSA subjects remains largely unclear [Kitahara et al 2006][Phillips et al 2008].

I hypothesized that 6 months of CPAP use reduces arterial stiffness in otherwise healthy subjects with moderate to severe OSA. In order to test this hypothesis, I performed applanation tonometry on normotensive patients with moderate to severe OSA at the time of diagnosis and repeated these measurements after 6months of CPAP treatment.

3.3.4.3 Methods

The methods have been described in details under section 2.

3.3.4.4 Results

Table 3-32 depicts the baseline characteristics of OSA subjects whilst Table 3-33 and 3-34 shows data on the responsiveness of indices of arterial stiffness to systemically administered GTN and Salbutamol, pre- and post-CPAP.

Pulse wave analysis and pulse wave velocity measurement was completed on 26 patients before and after CPAP treatment. Recorded/Objective data on the use of CPAP during follow up averaged 5 hours per night 5 nights per week. During follow up a significant post-CPAP drop in systolic ($p<0.001$) diastolic blood pressure ($p=0.024$) and the Epworth score was observed ($p<0.01$) [Table 3-32].

At baseline, compared with pre-CPAP measurements, indices of PWA (AA, AIx, AIx@75 and SEVR) were significantly reduced (all $p<0.05$) however PWV was comparable at before and after CPAP therapy [Table 3-33; 3-34]. Following Salbutamol administration, AA, AIx and AIx@75 were significantly reduced thorough out the 20 minutes of recording (all $p<0.05$) indicating reduction in arterial stiffness after CPAP therapy however PWV and SEVR remained unchanged ($p>0.05$) [Table 3-35]. After GTN administration, AA, AIx and AIx@75 responses were delayed compared to Salbutamol induced responses (significant improvement was only noted 20 minutes after GTN administration), whilst SEVR and PWV remained unchanged ($p>0.05$)[Table 3-36]. A significant change in baseline values were found between pre- and post-CPAP cohort [Table 3-37].

Post CPAP indices of arterial stiffness (including AA, AIx and AIx@75) were inversely correlated with duration of CPAP usage (r= -0.592, p=0.001; r= -0.592, p=0.001; r= -0.522; p=0.006 respectively).

3.3.4.5 Discussion

This study has three important findings: 1) significant post CPAP improvements in large-conduit arterial stiffness in patients with moderate to severe OSA, who were free from any cardiovascular comorbidities; 2) enhanced systemic arterial responses to endothelium-dependent (Salbutamol induced) and -independent (GTN) vasodilators in post-CPAP arm; 3) post-CPAP blood pressure reduction as a likely manifestation of improved arterial elastic properties.

AIx, AIx@75, SEVR and PWV are some of the direct and indirect indicators of central arterial stiffness and could be predictive of cardiovascular events in high-risk population [Wilkinson et al 2002] [Weber et al 2004] [Williams et al 2009] [Noon et al 2008] [Drager et al 2007 (b)]. These indices provide indirect information about left ventricle pressure dynamics, which has a profound contribution in the development of left ventricular hypertrophy, heart failure and target end-organ damage [Hashimoto et al 2007][Iketani et al 2000][O'Rourke et al 1996].

My observations of improved arterial stiffness (PWA) are consistent with previous CPAP studies in OSA subjects [Phillips et al 2008][Drager et al 2007 (b)][Kitahara et al 2006]. I have demonstrated that after 6 months of CPAP use arterial elastic propertice change positively as manifested by reduction in arterial augmentation. This study is one of its kinds as previous studies were relatively smaller and included OSA subjects with coronary artery disease, hypertension and other cardiovascular risk factors which could have confounded the study outcome. I ensured adequate female representation whilst some interventional study exclusively

enrolled males. Notably, females have a higher incidence of arterial stiffness, hence the findings from these investigations can not be extrapolated to the general population [Noon et al 2008] [Protogerou et al 2007] [Drager et al 2010].

I did not detect statistically significant changes in PWV which is in contrast with previous CPAP studies and can be explained with a number of reasons [Baguet et al 2009][Drager et al 2010 (a)][Chung et al 2011][Kitahara et al 2006]. For example I exclusively studied moderate to severe OSA population whereas previous investigations also included milder forms of the disease. Furthermore, compared to ankle-brachial technique, I used carotid-femoral approach to measure PWV and the difference of the techniques could introduce notable difference compared with previous findings [Kitahara et al 2006][Nagahama et al 2004]. Previous studies had loose exclusion criteria and the OSA subjects with cardiovascular disorders - which are independent and notable causes of increased PWV- were not meticulously excluded and could add significant bias [Jelic et al 2002][Baguet et al 2009]. Hence, I conclude that, in otherwise healthy middle aged population of moderate to severe OSA, CPAP may reduce arterial stiffness but changes in PWV are likely to be observed in the subjects with coexisting cardiovascular risk diseases.

I observed for the first time, post-CPAP enhancement in vascular responses to endothelial dependent and independent stimuli whilst using applanation tonometry. Macro-vasculature contains considerable quantities of vascular smooth muscle, which is directly influenced by the nitric oxide and sympathetic tone. CPAP has been demonstrated to accentuate nitric oxide production and diminution of sympathetic tone [Ip et al 2004][Ip et al 2000]. Both of these mechanisms contributes to reduce arterial stiffness and enhance vascular responsiveness [Waradekar et al 1996][Narkiewicz et al 1999][Mills et al 2006]. These hypotheses also partially explain my findings of inverse correlation between reduction in arterial stiffness and CPAP compliance.

In addition, I noted a reduction in peripherally measured systolic and diastolic blood pressure which is in accordance with the available data [Phillips et al 2008][Martínez-García et al 2007][Bazzano et al 2007]. Improvement in arterial atiffness and endothelial status has been implicated in the blood pressure lowering effect of CPAP. It is worth mentioning that both reduction in blood pressure and arterial stiffness are associated with improved survival in selected cohorts [Sawicki et al 1995]. Since this study has shown parallel reduction in arterial stiffness and blood pressure, it can be proposed that CPAP may improve cardiovascular prognosis [Nürnberger et al 2002].

Limitations

Ip et al had reported that the improvement in endothelial function is not sustained after stopping CPAP for 1 week, despite its previous use for several months [Ip et al 2004]. Therefore, inability to perform measurements at the end of the washout period may raise concerns of a carryover effect from previous treatment.

A strong relationship exists between BP and PWA and PWV. In a recent Explore study antihypertensive showed significant reduction in PWV and AIx with parallel reduction in the BP, suggesting BP reduction as one of the mechanism to reduce arterial stiffness [Boutouyrie et al 2010]. I demonstrated significant reduction in systolic and diastolic BP after CPAP which is in accordance with the available data. Additionally I also demonstrated significant reduction in baseline PWA and PWV parameters after CPAP. It can be questioned if such change is secondary to anti-hypertensive effect of CPAP. It largely unclear and this study do not answer this to the level of satisfaction. I investigated a relationship between the relative reduction in BP and its relationship with the relative change in PWA and PWV. I did not find any relationship and hence

it’s reasonable to assume that, this change is secondary to CPAP and not purely due to BP reduction. It can further be argued that CPAP led reduction in arterial stiffness is mediated by its antihypertensive effect. Clearly this needs more investigation to establish a causal relationship amongst CPAP, BP and arterial stiffness.

3.3.4.6 Conclusion

In summary, these findings suggest significant reduction in arterial stiffness, improved vascular reactivity and and reduction in blood pressure after prolonged CPAP therapy in moderate to severe OSA subjects. Considering available data on the relationship between arterial stiffness and cardiovascular morbidity/mortality, CPAP use may reduce overall cardiovascular risk in selected OSA populations.

Table 3-32: Patient Characteristics

	Pre-CPAP	**Post-CPAP**	**p**
n	26	26	
Age (Years)	49±10	49±10	0.719
AHI	31 (20-46)	Not recorded	
BMI (Kg/m2)	31±5	31±5	0.317
Epworth score	13±6	7±7	<0.0001
Systolic BP (mmHg)	143±17	132±12	<0.0001
Diastolic BP (mmHg)	83±13	79±7	0.024

Values expressed as Mean (Standard Deviation) or Median (Inter quartile range). AHI= Apnoea-Hypopnea Index; BMI= Body Mass Index; BP= Blood Pressure; CPAP= continuous positive airway pressure

Table 3-33: Indices of Pulse Wave Analysis and Pulse Wave Velocity before and after systemic administration of Salbutamol, as Median (Inter-quartile range)

Index	Pre-CPAP	Post-CPAP	p
Baseline 3			
AA	14 (10 – 21)	5 (0-19)	0.002
AIx	26 (20-31)	12 (0-32)	0.006
AIx@75	18 (10-29)	7 (-2 – 26)	0.007
SEVR	171 (138-196)	152 (140-184)	0.08
PWV	6 (5-7)	5 (5-8)	0.363
After 5 Minutes			
AA	22 (15-29)	4 (1-15)	0.004
AIx	25 (12-35)	10 (2-30)	0.01
AIx@75	20 (8-27)	5 (-4 – 25)	0.012
SEVR	168 (147-189)	158 (131-175)	0.023
PWV	6 (5-7)	6 (5-7)	0.763
After 10 Minutes			
AA	13 (7-20)	6 (0-15)	0.002
AIx	24 (14-31)	15 (0.5-29)	0.019
AIx@75	17 (7-28)	7 (-1 – 24)	0.011
SEVR	161 (149-183)	163 (135-178)	0.184
PWV	5 (5-7)	6 (5-7)	0.396
After 15 Minutes			
AA	14 (7-20)	7 (0.5-16)	0.008
AIx	22 (13-33)	14 (1-25)	0.023
AIx@75	16 (8-29)	5 (-3 – 25)	0.043

SEVR	168 (155-192)	154 (138-175)	0.023
PWV	6 (5-7)	5 (5-7)	0.841
After 20 Minutes			
AA	12 (7-22)	7 (0.5-16)	0.006
AIx	22 (15-33)	14 (1-28)	0.017
AIx@75	15 (7-28)	7 (-4 – 29)	0.042
SEVR	171 (151-184)	152 (133-183)	0.141
PWV	6 (5-7)	6 (5-7)	0.647

HT=Hypertension, CPAP= Continuous Positive Airway Pressure, AA=Aortic Augmentation, AIx=Augmentation Index, AIx@75=Augmentation Index at heart rate of 75/minute, SEVR=Sub-Endocardial Viability Ratio, PWV= Pulse Wave Velocity

Table 3-34: Indices of Pulse Wave Analysis and Pulse Wave Velocity before and after systemic administration of Glyceryl trinitrate, as Median (Inter-quartile range)

Index	Pre-CPAP	Post-CPAP	p
Baseline 4			
AA	15 (9 - 22)	5 (-0.5 – 18)	<0.0001
AIx	26 (17 - 33)	9 (-0.75 – 35)	0.003
AIx@75	19 (10 - 28)	4 (-1.25 – 28)	0.001
SEVR	167 (151 - 187)	150 (133 - 174)	0.021
PWV	6 (5 - 7)	5 (5 - 7)	0.808
After 5 Minutes			
AA	3 (-4 – 8)	-1 (-6 – 6)	0.271
AIx	5 (-7 – 14)	-2 (-10 – 10)	0.420
AIx@75	1 (-9 – 10)	-2 (-10 – 9)	0.429
SEVR	163 (145 – 184)	161 (140 – 175)	0.404
PWV	6 (5 - 8)	6 (5 - 7)	0.862
After 10 Minutes			
AA	1 (-4 – 9)	-4 (-6 – 3)	0.333
AIx	2 (-7 – 13)	-7 (-13 – 7)	0.399
AIx@75	-1 (-9 – 8)	-8 (-15 – 3)	0.338
SEVR	164 (143-182)	161 (149 – 178)	0.577
PWV	6 (5-8)	6 (5 – 8)	0.661
After 15 Minutes			
AA	2 (-4 – 7)	-2 (-9 – 3)	0.184
AIx	5 (-6 – 13)	-4 (-14 – 7)	0.166
AIx@75	-2 (-12 – 11)	-8 (-15 – 6)	0.146

SEVR	167 (145-185)	166 (145-175)	0.904
PWV	6 (5-7)	6 (5-7)	0.445
After 20 Minutes			
AA	4 (-3 – 12)	-2 (-6 – 5)	0.038
AIx	7 (-7 – 19)	-4 (-12 – 8)	0.05
AIx@75	4 (-11 – 16)	-8 (-16 – 8)	0.03
SEVR	165 (145-186)	162 (139-186)	0.339
PWV	6 (5-7)	6 (5-7)	0.071

HT=Hypertension, CPAP= Continuous Positive Airway Pressure, AA=Aortic Augmentation, AIx=Augmentation Index, AIx@75=Augmentation Index at heart rate of 75/minute, SEVR=Sub-Endocardial Viability Ratio, PWV= Pulse Wave Velocity

Table 3-35: Indices of Pulse Wave Analysis and Pulse Wave Velocity versus time after Salbutamol in Post-CPAP Obstructive sleep apnoea patients

	Pre-Sal	**5 min**	**10 min**	**15 min**	**20 min**	**P**
AA	5 (0-19)	4 (1-15)	6 (0-15)	7 (0.5-16)	7 (0.5-16)	0.998
AIX	12 (0-32)	10 (2-30)	15 (0.5-29)	14 (1-25)	14 (1-28)	0.995
AIx@75	7 (-2 – 26)	5 (-4 – 25)	7 (-1 – 24)	5 (-3 – 25)	7 (-4 – 29)	0.982
SERV	152 (140-184)	158 (131-175)	163 (135-178)	154 (138-175)	152 (133-183)	0.777
PWV	5 (5-8)	6 (5-7)	6 (5-7)	5 (5-7)	6 (5-7)	0.691

Values as Median (Inter-quartile range); $p<0.05$ is significant

CPAP= Continuous Positive Airway Pressure, AA=Aortic Augmentation, AIx=Augmentation Index, AIx@75=Augmentation Index at heart rate of 75/minute, SEVR=Sub-Endocardial Viability Ratio, PWV= Pulse Wave Velocity

Table 3-36: Indices of Pulse Wave Analysis and Pulse Wave Velocity versus time after Glycerol trinitrite in Post-CPAP Obstructive sleep apnoea patients

	Pre-GTN	5 min	10 min	15 min	20 min	P
AA	5 (-0.5 – 18)*^ #∞	-1 (-6 – 6)*	-4 (-6 – 3)^	-2 (-9 – 3)#	-2 (-6 – 5)∞	<0.0001
AIX	9 (-0.75 – 35)*^ #∞	-2 (-10 – 10)*	-7 (-13 – 7)^	-4 (-14 – 7) #	-4 (-12 – 8) ∞	<0.0001
AIx@75	4 (-1.25 – 28)*^ #∞	-2 (-10 – 9)*	-8 (-15 – 3)^	-8 (-15 – 6) #	-8 (-16 – 8) ∞	<0.0001
SERV	150 (133 - 174)	161 (140 – 175)	161 (149 – 178)	166 (145-175)	162 (139-186)	0.136
PWV	5 (5 - 7)	6 (5 - 7)	6 (5 – 8)	6 (5-7)	6 (5-7)	0.451

Values as Median (Inter-quartile range); $p<0.05$ is significant

* Significant difference between time 0 and 5 min

^ Significant difference between time 0 and 10 min

Significant difference between time 0 and 15 min

∞ Significant difference between time 0 and 20 min

CPAP= Continuous Positive Airway Pressure, AA=Aortic Augmentation, AIx=Augmentation Index, AIx@75=Augmentation Index at heart rate of 75/minute, SEVR=Sub-Endocardial Viability Ratio, PWV= Pulse Wave Velocity

Table 3-37: Comparison of the Baseline values in OSA groups at 4 time points; as Median (Inter-quartile range)

	Pre-CPAP		Post-CPAP		p
	B1	**B2**	**B3**	**B4**	
AA	15 (9-21)*^	14 (9-21)*^	5 (0-19)*	5 (-0.5 – 18) ^	<0.01
AIx	26 (20-32) *^	26 (19-33) *^	12 (0-32)*	9 (-0.75 – 35) ^	0.002
AIx@75	18 (11-31) *^	22 (13-31) *^	7 (-2 – 26)*	4 (-1.25 – 28) ^	0.007
SEVR	162 (138-194)*^	163 (145-187)*^	152 (140-184)*	150 (133 - 174)^	0.02
PWV	6 (5-7)	5.8 (4.8-7)	5 (5-8)	5 (5 - 7)	0.723

B1= Pre-CPAP Pre-Salbutamol Baseline 1; B2= Pre-CPAP Pre-GTN Baseline 2; B3= Post-CPAP Pre-Salbutamol Baseline 3; B4= Post-CPAP Pre-GTN Baseline 4; Sal= Salbutamol; GTN= Glycerol trinitrite; AA=Aortic Augmentation, AIx=Augmentation Index, AIx@75=Augmentation Index at heart rate of 75/minute, SEVR=Sub-Endocardial Viability Ratio, PWV= Pulse Wave Velocity

3.3.5 Effect of Continuous Positive Airway Pressure on Left Ventricle Systolic and Diastolic in obstructive sleep aponea population

3.3.5.1 Abstract

Background: Obstructive sleep apnoea (OSA) has been reported to cause left ventricle (LV) systolic/diastolic dysfunction and left atrium (LA) enlargement. I investigated the impact of continuous positive airway pressure (CPAP) on LV function and LA volumes in moderate to severe OSA subjects, using two-dimensional- (2DE) and real time three-dimensional echocardiography (RT3DE).

Methods: 40 newly diagnosed patients with moderate to severe OSA were studied at baseline however 37 patients with (apnoea-hypopnoea index- median 36 (inter quartile range 21-51) were available after a mean follow up of 6 months. All patients underwent 2DE, tissue Doppler imaging and RT3DE before and after CPAP therapy.

Results: I demonstrated significant post-CPAP enhancement in LV systolic function, both with 2DE and RT3DE. Post-CPAP tissue Doppler imaging detected marked improvement in LV filling characteristics (E/E' reduced from 9±4 to 8±2, p=0.02). Additionally, CPAP treatment led to a marked reduction in LA volume and LA volume index.

Conclusion: Long term treatment with CPAP improves LV systolic function and reverses LV filling abnormalities and LA structural deformation– partially or completely - in subjects with moderate to severe OSA.

3.3.5.2 Introduction

A number of observational and therapeutic studies have detected a higher frequency of left ventricular (LV) diastolic dysfunction in obstructive sleep apnoea (OSA) patients when compared to healthy controls [kim et al 2008][Alchanatis et al 2002][Hedner et al 1990]. The stiffness of the LV and resultant reduction in its compliance might cause left atrial (LA) overstretching and chamber dilation in order to maintain adequate LV filling. Unsurprisingly LA enlargement have been shown in OSA and may be relavant in the high prevalance of atrial fibrillation and stroke in OSA population [Kono et al 2007][Hirschler et al 2006]. LV systolic dysfunction and subsequent heart failure has also been reported in OSA, however the data is conflicting [Kim et al 2008][Kraiczi et al 2001] [Alchanatis et al 2002].

The data on the effects of continuous positive airway pressure (CPAP) on LV systolic/diastolic function in moderate to severe OSA are limited and conflicting. Whilst some report improvement in LV systolic/diastolic function (surrogated by conventional markers such as LVEF), other showed no post CPAP change [Alchanatis et al 2000] [Bendjelid et al 2005][Akar et al 2009][Haruki et al 2010][Bellone et al 2010][Alchanatis et al 2002]. One reason of such inconsistent results is the mere use of 2DE, where wide inter- and intra-observer variability is widely reported. Conversely, RT3DE has demonstrated its efficacy in determining LV structural and functional propertities [Von Ramm et al 1990][Shiota et al 1998]. However it has not been used in evaluating the effect of CPAP on LV structure and functions in subjects with moderate to severe OSA.

I hypothesized that prolonged CPAP use leads to improvement in LV systolic and diastolic function. In order to test this hypothesis I investigated otherwise healthy subjects with moderate to severe OSA and comprehensively studied LV structural and functional characteristics using 2DE as well as RT3DE before and 6 months after CPAP therapy.

3.3.5.3 Methods

The methods have been described in details under section 2.

3.3.5.4 Results

2DE and RT3DE were performed on the 40 patients prior to the initiation of CPAP treatment [Table 3-38]. After 6 months of effective CPAP therapy, 37 middle aged patients with were available for follow up. Recorded/objective data on the use of CPAP during follow up averaged 5.7 hours per night 5 nights per week. On the morning of follow up scans, the average heart rate was 72±11 beats per minute which was comparable with the pre-CPAP heart rate ($p=0.205$). During follow up a significant post-CPAP drop in systolic/diastolic blood pressure ($p<0.0001$ and 0.03 respectively) and the Epworth score ($p<0.01$) was observed [Table 3-38].

LV Systolic Function

Both, M-mode and modified Simpson's technqies demonstrated significant improvement LV diastolic dimensions and systolic parameters post-CPAP (all $p<0.05$) [Table 3-39]. RT3DE semi-automatic planimetry also complemented 2DE findings, where an increased LVEF was noted after CPAP therapy ($p=0.024$) [Table 3-39].

LV Diastolic Function

Table 3-40 presents diastolic indices provided by traditional echocardiography and TDI. Conventional indices of diastolic function improved after CPAP use with an increase in E ($p<0.0001$), E/A ($p<0.0001$) and reduction in the peak A velocity ($p=0.02$) [Table 3-40]. On tissue Doppler recordings, OSA patients had prolonged IVRT, greater E/E' ratio and attenuated

septal E' velocity at baseline, with marked improvement in these indices after CPAP therapy, especially reduction in E/E' ratio (p=0.02).

LA Volume and LA Volume Index (LAVI)

There was a significant reduction in LA volume and LAVI post-CPAP (p<0.0001 for both) whilst using 2DE and RT3DE techniques [Table 3-41].

3.3.5.5 Discussion

In this study, I have shown that CPAP therapy enhances LV systolic and diastolic functions in otherwise healthy patients with moderate to severe OSA, using 2-DE and RT3DE. The present study is the first study to use RT3DE to assess CPAP effect in moderate to severe OSA subjects.

My findings of CPAP led improvement in LV systolic function, are consistent with available data [Bendjelid et al 2005][Akar et al 2009][Haruki et al 2010][Bellone et al 2010][Alchanatis et al 2002][Scharf et al 1996]. For example, Alchanatis et al quantified LVEF in 29 OSA subjects and reported an increase in LVEF (p<0.001) after a 6 months treatment with CPAP [Alchanatis et al 2002]. Also consistent with my results, Haruki et al demonstrated a significant reduction in longitudinal, radial, circumferential LV strain after CPAP in OSA subjects [Haruki et al 2010]. Interestingly, Malone et al reported dose-dependent improvement in LV systolic function in non-OSA subjects with dilated cardiomyopathy, after 1 month of CPAP treatment [Malone et al 1991]. It must be noted that compared to all 2DE based methods, RT3DE allows more accurate LV volume assessment. This is particularly useful in OSA subjects where real time three dimensional imaging can provide better image dataset in a particularly challenging habitus in OSA subjects.

Improved LV pressure-flow dynamics, reduction in central blood pressure, correction of hypoxia and rhythmic intrathoracic pressure swings have been proposed as some of the mechanisms whereby CPAP improves LV systolic function [Bendjelid et al 2005][Haruki et al 2010].

I observed improved LV diastolic profile after CPAP therapy and this is consistent with the current data [Konermann et al 1996][Alchanatis et al 2000]. Several studies suggest that OSA leads to LV filling abnormalities by causing LV hypertrophy and/or fibrosis, even in the absence of hypertension; and that these changes are potentially reversible with CPAP [Alchanatis et al 2000][Konermann et al 1996][Malone et al 1991][Krieger et al 1989][Buda et al 1991]. For example Alchanatis et al observed significant reduction after 14 week treatment with CPAP on OSA population [Alchanatis et al 2000], whilst Konermann et al detected increased E/A ratio (rose by 9.3% compared to baseline; $p<0.05$) after short term CPAP therapy in patients with severe OSA [Konermann et al 1996]. However compared to the present study, most previous were smaller and did not investigate tissue Doppler indices which are more sensitive and accurate diastolic parameters.

I demonstrated significant drop in systolic and diastolic blood pressure after CPAP. It is noteworthy that reduction in blood pressure and improved systemic vascular resistance has been implicated as the underlying mechanisms for CPAP led improvement in diastolic dysfunction [Brooks et al 1997].

Abnormal LV relaxation pattern and changes in cardiac visco-elastic characteristics and direct exposure of LA to raised LV pressure may augment atrial wall tension resulting in over-stretching and ultimately the LA dilatation [Greenberg et al 1979] [Dent et al 2001]. Due to LA geometric changes secondary to individual anthropometric differences, I corrected LA volume for body surface area and reported as LAVI [Lang et al 2005][Pritchett et al 2003]. I demonstrated post-

CPAP reduction in LA volume and LAVI in otherwise healthy normotensive patients with moderate to severe OSA. These observations are in accordance with limited data [Khan et al 2008].

I demonstrated CPAP related changes in LA remodelling using RT3DE for the first time in moderate to severe OSA subjects. Oliveira et al [Oliveira et al 2009] investigated LA passive emptying fraction using RT3DE in OSA but failed to detect structural LA changes. RT3DE is particularly useful considering that LA enlargement occurs eccentrically which reduces the sensitivity of 2DE methods. It should be borne in mind that, LA remodeling is increasingly viewed as a surrogate marker for LV diastolic dysfunction, atrial fibrillation and an independent predictor of cardiac failure [Pritchett et al 2005] [Takemoto et al 2005]. Therefore in our study of patients *without* structural/functional heart disease, reduction in LAVI may reflect attenuated risk of cardiovascular morbidity.

Limitations

After CPAP as described in the results, I demonstrated a significant improvement in systolic and diastolic parameters with concurrent BP (systolic and diastolic) reduction. It is difficult to say if this BP reduction was the underlying factor for such an improvement in LV dynamics, even if my analysis does not show a relationship between a change in the BP and the change in such parameters. It is one of the important limitations of this study and is very difficult to answer, considering a well known anti-hypertensive effect of CPAP in OSA. Logical reasoning would support such correlation but statistical analysis for the present study does not back up such hypothesis about BP reduction led change in LV structural and functional parameters.

Many suggest that the negative LV remodelling is predominantly due to OSA's ventilatory effects ie dramatic and exaggerated changes in pre/after load and autonomic surges causing LVH.

Considering that, a relief from such a ventilatory pressure due to OSA through CPAP treatment can translate into enhanced LV dynamics. Again, this can not be answered in the present study as the AHI was not measured during the follow up due to unfavourable logistics. A study investigating the pulmonary pressures during apnoea and hypopnoeas and after CPAP (in OSA) and there relationship with change in LV function would probably provide the answer. This study can be regarded as a pilot study for future and it focussed more on the general aspects of the disease.

OSA subjects in this study were obese and obesity could alter diastolic characteristics on its own merit [Mureddu et al AJC 1996]. However OSA patients had comparable body mass index pre- and post-CPAP. I did not check the agreement between 2DE and RT3DE outcomes for a given index. Observational model is another limitation of this study. However in order to ensure uniformity all the measurements and recordings were performed and analyzed by the same operator. Furthermore, inter- and intra-observer variability was satisfactory (less than 10%).

3.3.5.6 Conclusion

Effective treatment with CPAP improves the LV diastolic and systolic function in moderate to severe OSA subjects. The beneficial effect on the LA remodelling may have important implications as this may reflect diminuted risk of atrial arrhythmias (such as atrial fibrillation) in compliant OSA patients. Large and blinded studies would be required to evaluate deeper impact of CPAP on LV functional properties in patients with moderate to severe OSA.

Table 3-38: Patient Characteristics

	Pre-CPAP	Post-CPAP	p
n	37	37	
Age (Years)	49±10	49±10	0.451
AHI	36 (21-51)	Not recorded	
BMI (Kg/m2)	34±7	34±8	0.180
Epworth score	13±6	5±6	<0.0001
Systolic BP (mmHg)	144±16	133±13	<0.0001
Diastolic BP (mmHg)	83±11	80±7	0.025

Values expressed as Mean (Standard Deviation) or Median (Inter quartile range). AHI= Apnoea-Hypopnea Index; BMI= Body Mass Index; BP= Blood Pressure, CPAP= continuous Positive Airway Pressure

Table 3-39: Systolic Indices before and after Continuous Positive Airway Pressure Therapy

	Pre-CPAP	Post-CPAP	p
n	37	37	
M-mode Method			
EF (%)	64±7	74±8	<0.0001
LVEDd (cm)	4.9±0.6	5.2±0.6	<0.0001
IVDd (cm)	1.1±0.2	0.99±0.2	<0.0001
PWd (cm)	1.2±0.2	1.1±0.2	0.016
LVEDV (ml)	114±34	136±40	<0.0001
FS (%)	35±5	44±7	<0.0001
Modified Simpson's Method			
EF (%)	65±9	70±7	0.007
RT3DE			
EF (%)	66±8	71±9	0.024
LVEDV (ml)	86±25	93±27	0.05

Values are described as Mean (Standard Deviation). P <0.05 was considered statistically significant. RT3DE= Real Time 3-Dimensional Echocardiography; EF= Ejection Fraction; FS= fractional Shortening; IVSd= Interventricular septal dimension in diastole; PWd= Posterior Wall dimension in diastole, LVEDd= Left ventricular End-Diastolic dimension; LVEDV= Left Ventricular End-Diastolic Volume, CPAP= continuous Positive Airway Pressure

Table 3-40: Diastolic Indices before and after Continuous Positive Airway Pressure Therapy

	Pre-CPAP	Post-CPAP	p
n	37	37	
E (m/sec)	52±12	64±12	<0.0001
A (m/sec)	53±14	48±12	0.016
E/A	1.0±0.4	1.4±0.4	<0.0001
E' (sep) (m/sec)	6±2	8±2	<0.0001
E/E'(sep)	9±4	8±2	0.02
IVRT (ms)	0.09±0.02	0.07±0.02	<0.0001

Values are described as Mean (Standard Deviation). P <0.05 was considered statistically significant. E= Mitral early inflow peak velocity, A= Late inflow peak velocity, sep E'= septal E prime, IVRT=Isovolumetric relaxation time, CPAP= continuous Positive Airway Pressure

Table 3-41: Left atrial Volume Index using Two-Dimensional and Real time 3-Dimensional Echocardiography before and after Continuous Positive Airway Pressure Therapy

	Pre-CPAP	**Post-CPAP**	**p**
n	37	37	
Ellipsoid Method	26±9	21±6	<0.0001
Area-length Method	28±9	23±6	<0.0001
RT3DE	26±8	22±7	<0.0001

Values are described as Mean (Standard Deviation). P <0.05 was considered statistically significant.

CPAP= continuous Positive Airway Pressure, RT3DE= Real time 3-Dimensional Echocardiography

3.3.6 Impact of Continuous Positive Airway Pressure Therapy on Myocardial perfusion in Moderate to Severe Obstructive Sleep Aponea

3.3.6.1 Abstract

Background: Obstructive sleep apnea (OSA) has been implicated in the initiation and progression of cardiovascular diseases owing to the impairment of myocardial perfusion. I hypothesized that myocardial perfusion improves after long term continuous positive airway pressure **(**CPAP) therapy in subjects with moderate to severe OSA.

Methods: Myocardial blood flow was quantified using myocardial contrast echocardiography in 36 otherwise healthy subjects with moderate to severe OSA, before and after CPAP treatment. Real-time MCE dataset were acquired in three apical views at rest and after dipyridamole stress.

Results: On average patients were middle aged (49 years ±10) and obese (BMI= 34±8 Kg/m^2). Basal and hyperaemic myocardial perfusion responses improved significantly ($p<0.001$ for all) after 6 months of CPAP therapy.

Conclusion: Long term CPAP therapy significantly improved myocardial perfusion in otherwise healthy subjects with moderate to severe OSA.

3.3.6.2 Introduction

Untreated OSA is an independent association with cardiovascular disorders and predictive of worse cardiovascular outcome [Peker et al 2000][Marin et al 2005] [Young et al 1993][Shahar et al 2001]. Subclinically impaired myocardial perfusion has been demonstrated in untreated OSA using magnetic resonant imaging [Nguyen et al 2010]. Interestingly, a high high prevalence of previously undiagnosed OSA in patients admitted with acute myocardial infarction has also been reported [Lee et al 2009]. Reassuringly however, CPAP use has shown promising results in reversing myocardial perfusion patterns with a suggestion that such enhancement may be secondary to improvement in microvascular function located in the sub-endocardial layer [Nguyen et al 2010][Marini et al 2010]. Indeed regular and efficient use of CPAP has shown to reverse ED leading to reduction in systemic arterial blood pressure in OSA patients [Bazzano et al 2007].

However, MCE has not been utilized to detect CPAP led changes in myocardial blood flow patterns. It must be noted that MCE has a close agreement with other perfusion assessment imaging tools such as MRI and SPECT [Wei et al 1998][Peltier et al 2004][Moir et al 2006] [Di Bello et al 2004]. Perhaps most importantly, the available data is limited and no previous study has focused on healthy subjects with *moderate to severe* OSA.

I hypothesized that the long term CPAP therapy may improve myocardial perfusion - as assessed by MCE - in subjects with moderate to severe OSA. In order to test this hypothesis I performed MCE on otherwise healthy subjects with moderate to severe OSA before and after 6 months of the CPAP use.

3.3.6.3 Methods

The methods have been described in details under section 2.

3.3.6.4 Results

Table 3-42 shows patients' characteristics at baseline and follow up whilst Table 3-43 show indices of myocardial perfusion, pre- and post-CPAP treatment.

MCE was performed on 40 patients prior to the initiation of the CPAP therapy. During follow up duration two patients failed to tolerate CPAP, one developed atrial fibrillation and one withdrew from study. Therefore, after 6 months of CPAP therapy, 36 middle aged (49±10 years) patients with reasonable female gender representation (20%), were available for follow up. All subjects complied with CPAP and use of CPAP averaged 5.4 hours per night 5 nights per week [Table 3-42].

A significant reduction was noticed at follow up in systolic ($p<0.0001$), diastolic blood pressure ($p=0.04$) and Epworth score ($p<0.001$) [Table 3-42].

At rest Post-CPAP segmental blood volume fraction (α) and the blood flow velocity (ß) were significantly greater compared with pre-CPAP {('α' Pre-CPAP 21±6 versus post-CPAP 25±2; $p=0.001$) and ('ß' Pre-CPAP 1.2±0.5 versus post-CPAP 1.6 ±0.4; $p<0.0001$)}. The product of the 'α' and 'ß' at rest, regarded as myocardial blood flow at rest (FR), was also statistically greater in follow up group ($p<0.001$) [Table 3-43].

The *post-stress* (induced by Dipyridamole infusion) 'α' was 22 ±6 before CPAP treatment which rose to 29 ±2 after 6 months of treatment ($p<0.001$). The post-stress change in 'ß' was even more pronounced, which increased from 1.9 ±1 to 4.8 ±1 after CPAP ($p<0.0001$) and therefore unsurprisingly the 'FS' was also estimated significantly higher after CPAP ($p<0.001$) [Table 3-43].

The MBFR (ratio of the ‘FS’ and ‘FR’) and was greater in post-CPAP group compared to pre-CPAP indicating significant reversal of the myocardial perfusion after CPAP treatment respectively ($p<0.0001$) [Table 3-43].

3.3.6.5 Discussion

This is the first study to provide a comprehensive evaluation of subclinical CVD using MCE in healthy patients with moderate-severe OSA. OSA Patients had abnormal MBFR, suggesting these patients have microvascular disease and endothelial dysfunction. These abnormalities improved after 6 months of effective CPAP, providing further evidence that OSA may contribute to the development of cardiovascular diseases.

Microvascular disease and endothelial dysfunction are the earliest manifestations of coronary heart disease and can be found in patients without obstructive coronary artery disease. Indeed subclinical coronary microvascular dysfunction has been previously demonstrated in patients with cardiovascular risk factors. For example, coronary flow reserve was reduced by 21% in smokers and normalized with vitamin C administration [Kaufmann et al 2000]. Similarly impairment in coronary flow reserve has also been shown in asymptomatic subjects with hypercholesterolemia and angiographically normal coronary arteries with demonstrated reversibility with cholesterol lowering strategies [Dayanikli et al 1994] [Gould et al 1994]. Similarly, in the present study, patients with OSA with no known coronary or myocardial disease had impaired myocardial perfusion which improved after CPAP, suggesting that the presence of microvascular disease is due to OSA. To our knowledge, this is the first study to show that patients with OSA have microvascular disease measurable by MCE, which improves with therapeutic CPAP.

Several mechanisms have been suggested to explain the relationship between OSA and vascular dysfunction [Lanfranchi et al 2001]. Evidence suggests that recurrent apnea-hypopneas in OSA

patients are associated with repetitive cycles of hypoxemia and reoxygenation, enhancing production of reactive oxygen species. This leads to increased intra cellular oxidative stress and breakdown of NO, resulting in vascular dysfunction. The relationship between apnea and vascular dysfunction was further supported in a recent study, which showed that regional myocardial perfusion defects were present during periods of apnea in patients with OSA without obstructive coronary disease but were not present during daytime SPECT [Orea-Tejeda et al 2003].

Correction of hypoxia by addressing OSA related ventilatory dysfunction may be underlying reason for CPAP led improved perfusion. This is also reported to improve oxidative stress and endothelial dysfunction [Lattimore et al 2006] [Imadojemu et al 2002][Ip et al 2000] [Kato et al 2000]. For example, Mak et al noted normalization of coronary flow reserve shortly after the initiation of CPAP therapy in a patient with intermediate epicardial arterial lesion [Mak et al 2010].

Limitations

I have derived that the enhancement in myocardial perfusion in otherwise healthy OSA subjects is secondary to reduction in total vascular resistance, resulted from CPAP led improvement in endothelial status. However this conclusion is indirect as we did not measure coronary vascular resistance directly. I suggest that CPAP effects on the coronary vascular resistance and myocardial blood flow should be investigated concomitantly in moderate to severe OSA.

The observational study design is a limitation of this study. In order to ensure uniformity, all the measurements and recordings were performed and analyzed by the same operator. Furthermore, we demonstrated satisfactory inter- and intra-observer variability (<10%). Finally, these observations are limited to healthy middle aged subjects with moderate to severe OSA and cannot be extrapolated to patients with milder severity or OSA with co-existing disease.

Myocardial perfusion enhanced after CPAP along with the concurrent BP reduction. As the myocardial perfusion is directly related to coronary microvasculature which is closely related with the endothelial health, I suggest that such change in perfusion is secondary to improved endothelial status after CPAP. Improvement in FMD as a surrogate of ED has been described elsewhere in this thesis. ED improvement is multifactorial and has been reported with CPAP with and without BP reduction. It is very reasonable to question if BP reduction is a dominant contributor is such change. I did not note any correlation between BP change and MBFR change suggests no such link. Nevertheless I did not demonstrate a direct correlation between marker of ED and change in MBFR. This unresolved and unanswered aspect of this study needs to be explored in future studies.

3.3.6.6 Conclusion

In summary, this novel study shows that CPAP led changes in myocardial perfusion in moderate to severe OSA in otherwise healthy humans. Our observations need further study or clinical trials to determine the prognostic implications of using CPAP in a large population of patients with OSA.

Table 3-42: Patient Characteristics

	Pre-CPAP	**Post-CPAP**	**p**
n	36	36	
Age (Years)	49 (10)	49(10)	0.369
AHI	36 (20-50)	Not recorded	-
BMI (Kg/m2)	34 (8)	34 (8)	0.103
Epworth score	13 (5)	6 (7)	<0.0001
Systolic BP (mmHg)	143±16	133±12	<0.0001
Diastolic BP (mmHg)	83±12	79±8	0.04

Values expressed as Mean (Standard Deviation) or Median (Inter quartile range). P<0.05 was considered Significant

AHI= Apnoea-Hypopnea Index; BMI= Body Mass Index; BP= Blood Pressure; CPAP= Continuous Positive Airway Pressure

Table 3-43: Indices of Myocardial Perfusion

	Pre-CPAP	Post-CPAP	p
n	36	36	
Segments	4	4	
FR	26 (13)	41 (12)	<0.0001
FS	43 (26)	134 (28)	<0.0001
MBFR	1.7 (0.7)	3.5 (1.5)	<0.0001

Values expressed as Mean (Standard Deviation). P<0.05 was considered Significant. OSA= Obstructive Sleep Aponea; HT= Hypertension; FR= Myocardial Blood Flow at Rest; FS= Myocardial Blood Flow at Stress; MBFR= Myocardial Blood Flow Reserve; CPAP= Continuous Positive Airway Pressure

Section 4 Summary and Conclusion

4.1 Summary of Findings

In this thesis, I have comprehensively investigated micro- and macro-vascular dysfunction in OSA population, with an emphasis on the coronary circulation. I compared moderate to severe OSA subjects with hypertensive and healthy controls; however only OSA subjects were followed up after 6 months of CPAP teartment.

In chapter 3.2.1, I have demonstrated that enodhtlial-dependent and -independent FMD is impaired in OSA and hypertensive subjects compared with healthy subjects. However, the results were comparable between OSA and hypertensives. This clearly demonstrated presence of macro-vascular dysfunction in OSA and is consistent with the previous studies. After CPAP treatment for 6 months, I noted significant improvement in brachial artery reactivity as described in chapter 3.3.1.

I studied microvascular perfusion patterns in OSA using laser Doppler flowmetry, in chapter 3.2.2. I noted impaired ACH and SNP induced responses in OSA as well as in hypertensives compared with the healthy controls. Post-CPAP perfusion parameters enhanced significantly in OSA cohort and have been described in chapter 3.3.2.

I did not find a statistically significant difference in parameters of arterial stiffness, amongst the groups. In chapter 3.2.3, I demonstrated comparable markers of macro-vascular stiffness such as AA, AIx, AIx@75, SEVR and PWV amongst the three groups. Thus, arterial stiffness is more pronounced and obviously demonstarable in diseased OSA subjects i.e with coexisting cardiovascular diseases. Nevertheless, CPAP therapy led to a dramatic change in these markers after 6 months and this has been reported in chapter 3.3.3.

In chapter 3.2.4, I found no difference in EPC and CEC distribution in OSA compared with controls. Also, no significant change was noted after CPAP therapy in either of the cell strains or

their ratio as described in 3.3.4. Indeed functional aspects may alter after CPAP (which were not explored in the present study) but it can be argued that disturbed distribution patterns may only occur in OSA *with* cardiovascular diseases.

I studied the impact of OSA on cardiac remodeling using convensional 2DE and 'state-of-the-art' RT3DE. It must be noted that RT3DE has never been used for this specific purpose before in moderate to severe OSA population. I have shown preserved systolic yet impired diastolic function in OSA compared with healthy subjects as described in chapter 3.2.5. This is consistent with available data. I also demonstrated enlarged LAVI (owing to chronic diastolic dysfunction), which may explain high prevalence of atrial fibrillation in OSA population. CPAP was shown to have a discernible impact on LV remodeling, as evident by improvement in diastolic impairment and reduction in LAVI. These findings may have implications in exploring the AF management strategies in moderate to severe OSA subjects as shown in 3.3.5.

I studied myocardial blood flow patterns using myocardial contrast echocardiography and found impaired myocardial perfusion compared with the healthy subjects. MCE has never been performed before in OSA cohort. My findings reported in chapter 3.2.6 suggest microvascular dysfunction in moderate to severe OSA subjects. I demonstrated marked enhancement in myocardial blood flow responses and perfusion indices after 6 months of CPAP treatment, chapter 3.3.6.

4.2 Suggestions for Future Studies

The studies described in this thesis are novel, owing to its design and techniques used. Moderate to severe OSA subjects have never been compared with healthy and 'disease' (hypertensive) controls simultaneously. I focused on moderate to severe OSA specifically, as it is the primary target for CPAP treatment. Similarly my reported studies on FMD, LDF, flow cytometry and arterial stiffness are largest of their kind, albeit these have been performed on OSA cohorts previously. Most importantly, for the first time I used RT3DE and MCE in moderate to severe OSA subjects for LV assessment.

However these studies have limitations such as exclusion of mild OSA. These limitations should be addressed in future studies. Also, most 'real life' OSA subjects have complex clinical profile where they have conditions such as hypertension and coronary artery disease. It it is possible the subjects with such confounding variables may have different perfusion patterns and hence should also be studied in comparison with matched controls.

My OSA subjects were non-hypertensive although the average blood pressure was higher than the cut-off for the healthy population. The blood pressure readings were taken in the community, specialized clinic and during the study and were found to be in the normotensive reange. I feel that the average blood pressure could be higher due to an element of white coat hypertension in OSA subjects. Indeed the possibility of concealed hypertension can not be ruled out completely, as we did not perform a screen 24 hour blood pressure minitoring prior to the study. However, I would like to emphasize here that I took utmost care to exclude hypertension in OSA subjects. Future studies must ensure to enroll only subjects with desirable average blood pressure on ambulatory blood pressure.

Some of the observations reported would need further exploration. For example, I report impaired endothelial independent responses in OSA compared to healthy subjects. Notably, impaired endothelial independent responses have been described in smokers but this has never been described before in moderate to severe OSA subjects. Contrary to available data, I did not notice any changes in flow cytometric analysis and in the arterial elastic properties. I suggest largers yet similar studies to confirm or negate our results.

It is known that OSA subjects have a high incidence of AF compared to healthy subjects. It has also been reported that the rate of recurrence is higher in electrically cardioverted or electrophysiologically ablated AF subjects with OSA. I reported CPAP led reduction in LA volumes, improvement in diastolic impairement and systemic blood pressure, all of which are important risk factors for AF. A potential reduction in the cumulative incidence or arrhythmia burden (eg. by continuous monitoring) of AF can be derived in CPAP treated subjects. Similarly, it can also be hypothesized that the AF related ischaemic stroke rate may also fall in OSA subjects. This may have important prognostic and logistic implication. I suggest that occurance patterns of AF should be prospectively re-studied in CPAP treated OSA to observe if CPAP led cardiac changes translate in a better morbidity.

MCE has never been previously performed in OSA previously. Hence the present study can be used as pilot for future projects. Clearly these data showed subclinically impaired myocardial perfusion. However, it should be explored if it determines poorer outcome in untreated OSA subjects. Circumstantial evidence supports the presence of cardiovascular disorders in OSA more than non-OSA subjects, but do the epidemiological patterns correlate with the impaired myocardial perfusion in OSA? I demonstrated a significant improvement in myocardial perfusion with CPAP therapy. It would be interesting to examine CPAP induced enhanced cardiac perfusion translates into prognostic benefits.

4.3 Conclusion

OSA is an important public health condition worldwide. It is strongly associated with cardiovascular disorders. Available evidence supports higher incidence of heart failure, CAD, hypetrnesion, and stroke in OSA subjects. Investigations of peripheral and coronary vasculature proved that systemic endothelial dysfunction exists in OSA subjects. In coronary vasculature, endothelial dysfunction manifests as impaired myocardial perfusion which may lead to subclinically ischaemic myocardium and ultimately heart failure. OSA also causes cardiac remodeling i.e. LVH and LA enlargement, which may have arrhythmic potential such as high incidence of AF in OSA.

Perhaps the most significant observation of this study is that CPAP reverses endothelial dysfunction and thus may enhance myocardial perfusion as shown by the MCE. Such positive changes in peripheral and myocardial flow patterns may be implicated into an improved clinical and prognostic outlook with CPAP. This thesis studies a comprehensive assessment of macro- and micro-vascular (dys)function in OSA and provide pathophysiological insights into the potential benefits of CPAP therapy in OSA.

Section 5 Appendices

5.1 Standard operating Procedures (SOPs)

5.1.1 Flow Mediated Dilatation (FMD)

Updated by Mehmood Butt, 2008

Introduction

Endothelium-dependent vasodilatation of the brachial artery occurs in response to increased flow. This response can be assessed using ultrasound; a forearm cuff is used to occlude the vessel downstream. After cuff release, reactive hyperaemia occurs and increased blood flow causes nitric oxide (NO) release from the endothelium and consequent endothelium-dependent vasodilatation. The endothelium-independent response can be assessed by administration of a systemic NO donor such as GTN. Abnormalities in FMD have been found in patients at risk of atheroma before evidence of actual atheroma has developed, and it is also a predictor of future cardiovascular events, at least in hypertensive patients [Chong et al 2003][Celermajer et al 1992].

Equipment

You will need:

- Patient (!)
- An available vascular scanner (for around 45 minutes at least)
- Echo jelly (the tubs are refillable)
- ECG monitoring electrodes

- Apple Mac MO disk or VCR cassette; ideally both
- Timer (can use clock on echo machine if not available)
- Marker pen suitable for skin
- Manual sphygmomanometer (capable of pressures up to 300mmHg)
- GTN spray

1. Preparation

a. you may need to turn all the equipment on: the source projector box is located behind the computer desk: switch this on first.

b. Turn on echo machine (switch on the front of the machine about half way down, towards the left)

c. Insert an MO disk into the disk reader attached to the Apple Mac laptop (if you want to save your images using echopac).

d. Turn on the AppleMac laptop (button at top right of the keyboard); if you are asked for a password at any time press space bar then enter

e. open echopac using the icon on the desktop if it isn't already open (click on 'finder' at the top and select echopac if echopac is open but hidden, ie running in the background).

f. Systems should connect automatically

g. Allow the subject to rest in the room for about 15 minutes before scanning

h. Place the ECG leads on the patient

i. Press 'patient ID' on the echo machine console (towards the left) and key in your patient's information. (Or you can do this from echopac)

2. On the echo machine, select the probe

a. (To select options using the menus, use the black tracker ball to move the arrow over the option, then select by pressing any part of the white area surrounding the tracker ball)

b. Press ‘probe’ on left side of echo machine console

c. Select 10MHz; then select arterial limb

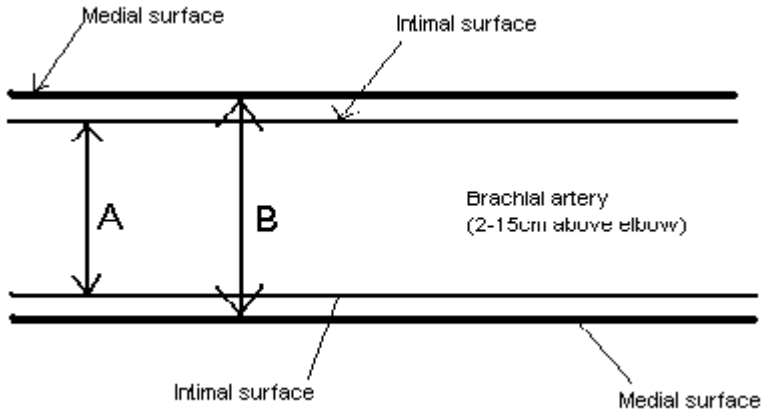
d. Use the vascular scanner probe (10MHz), not the echo probe – (the one you want is rectangular, about 5cm length, not the smaller echo probe)

3. Label the image (using the ‘text’ button towards the left of the console) as ‘baseline 1’. Scan about 2-15cm above elbow (it doesn’t matter much where so long as you get the same segment of artery for baseline and measurement scans). Identify the artery with colour doppler if necessary (button marked CFM on echo machine) – arterial flow is obviously pulsatile and typically very bright on colour flow (it may be blue or red); once identified as arterial, it is easiest to turn colour flow off again in order to see the arterial wall clearly. Try to find a section where a long segment is clearly visible, ideally with endothelium seen on both superficial and deep walls of the vessel. Because you take several measurements (before and after FMD and GTN), it is worth trying to note some reference points near the segment being measured; this makes it much easier to be sure you are measuring the same segment in subsequent measurements. It’s also worth finding a vessel that is easy to identify because the later scans are time dependent and you will be under some pressure to find the same vessel quickly.
4. When you have found a good section of vessel, mark the skin clearly so that you can easily place the echo probe in the same position.
5. Freeze the image (button to the right of the console) when you have a clear segment seen for several seconds, and you can then replace the probe in the machine. Scroll backwards and forwards (using the tracker ball) to use the R wave of the ECG cycle so that subsequent readings

are taken in the same part of the cycle, and so that the image quality is optimal. Press 'image store' to send the image to echopac (allows someone else to analyse the image without your measurements being visible); you may also wish to videotape the image for later analysis (press VCR record once to start recording and again to stop recording).

6. Using 'caliper' button, measure 5 readings from leading edge to leading edge of the vessel. Move the cross cursor to the first point, press the white area around the tracker ball once you are happy with position, then move to the second area and click again. The machine then displays the distance. Select repeat measurement to take 5 readings. Once you have the readings, either note them down or send the image with the readings visible to echopac; take the average of the 5 readings as your measure. Different people use different places to measure: the important thing is to be consistent between readings and between patients. In the figure 4-1, both A and B are acceptable so long as the same method is performed in all patients.

Figure 4-1



7. Place the cuff on the forearm and inflate to 250-300mmHg (must be at least 50mmHg above systolic blood pressure; warn the patient that it will be uncomfortable! Start timing. Keep an eye on the pressure in the sphygmomanometer: it may drop a little with time and you will need to

keep it above 250mmHg by pumping more air into the cuff. You can now unfreeze the echo image; delete the label with the ‘page erase’ button to the left of the console. Then label the image ‘FMD’ or similar in preparation for the next image.

8. At 4½minutes, deflate the cuff rapidly.
9. Repeat the scan 30-90 seconds after cuff deflation. It is worthwhile looking for the artery soon (immediately) after cuff deflation so that you can be sure to find the artery within this time, then holding the probe steady until your 30-90seconds is reached. Try to identify the same segment as you scanned for the first baseline (compare the image you are getting with the image displayed on the mac which you sent to echopac), then freeze the image and repeat the measurement as before, again using the R wave of the ECG cycle.
10. Wait 15 minutes to allow the effect of the FMD to wear off. (You can use this time to gather other information such as history, drug use, or measuring other things such as carotid IMT; make sure you are not doing anything that might upset vasomotor function though (such as blood pressure, venesection)
11. Repeat the baseline measurement as before, on the R wave of the ECG cycle. Label as ‘baseline 2’ and record the image.
12. Give 2 puffs of sublingual GTN (warn the patient they may get a mild headache / feel dizzy). Prepare for the next image by labelling ‘GTN’ or similar
13. Repeat the scan after 4½ minutes, measuring on the R wave of the ECG cycle, and record the image to echopac / VCR.
14. Clean jelly off the patient and thank them!
15. The echopac images are stored in the buffer and need to be saved – save to your MO disk using the button at the bottom of the screen.
16. Clear up the room: wipe jelly from the probe, make sure the GTN is on the shelf out of reach of children etc., tidy up the examination couch, turn off equipment if not going to be used shortly. Bear in mind that the equipment is expensive and used by a lot of people...

Potential problems

This technique takes practise – you should ideally scan about 50 people before recording results formally! It is worth trying quite hard to get a good image, and be scrupulous in technique in trying to identify the same segment of artery each time. The second scan (FMD / GTN scan as opposed to baseline scans) is a bit pressured as there is a time factor involved. As previously mentioned, try to find the relevant segment of artery early to give you more time to look. If you don't have time to actually measure the vessel diameter before the next reading is due, you can measure it later from echopac (so long as you have sent the image) so don't worry. It is quite easy to forget to send one of the images to echopac – be sure you have recorded one image from each scan. The VCR recorder is on / off with each press of the button so make sure you actually stop recording after you have captured enough information (otherwise you will be stopping the recording next time you try to record something!).

If echopac does not detect the echo machine, turn everything off and start again.

If the Mac crashes, you may need to reset it: the only really reliable way to do this is to disconnect the power supply and transiently remove the battery. It's not very good for the computer and all unsaved data will be lost so give the machine time to see if it can recover itself before you resort to this. It is vital to label all images as sometimes echopac seems to mix up the order it displays the images (so, for example, the baseline may appear either before or after the measurement).

5.1.2 Laser Doppler flowmetry (LDF)

Updated by Mehmood Butt and Andrew Blann 2008

Health and Safety / COSHH

The only major hazard in this procedure is the risk of damage to the retina due to the laser. Therefore avoid eye contact. Some subjects may be allergic to Ach & SNP. Some may respond adversely to the electrical current. Obtain consent.

Introduction

The function of large arteries such as the aorta, brachial and femoral can be assessed by their dilation response to changes in blood flow (i.e. flow mediated dilatation). This assessment is not possible in the study of small arteries and arterioles, often within vascular beds such as the skin. However, the recent development of perfusion imaging can assess these small arteries. The present SOP is to enable this assessment. Laser Doppler perfusion imaging is based on four separate technologies:

- (a) the Doppler shift phenomenon (change of the wavelength of the moving object proportionally the speed of the object, i.e., blood cells),
- (b) the ability of the Laser imaging system to detect this phenomenon,

- (c) perfusion of skin with pharmacologically active drugs (acetylcholine [Ach] and sodium nitroprusside [SNP]) that will alter small blood vessels close to the surface of the skin, and
- (d) the electrical delivery of these agents into the skin (ionotophoresis).

Thus the method allows an evaluation of the perfusion of surface tissues (e.g. skin) in real time scale. Changes in skin blood flow can be determined in response to various stimuli. Vasoactive substances can be used to alter the flow of blood in the skin vessels if delivered by an appropriate means. Iontophoresis is a delivery method that uses the charge of a charged vasoactive substance to allow it to be driven through the skin using an electric current. Sodium nitroprusside is a vasoactive substance with a negative charge that can be delivered through the skin to the microcirculation; once it has passed through the skin it acts as a nitrate donor and therefore acts directly on the vessel's smooth muscle to cause vasodilatation. Thus it is independent of the endothelium in its vasodilatory action. Acetylcholine on the other hand is a positively charged molecule that can similarly be delivered to the subcutaneous tissues by iontophoresis but it acts on the endothelium rather than on the smooth muscle wall, causing endothelial release of nitric oxide. Thus acetylcholine acts in an endothelium-dependent way on the vessel wall to produce vasodilatation. The vasoactive substances are provided close to the skin in a small perfusion chamber that also acts as an electrode. A second electrode is required to complete the circuit, and this is attached to a nearby section of skin.

Test solutions

a) 2% acetylcholine chloride (Ach) [Sigma-Aldrich]

b) 1% sodium nitroprusside (SNP) [Sigma-Aldrich]

Both must be prepared in sterile filtered distilled water (Sigma-Aldrich) and appropriate volumes (approximately 1ml aliquots) stored in stored top plastic tubes a fridge at 4°C up to 1 month.

These solutions are transferred from the plastic tubes to the iontophoresis chamber with a plastic disposable transfer pipette (stores in preparation room and the laboratory). Ensure the reagents are allowed reach room.

Preparing the Laser Doppler Flowmeter (LDPI II, Perimed, Sweden).

The laser requires a 20 minute warm up period so do this well before the subject is assessed.

1. To switch on the LD, press the green button on Blue Box (Laser Doppler block)

2. Turn on the computer (button front panel).

3. Start LDPIwin 2.6 icon (Laser Doppler software) on the desktop

4. Check settings of scanning in **Measurement dialog** if necessary. The settings should be as:

    (1) Repeated Mode;

    (2) Image format: 25 x 25 (width and height);

    (3) Start Method: Manual;

    (4) Resolution: High;

    (5) Threshold: 6.2

The equipment should now be ready.

Iontophoresis and Data acquisition

1. For the assessment the patient is laying on the couch comfortably in a constant room temperature room for 20 minutes before the start of measurements. All measurements should be performed in dim light. The forearm is placed on a supportive cloth/pillow.

2. A hairless area of forearm skin in the non-dominant arm is cleaned with a 40% ethanol swab and the iontophoresis chamber (Perimed UK) placed over the skin.

3. The reference electrode is placed over the volar aspect of the ipsilateral wrist 15 cm from the chamber.

4. The chamber is then filled with about 0.5 ml Ach (using transfer pipette) with ensuring that no air bubbles are visible below the glass cover. The anode (red) is attached to this and the cathode (black) to the other (wrist) electrode.

5. Iontophoresis is not commenced until three complete baseline readings had been taken:

- press **New** following **Start** to begin scanning
- press **Continue** to acquire more scans or **End** to finish acquisition
- save results obtained.

The computer driving the laser scanner is pre-programmed to scan the area of the iontophoresis chamber using high resolution settings (see above).

6. During baseline readings the iontophoresis power pack is prepared. Switch on with the left hand knob rotating clockwise. Set the unit to deliver 0.1mA over 1 minute (total charge delivered 6mC) as a continuous current. There is a spare battery in the grey Periiont box.

7. Apply the current and acquire perfusion scans for 6 min following ACh delivery (maximal response is usually seen at 2-3 min.). Store the data in an appropriate file on the computer.

8. After the ACh readings are completed, the patient rests if required (5- 20 minutes) then proceeds to SNP measurement.

9. The chamber is removed and a clean iontophoresis chamber placed approximately 4cm further away from the wrist electrode (so that the iontophoresis electrode did not lie within the path of the current from the previous recording).

10. The same procedure is used for SNP (about 0.5 ml) except that the electrode polarity is reversed. Acquire perfusion scans for 10 min following SNP delivery (maximal response is usually seen at 5-6 min.).

11. Complete the procedure by wiping excess fluids from the subject, disposing of waste material etc. Power down the LD and switch it off.

Analysis of results

After the readings had been taken, the images are analysed using the dedicated software package provided by the manufacturer.

1. To select area of interest (i.e. the area of chamber that had vasoactive substance directly in contact with the skin) to be analysed use **Create Elipse Tool** and **Pointer Tool**.

2. Press right mouse button and select **Unlink POI on all images**

3. Adjust position of the area of interest on all consequent scans (i.e. for each time point)

4. Save changes

5. Select **Export** and **Images and calculations to Excel** to export results to Microsoft Excel; the values for each time point are used to determine the peak response (in terms of absolute perfusion units, and also % change from baseline), and area under the curve for the perfusion over the 10 minute experiments.

6. Save results obtained and print them if necessary.

7. Close software

8. Turn off the computer and switch off Blue Box (Press green button).

5.1.3 Flow Cytometry

Written by Patrick Goon and Timothy Watson, August 2006

Updated by Mehmood Butt 2008

Introduction

The potential importance for EPC/CECs in the physiological response to endothelial damage has led to their investigation in numerous disease settings. For example EPCs have been shown to inversely correlate with cardiovascular (Framingham) risk scores. Similarly CECs have been described to represent endothelial damage. This has led to speculation that the ability to enhance their numbers may allow for a novel approach to modulate various disease states.

The definition of these cells has continued to evolve. Currently cited markers include CD34, CD146 and Kinase Domain Receptor (KDR). Importantly, as these cells mature, the cell surface markers change. In addition, other cell lines such as monocytes and tissue resident stem cells may cross-differentiate, adding to the complexity of defining this cell population.

This method describes enumeration of KDR+, CD34+, CD45- cells represent EPCs whilst CD146+, CD34+ and CD45- cells represent CECs.

Materials

1) BD “FACS Flow” Running solution [Becton Dickinson Catalogue No. 342003]

   Order 6 x 10L containers and keep in stock

2) BD “FACS Rinse” Rinsing Solution [Becton Dickinson Catalogue No.

   Order 1L containers only

3) BD “FACS Clean” Cleaning Solution [Becton Dickinson Catalogue No.

   Order 1L containers only

4) BD Falcon tubes[Becton Dickinson, Catalogue No. 352054]

   Order 10 packs and keep in stock

5) BD Lysing solution [Becton Dickinson Catalogue No. 349202]
6) Distilled water [make >10L at a time using laboratory distilling equipment in HTVBU lab]
7) Phosphate Buffered Saline tablets. [Sigma Catalogue No. P4417]
8) Sodium Azide [Sigma Catalogue No.
9) Bovine Serum Albumin - 100g [Sigma Catalogue No. A-3912]
10) CD45 – FITC conjugated monoclonal antibody - 100 tests [BD Catalogue No. 555482]
11) CD34 – PE Cy5 conjugated monoclonal antibody - 100 tests [BD Catalogue No. 555823]
12) CD146 – PE conjugated monoclonal antibody - 100 tests [Biocytex, France Catalogue No. 130-080-801]
13) KDR – PE conjugated monoclonal antibody - 100 tests [R&D, UK Catalogue No. 130-080-801]
14) Mouse Serum – 5ml [Sigma Catalogue No. M5905-5mL]
15) Octagam (2.5 g in 50 mls PBS) [Octapharma Ltd]
16) Blue pipette tips [Appleton Woods Catalogue No. HTL160]
17) Yellow pipette tips [Appleton Woods Catalogue No. TG701]
18) 15ml Centrifuge tubes [Sarstedt Catalogue No. 62.554.502]

Supplier contact details:

Becton Dickinson (UK)

Between Towns Road

Cowley

Oxford ,Oxfordshire

OX4 3LY

Fax: 01865 781578

Appleton Woods Ltd

Lindon House, Heeley Road

Selly Oak, Birmingham B29 6EN

Tel 0121 472 7353, Fax 0121 414 1075.

Freefax orderline 0800 387 462

Sarstedt Ltd.,

68 Boston Road, Beaumont Leys,

Leicester LE4 1AW, United Kingdom

Tel.: +44 1162 359023,

Fax: +44 1162 366099

Octapharma Ltd

6 Elm Court,

Copse Drive,

Coventry.

CV5 9RG

Telephone: +44 (0) 1676 521000

Fax: +44 (0) 1676 521200

3. Procedures

3.1 General Preparation

3.1.1 Lysing solution.

Make from 50ml concentrate diluted with 450ml distilled water in ½ litre bottle. Store in fridge.

3.1.2 Wash solution

Make up 1 litre phosphate buffered saline (5 PBS tablets + 1 litre distilled water). Sterilize in autoclave (120C for 2-3hours). When ready to use add 5g =bovine serum albumin and 0.5g Sodium Azide. Mix thoroughly and store in fridge.

[Caution COSHH Data…]

3.1.3 Mouse Serum 10%

[Dilute 0.5 ml mouse serum in 4.5ml PBS and store in fridge. Remainder can be stored in freezer until required.]

3.2 Sample Preparation

3.2.1 Gently vortex the EDTA or citrate blood sample. Take 100 mL and add to a 15ml centrifuge tube. Add 10ml pre-diluted BD lysing solution. Put sample on automated mixer for 10 minutes. Then centrifuge 700g for 5 minutes

3.2.2 Decant supernatant and add 10mls of PBS/BSA Solution. Vortex gently to resuspend pellet. Then centrifuge once again at 700g for 5 minutes.

3.2.3 Discard supernatant. Add 20 uL Octagam (blocks Fc receptor) and 200 uL of 10% mouse serum (prevents non-specific binding). Gently flush in to and out of pipette tip to re-suspend pellet. Leave to stand at room temperature for 20 minutes.

3.2.4 Add 10uL each of CD146, KDR, CD34, and CD45 fluorochrome labelled antibodies. Flush into and out of pipette tip to ensure thoroughly mixed and then gently vortex. Stand in the dark at room temperature for 20 minutes.

3.2.5 Add 10ml PBS/BSA and centrifuge once again at 700g for 5 minutes. Discard Supernatant.

3.2.6 Add 200 uL 2% paraformaldehyde solution and flush into and out of pipette tip, followed by gentle vortex to ensure thoroughly mixed. Store sample in dark at 4C until ready to be processed (note sample must be processed within three days)

3.2.7 Carefully disconnect the waste container and empty contents down sink with plenty of water. Add approximately 20ml concentrated household bleach and reconnect container.

3.2.8 When the indicator light changes from “Not ready” to “Standby”, pressurise the unit (takes about 20-30 sec) by moving the “Vent Valve” switch to the down position

3.2.9 Any excess air trapped in the sheath filter can, if necessary, be cleared by venting through the bleed tube (the dead-ended rubber tube with a cap).

3.2.10 Ensure that a Falcon tube approximately 1/3 full of distilled water is positioned over the needle sheath and that the swing arm is positioned under this tube. Turn the selection dial to the DRAIN position. A squirt of FACS Flow solution will travel along the rubber tubing and bubbles will appear in the Falcon tube. Continue this process for 30 secs.

3.2.11 Now turn the selector dial to the FILL position for another 30 secs. If further bubbles are seen in the Falcon tube, repeat steps 6 and 7.

3.2.12 Finally turn the selector dial to the RUN position and run distilled water for a further 5 minutes. This process ensures the machine is clean prior to running samples and helps minimise blockages.

3.2.13 The machine is now ready to run samples.

3.3 Running Samples.

Note. It is best to learn this from an experienced operator as the intricacies of the Cell Quest software are complex.

3.3.1 Click Apple icon in top left of screen and open CellQuest Pro.

3.3.2 Close current document, then open the EPC enumeration document located within the Experimental Protocols folder

3.3.3 Click 'Connect to Cytometer', located under the 'Acquisition' menu.

3.3.4 Under the 'Cytometer' menu, click 'Cytometer Settings'. Change settings to EPC Settings, also located within the Exp Protocols folder. Make sure to click 'set' prior to clicking 'done'.

3.3.5 Click the 'Acquisition menu' once more and click 'show browser'. Enter the file name and number for data to be stored under and choose an appropriate location to save data to.

3.3.6 Each sample now needs dilution with 600mL PBS/BSA to make a sample volume of approximately 1.1ml. This should be mixed thoroughly and transferred to a BD Falcon tube. Open swing arm at bottom right of the cytometer and replace the Falcon tube with the sample to be run. Replace the swing arm under the Falcon tube.

3.3.7 Click Acquire on the browser menu. The sample will now run for 10mins, 12 seconds. Cell events will be displayed on the screen throughout the process.

3.4 Shut-down procedure

3.4.1 Fill two Falcon tubes (already labelled for use and kept on top of cytometer) half full with FACS Rinse and distilled water respectively.

3.4.2 Install FACS Rinse tube (essentially a mixture of detergent and water) over the aspiration sheath. Leave the support arm out at 90 degrees for approximately 1 minute. This cleans the outer

portion of the aspiration sheath. The fluid will be rapidly aspirated, so ensure that the tube doesn't empty completely.

3.4.3 Now replace the side arm under the Falcon tube and aloe to run for approximately 5 minutes. This cleans the inner portion of the aspiration sheath and the FACS machine itself.

3.4.4 This step is only to be performed once per week. Repeat steps 2 & 3 with a Falcon tube containing 1/3 FACS Clean solution

3.4.5 Repeat steps 2 and 3 with distilled water. Once step 3 is complete, Leave the sheath in the BD Falcon tube containing distilled water and turn the selection dial to STANDBY and depressurize the machine by moving the "Vent Valve" switch to the up position. The machine will hiss as it depressurizes.

3.4.6 Leave the machine on for a further 10 minutes to allow the laser lamp to cool. Turning the machine off prematurely will result in the lamp cracking.

3.4.7 Finally power down the FACScan and Apple Mac.

Note: * If subsequent use of the FACScan is anticipated within the next hour or so, perform all the shut-down steps but leave the machine on in STANDBY mode at step 6.

3.5 Monthly maintenance

This maintenance schedule should be performed on a monthly basis to ensure optimal running of the FACScan machine.

3.5.1 Perform daily shut-down procedure as above first, to ensure system is relativel clean already.

3.5.2 Depressurize sheath container (FACS Flow container), carefully disconnect and replace container with the dedicated sheath container marked FACS Rinse (kept on bench next to machine). This container should be approximately half full with FACS Rinse.

3.5.3 Disconnect the white tube from the sheath filter and replace this with the white tube from the sheath container. This step bypasses the filter ensuring that FACS Rinse does not enter this part of the machine.

3.5.4 Install a Falcon tube containing FACS Rinse (about 3ml) and pressurize the system.

3.5.5 Turn selector to DRAIN (30 seconds) and then FILL (30 seconds). Repeat this step twice.

3.5.6 Turn the selector dial to run and leave for 20 minutes.

3.5.7 Turn selector to Standby and depressurise the machine (vent valve up position). Repeat steps 4 to 6, **this time with the sheath container for FACS Clean (marked, again should be half full) and a Falcon tube also containing FACS Clean.**

3.5.8 Turn to standby and depressurise the system once more.

3.5.9 Remove the Falcon tube and replace sheath container with FACSFlow container. Make sure that the probe is thoroughly rinsed with distilled water prior to reinserting!

3.5.10 Reconnect white filter tubes to their original positions and install a Falcon tube of distilled water over the aspiration sheath.

3.5.11 Repressurise the system (vent valve down) and turn selector dial to DRAIN (30 secs), then FILL (30 secs) twice as before.

3.5.12 Turn selector dial to RUN and leave for 10 minutes.

3.5.13 Finally, de-pressurize machine (vent valve up) and select STANDBY mode.

3.5.14 Allow laser lamp to cool for 10 minutes, then power down machine.

5.1.4 Pulse wave analysis (PWA) and Pulse wave velocity

Written by Sern Lim 2008

Updated by Mehmood Butt 2010

Introduction

Left ventricular (LV) contraction generates a forward travelling pressure wave. Some of this pressure wave will be reflected at sites of impedance mismatch (eg: bifurcations and sites of constriction). Increased arterial stiffness results in earlier return of the reflected pressure wave in systole rather than diastole. The reflected pressure wave will increase systolic blood pressure, termed pressure augmentation and may be expressed as the augmentation index. Increased arterial tone, such as that associated with endothelial dysfunction also increases the reflected wave and the augmentation index. The analysis of the arterial pressure wave, termed pulse wave analysis (PWA) may therefore provide information on the functional properties of the arterial system. The administration of salbutamol and GTN also allows assessment of endothelial-dependent and independent function as NO-mediated vasodilatation reduces arterial wave reflection. The relative change in augmentation index to salbutamol and GTN provides a measure of systemic endothelial function.

<u>Equipment</u>

You will need:

- Patient (!)

- SphygmoCor with pressure transducer (Millar)
- Laptop computer with SphygmoCor software (for around 45 minutes)
- Power cables and USB connection cables
- ECG monitoring dots
- Omron BP recorder
- Salbutamol inhalers and GTN spray

Preparation

a. Allow the subject to rest in the room for about 10 minutes before scanning. The test must be done in a supine position.
b. While the patient is resting, you should connect the power cables to the SphygmoCor machine (at the back) and the laptop computer. The computer should then be connected to the SphygmoCor machine (use the USB connection at the back). The ECG cables should be connected to the SphygmoCor machine (in front where it says 'ECG').
c. Switch on the SphygmoCor machine first. The lights in front will blink. When the lights stop blinking and green light stays on 'ready', turn on the computer. This sequence will allow the software in the computer to recognise the SphygmoCor.
d. The SphymoCor icon should be on the computer desktop. Double-click to activate the software. A warning will come on the screen, just click 'allow'.
e. You will see PWA and PWV on the top left of the screen. Click on PWA for pulse wave analysis.
f. Click 'create new' on the top right of the screen. You will need to enter the patient details in the relevant boxes (eg: date of birth) and click 'update'.
g. Click on PWA on the top left for pulse wave analysis and then 'study' on the top left of the screen. Choose radial or carotid by ticking the box. Measure the patient's brachial blood pressure (Omron). Take 3 measurements and use the average of the last two readings.

h. Enter BP measurements. Height and weight if you have the data.

i. Place the ECG leads on the patient and click ‘Capture data’ on the top right of the screen. You are now ready to go!

In the drawer of the tonometer machine, you will find the Millar tonometer with a plastic cap to protect the high fidelity tip (this cap is important, DO NOT lose it!). The tonometry should be connected already. DO NOT disconnect the tonometer. Remove the plastic cap and it is ready for use.

Feel for the radial or carotid pulse (depending on which you want to measure) and place the tonometer over the pulsation. You will see a pressure trace on screen. The screen will automatically adjust the scale to accommodate the pressure trace. You need to make sure the trace is consistent for at least 12 seconds. To obtain the measurement, press the spacebar on the laptop. The machine will not take the last 2 seconds of recording to give you time to let go and press the spacebar.

The computer screen will then change to the data screen with the ensemble-averaged pressure trace (radial or carotid depending on the one you have chosen) shown and the derived central arterial pressure trace. First thing to do: check the quality control box on the top left of the screen. If this number is red, then the data is of insufficient quality and the measurement will need to be repeated.

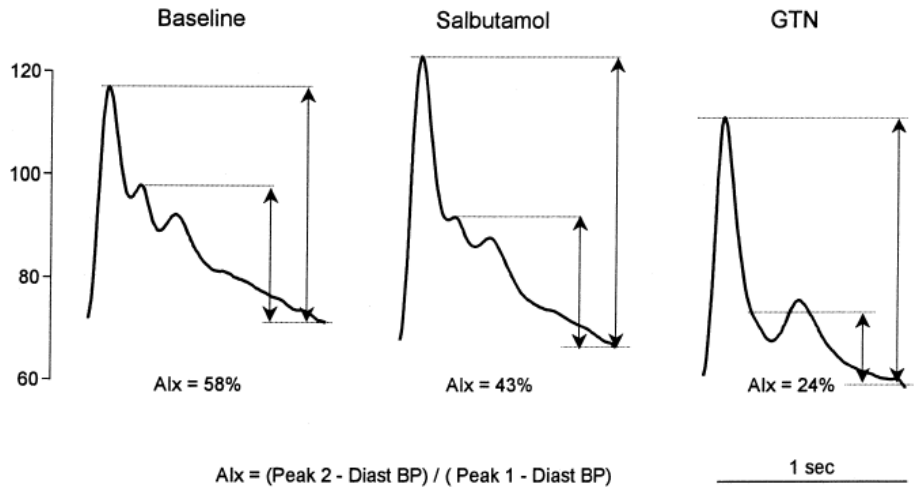
The data will be available at the bottom of the screen. At the top right of the screen, click ‘Export’ to save the data in your folder. You can store the data as a text file or jpeg (the screen shot). I would suggest you store both. If you are not doing endothelial function assessment with the SphygmoCor, then your study is done!

If you want to assess endothelial function, you will need to assess endothelium-dependent and independent vasodilatation. Endothelium-*dependent* vasodilation is assessed by giving 2 puffs of salbutamol (inhaler). Blood pressure (the other arm) and PWA measurement should be repeated at 5, 10 and 15 and 20 minutes to look for maximal decrease in augmentation index.

This is then followed by 2 puffs of sublingual GTN (warn the patient they may get a mild headache / feel dizzy) to assess endothelium-*independent* vasodilatation [Figure]. Similarly, BP and PWA measurement should be repeated at 5, 10, 15 and 20 minutes.

Make sure you label the files (eg: salbutamol and GTN) when you are storing the data.

Figure 4-2



Potential problems

1. The high fidelity tonometer is very sensitive to pressure (that is what it is meant to do!). You will need practise to achieve a consistent level of pressure in order to obtain reproducible readings. It is generally more difficult for carotid artery compared to the radial artery.
2. To avoid any problems with the computer, it should NOT be used for any other reasons (eg: surfing the internet). The computer and the tonometer (plus ALL the cables and ECG leads) should ALWAYS be kept together.
3. Every operator should create a folder for him/herself and store ALL the data in that folder. This should avoid confusion and the relevant data may be easily retrieved.

5.1.5 Echocardiography

Two-dimensional, Three-Dimensional and Myocardial Contrast Echocardiography

Written by Mehmood Butt & Girish Dwivedi, 2008

Introduction

Two-dimensional echocardiography (2DE) has been used for many years for gaining structural and functional information. However, it has its own limitation especially with regards to volumetric analsyis. Lately real time three-dimensional echocardiography (RT3DE) has been introduced which have improved the diagnostic yield in subjects with asymmetric cardiac geometry.

Myocardial contrast echocardiography (MCE) is destruction-replenishment imaging. The failure of echocardiography to give diagnostically useful information in a significant proportion of patients has led to the development of specific contrast agents (microbubbles) to enhance imaging. The significant acoustic difference created between microbubbles and blood allows for their easy detection using specific ultrasound imaging techniques. The current generation of ultrasound contrast agents comprises ‘microbubbles’ of a high-molecular-weight gas encapsulated in a ‘shell’ of phospholipid or protein. Microbubbles are hemodynamically inert and remain entirely within the intravascular space. The development of new ultrasound contrast agents and imaging techniques have made possible the assessment of myocardial perfusion, permitting simultaneous assessment of global and regional myocardial structure, function and perfusion.

Equipment

You will need:

- Cooperative subject (Always ensure 24hrs of caffeine free interval prior to the test).
- Echocardiography machine (IE33) free (for around 60-90 minutes at least)
- Echo jelly (the tubs are refillable)
- ECG monitoring electrodes
- Philips computer with excelera software
- Timer (can use clock on echo machine if not available)
- Sphygmomanometer
- Contrast agent (Sonovue 3-4 vials per patient)
- Dipyridamole (Persantin 0.56mg/kg body weight)
- Aminophylline as an antidote (50-100 mg)
- Resuscitation trolley fully equipped with O2 source (tubing attached)

Method

j. Turn on echo machine (button located on the main body of the machine behind the main screen)

k. Turn on the Philips computer with excelera

l. Systems should connect automatically

m. Allow the subject to rest in the room for about 5 minutes before scanning

n. Place the ECG leads on the patient

o. Press 'patient ID' on the echo machine console (towards the left) and key in your patient's information.

p. Place an IV cannula and take necessary blood tests (pre MCE flow cytometry EDTA sample along with a yellow, and a blue top tube).

On the echo machine

2DE

i. Select the probe (S5)
ii. Set the background gains at a level so that minimal tissue signals are seen
iii. Standard para-sternal long axis, para-sternal short axis, apical 4 chamber, apical 3 chamber, apical 2 chamber, epigastric and supra-sternal images are visualized and stored.

RT3DE

a. Select the probe (X3)
b. Select Grey scale 8 and chrome 3 on the contrast option.
c. Standard apical 4 chamber, apical 3 chamber and apical 2 chamber images are visualized and stored.

MCE

1. Select the probe (S5)
2. Select the protocol 'GD' perfusion
3. Select physio on left screen and press 'trigger imaging' on button
4. Optimise delay to the end of 'T' wave in your patient
5. Press contrast option on the right hand screen
6. Select Grey scale 8 and chrome 3 on the contrast option.

Prepare the infusion pump

a. Prepare 2-3 (3 for obese and less for thin subjects) vials of Sonovue (preparation method in the Sonovue box) and withdraw in a syringe provided by Bracco
b. Connect this syringe to the connecting tube provided and place horizontally in the pump.
c. Close the cover of the pump and activate the pump (it will gently rotate for around 90 seconds)
d. Press purge to get rid of the air in the connecting tube
e. Connect the free end of the tube to three way tap attached to IV cannula.

Imaging

a. Set the background gains at a level so that minimal tissue signals are seen
b. Set the focus at the level of mitral valve
c. Start Sonovue (already put in the infusion pump) infusion at the 70-90 ml/hour
d. It is worth optimizing the machine settings again to obtain best possible myocardial opacification with minimal attenuation.
e. MCE is usually performed in the 3 standard apical (4, 2 and 3 chamber) views. Obtain at least two set of images for each view during rest imaging. Infusion of contrast agent can be stopped after rest imaging.
f. Observe the replenishment for at least 15 cardiac cycles following high mechanical index (1) microbubble destruction. During this ECH should be triggered, press acquire (on main button panel on the echo machine) and then flash (on right hand ECHO display LCD screen) to achieve this.
g. After obtaining rest pictures, checking the blood pressure and auscultating lungs (to rule bronchospasm), administer dipyridamole (dose calculated as per body weight) through a three

way cannula over 4 mins. It is important to look at the ECG rhythm and measure the BP at least twice during the infusion.

h. Restart contrast infusion 90 seconds after finishing the dipyridamole infusion.

i. Repeat the MCE as described during the rest imaging to obtain stress images.

Drug calculation and administration

a. Dipyridamole is available in 2 ML vials (10mg/2ML). For stress following formula is used:

0.56mg x body weight in kilograms

b. Calculate the dose required and draw in a 10-20 cc syringe using green needle.

c. Draw 10ML normal saline in another 10ML syringe separately as ‘flush’.

d. Take blood pressure reading before injecting dipyridamole.

e. Once images have been taken at rest, dipyridamole is given over a period of 4 minutes in a large peripheral cannula.

f. Flush the cannula with10ML of saline.

g. During the injection continue to monitor the ECG of monitor and take blood pressure (from other arm) every 2 minutes or more frequently if necessary.

h. If any alarming symptoms/signs (such as anaphylaxis, significant drop in blood pressure, induction of arrhythmias, any neurological signs, wheeze and/or cough) stop infusion.

i. Management of possible complications is given in another section.

Last but not least

a. Clean jelly off, take the ECG leads off the patient and thank them!

b. Clear up the room: wipe jelly from the probe; get rid of sharps, tidy up the examination couch, and turn off equipment if not going to be used shortly. Bear in mind that the equipment is expensive and used by a lot of people...
c. Always check contraindications to the drug use.

Potential problems

This technique takes practise – you should ideally be competent in normal 2D echocardiography before attempting to perform MCE. It is always helpful to put the cannula in a large vein for contrast infusion. Be liberal with the use of aminophylline if you suspect any problems following dipyridamole infusion. Try to finish obtaining MCE images before giving dipyridamole. However, as the patient safety comes first, if the situation demands, administer IV aminophylline (slowly) even if you have to abandon the test. Keep plasma expanders (or normal saline) and IV atropine handy, as rarely subjects become hypotensive and bradycardic following dipyridamole infusion.

Aminophylline indication and administration

Should severe chest pain or brochospasm occur, parenteral 75 to 100 mg aminophylline may be administered by slow intravenous injection; repeated if necessary. If aminophylline does not relieve chest pain symptoms within a few minutes, sublingual nitroglycerin may be administered. If chest pain continues despite use of aminophylline and nitroglycerin, the possibility of myocardial infarction should be considered.

Management of specific complications

Anaphylaxis:

Stop infusion and imaging immediately.

Secure Airway and start 100% Oxygen.

Get good peripheral line and start IV normal saline.

Treat with subcutaneous adrenaline, IV hydrocortisone 200mg and 10mg Piriton IV.

Hypotension:

Stop infusion and imaging immediately.

Secure Airway and start 100% Oxygen.

Get good peripheral line and start IV normal saline.

Aminophylline can be given also.

Bronchospasm:

Stop infusion and imaging immediately.

Secure Airway and start 100% Oxygen.

Get good peripheral line and start IV normal saline.

Nebulize with 5mg salbutamol. And give 200mg of IV hydrocortisone.

Aminophylline can be given also.

Cardiac arrest or Peri-arrest situation:

To be dealt with according to current resuscitation council guidelines.

5.2 Publications arising from this Thesis

1. Butt M, Dwivedi G, Shantsila A, Khair OA, Lip GY. Left ventricular systolic and diastolic function in obstructive sleep apnea: impact of continuous positive airway pressure therapy. Circ Heart Fail. 2012 ;5:226-233.

2. Shantsila A, Dwivedi G, Shantsila E, Butt M, Beevers DG, Lip GY. A comprehensive assessment of cardiac structure and function in patients with treated malignant phase hypertension: The West Birmingham Malignant Hypertension project. Int J Cardiol. 2011 Dec 20. [Epub ahead of print]

3. **Butt M**, Khair OA, Dwivedi G, Shantsila A, Shantsila E, Lip GYH. Myocardial perfusion by myocardial contrast echocardiography and endothelial dysfunction in Obstructive Sleep Aponea. Hypertension. 2011;58:417-424.

4. Shantsila A, Dwivedi G, Shantsila E, **Butt M**, Beevers DG, Lip GY. Persistent macrovascular and microvascular dysfunction in patients with malignant hypertension. Hypertension 2011;57:490-496

5. **Butt M**, G. Dwivedi, OA. Khair, R. Haynes, GYH. Lip. Impaired Myocardial Blood Flow Reserve in Obstructive Sleep Apnea as Assessed by Myocardial Contrast Echocardiography: Effects of Continuous Positive Airway Pressure Therapy. Arterioscler Thromb Vasc Biol 2010;30:e276

6. **Butt M**, Dwivedi G, Blann A, Khair O, Lip GY. Endothelial Dysfunction: Methods of Assessment & Implications for Cardiovascular Diseases. Curr Pharm Des. 2010;16:3442-3454

7. **Butt M**, Dwivedi G, Khair O, Lip GY. Obstructive sleep apnea and cardiovascular disease. Int J Cardiol. 2010;139:7-16

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5.3 Presentations (Oral and Posters)

1. **M. Butt**, G. Dwivedi, O. Khair, A. Shantsila, A. Blann, GYH. Lip. Left ventricular systolic and diastolic function in obstructive sleep aponea: impact of continuous positive airway pressure therapy. *European Society of Cardiology Congress, Paris, France 2011.*

2. G. Dwivedi, **M. Butt**, A. Blann, GYH. Lip. Impaired myocardial perfusion is an independent predictor of chronic diastolic dysfunction in obstructive sleep apnoea. *European Society of Cardiology Congress, Paris, France 2011.*

3. A. Shantsila, G. Dwivedi, **M. Butt**, A. Blann, DG. Beevers, RP. Steeds, GYH. Lip. Is vascular ventricular coupling in malignant phase hypertension abnormal? An echocardiographic assessment of arterial and systolic elastance in patients with malignant hypertension. *European Society of Cardiology Congress, Paris, France 2011.*

4. **M. Butt**, G. Dwivedi, OA. Khair, GYH. Lip. Impaired Myocardial Blood Flow Reserve in Obstructive Sleep Apnea as assessed by Myocardial Contrast Echocardiography: Effects of Continuous Positive Airway Pressure Therapy. *European Society of Cardiology Congress, Stockholm, Sweden Aug 2010.*

5. **M. Butt**, G. Dwivedi, OA. Khair, GYH. Lip. A comparison of Left Atrial Volumes evaluated by 3D &2D Echocardiography in Obstructive Sleep Apnea with Hypertensive and Healthy Controls. *European Society of Cardiology Congress, Stockholm, Sweden Aug 2010.*

6. **M. Butt**, G. Dwivedi, OA. Khair, R. Haynes, GYH. Lip. Impaired Myocardial Blood Flow Reserve in Obstructive Sleep Apnea as Assessed by Myocardial Contrast Echocardiography: Effects of Continuous Positive Airway Pressure Therapy. *Arteriosclerosis, Thrombosis and Vascular Biology Conference, San Francisco, April 2010.*

7. **M. Butt**, G. Dwivedi, OA. Khair, R. Haynes, GYH. Lip. A comparison of Left Atrial Volumes evaluated by 3D & 2D Echocardiography in Obstructive Sleep Apnoea with Hypertensive and Healthy controls. *Heart Failure 2010 Conference (European Society of Cardiology) Berlin, May 2010.*

8. **M. Butt**, G. Dwivedi, OA. Khair, R. Haynes, GYH. Lip. Improvement in left Atrial Volume as assessed by 3D and 2D Echocardiography in Obstructive Sleep Apnoea: Effects of Continuous Positive Airway Pressure Therapy. *Heart Failure 2010 Conference (European Society of Cardiology) Berlin, May 2010.*

9. **M. Butt**, G. Dwivedi, OA. Khair, R. Haynes, GYH. Lip. Impaired Myocardial blood flow reserve in Obstructive Sleep Apnoea as assessed by Myocardial Contrast Echocardiography: effects of Continuous Positive Airway Pressure Therapy. *Heart Failure 2010 Conference (European Society of Cardiology) Berlin, May 2010.*

10. **M. Butt**, G. Dwivedi, OA. Khair, R. Haynes, GYH. Lip. Is there any improvement in 3D and 2D Echocardiography determined Left Atrial Volume following Continuous Positive Airway Pressure treatment in patients with Obstructive Sleep Aponea? *British Cardiovascular Society, Manchester, Jun 2010.*

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