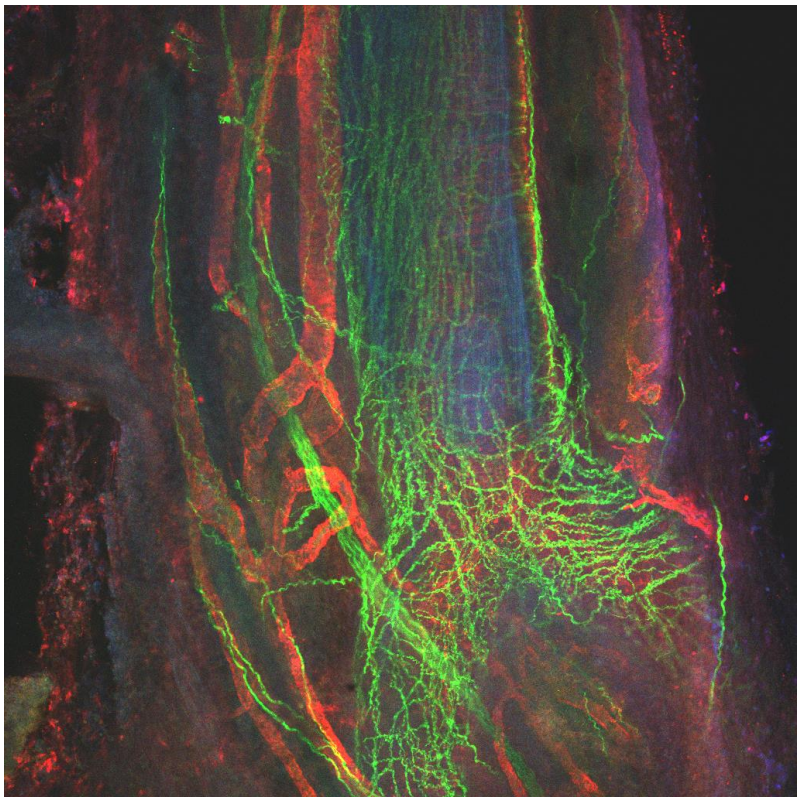
An integrated understanding of the effects of sleep disordered breathing on the physiology of the developing child.



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Doctor of Philosophy

2016

Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text. I give consent for the digital version of the thesis to be made available on the web, via the University's digital research repository, the library catalogue and also through web search engines. The copyright to Chapter 2 and 3 are held by the respected journal.

Anna Kontos

Acknowledgements

I would like to express my sincere gratitude to all the people that have assisted me through this journey. In particular I would like to thank:

- The University of Adelaide, who gave me this opportunity.
- The children and their families who have supported these studies. Their commitment and involvement has allowed me the opportunity to better understand this disorder and I hope that future generations will benefit from the finding of this thesis.
- My primary supervisor, Professor John Declan Kennedy, for his support, encouragement, and commitment to my work and his ever positive outlook, enthusiasm and wisdom. I was very fortunate to a have a primary supervisor who has a great passion and commitment to the research and to his patients. He made the process easy and enjoyable. It has been a great joy and an honor to work with him and his Associates from the University of Adelaide's Paediatrics Discipline, including Professor Jennifer Couper, Professor Antonio Ferrante and A/Professor Simon Barry.
- My co-supervisor, Professor Kurt Lushington, for his encouragement, and continued input and support for all aspects of the work, including writing and statistical analysis. In particular I would like to thank Prof Lushington for including me in many endeavors that have enhanced my abilities as a researcher, broadened my scope of interest and increased my network of friends and associates, which include the very many great researchers at the University of South Australia.
- My co-supervisor Dr James Martin, for his positive praise, support and input to this thesis. Also I'd like to especially thank Dr Martin in his capacity as Head of Department, Respiratory Medicine, Women's and Children's Hospital. Without his support and that of his excellent staff, Beverley Brodie, Paula Mitchell, Andrew Tai and Greg Smith I could not have accomplished the work covered in this thesis.
- Associate Professor Mathias Baumert and Dr Scott Willoughby for their input and continued support and encouragement. Thank you for contributing your knowledge and

for the long discussions about the different aspects of research. A special thank you to Scott, who would deliver the Multiplate system to the hospital, on a Friday afternoon.

- Yvonne Pamula – Manager of the Sleep Disorders Unit, WCH, for her organisation of the data and her encyclopedic, in-depth knowledge regarding sleep and sleep related measurements.
- Dr Cameron van den Heuval – Previous Senior Researcher – for data contributed to this thesis.
- Members of the Women's and Children's Sleep Laboratory past and present, Charmaine O'Reilly, Chrisine Cliff, Luke Chesson, Scott Coussens, and Alex Chatburn.
- The Sleep Research Team, Tyler Grummett, Kostas Kontos, Paulis Pignata and Tony Pignata.
- Professor David Parson and the CF Gene Research Team, WCH, Trish, Martin, Nigel, Bernadette, Corinne, Harsh, Ally and especially Chanetelle McIntyre.
- Jessica Carlson-Jones and Benlee Yap.
- The Robinson Research Institute, staff and colleagues.
- Dr Quenten Schwarz and Xiangjun Xu (Jun).
- Ryan Green, University of South Australia.
- Professor Antonio Ferrante, Immunology – University of Adelaide.
- Dr Elke Sokoya – Flinders University – Cerebral Vascular Laboratory.
- David Fletcher – Biomedical Technology – WCH.

Finally, I would like to dedicate this thesis to my two beautiful and supportive children, Anthea and Harry and my wonderful parents Anthoula and Konstantinos Kontos who have gone above and beyond to make sure I complete my studies.

I would also like to dedicate and acknowledge the work of the late Mal Bakewell, biomedical engineer and good friend who passed away during my candidature.

The studies for this thesis were supported by the Australian Postgraduate Award, University of Adelaide Children's Research Council, Channel 7, and WCH Foundation.

Summary

The role of Sleep Disordered Breathing (SDB) in adult hypertension and cardiovascular morbidity is well established and increased blood pressure and cardiac remodeling has been demonstrated in children with severe SDB. There is less evidence suggesting that milder forms of SDB are associated with cardiovascular anomalies. Therefore this thesis contains a series of studies which compares the underlying physiology, particularly vascular function in children with mild SDB with aged matched healthy non-snoring children. Children with SDB were recruited at the Women's and Children's Hospital, through the Ear, Nose and Throat surgical waitlist, prior to treatment to resolve their SDB. All children underwent an overnight sleep study to assess their SDB severity for each study.

Chapter 1 is a literature review which provides a brief summary of the autonomic nervous system and the physiology of sleep. A synopsis of the disorder is also presented including information on diagnosis and treatment of the disorder. An in depth assessment of the literature pertaining specifically to studies measuring cardiovascular, autonomic and inflammatory response in children with SDB is summarized and the physiological pathways that lead to hypertension are explained.

Chapter 2 assesses Flow Mediated Dilatation, a validated measure of endothelial function in healthy children ranging from 6 – 16 years. At the time of publication there were no studies using FMD in children as young as 6 years. This study shows a positive relationship between the time to reach maximal dilation after ischemia in the brachial artery and both age and body habitus. Younger children reached maximal dilation by

35s while older children reached maximal dilation by 60s+. This paper is important as it helped to determine the age range and analysis for the following study.

Chapter 3 outlines the results of a study in young children with primary snoring aged 5-9 years using FMD. We showed that both resting and hyperaemic velocity time integral (VTi; area under the curve of velocity over ejection time) was significantly higher in children referred for evaluation of SDB (n=23) compared with healthy matched controls (n=11). Other groups report similar results in blood flow velocity measured in middle cerebral arteries using transcranial Doppler in children with primary snoring. We also found that the brachial artery took approximately 20 seconds longer to fully dilate after induced hyperaemia in the children with SDB compared with controls. Of note is that both children with SDB and controls in our FMD study demonstrated similar rates of dilatation in the initial 30s, suggesting that endothelial function is preserved, but after 30s the groups diverged and children with SDB took longer to reach maximum dilatation. The time delay in maximum dilatation suggests augmented cell-to-cell connectivity as the dilatation process moves from the endothelial cells through smooth muscle, ending in the adventitial layer.

Having defined vascular changes in medium sized vessels, in Chapter 3, Chapter 4 investigates the vascular effects of SDB on the heart and major vessels using cardiac MRI in children aged 5-14 years including primary snorers/mild SDB and non-snoring controls. This study found a significant increase in peak systolic blood flow velocity in the ascending aorta in children with SDB (n = 12) compared to controls (n = 7). The increase in blood flow velocity was similar to increases reported by other groups (20-30%) in cerebral blood flow and by our own group (Chapter 3).

A blood sample was also analysed from the children in this study. We used Flow Cytometry to measure cytokine expression of markers of inflammation - Tumor Necrosis Factor alpha (TNFα) and interferon gamma (IFNγ) which are known indicators of cardiovascular disease. We found that there was a significantly higher level of $CD8^+$ cells expressing both TNFα and IFNγ. Consistent with the paradigm that cardiovascular changes are coupled with an increased inflammatory state, we also found a strong positive association between these immune-markers and peak blood flow velocity in the ascending aorta and $CD8^+$ cells expressing IFNγ and TNFα. Our findings support the interrelationship between mild SDB, abnormal vascular function and increased inflammation. While increased inflammatory pathways have been reported in children with severe OSA, this is the first time it has been shown in children diagnosed as primary snorers/mild SDB.

Chapter 5 details the relationship between vascular changes observed in Chapter 3 (increased blood flow velocity and time to maximal dilatation in the brachial artery) in relation to autonomic function. We measured sympathetic overactivity using digital pupillometry, and found that it was closely associated with the haemodynamic changes we observed on FMD evaluation of the brachial artery of children with mild SDB. We further undertook the novel step of examining the effect of sympathetic overactivity on the arterial wall by studying the dorsal lingual artery of children undergoing adenotonsillectomy, the accepted treatment for SDB. We found that sympathetic nerve fibre density of the vessel (measured by immunohistochemistry) strongly correlated with the degree of sympathetic overactivity on pupillometric testing and the functional measure from the FMD. To our knowledge this is the first time that functional vascular changes have been correlated to vascular histology in humans.

We also explored the association between the sympathetic nerve fibre density brachial artery blood flow velocity and FMD parameters, pupillometry and platelet aggregation (using whole blood samples). Platelet aggregation is a measure of endothelial integrity. Our results show that the children with increased brachial artery blood flow velocity also exhibited increased platelet aggregation in response to the collagen antigen, suggesting endothelial damage. Sympathetic nerve fibre density was also positively associated with increased platelet aggregation, in particular with the aggregation velocity. In addition, there was strong association between the pupillary light reflex variables and the platelet aggregation.

Chapter 6 is an overall discussion that amalgamates the findings of each chapter and provides recommendation for future work and consideration.

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List of Abbreviations

BMI	Body Mass Index
OSA	Obstructive Sleep Apnoea
CPAP	Continuous Pulmonary Airway Pressure
CVD	Cardiovascular Disease
SDB	Sleep Disordered Breathing
CSF	Cerebral Spinal Fluid
EEG	Electroencephalogram
ANS	Autonomic Nervous System
REM	Rapid Eye Movement
NREM	Non-rapid Eye Movement
AASM	American Association of Sleep Medicine
SWS	Slow Wave Sleep
CAP	Cyclic Alternating Pattern
ECG	Electrocardiogram
CNS	Central Nervous System
SNS	Sympathetic Nervous System
PNS	Parasympathetic Nervous System
CO2	Carbon Dioxide
O2	Oxygen
RVLM	Rostral Ventral Lateral Medulla
NA	Noradrenaline
BRS	Baroreflex Sensitivity
HRV	Heart Rate Variability

Pcrit	Critical Pressure
BAT	Brown Adipose Tissue
BRAC	Basic Rest Activity Cycle
AHI	Apnoea Hypopnea Index
OAHI	Obstructive Apnoea Hypopnea Index
SpO_2	Oxygen desaturation
SDSC	Sleep Disturbance Scale for Children
Ang II	Angiotensin II
FMD	Flow Mediated Dilatation
VTi	Velocity Time Integral
PSV	Peak Systolic Velocity
HR	Heart Rate
RAS	Renin Angiotensin System
ED1	Endothelin 1
PLR	Pupil Light Reflex
IFNγ	Interferon Gamma
TNFα	Tumor Necrosis Factor Alpha
ECM	Extracellular Matrix Protein
SMC	Smooth Muscle Cells
IH	Intermittent Hypoxia
PTT	Pulse Transit Time
SNFD	Sympathetic Nerve Fibre Density
NPi	Neuronal Pupillary Index

Publications, Posters and Presentations Arising from this Work

Publications

Kontos, A., et al., Flow-mediated dilatation, using time course data, shows maturation of the brachial artery from young children to mid-adolescents. Clin Exp Pharmacol Physiol, 2015. **42** (3): p. 240-5.

Kontos, A., et al., Delayed brachial artery dilation response and increased resting blood flow velocity in young children with mild sleep-disordered breathing. Sleep Med, 2015. **16** (12): p. 1451-6.

Posters

Kontos A., Schwarz Q, Green R, Wabnitz D, Martin AJ, Xu X, Lushington K, Sokoya E, Baumert M, Willoughby S, Ferrante A, La Forgia M, Kennedy JD. "Is sleep disordered breathing in children a precursor of cardiovascular disease?" ESRA 2016.

Presentations

Kontos A, Schwarz Q, Green R, Wabnitz D, Martin AJ, Xu X, Lushington K, Sokoya E, Baumert M, Willoughby S, Ferrante A, La Forgia M, Kennedy JD. ESRA 2016. The relationship between vascular resistance and sympathetic nerve fibre density in children with sleep disordered breathing. Presented at ASA 2016.

Kontos A, Martin AJ, Schwarz Q, Green R, Wabnitz D, Xu X, Lushington K, Sokoya E, Baumert M, Willoughby S, and Kennedy JD. Increased resting brachial artery blood flow velocity associated with increased sympathetic nerve fiber density on the dorsal lingual artery (Tonsil) in children with sleep disordered breathing. Presented at the IPSA, 2016

Kontos A, Willoughby S, van den Heuvel C, Kennedy JD, Martin AJ, , Hodge G, Worthley M, Chin A, Nelson A, Teo K, Baumert M, Pamula Y, Lushington K. Increased aortic blood flow velocity and inflammation in children with sleep disordered breathing. Presented at the IPSA, 2016

Kontos A, Pamula Y, Martin AJ, Gent R, Lushington K, Baumert M, Willoughby S, Couper J and Kennedy JD. Delayed brachial artery dilation response and increased resting blood flow velocity in young children with mild sleep disordered breathing. ASA, 2015.

Chapter 1:

Literature Review

Introduction

1.1 The Importance of Sleep

1.1.1 What is Sleep?

Sleep is an essential part of human existence and, although a clear purpose of sleep is debated by many, the overwhelming consensus is that sleep acts as a restorative and conservation mechanism accompanied by a comparative loss of consciousness.

"Sleep is commonly looked upon as a periodic temporary cessation, or interruption of the waking state, which is the prevalent mode of existence for the healthy human adult."

Sleep and Wakefulness, Kleitman, 1963

Kleitman explains sleep as 'a state of being' that is required by all to maintain a healthy body and mind. Dement, however further clarifies that, '*sleep erects a perceptual wall between the conscious mind and the outside world,*' and that '*a defining feature of normal sleep is that it is immediately reversible*' (p.17) (1).

There are many theories of sleep. Collectively, these theories suggest that sleep is a multifunctional process and possibly the most complex biological mechanism. This is

because sleep requires great counter operative changes to occur for the process to be maintained uninterrupted over long periods of time. Only five theories will be briefly reviewed here, however a comprehensive evaluation of the major theories of the function of sleep can be sourced from the "Principles and Practice of Pediatric Sleep Medicine", Chapter 1, *The Function, Phylogeny and Ontogeny of Sleep*, by Stephen H. Sheldon.

1.1.1.1 Restorative Theory

The restoration theory was first proposed by Sherington in 1946, and explains the function of sleep as a state required to promote tissue growth and repair. This theory suggests that many somatic and cerebral deficits associated with the awake state are restored/repaired during sleep. This allows for the recuperation process to occur and for normal daytime function to continue. Evidence to support this theory includes: the rebound effect of increased slow wave sleep after sleep deprivation, the relationship between increased body mass with total sleep time duration; the release of growth hormones, and reduction of catabolic steroid release occurring in the first few hours of sleep. Lymphocyte proliferation and bone growth also increases during sleep and further supports the idea that sleep acts as a protecting and recuperative (repair) period.

1.1.1.2 Energy Conservation Theory

This theory simply explains sleep as a mechanism to conserve energy, as in mammals, total sleep time is inversely correlated with metabolic rate. It proposes that sleep imposes periods of 'compulsory' rest and reduces the activity cycle. However, themoregulation continues during sleep in large mammals and the metabolic drop is only approximately 8-10% suggesting that as a 'conserving mechanism' sleep is very inefficient.

1.1.1.3 Learning Theory

Recent debate in the sleep field suggests that sleep serves as an 'ongoing support for neuronal function in the brain', playing an integral role in learning and memory consolidation (2). The relationship between sleep and memory consolidation is well recognized however the exact role sleep plays is not well defined, in that the underlying aspects of sleep (e.g. mechanisms) that aid cognition and memory remain unknown.

1.1.1.4 Synaptic Efficiency

Many studies have looked at the effects of sleep and sleep deprivation/restriction on the structure and function of synapses (3). These studies which include, molecular, electrophysiological and structural approaches have shown that even a few hours of sleep loss per night can alter the size, number and composition of excitatory synapses and also changes their rate of transmission. In addition, studies show that sleep loss affects the rate of long-term potentiation which may underlie the cognitive and memory deficits reported in studies looking at the impact of sleep loss/deprivation. For more details readers are directed to a recent review by Cirelli, (3).

1.2.1.5 Metabolic Clearance

More recently, Nedergaard, propose that sleep acts as a state that promotes metabolic and neurotoxic clearing from the brain. She noted that the brain produces large amounts of neurotoxins and metabolites but has no lymphatic vessels for their clearance, as occurs elsewhere in the body. Cerebral spinal fluid (CSF) and interstitial fluid act as a sink for waste (neurotoxins and metabolites) that need to be transferred out of the brain. They demonstrated that CSF clearance occurs via the interaction between glial cells and arteries and for interstitial fluid via veins. Known as the 'glymphatic system' the process is dependent on astrocytic aquaporin-4 water channels which absorb analogous

to lymphatic vessels. These channels eventually drain into the cervical lymphatic system and the metabolites are excreted via the kidneys or broken down in the liver.

Using a fluorescent tracer injected into the subaracnoid CSF, researchers measured the glymphatic CSF influx in awake and sleeping rats (4). They noted a 60% increase in the cortical interstitial space in the sleep state of the rat and that there was a twofold faster clearance of tracer during sleep compared to awake state, which they suggest is evidence of metabolic/waste clearing during sleep. Furthermore they showed that the changes associated with the increased rate of cortical interstitial space/CSF fluid clearance were dependent on inhibition by the adrenergic signalling in the arousal mechanisms (*locus coeruleus* pathways). An adrenergic antagonists was microinjected into the cisterna magna to simulate what happens in sleep (reduced SNS activity). This induced a similar influx of CSF in the brain as was observed during sleep and their further investigation demonstrated that adrenergic signalling plays a pivotal role in modulating both the cortical neuronal activity and the volume of the interstitial space. They also discovered the phenomenon known as CSF pulsation which indicates that the brain receives a much greater blood supply and actually 'throbs' like a beating heart, which they suggest also plays a part in the clearance mechanism. This theory of sleep as a function of neuronal metabolite clearance fits with both the 'the learning theory', which suggests that sleep aids normal brain function, and development and also the 'restorative theory' proposed by Sherington.

As a whole these theories on the 'purpose of sleep' suggest that sleep is a critical determinate of health and wellbeing especially in childhood, where all the foundations of good health are set. Conversely, ongoing deviation from 'normal' sleep in childhood

is potentially accompanied with poor long-term health outcomes when you consider children spend approximately 50% of their childhood years asleep.

Sleep is easily recognized. Humans sleep in a recumbent position, their body becomes immobile, the eyelids close and deviate upwards and outwards and proprioception and other reflexes disengage (1). Sleep in other animals varies with some sleeping in the standing position, others with their eyes open while others hanging from their feet (1). The amount of time spent sleeping also varies, with the average human adult sleeping for 8 hour overnight, while other species such as the rats, considered polyphasic sleepers, only sleep for short periods of 1 – 2 hours. Newborn babies spend in excess of 16 h/24 h sleeping, but this decreases with age, with the time spent sleeping by the 6^{th} decade decreasing to an average of 6.5 hours.

A significant transition occurs in the underlying physiology during sleep with a systemic relaxation of muscle tone and a reduction in heart rate and blood pressure (5). Also, both respiratory rate and body temperature decrease, while melatonin secretion from the pineal gland increases (6). The rapid, low voltage, beta wave oscillations observed in electroencephalogram (EEG) while awake become slow, the eyes close and higher strength alpha waves are observed and then gradually replaced by lower-frequency theta wave (1). The mind wanders and at this point a transition from the awake state to the sleep state occurs.

1.1.2 Sleep Physiology

1.1.2.1 Sleep Architecture/ Staging

Sleep is first evident in the human fetus and premature infants from as early as the 25^{th}

- 32nd week of gestation (7-9). Sleep is an active and rhythmic neural process that involves crucial changes in the autonomic nervous system (ANS) regulation of cardiovascular tone and respiration (Figure 1.1). The ultradian rhythm in sleep was first characterized by Aserinsky and Kleitmann when they observed bursts of eye movement alternating every 90-110min throughout sleep. The cyclic succession of brain activity during sleep has been classified into two main states of vigilance, rapid eye movement sleep (REM) (Table 1) and non-rapid eye movement sleep (NREM). The sleep 'stages' were first classified and used to 'score' staging in accordance with rules devised by Rechtschaffen and Kales, based on the waveforms evident in the EEG in 1968 (10).

NREM sleep is further classified into four sleep stages (1-4), each with evidence of distinct psychophysiological changes, manifested in the EEG. The states are characterised by progressively slower frequencies and higher voltage activities that correspond to successively deeper states of sleep (Table 1.1). In 2007, the American Association of Sleep Medicine (AASM) amalgamated stages 3 and 4, and together constitute slow wave sleep (SWS) (11). NREM sleep is often referred to in the literature as synchronized sleep as the wave forms recorded from different sites on the scalp show synchronicity, be they slightly phase delayed. NREM sleep makes up the largest proportion of sleep, collectively accounting for 75%, most of which is spent in SWS. SWS is more evident in the first half of the sleep period (Figure 1.1).

Also evident in NREM sleep is the spontaneous appearance of cyclic alternating pattern (CAP) in the EEG trace. Considered a maker of sleep instability, the transient oscillations in the EEG, (A phase of the cycle) are followed by intervals of background activity (B phase of the cycle) (12). These phases are further subtyped by their level of synchronicity between the different regional EEG signals, (A1, synchronized, A2 both

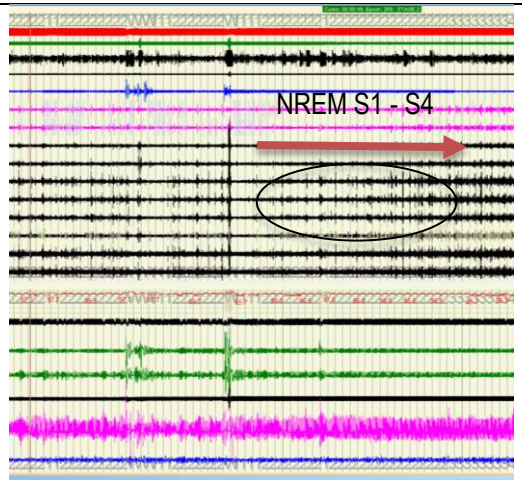
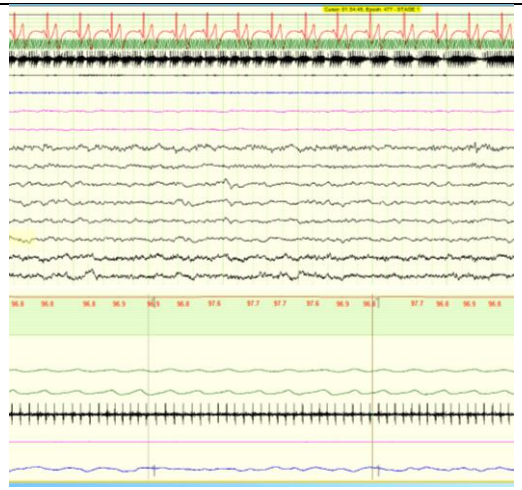
synchronized and desynchronized and A3 only desynchonised). A1 subtypes are associated with the build-up and maintenance of SWS, and are proposed to protect sleep continuity, while both A2 and A3 are involved in increased autonomic activity and are associated with arousability and the REM like state (13). The origin of the CAP signal, is proposed to involve the autonomic nervous system (ANS). Ferri et al. showed that periods of CAP during NREM sleep were associated with increased vigilance as measured using heart rate variability (spectral analysis for continuous electrocardiogram (ECG) recording) compare to parts of NREM with no CAP (14), suggesting that the origin of CAP involves activation of ANS pathways.

REM sleep accounts for the remaining 25% of sleep and is characterized by bursts of eye movements, back and forth under closed eyelids, muscle atonia, desynchonised low amplitude and high frequency EEG signals. Increased burst of theta signals, variability in heart rate, blood pressure and an irregular respiratory rate, increased bursts of sympathetic activity and increased upper airway resistance (Table 1.1) also occur in REM sleep. It is often referred to as desynchonised sleep or even paradoxical sleep and becomes more prevalent as sleep duration is increased. (Figure 1.1)

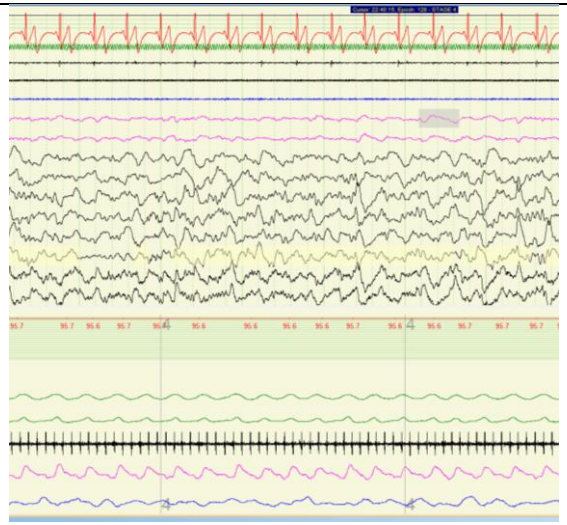
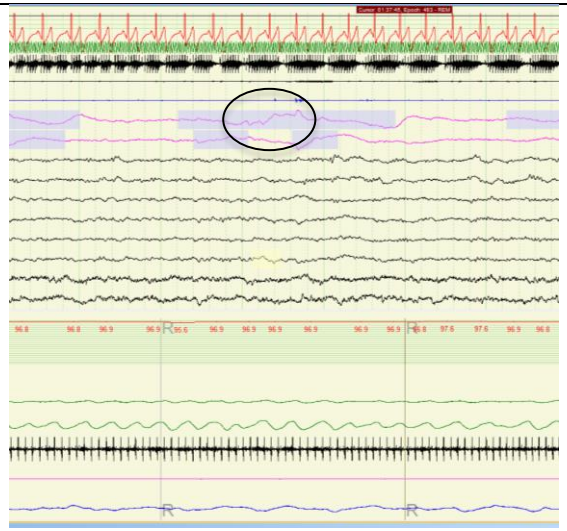
Figure 1.1: Shows a hypnogram from a child, blocks of blue denote slow wave sleep (SWS), green, Stage 2 NREM sleep, yellow Stage 1 NREM sleep and in red, rapid eye movement sleep (REM). Notice the large blocks of SWS at the beginning of the night and how it becomes less frequent as the night progresses while REM sleep increases with sleep duration.

In adults the circadian rhythm (24 hour cycle) has a pivotal influence on sleep onset and sleep. Infants are born with an active superchiasmatic nucleus, (the circadian rhythm pattern generate in the brain) and initially their circadian rhythm is entrained to that of their mothers. By one month, infants begin to acquire their own circadian rhythm, however sleep remains polyphasic (ultradian pattern), with repeated awakenings, and feeding occurring throughout the day and night (15). As infants mature, sleep architecture and time spent asleep also changes dramatically, reflecting the ongoing development of the central nervous system (CNS) and its respective interaction with the environment and also sleep experience (15). Three states of sleep are characterised in newborn infants (16). They include ‘active sleep’, characterised by an irregular heart rate, rapid eye movements and low voltage irregular/ mixed EEG activity, ‘quiet sleep’, characterised by a regular heart rate and respiratory rate, high amplitude mixed frequency EEG and ‘intermittent sleep’, which is a combination of the two states (evident within and epoch (30s) of EEG).

Table 1.1: Shows the PSG derived parameters indicating EEG/ECG/EMG differences between Stage 1 – 4 NREM sleep and REM sleep

Non-Rapid Eye Movement Sleep (NREM)		• NREM sleep is predominately regulated by parasympathetic nerve activity. • Heart rate decreases. • Breathing deepens and is regular. • Sympathetic tone is attenuated. • Blood pressure also decreases as the body transitions from S1 to SWS. • It makes up 75% of sleep in normal children with most time spent in S3 and S4. • As the sleep progresses from Stage 1-4 the signals become more synchronized.
Stage 1		• Evident at the beginning of sleep, light sleep. • Small proportions throughout 'normal' sleep cycle. • Low voltage, mixed frequency, highest amplitude in 2-7 Hz band. (can reach values of 200mV and can have vertex sharp waves). • EMG shows lower tone than wakefulness. • Less than 50% alpha and mixed frequency is used to classify S1.

Stage 2		• An absence of slow wave. • Evidence of both sleep spindles in the 12-14Hz band. • K complex waveforms are the main characteristics of Stage 2. • K complexes are sharp negative waveforms alternating with a slower positive surge.
Stage 3		• 20-50% wave in the 2Hz band or slower and an increase in amplitude (voltage), to 75mV (delta waveform). • Both spindles and K complexes occur in Stage 3, however at a much lower rate than Stage 2. • Cyclic alternating pattern, CAP.

Stage 4		• Similar to stage 3, however the signal shows more than 50% of waveforms in the 2Hz and slower and amplitude greater than 75mV (delta). • Muscle tone is reduced but not absent and cerebral blood flow is decreased to the frontal cortex. • There is greater synchronization between electrodes on the EEG. • Heart rate, blood pressure and respiratory rate are decreasing and upper airway resistance is increasing as time progress into S4. Collectively S3 and S4 are classified as Slow Wave Sleep (SWS).
Rapid Eye Movement (REM) sleep		• Characterized with generalized muscle atonia. • Increased cerebral blood flow to the frontal cortex. • Desynchronized EEG and variability in heart rate, blood pressure and respiratory rate. • Upper airway resistance increases as does sympathetic modulation. • Burst of eye movement, back and forth, under closed eyelids. Highlighted in purple. • The signal are more desynchronized compared to NREM sleep.

1.2 Sleep Disordered Breathing (SDB): Reaching a New Set-Point

During sleep the upper airway patency is maintained by the balance of multiple interrelated physiological components, including the pharyngeal dilatory muscle tone, neurogenic tone, upper airway resistance, pharyngeal collapsibility, and intrapharyngeal pressure generated by the inspiratory muscles (17). Interruption of the balance between these components can result in narrowing or total obstruction of the airway causing an increase in airway resistance. This results in increased effort required of the inspiratory muscle to maintain the airway of sufficient patency to allow adequate gas exchange.

Snoring is a primary indicator that there is resistance to airflow and is evidence of SDB. SDB, affects the respiratory load and its severity ranges from the most prevalence and least severe, known as primary snoring, to obstructive sleep apnoea where there is sufficient disruption to sleep and changes to critical ventilation parameters (see 1.6.3.2 for more details). As respiration is an essential physiological function, any deviation from its optimum pattern of functional processes alters the physiological mechanisms which are designed to maintain homeostasis at a constant *milieu*. When the deviation is chronic, occurring on most nights, the set-point to meet homeostasis changes and a different pattern of physiological interactions are induced. Overtime, these recruited mechanisms become established and a new trajectory for the underlying systems develops. The transitory change is termed allostasis, from the Greek words '*allo*' meaning other and '*stasis*' meaning state. Allostasis occurs all the time, when seasons transition, the body responds, e.g. by increasing fat storage during winter (thermal insulator and energy source) to protect us from the harsh cold weather and when food supply is limited. When spring comes, the extra kilos are shed, food is abundant again and we transition back to our summer selves. But what if winter continued longer than 3 months? What if winter did not come at all? Our bodies would adapt to the new norm and the new state would be the one that

was optimum for survival at that time. However, the long-term repercussions of this new state may in itself cause changes that are detrimental to the underlying physiology in the longer term. This unfavorable outcome is a result of allostatic overload and SDB can be viewed as an example of allostatic overload.

1.2.1 Homeostasis, Allostasis, Allostatic Load and the Autonomic Nervous System

'Homeostasis', first proposed by Canon in 1926 are the processes involved in the coordination of physiological responses that maintain the '*milieu interieur*' of the organism within an optimal range required to sustain life. Typically these processes include the essential functions of life, such as pH balance, body temperature, glucose levels and oxygen tension. Allostatic processes support homeostasis and also promote the adaptation to stress exposure. In humans, the processes involved in establishing the new set-point are dictated by the central nervous system (CNS), which governs the contraction of striated and smooth muscle, and neuroendocrine/exocrine function. Activation of these processes can either amplify or reduce a particular process, for example, in blood vessels the 'amplification' may result in the dilation of blood vessels, in a particular vascular bed in response to reduced circulating oxygen or as is the case in brown adipose tissue vasculature in mammals during cold exposure(18). While at the same time a reduction in vascular compliance and vasoconstriction may occur to reduce blood flow to a specific tissue, for example the skin during cold exposure. These process occur as a concerted response to preserve the homeostatic balance and to optimise survival.

1.3.2 Autonomic Nervous System (ANS)

As the main focus of this thesis is the alteration in function of the autonomic nervous system during SDB, the next section will give a brief synopsis of the ANS and the function of these components during normal sleep.

The main regulatory arm of the CNS involved in allostasis is the autonomic nervous system (ANS). Although not completely autonomous, the system simultaneously executes both excitatory and inhibitory activation of a wide range of tissues, target organs, and blood vessels. Single unit cells, such as mast cells, are also influenced by ANS activation. The control of the ANS is complex and works as a group of interconnected reflex arcs which are primarily centrally mediated by the hypothalamus and lower brain stem nuclei (19). The ANS regulates the body's visceral functions including respiration, cardiovascular function, thermoregulation, neuroendocrine secretion, gastrointestinal and genitourinal functions. The regulation of these functions are mediated through efferent nerves, which respond to moment-by-moment status changes via the activation of feedback afferent sensory relays from chemoreceptors, mechanoreceptors, nocioceptors, thermoreceptors, baroreceptors and osmoreceptors. These afferent feedback loops terminate in the CNS where the reflex arcs are regulated. The ANS exerts its control via two efferent pathways, the sympathetic nervous system (SNS), considered the energy recruitment component and parasympathetic nervous systems (PNS), the restorative component (20). They are comprised of preganglionic neurons in the brain stem and spinal cord and postganglionic neurons, which synapse at the target organ (see Figure 1.2) (21).

The role of the SNS, often misunderstood and misrepresented, is to facilitate the moment-to-moment regulation of cardiovascular function during periods of high excitation (extreme stimuli) but it is also active in periods of rest, not just during 'fight or flight mode' (22). Despite many textbooks describing the components of the ANS in normal conditions as existing in a harmonious state of balance, the reality is that this is not the case. Throughout the day and night these components (SNS and PNS) work at different levels of activation and are not necessarily in balance or even synchronized. The different activation levels of the two components give

rise to different patterns of behavior. An example of this is clearly demonstrated during sleep onset where the components of the SNS become less active, evident in vasodilation in the cutaneous vasculature, an indicator of SNS inhibition and somatic cooling, while the PNS dominates, reducing the heart rate, deepening respiratory effort and prolonging each breath.

A major function of the sympathetic nervous system is the regulation of arterial pressure. It does this by rapidly adjusting vascular compliance by increasing/decreasing the diameter of resistance arteries and reducing sodium and water excretion by the kidneys (23). This allows the body to maintain adequate perfusion of organs in order to meet their metabolic requirements. Unlike motor neurons which activate when required to execute a purpose, the sympathetic nerves are always active to a varying degree and, hence, all innervated blood vessels remain under some degree of continuous constriction (22).

Sleep is a major process where there is considerable change in ANS function compared to the awake state (24). The effect of chronic SDB disrupts the normal pattern of sleep and results in sympathetic over-activation, hypoxia, fragmented sleep, and intrathoracic pressure swings. These changes have be independently associated with atherogenesis and the development of cardiovascular disease (CVD) (25, 26). Even the acoustic vibration from snoring has been suggested as a possible contributing factor by adding to the allostatic imbalance, (27) which is thought to increase the risk of cardiovascular dysfunction. Research in adults and children has demonstrated that SDB is associated with sympathetic over-activation, systemic vascular irregularities including endothelial dysfunction, inflammation and the production of reactive oxygen species (28). These physiological changes are also all factors implicated in the development of cardiovascular disease.

SDB during childhood, where sleep is an important part of development and growth, may act as an increased burden on the underlying physiology by changing the ‘normal’ set points in the

system. Over time, this allostatic overload will result in the development of pathological maladaptive processes, involving the ANS pathway, most of which will affect the development of the cardiovascular system.

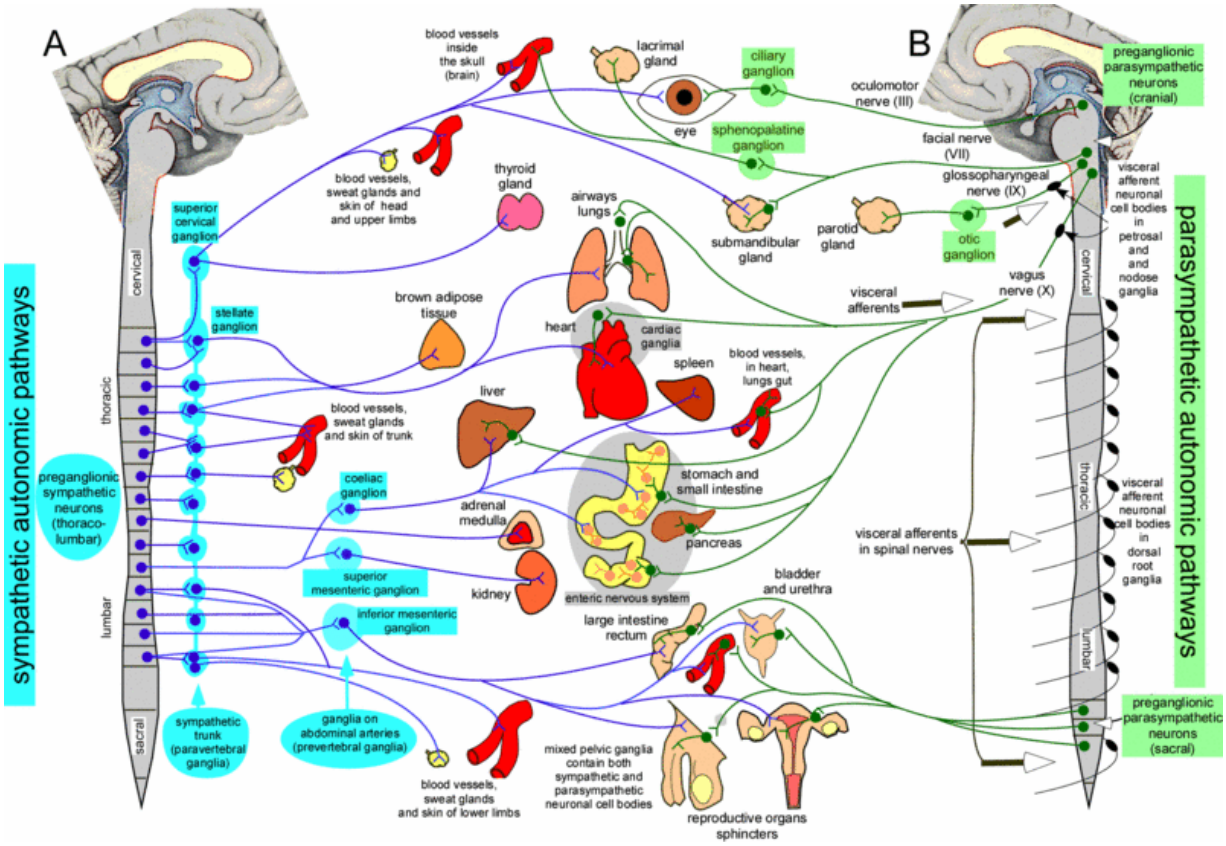


Figure 1.2: Autonomic neuronal output of (A) The sympathetic nervous system and (B) parasympathetic from the Central Nervous System.

Blessing & Gibbons Scholarpedia http://www.scholarpedia.org/article/Autonomic_nervous_system)

1.3 Autonomic Function and Sleep

To assess the effect of SDB on the cardiovascular and ANS function in children we need to understand the typical function of these systems during sleep. As previously mentioned, regular sleep involves cyclical changes throughout the night, which are coupled to different states of vigilance, associated with changes in autonomic function. Heart rate, stroke volume, cardiac output and blood pressure are reduced during sleep and also show an alternating pattern with changes in sleep stages. Although the purpose of these 'state' changes is still debated, what is clear is that the underlying mechanisms (blood pressure, heart rate) involved in these changes are affected in both adults and children with SDB (29). A detailed evaluation of autonomic changes during sleep is outlined in Chapters 3, 4 and 5 of Pier Luigi Parmeggiani's, *Systemic Homeostatsis and Poikilostasis in Sleep. Is REM Sleep a Physiological Paradox? (30)*.

1.3.1 Blood Pressure

Regional blood flow is determined by both perfusion pressure (arterial pressure) and resistance to flow in the specific region (31). Arterial pressure measurement consists of two parts, the maximal pressure during the ejection phase (systole) of the heart cycle (systolic pressure) and the minimal value measured at the end of ventricular (diastole) (diastolic pressure). Although, blood pressure can fluctuate depending on behaviour, it is, however, tightly regulated and is maintained within a narrow range via a complex network of both short- and long-term feedback/feedforward control systems (32). The factors involve (i) the feedforward 'central command' mechanisms, which include the ANS innervation to the heart and blood vessels and are part of the generalized adaptive physiological response; ii) the intrinsic mechanisms which include size and location of the sinus node and structural components of the heart and vasculature (smooth muscle cells, elastin, collagen fibers); (iii) circulating hormones; (iv) local factors such as metabolites (ATP, lactate and pH) and endothelial cell integrity (31) and (iiv)

receptors involved in the feedback/reflex including the baroreceptors, which are sensitive to changes in arterial pressure and also receive input from peripheral vasculature chemoreceptors which respond to change in CO_2/O_2. Also included in the network are respiratory mechanoreceptors (lung afferents) (32) and vestibular receptors (inner ear) (33) which relay information of positional change. Together these components allow for the moment-by-moment regulation of arterial blood pressure (34) and make up the 'baroreflex'. The baroreflex provides an ongoing buffering system to dampen the effects of sudden fluctuations in blood flow caused from emotional stress, exercise and orthostatic change (34) (Figure 1.3) and is pivotal for the body's ability to meet the metabolic requirement of each region, which may vary greatly (31).

The baroreflex maintains both cardiac output and total peripheral resistance via the activation of 'baroreceptors' which are mechanoreceptors sensitive to stretch. These afferent nerve terminals are located in a small dilation in the adventitia of the internal carotid known as the carotid sinuses (transduces the signal via the glossopharyngeal nerve) and aortic arch (transduces the signal via the vagus nerve and/or aortic depressor nerve) (35). There are also baroreceptors in both atria, at the entrance of the major veins (superior, inferior vena cavas, pulmonary) called cardiopulmonary receptors. An increase in arterial pressure, deforms the luminal wall and activates the baroreceptors, causing a depolarization and an increase in firing to the nucleus of solitary tract, which responds by initiating a sympathoinhibitory response via interneurons in the caudal ventral lateral medulla, that release γ-aminobutyric acid (GABA). This then inhibits the sympathoexcitatory neurons located in the rostral ventral lateral medulla (RVLM), which controls the tonic and reflex control of arterial blood pressure. The neurons of the RVLM produce noradrenaline and project direct to the intermediolateral cell column, which activates vasoconstriction to the muscle, mesenteric and renal blood vessels. The inhibition of

the RVLM neurons initiates the vasodilation of peripheral vessels, by withdrawal of the vasoconstriction, and hence minimizes the arterial pressure load and the pressure decreases. At the same time the NTS regulates the release of arginine vasopression and also regulates the heart via the activation of vagal preganglionic cholinergic neurons to the nucleus ambiguous. These neurons inhibit the automatism of the sinus node and influence the beat-to-beat innervation of the heart (34). The summation of these activations result in changes in vasomotor and cardiomotor nerve activity and buffers the initial change to arterial pressure.

The primary effect of the baroreflex is on total peripheral resistance via the RVLM's activation of sympathetic vasoconstrictor neurons, arising in the submesencephalic brain stem region, including rostral preoptic region and raphe in the medulla (36). The preganglionic sympathetic neurons release acetylcholine, which elicits a rapid excitation of noradrenergic neurons in the sympathetic ganglia. They then release noradrenaline (NA) at their terminals located at the media/adventitia interface of resistance blood vessels. The NA then activates α-1 adrenergic receptors on the smooth muscle cells causing the muscle fibres to contract and vessels to constrict (37). The baroreflex activation alters peripheral vasomotor activity and blood flow in an organ-specific manner, with changes in the vascular bed of the kidneys, gut, skeletal muscle and human skin vasculature (38).

Baroreceptor sensitivity (BRS) is functional in the latter weeks of gestation, and increases gradually as children reach puberty. In children both systolic and diastolic blood pressures increase with age and size, including height and less so BMI (39). BRS starts to decline after the second decade and is reported to decrease to almost zero in the 7th-8th decades (39). Vascular compliance is an important determinant of BRS, with reduced vascular compliance and arterial wall stiffening and in particular carotid distensibility associated with reduced BRS

(40). Reduced BRS results in a decrease in the capacity to response to changes in blood pressure (41).

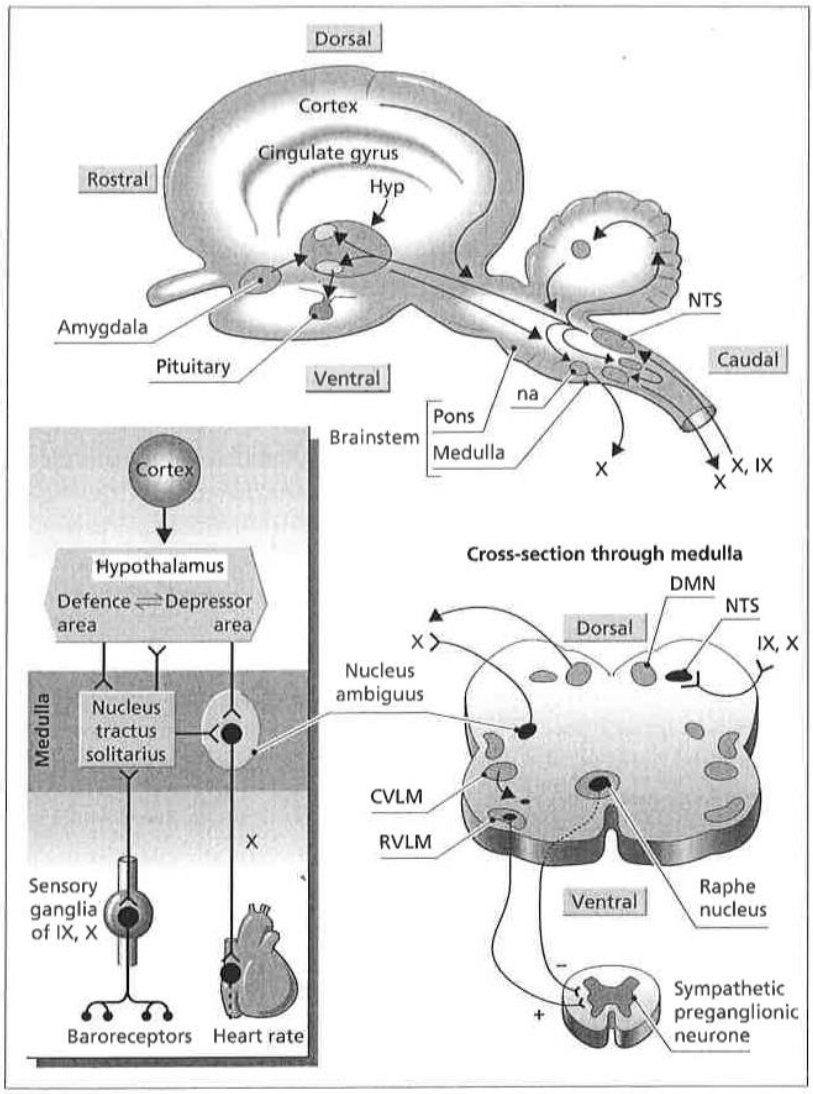


Figure 1.3: The Baroreflex neural networks. In normotensive humans increased peripheral blood pressure triggers baroreceptors. The afferent signal travels to the brain stem and triggers the reflex response, which includes decrease heart rate through the vagus nerve and also inhibits the sympathetic preganglionic neurons to the heart, reducing ionotropic force (reducing stroke volume and cardiac output) and dilation of blood vessels, causing a reduction in blood pressure (pg 43) (42).

1.3.1.1 Blood Pressure During Sleep

In humans, blood pressure is reported to reduce by 15-20mmHg during synchronized NREM sleep (24). The largest decreases of arterial blood pressure occur as sleep stages transition to the deeper stages of sleep (S3/4) (43). There is evidence that arousals during NREM sleep result in phasic increases in blood pressure (44) while K complexes, which are evident in Stage 2 and occasionally Stage 3, are also followed by a transient increases in blood pressure (45). Spectral analysis of continuous blood pressure assessments has shown a fall in the power of low frequency blood pressure variability during NREM, which is reversed during REM sleep (5).

During desynchronized REM sleep blood pressure oscillates, both increasing and decreasing, with levels reported below that of NREM and at times even higher than during awake. It has been proposed that the reduction in blood pressure during synchronized NREM sleep is as a result of reduced cardiac output. The changes experienced during desynchronized REM sleep, however, result as a consequence of both changes of total peripheral resistance and cardiac output (46), which suggests that the blood pressure increases and decreases experienced during REM sleep are mediated from autonomic control, e.g. periodic sympathetic activation.

1.3.2 Heart Rate

Heart rate is primarily determined by the depolarization of the Sino Atrial node (SA), the atrioventricular node, the intrinsic properties of myocardial cells, the geometries of the heart, compliance of the heart and vasculature and the Purkinje tissue (47). The heart rate reflects the net effect of the parasympathetic nervous system, which slows the heart rate, and sympathetic nervous system, which increases it. The sympathetic neurons effect the innervation all the tissue of the heart via the release of NA and activation of cardiac β1-adrenoceptors while the

parasympathetic nervous system only innervates the SA and atrioventricular nodes, the atria and the ventricular conducting system, via the release of acetylcholine acting on nicotinic receptors (42). Sympathetic stimulation of the cardiac muscle also increases the rate of dromotropy, (transmission in cardiac conductive tissue) and inotropy (force of ventricular contraction). These actions act in unison to ensure that the level of adjustment in cardiac function meets the moment-to-moment requirements of the body. The rate of transmission, within the baroreflex maintains the arterial pressure and is a key driver in maintaining cardiac output, while reflex adjustment also occurs in response to stretch from the great veins and atria by an increased in venous return (42). This results in an increase in heart rate so that output matches inflow with little change in contractility. Respiration also plays an important part in modulating heart rate. Increased tidal volume activates stretch receptors in the lung, which results in tachycardia and an increase in blood flow to meet respiratory demand. Activation of chemoreceptors (See 1.5.4) leads to a reduction in heart rate (bradycardia) via the medullary vagal output, with drops evident during periods of hypoxia, reducing cardiac output and increases the chances of survival.

1.3.2.1 Heart Rate During Sleep

Heart rate decreases when transitioning from awake to the sleep state, with the biggest decrease occurring in SWS (48). Baust & Bohnert demonstrated that the reduction in heart rate seen during the transition from the awake to synchronized NREM sleep is caused by an increase in vagal output (PNS) (49). More recent studies suggest that the fall in heart rate is in part attributable to the circadian cycle (50) (51).

In REM sleep, phasic increases in heart rate are evident. Iwamura et al., reported swings in heart rate during, desynchronized REM sleep that persisted after vagotomy thereby concluding

that the SNS innervation is responsible for the alternating heart rate. The fall in heart rate during REM are mediated by reduced SNS activation with reciprocal changes in vagal activity.

1.3.2.2 Heart Rate Variability (HRV)

The effects of SDB on the ANS can be evaluated using spectral analysis of the overnight electrocardiogram (ECG), known as heart rate variability (HRV). The analysis involves the use of algorithms that determine beat-to-beat fluctuations both in relation to time (time domain) and frequency (frequency domain) quantify the power spectral analysis of ECG segments (52) (*See Appendix 3 for full list of HRV variables*). Low frequency (LF) represents both parasympathetic and sympathetic activities while high frequency (HF) represents parasympathetic activity and the function of the vagus nerve. The ratio of LF to HF represents sympathovagal balance. Measuring HRV during sleep is the ideal time to assess the 'baseline' function of the ANS, in particular the PNS.

1.3.2.3 Heart Rate Variability During Sleep

For an in depth evaluation of HRV variables and their relevance to sleep and SDB, readers are directed to a comprehensive review by Stein and Pu (2012). In short, as sleep progresses to deep sleep the R-R frequency decreases and the HRV decreases with the biggest reduction associated with SWS with an increase in HF (increased PNS) and decreased LF (decreased SNS) and hence decreased LF/HF ratio. Conversely during REM sleep the reverse occurs in the frequency domain variables, with decreased HF (reduced PNS) and increased LF (higher SNS) and increase LF/HF ratio. The increase in HF during SWS is associated with higher respiratory regulation (53) Aging has an effect on HRV during sleep with an increase in HR and lower HF during NREM sleep occurring as we age, suggesting a reduction in cardiac vagal activity during sleep as we age (39).

1.3.3 Respiration

Respiration is regulated by two separate neural mechanisms. One set of mechanisms controls autonomic respiration, experienced while we are asleep and the other mediates voluntary control. The autonomic control arises in the pacemaker cells in medulla. The rhythmic activation from the pacemaker cells is coupled to the motor neurons in the cervical and thoracic spinal cord, which innervate the inspiratory muscles (54). The cervical motor neurons activate via the phrenic nerve to contract the diaphragm and initiate inspiration, while the thoracic motor neurons activate the external intercostal muscles, and the signal transduces to the internal intercostal and other expiratory muscles.

1.3.3.1 Respiration During Sleep

Respiratory rate decreases and becomes shallower as mammals enter the early stages of NREM sleep from quiet waking. As NREM sleep stages progress respiration becomes more regular with the appearance of definite expiratory pauses, a small increase in tidal volume and a larger decrease in respiratory rate, resulting in reduced minute ventilation. The decreased frequency is accompanied by reduced oxygen uptake and a 10-20% reduction in carbon dioxide production, indicating a decrease in metabolic rate during NREM, especially in SWS. Also evident are periodic fluctuations in the depth of breathing, especially in the early hours of NREM sleep. There is a successive reduction in tidal volume with each inspiration that ends in an expiratory apnoea and terminates with an exaggerated inspiration. This cycle repeats itself and is similar to the classical Cheyne-Stokes pattern of respiration. A 15-30% fall in alveolar ventilation is indicated by a mild increase in alveolar partial pressure of CO_2 ($PACO_2$) and a similar increase in arterial partial pressure of CO_2 ($PaCO_2$), which is paralleled with small decreases in PaO_2 and PAO_2 and minor decrease in arterial pH (24). This results in both mild hypoxia and hypercarpnia throughout sleep, regardless of sleep stage.

Respiration during REM sleep is variable, with periods of hyperventilation (rapid breathing) interspersed with moments of regular and reduced respiration and apnoeas of varying lengths. Hence both the respiratory volume and rate can increase and decrease throughout REM sleep. Minute ventilation phasically increases to levels measured during the awake state but overall it remains lower. The degree of hyperventilation is thought to be associated with the level of muscle activity and eye movements that occur periodically during REM sleep (24) and suggests that respiratory changes are secondary to internal changes in neuronal activity. It has been proposed that the hypoventilation during REM sleep is associated with reduced ventilatory drive rather than a change in mechanical factors, such as reduced upper airway patency (55). In addition, $PaCO_2$ measurements decrease while PaO_2 remains the same and can in some cases increase during REM sleep. The irregular breathing pattern is not affected during hypercapnia and is insensitive to afferent input including metabolic alkalosis, vagal block, and denervation of peripheral chemoreceptors (56).

During sleep, respiratory parameters are critically dependent on both chemical and mechanical feedback reflexes (24). When using mechanical ventilation to progressively increase tidal volume and thereby reduce circulating CO_2 levels, this results in apnoea events. Conversely, increasing inspired CO_2 levels eliminates both central and periodic breathing (57) because of the induction of increased respiratory drive. Gleeson et al. showed that in humans the level of circulating O_2 and CO_2 determines the ventilatory response and in particular, that the level of CO_2 was the primary respiratory driver (58). Sleep stage also plays an important role in the sensitivity of the respiratory control system, with decreased ventilation and increased $PaCO_2$ during NREM sleep (24). Burlow et al., demonstrated that ventilation response to changes in CO_2 is significantly reduced in NREM compared to awake (59).

In sleep the airway patency is reduced, possibly due to the global reduction of neuronal activity. The collapsibility of the upper airway, a major issue in adults with SDB and possibly in children with SDB too, can be measured using pharyngeal critical pressure (P*crit*) and negative expiratory pressure (NEP) techniques (60). P*crit* is the critical pressure where maximal inspiratory airflow occurs while asleep and is the relationship between pressure and airflow through a dynamic tube (*Starling resistor*) (60).

In NREM sleep, motor output to the respiratory pump muscles and upper airway is drastically reduced, resulting in a hypoventilation, with increases of between +2 and +8 mm HG arterial partial pressure of CO_2 (P_{aCO2}); a reduction in upper airway muscle compliance (hypotonicity), increased P*crit* and airway collapsibility in health adults (57). In adults, both tonic (postural) and phasic (inspiratory related) innervation are reduced during REM sleep as measured with electromyogram of the intercostal muscles (61). This alters chest wall compliance and reduces tidal volume. These experiments demonstrate the effects of the both the chemical and mechanical feedback reflexes on breathing patterns.

1.3.4 Chemosensitivity

Peripheral chemoreceptors in the carotid and aortic bodies are neurosecretory glomus cells. Carotid body chemoreceptors are sensitive to decreased O_2, increased CO_2 levels and decreases in pH in the circulating arterial blood, (62, 63) while aortic chemoreceptors are only sensitive to decreased O_2 and increased CO_2 levels. Activation of the chemoreceptors in the carotid bodies results in the release of neurotransmitters, acetylcholine, nitric oxide (NO) (64) and carbon monoxide (CO) (65). This transduces a signal that travels via the sinus nerve (carotid body) and aortic depressor nerve (aortic body) or chemoreceptor fibres linked with the vagus nerve. Activation in the carotid sinus simultaneously activates baroreceptors and the signals

travel via the glossopharyngeal nerve (38). These signals travel to the brain stem via the vagus and depending on the type of stimuli (pH, O_2, CO_2) will evoke a respiratory correction and regulate tissue perfusion accordingly. Central chemoreceptors, in the brain (medulla, NTS, locus coeruleus, hypothalamus) also detect changes in arterial CO_2 and regulated cerebral blood flow, pH and brain metabolism (66). The response to chemoreceptors is fast and modulates respiration on a breath-to-breath basis. In addition, activation is associated with regionally selective vasoconstriction, with bradycardia, reduced cardiac output and sometimes hypertension (p. 122) (38). In the awake, normal breathing state, arterial CO_2 (via pH) remains the primary determinant of ventilation.

1.5.4.1 Chemosensitivity in Sleep

Early studies by Brebbia and Altshuler showed O_2 consumption is decreased between sleep compared to wakefulness which demonstrates the reduction of metabolic rate associated with sleep. They found that O_2 consumption was reduced in all sleep stages compared to wakefulness and that O_2 consumption in REM sleep was higher than NREM consumption, which reduces as NREM stages of sleep increase (S1-3/4). However the reduction in O_2 is only small and does not significantly alter saturation of haemoglobin (24). As previously mentioned, respiratory drive decreases in NREM sleep and circulating CO_2 increases the most during SWS by between 10-20% (67). Ventilation response to increased CO_2 is also moderately reduced in NREM sleep. This suggests that chemoreceptors (central and/or peripheral), which would normally activate to correct the increased concentrations of CO_2 are blunted compared to the awake state and REM sleep. In further support of reduced chemosensitivity in NREM sleep, Berthon-Jones and Sullivan using 7% CO_2 in 40% O_2, a rebreathing test, that was administered via nasal mask to evoke arousals in different sleep stages showed that males aroused at greater alveolar P_{CO2} during NREM sleep, compared to REM sleep and awake state. They found no differences in women in either sleep state compared to awake (68).

Selective denervation of the carotid chemoreceptors in the cat causes a decrease in arterial blood pressure during REM sleep (usually increased) but not denervation of baroreceptors. This suggests that the carotid chemoreceptors play a vital role in maintaining/increasing arterial pressure during REM sleep. It may be the case that the drop in ventilation during NREM sleep and consequential increase in hypercapnia is part of the centrally generated sleep state that, over time, precipitates a level (CO_2 increase vs O_2 decrease) required to activate the chemoreceptors and hence reverse the consequences of time spent in the NREM sleep state and transitioning back to REM (69). The increase in circulating CO_2 acts as a vasodilator, contributing to reduction of vascular resistance, which occurs predominately during SWS (24).

1.3.5 Blood Flow, Peripheral Resistance and Thermogenesis

Global, blood flow to the brain has been shown to be decreased during both REM and NREM sleep compared to awake. This suggests that cerebral autoregulation is altered during sleep, possibly resulting from systemic haemodynamic depression (reduced cardiac output and reduced total peripheral resistance occurring together) (30). Total peripheral resistance is reduced both in REM and NREM sleep compared to the awake state (70) and may account for the difference in global cerebral blood flow during sleep. Global cerebral blood flow is greater in REM sleep compared to NREM sleep by approximately 17% (71). Paradoxically, some areas of regional flow in the brain are increased while others are significantly reduced during REM sleep compared to pre-sleep (Table 1.2). The increase in blood flow is not associated with changes in perfusion pressure, circulating CO_2 or cerebral metabolic rate (72). The surges of regional brain blood flow coincident with periods of phasic REM and peaks in diaphragmatic EMG activity and possible phasic sympathetic activity. In the cat, the fall in arterial blood pressure during desynchronised REM sleep results from vasodilation of the splanchnic and

renal beds, while a reduction in muscle vascular conductance (vasoconstriction) occurs at the same time in peripheral vessels (73).

During synchronized NREM sleep, in humans, and other mammals (rhesus monkey, rabbit and cat) skin temperature measured on the ear (pinna), hand/fingers is similar to the awake state. However contradictory reports of both hand volume and skin temperature have been reported is desynchronized REM, with one report suggesting increased hand volume and temperature, indicating considerable vasodilation during REM sleep (74). Conversely, others have reported peripheral vasoconstriction in humans during REM sleep. NREM sleep, particularly slow wave sleep, is accompanied with bradycardia and small reduction in cardiac output, reduction in sympathetic tone in the muscles and reduction in systemic vasculature resulting from reduced systemic vascular resistance. Studies in the cat and humans have shown peripheral conductance and aortic flow are slightly reduced in synchronized NREM sleep, however during desynchronized REM sleep peripheral conductance was increased by 9% and, hence, results in a decrease in total peripheral resistance (30).

A small increase in hypothalamic temperature (0.2℃) during REM sleep has been described by Parmegianni et al., (30). They propose that the increase in hypothalamic temperature (pre-optic region specifically) is possibly due to increased metabolic heat produced from nervous tissue, arterial blood flow and the arterial blood temperature. They also speculate that the rise is associated with alterations of systemic haemodymanics observed through REM sleep, including the vasoconstriction to the cutaneous vessels and decreased cardiac output. More recently Ootsuka et al., speculated that the increase in brain temperature (hypothalamus) during REM sleep is associated with brown adipose tissue thermogenesis, which they showed in rats, follows a 90 – 110min ultradian cycle during the dark phase of the circadian cycle (when rats

are more active) (75). The authors proposed that the BAT ultradian rhythm is part of the Basic Rest Activity Cycle (BRAC), first proposed by Kleitman in the 1960s. Recent PET scan studies have shown that active BAT is still present in adults, however, whether the BAT ultradian rhythm plays an active role in the sleep cycle in humans is yet to be investigated (76).

Table 1.2: Compares autonomic and cardiovascular function between the awake state and different sleep stages

Cardiovascular Parameter	REM	NREM
Blood Pressure Baroreflex Sensitivity	• Tonic ↑ and Phasic ↑ ↓ • Tonic↑↑ and Phasic↑↓	• ↓
Heart Rate HRV	• Tonic ↑ and Phasic ↑↓ • HF ↓ • LF ↑	• ↓ • HF ↑ • LF ↓
Breathing	• Respiratory Volume and Frequency unstable	• Respiratory Volume and Frequency stable
Cerebral Blood Flow (77)	• ↓ Global flow compared to pre-sleep awake,↑ compared to post –sleep awake. • ↑ flow in the pontine tegmentum, paralimbic areas, hippocampus, posterior cingulate cortex. • ↑ to the dorsolateral prefrontal and frontal opercular cortices inferior partietal lobule	• Global 26% ↓ (Stage3/4) - in caudate, midbrain, pontine tegmentum, basal forebrain, anterior hypothalamus- preoptic, inferior cerebellar hemispheres
Cutaneous Blood Flow	• ↑ and ↓ (phasic)	• Same
Mesenteric Blood Flow	• ↑ blood flow when blood pressure increases ↑ 28% conductance ↑ Vasodilation	• Same flow, small increase in conductance, • Vasodilation
Muscle Blood Flow	• ↓17% Iliac conductance • Vasoconstriction	• Same Iliac conductance
Renal Blood Flow	• 20% ↑ in conductance • no change in blood flow	• Same

N.B. ↑ denotes increased, ↓ denotes decreased.

1.4 Sleep Disordered Breathing in Children

Thirty years ago, Guilleminault et al., were first to describe SDB in children using polysomnography. They described similar abnormalities to those observed in adults with SDB such as respiratory pauses, restless sleep, difficulty breathing and oxygen desaturation. They also noted excessive daytime sleepiness, increased sweating, abnormal weight (both over and under-weight), morning headaches, nocturnal enuresis (betwetting) and decreased school performance in children with SDB. More importantly, they were the first to report a '*progressive development of hypertension*' in children who demonstrate '*loud snoring interrupted by pauses during sleep*' (78). SDB occurs in all age groups including preterm babies and adolescents, however the disorder is most common in the 2-5 year age group (79).

1.4.1 Prevalence

Although there are many epidemiological studies in paediatric SDB, a clear picture of the number of children affected is still to be definitively determined. The results of prevalence studies now available from North and South America, Europe, Australia, Africa and Asia collectively demonstrate that SDB in children is a common disorder which affects both developed and developing countries (80). Prevalence varies from country to country and even within country reports. These discrepancies are due to inconsistent measuring methods (breathing during sleep, clinical setting, parental questionnaire), small sample sizes and consistency with standardized classification of habitual snoring and also OSA. In a recent 'technical support report', prevalence of habitual snoring was reported at between 1.5% to 27.6% using subjective measures (parental reporting/questionnaires) in studies from around the world (See Table 1.3). The working committee concluded that at the severe end of the disorder, between 1.2%-5.7% of children have OSA (using data of three general paediatric

population studies (81-83)). The authors supported the need for more population based studies to further delineate gender, age and race distribution in SDB (84). Despite the range in prevalence reported, what is clear, is that SDB is a common, worldwide, paediatric disorder.

Table 1.3: Prevalence of SDB around the World adapted from Marcus et al, 2012 (84) and Lumeng and Chervin, 2008.

Region/Country	No of Participants	Frequency	Reference	Age Range	Prevalence
Asia/Oceana					
Australia	996	>4 /wk	Zhang, 2004 (85)	4 – 12	15.2
Australia	974	> 4/ wk	Lu, 2003 (86)	2 – 5	10.2
Australia	1650	Yes/no	Valery, 2004 (87)	0 – 17	14.2
Hong Kong	3047	> 6 / wk	Ng, 2005 (88)	6 - 12	10.9
Hong Kong	200	> every nt	Ng, 2002 (89)	6.4 (4)	14.5
Singapore	10279	> 3 / wk	Chng, 2004 (90)	4 – 7	6.0
Thailand	1008	Most nts	Anuntaseree, 2001 (91)	6 – 13	8.5
Thailand	755	Most nts	Anuntaseree, 2005, (92)	9 – 10	6.9
Korea	3871	> 3 / wk	Shin, 2003 (93)	15 – 18	11.2
New Zealand	1585	Yes/No	Mitchell, 2003 (94)	1 – 6m	26.1
Taiwan	179	Yes/No	Liu,2004 (94)	10 – 19 y	26.3
China	5979	?	Liu, 2005 (95)	2 – 12 y	5.6
Europe					
Belgium	3045	Yes/No	Spruyt, 2006 (96)	6 – 13 y	2.5
France	25703	Often	Delasnerie-Laupretre, 1993 (97)	17 – 20 y	4.6
Germany	1144	Frequently	Schlaud, 2004 (98)	9.6 y	10.1
Greece	3680	Always	Kaditis, 2004 (99)	1 – 18 y	4.2
Greece	1821	> 3 / wk	Alexopoulos, 2006 (100)	5 - 14	7.4
Iceland	454	Often	Gislason, 1995 (101)	6mo – 6 y	3.2
Italy	895	Always	Brunetti, 2001 (102)	3 – 11 y	4.9
Italy	2209	Often	Corbo, 2001 (103)	10 – 15 y	5.6
Italy	447	Often	Castronovo, 2003 (104)	3 – 6 y	34.5
Italy	1615	Often	Corbo, 1989 (105)	6 – 13 y	7.3
Portugal	976	Frequently	Ferreira, 2000 (106)	6 – 11 y	8.8
Russia	200	Yes/No	Kelmanson, 2000 (107)	2 - 4 mo	5
Spain	100	Often	Sanchez-Armengol, 2001 (108)	12 – 16 y	14.8
Sweden	325	Always	Hultcrantz, 1995 (109)	4 y	6.2
Sweden	1844	> 3 / wk	Smedje, 1999 (110)	5 – 7 y	7.5
United Kingdom	6811	Almost always	Kuehni, 2008 (111)	1 - 4	7.9
United Kingdom	782	Most Nts	Ali, 1993 (112)	4 – 5 y	12.1
United Kingdom	245	Sometimes	Owen, 1996 (113)	0 – 10 y	27.0

Africa/Middle East					
Iran	2900	> 3 / wk	Bidad, 2006 (114)	11 – 17 y	7.9
Nigeria	909	snore	Alabi 2012 (115)	3 – 16 y	34.2
Turkey	1211	Always	Kara, 2002 (116)	6 – 13 y	2.4
Turkey	1198	> 3 / wk	Sogut, 2005 (117)	3 – 11 y	3.3
Turkey	2147	Often	Ersu, 2004 (118)	5 – 13 y	7.0
Turkey	1784	Often	Akcay, 2006, (119)	4 – 17 y	4.1
North America					
United States	1014	Always	Johnson, 2006 (120)	13 – 15 y	6
United States	1038	> Half time	Archbold, 2002 (121)	2 – 14 y	17.1
United States	5728	>3 / wk	O'Brien, 2003 (122)	5 – 7 y	11.7
United States	944	> 3 / wk	Montgomery-Downs 2006 (123)	2wk – 2 yr	5.3
United States	1494	> 1 / wk	Goodwin, 2003 (124)	8 – 11 y	10.5
United States	3019	> 3 / wk	Gottlieb, 2003 (125)	5 y	12
United States	141	Yes/No	Weissbluth, 1984 (126)	4 – 8 mo	12.0
South America					
Brazil	988	Frequent/always	Petry, 2004 (127)	9 – 14 y	27.6
Argentina	2210	Frequently	Perez-Chada, 2007, (128)	9 – 17 y	9
Ecuador	806	Often/ Always	Tafur, 2009 (129)	6 - 12	15.1

1.4.2 SDB Risk Factors

The etiology of SDB is multifactorial (25). Factors that affect airway flow include the skeletal and soft tissue anatomy of the upper airway, neuromuscular tone of the pharyngeal striated muscles and suction pressure generated by the inspiratory pump muscles. Studies in both adults and children have demonstrated significant differences between the upper airway of children with SDB and non-snorers and even considerable variability between those with SDB (130). This section will focus on enlarged tonsils and adenoids tissues which is the main risk factor of SDB in childhood, however a description of the other upper airway factors and further evaluation of the risk factors in children with SDB is supplied in *Appendix 1*.

1.4.2.1 Tonsils and Adenoids

The soft tissue of the upper airway consists of the palantine tonsils, adenoid, fat pads (located in the parapharyngeal area) and musculature. Although the size and composition of soft tissue

is primarily determined by genetic factors, other factors include inflammation, infection and infiltration of various storage/metabolites can alter their size (131). It has been proposed that the mechanical movement (vibration from snoring) of the tonsil may cause hypertrophy of the tissue and inflammation of mucosa, increasing the size of the tonsils and hence further obstructing the airway in children with SDB (132). Adenoid and tonsillar hypertrophy remains the major cause of sleep disordered breathing in children (133), as they narrow the pharynx.

Located at the roof of the nasopharynx, the adenoids are a mass of lymphoid tissues, that secrete mucus as part of a protective mechanism, and traps inhaled infectious agents via the nose, transferring them to the pharynx (134). In addition, the lymphatic tissues produce antibodies and act as an immune barrier. They are absent at birth but become noticeable by 6 months, reaching a maximal size between the ages of 2-10 years. The tissue then progressively decreases in size as children reach adulthood. Normal adenoid size ranges between 7 – 12mm and adenoids larger than 12mm are considered abnormal and in children with SDB, the adenoids are often significantly larger (mean = 13.46mm) compared to age matched controls. Enlarged adenoids in children can result in nasal obstruction, mouth breathing, ongoing nasal congestion, middle ear infection, snoring and fragmented sleep.

The palantine tonsils are soft tissues located in between the glossopalantine and pharyngopalantine arches. They are aggregated lymphoid tissue encased in a mucus membrane, which also act as an immune protective barrier. The adenoid and tonsils continue to grow throughout childhood and reach their maximal size just before the onset of puberty. In the clinical setting, children with OSA are more likely to have enlarged palantine tonsils compared to children without OSA. Inflammation induced adenotonsillar hypertrophy, leading to clinical OSA, has been noted in children under 1 year of age (135) and chronic infections are thought

to promote lymphoid hyperplasia, and increase their size (28). Although the link between enlarged T&A and SDB is well accepted, and their removal by adenotonsillectomy (T&A) reduces their SDB severity, what is less understood is the lack of association between the size of the tonsils and adenoids assessed both clinically and using imaging and the severity of SDB.

Several studies have demonstrated the efficacy of adenotonsillectomies to treat snoring and OSA using both parental report measures and polysomnography (136). However why approximately 33% of treated children who have no other risk factor (e.g. obesity etc.) continue to snore has not been effectively addressed (137). This suggests that the role of the upper airway characteristics (e.g. muscle tone, muscle composition) need further examination.

1.5.3 SDB Diagnosis

1.5.3.1 Clinical Assessment

The most widely used method to diagnose paediatric SDB is a clinical history and physical examination, with more than 90% of T&A undertaken on this basis with no objective testing employed. Large tonsils and adenoid tissues upon examination and the parental report of snoring, restless sleep, breath holding plus excessive daytime sleepiness, hyperactivity and/or aggressive behaviour would be enough to proceed to surgical intervention even when the polysomnography (PSG, see 1.5.3.2) is normal (138). However, history and physical examination are considered to have both low sensitivity and specification with a predictive accuracy of 30-50% (139) and cannot accurately determine the level of severity of SDB. In a meta-analysis determining the reliability of clinical evaluation alone to predict SDB in children versus polysomnography from 12 studies, Breitzke et al., concluded that the otolaryngologists were more likely to clinically over diagnose OSA compared to using PSG, OSA criteria (140).

The current guidelines of the American Association of Sleep Medicine (AASM), advocate that

the best diagnostic tool to assess SDB severity is an overnight polysomnography (PSG), which simultaneously measures a suite of physiological parameters known to be alter during sleep in children with SDB and especially during respiratory stress (84).

1.5.3.2 Polysomnography (PSG), Overnight Sleep Studies

Polysomnography (PSG) is a simultaneous overnight assessment conducted in a clinical setting which requires the evaluation of multiple physiological parameters including EEG (usually measured at 6 locations, C3-A2, C4-A1, and F3-A2 F4-A1, O1-A2 and O2-A1), left and right electrooculogram (EOG), sub-mental, diaphragmatic and leg electromyogram (EMG) and heart rate by electrocardiogram (ECG). These signals, as well as continuous infra-red video recording, are used to determine sleep staging. (141) Oronasal airflow by thermistor and nasal pressure, respiratory movements of the chest and abdominal wall using uncalibrated respiratory inductive plethysmography (RIP) and arterial oxygen saturation (SpO_2) by pulse oximetry are used to measure respiratory events. (141) Position sensors are also used to determine body orientation during sleep as obstructive events are more common in the supine position compared to the lateral prone positions. (142) Children are continuously monitored via infrared camera by a pediatric sleep technician, who also documents observations of sleep behavior, which include the presence or absence of snoring.

Considered the gold standard method of assessment for SDB, PSG differentiates between primary snoring and OSA and hence can be used to determine SDB severity. In addition, it can identify central apnoea (cessation of breath but no obstruction) and also can evaluate the efficacy of treatment. (143) Obstructive apnoeas, are classified as a cessation of airflow of at least two breaths, with or without oxygen desaturation and typically followed by an exaggerated recovery breath, known as a hypopneas (25). Apnoeas result in a significant oxyhemoglobin desaturation and terminated by a CNS evoked arousal. An obstructive

hypopnea, is a lesser reduction in ventilation, and is characterized as a 50% decrease in in airflow associated with a 3-4% desaturation and/or arousal. Normative sleep data in children have shown that obstructive hypopneas and apnoeas are rare and that the desaturation level seldom drops below 90% (144). The most commonly used measurements of severity of SDB are derived from the PSG variables. They include the Apnoea Hypopnea Index (AHI), and the Obstructive Apnoea Hypopnea Index (OAHI) are the number of episodes per hour of sleep and Oxygen Desaturation Index (ODI). These values are then used to classify the severity.

1.5.3.3 Determining Severity From Polysomnography

The spectrum of SDB severity in children is classified into three distinct categories. As the categories increase in severity so does the detrimental physiological impact of the disorder (145). They include:

- Primary snoring, where there is parental report of frequent snoring but less than 1 apnoea or hypopnea per hour of sleep measured during an overnight PSG; no abnormal gas exchange (hypoxia, hypercarbia) or sleep fragmentation (145) (146). Although considered a benign condition, recent reports of reduced neurocognition and abnormal blood flow (147) and vascular function (148) challenged this accepted assumption.
- Upper airway resistance syndrome, frequent snoring, flow limitation (progressive increased inspiratory negative intrathoracic pressure) (149) and frequent respiratory effort related arousals (RERA), resulting in sleep fragmentation without gaseous exchange abnormalities (11), no or few apnoeas, hyponeas or oxygen desaturation.
- Obstructive sleep apnoea syndrome (OSA) at the severe end of the continuum is further divided in to three sub-categories, (mild, moderate and severe) which are indicative of the number of obstructive events and sleep fragmentation. Specifically it is characterized by at least 1-5 (mild), or 5-10 (moderate), and in excess of 10 (severe) events of either partial or

total upper airway obstruction (apnoeas/hypopneas) per hour of sleep, abnormal ventilation, increased arousal and abnormal sleep architecture (145).

One limitation of the current AASM scoring criteria is that it does not take into account the association between the severity of OSA and the level of oxygen desaturation (both number, duration and percentage reduction). Many studies, in both adults and children have negative associations between increased oxygen desaturations, neurocognition and cardiovascular outcomes.

1.5.3.3 Obstructive Events, Sleep Staging and Sleep position

The general consensus is that 50% of obstructive events and their duration occur during REM sleep compared to NREM sleep in children with OSA (which constitutes approximately 22% of total sleep time) (150-153). The opposite prevalence is found in adults with OSA, with the majority of events reported in NREM sleep. It has been suggested that the reduction in muscle tone and increased arousals in response to an apnoea during REM sleep increases the occurrence of obstructive events.

Many studies in adults report that most obstructive events occur in the supine position compared to both lateral and prone position. More than 30% of SDB in adults is considered 'positional OSA', where obstructive events are only evident in the supine position. Most is related to obesity (154). It has also been postulated that the impact of gravitational force may push the soft tissues (tongue, soft palate) to the back of the throat, minimizing the size of the airway (154). Zhang et al., showed that AHI was higher in children with OSA that sleep in the supine position compared to children with OSA that slept in the lateral positions. Despite more obstructive events in the supine position, they observed that sleep architecture was not effected

and that supine obstruction and AHI were more associated with age, (with less of an association between position and obstruction in younger children). They also showed that oxygen saturation nadir, an important criteria in diagnosing OSA that is not currently recognized in the current AASM scoring rules, was independent of sleep position. They also recommended that children with SDB not sleep in the supine position. Verginis et al., also showed that up to a third of children showed a predominance of NREM sleep obstructive events, especially in older children, which coincided with higher arousal indices and less severe desaturation episodes (151). This suggests that the profile of SDB changes as children mature and increase in body habitus.

1.5.3.4 Efficacy of Polysomnography

Both the economical cost and validity of PSG measurement was questioned by authorities in paediatric sleep (142). The main concern is the lack of association between the PSG outcomes and adverse effects such as neurocognition and behavioural function, which are considered the main sequelae of paediatric SDB. Brietzke et al., proposed that either the current PSG thresholds need to be redefined and/or new testing criteria needed to be developed (possibly in conjunction with the PSG) that link both the adverse effects of SDB and measures derived from the PSG. They also highlighted the growing number of studies that showed children who were classified as primary snorers, without hypoxemia/hypercapnia, and obstructive events, using the PSG, were also likely to exhibit neurocognitive deficits and learning difficulties (140).

Further to this argument is the cost associated with such a labour intensive test which requires the Sleep Technician to monitor the child overnight (12 hour shift), a technician to score the test (2-6 hours) and a sleep physician to report the test. There is also the added cost of consumables, all of which makes it a very expensive test that can only be accessed by few. Stradling and Davies argued that the considerable variability in sleep apnoea from night to

night and even over the longer time course (from month to month, year to year) means that SDB severity is not accurately assessed with a single nights of PSG (155).

1.5.4 Overnight Oximetry

A cheaper and mobile option that can be used in the home setting to diagnose SDB is the pulse oximetry. It is currently used by many medical sleep facilities where PSG is not available. Pulse oximetry measures continuous changes in oxygen saturation throughout the night and has been shown to have a 97% positive predictive value in children aged over 12 months, however a negative result cannot rule out OSA in as many as 50% of those with a negative result (156). Nixon et al., claim that the overnight pulse oximetry can be used to estimate the severity of OSA and, hence, reduce the diagnostic and therapeutic process in the children that need intervention the most (157). A recent evaluation of the literature by Kaditis, Kheirandish-Gozal and Gozal has highlighted both the positive and negative aspects of using oximetry in children suspected of having SDB (158). They propose that specific guidelines i.e. those using the McGill oximetry score are followed when using oximetry to diagnose SDB severity.

1.5.5 Questionnaires and Parental Report

A comprehensive study by Spruyt and Gozal in 2011, evaluated 183 subjective tools (questionnaires) used to assess sleep and behaviour in children (159). Only two questionnaires, which are currently used worldwide, will be discussed here, however, it is recommended that the aforementioned reference is used for further information of the available tests and their relative reliability/validity.

1.5.5.1 The Sleep Disturbance Scale for Children (SDSC)

The Sleep Disturbance Scale for Children (SDSC) is a questionnaire devised by Bruni et al. in 1996. The SDSC has good re-test reliability and validity (160). It is quick to complete (10 min)

and consists of 26 questions which are then amalgamated to produce 6 subscales that include Disorders of Initiating and Maintaining Sleep, Sleep Disordered Breathing, Disorders of Arousal, Sleep-wake Transition Disorders, Disorders of Excessive Somnolence and Sleep Hyperhidrosis (161). The scores are measured on a 5-point Likert scale and the questionnaire is aimed for children aged 6.5 – 15.3 years. Importantly, the subscales comply with the categories and diagnostic classification of sleep and arousal disorders set by the Association of Sleep Disorders Centres and the Psychophysiological Study of Sleep (159).

1.5.5.2 Quality of Life

Studies have shown that children with SDB have significantly reduced quality of life in all severity types (143, 162) which improves once children receive T&A surgery to resolve the obstruction (163, 164). Quality of life questionnaires have been devised and translated so they can be used worldwide. The Total OSA-18 is a questionnaire completed by caregivers that is specific for SDB, and consists of 18 items grouped in five domains. Scores for each item range from 1-7, with lower collective scores indicating better quality of life. The last question is a global rating for quality of life where lower score equate to lower quality of life. Although these questionnaires are important in expressing the impact of SDB on sufferers and their families, they are not tools that can accurately determine the severity of SDB (143) and are recommended to be used in conjunction with the PSG parameters and questionnaires such as the SDSC.

1.6 SDB Non-Cardiovascular Deficits

SDB in children has been reported to reduce daytime functioning. Excessive daytime sleepiness, neurocognitive impairment and behavioral issues have been shown by parental report and cognitive testing measures (165). Sleep fragmentation and intermittent hypoxia during critical periods of development in early childhood such as during neuronal pruning and myelination of neuronal fibers (6 months - 3 years +) may have long-term consequence to brain

function. (166) For a more comprehensive evaluation of non-cardiovascular deficits, readers are directed to *Appendix 2*.

1.7 SDB Treatment and Management

1.7.1 Adenotonsillectomy (T&A)

Adenotonsillectomy was initially primarily performed to treat recurrent infection from streptococcal tonsillitis. With the increased awareness of SDB in children, it has now become the main initial therapy, with more than 50% of cases performed in the US, for obstructed breathing (167).

In children with adenotonsillar hypertrophy and SDB, T&A is the most effective treatment. Benefits included better sleep quality, daytime behaviour and quality of life. A comprehensive meta-analysis of the literature using PSG to evaluate the efficacy of T&A as treatment for OSA showed that AHI reduced by 12.4 events per hour and oxygen desaturation index decreased by -7.5% per events per hour as did arousal index by 6.1 events per hours (168). Using a random models-effects analysis, researchers showed that 51% of patients had an AHI < 1 and 81% had an AHI < 5 post surgery. Only 34% of obese children reduced the AHI to levels less than one, however this was not significantly different from values reported in the non-obese group. When data was set using an AHI < 5 as an improvement index analysis, only 61% of obese children compared to 87% of non-obese children reduced their respiratory events to this level. Overall, this suggests the efficacy of the treatment benefited most children with SDB, however, consistent with the consensus in the literature that suggests that obesity is a risk factor for ongoing obstruction despite T&A.

Also, published this year are the results of Cochrane ENT Group, which evaluated T&A or just tonsillectomy as treatment for SDB, compared to watch and wait policy or CPAP (169). They

concluded that children who had surgery reported better quality of life and less symptoms compared to those managed by watch and wait. Quality of life remained the same between children who had surgery and children using CPAP at 12 months. The results of the Epworth Sleepiness Scale were similar at 6 months between groups, however, at 1 year the children who received surgery reported lower scores of sleepiness, compared to children using CPAP and watch and wait.

Complications of T&A surgery for paediatric SDB are as uncommon but increased in children with severe SDB compared to surgery performed for other purposes. Delayed discharge has been reported by groups in 1.3% - 2.3% of SDB paediatric patients and 3.2% – 3.7 % have secondary complications requiring readmission (170). Non-respiratory complications are the least reported complications, they include dehydration, hemorrhage and fever. While respiratory complications are reported in 5% - 23% of patients with SDB post operatively and may reflect children who do not immediately respond to the treatment.

In the joint Position paper produced in 2008, by the Paediatric and Child Health Division of the Royal Australasian College of Physicians and the Australian Society of Otolaryngology Head and Neck Surgery, which reviewed current Tonsillectomy and Adenotonsillectomy in children in Australia and New Zealand, it was estimated that the overall adenotonsillectomy rate is approximately 3-7 per thousand, however, the epidemiological data suggested that the rate should be closer to 20 - 30 per thousand. This suggests that Australian children with SDB are severely under-diagnosed and under-treated (171).

1.7.2 Nasal steroids and Antileukotrienes (Anti-Inflammatories)

Nasal steroids have been recently introduced as a treatment for SDB in children as it can reduce the size of the adenoid and tonsils. The efficacy of nasal spray therapies, however, is

questionable. One study showed no change after a 5-day course of prednisone therapy in SDB severity, however, Demain and Goetz reported a reduction in adenoidal size and improved symptoms of nasal airway obstruction after 24 weeks of topical nasal corticosteroid use (172). Brouilette et al., showed that six weeks of regular use of fluticasone, a nasal glucocorticoid spray decreased OAHI by five events per hour in compared to placebo. (173)

Montelukast, a Leukotriene (LT) modifier, acts as a LT receptor antagonist. It is used to prevent the inflammatory response in asthma and allergic rhinitis in young children (174). In an elegant series of experiments Goldbart et al., showed that Montelukast given over a 16 week period reduced both adenoid size (measured using lateral X-ray films) and obstruction (measured using PSG) in children with mild SDB. Furthermore they showed a higher abundance of the LT1 and LT2 receptors in the tonsil tissue of children with SDB compared to non-snoring controls, indicating increased inflammation in this tissue. They hypothesized that LT production in the tonsillar tissue may promote cell propagation signaling pathways and hence lead to hyperplasia of the lymphoid tissues. They suggest that the use of the LT blocker retards to proliferation signaling and results in the reduction of tissue volume. (174)

More recently, Kheirandish et al., showed that the combined use of Montelukast and intranasal budesonide in children with residual OSA (post T &A treatment) was effective in improving and in some cases normalizing the SDB (175).

1.7.3 Continuous Positive Airway Pressure (CPAP) / Bi-level Positive Airway Pressure (BiPAP)

CPAP is the most common treatment utilised in adult SDB, however it is less commonly used in children. It is mainly used when children cannot be treated surgically (some craniofacial

disorders), or for residual OSA when surgery (T&A) has not successfully resolve the upper airway obstruction (176). CPAP involves the application of continuous positive pressure to increase ventilation throughout the night by splinting open the upper airway (177). A continuous flow of air distends the airway via a nasal/nasal oral/ face mask using a small compressor. It acts as a pneumatic splint and maintains airway patency, increases the residual capacity and prevents oxygen desaturation even when obstruction may occur. Studies have shown improvements in PSG parameters, oxygen saturation levels, and reported sleepiness and snoring in children using CPAP (178). BiPAP is similar to CPAP in that it delivers an inspiratory pressure, however expiratory pressure is lower, therefore, reducing respiratory work. Randomised studies have shown no significant difference in benefits or for adherence between methods. CPAP use in children requires ongoing review of pressure as the child grows (176, 179), as the pressure of the mask can cause changes to the growth profile of the midface (180).

1.7.4 Orthodontic Treatment

The association between the craniofacial and orthodontic development with SDB has only recently been appreciated. A narrow upper airway associated with maxillary constriction and mandibular retrusion is common in children with SDB. Some dental treatments are being offered in children with this profile, including oral devices such as rapid maxillary expanders (RME), mandibular retro-positioning and modified monoblock (176). Depending on the degree of irregularity in alignment RME methods recommended include orthodonitic, orthosurgical and surgical expansion. Orthodontic expansion uses a fixed oral appliance and expansion screw anchored on selected teeth. Periodically the expansion screw is adjusted to expand the midpalatal suture causing the maxillary bones to increase their span. This process can take up to 1 year and can improve nasal breathing (176). Recent post treatment studies have shown

that the positive effects of RME are still evident after 3 years, however, residual SDB was evident in nearly 70% of the cohort (181).

1.8 The Cost of Sleep Disordered Breathing

In adults, SDB is a substantial economic burden both to the individual and greater community. Health care utilization is a powerful determinant of morbidity in both adults and children. The increase in early onset cardiovascular morbidity in the adult cohort is coupled with a loss of occupational productivity, decrease in quality of life, increased risk of motor vehicle accident and dependence on social welfare both for living and medical expenses (182). Undiagnosed SDB individuals use twice the amount of healthcare resources, including physician services compared to controls. A cross-sectional study in the US, found that in the year prior to diagnosis, patients with OSA spent $2720 annually, compared to $1384 for non-snoring, age and sex matched controls. Also, they showed an association between AHI and annual medical costs (183).

In children, Tarasiuk et al., reported a similar pattern of overutilization of Canadian health care services prior to diagnosis of OSA in children with almost a two fold increase in annual cost per year compared to control children (184). Children with OSA had 40% increase in hospital visits within the first 4 years and by the second year of life drug prescriptions were 70-200% higher in OSA groups compared to controls (184). The main cost burden increase in children under 5 years was associated with in-patient admissions and emergency room visits, which accounted for 50% of the annual costs. It was suggested by the authors that the costs attributed to lower respiratory tract disease may actually arise from under-diagnosed OSA and that there was a need for earlier diagnosis and treatment.

1.9 Association Between SDB and Cardiovascular Disease

1.9.1 Cardiovascular Dysfunction in Adults (Summary)

The negative effects of SDB on cardiovascular function in adults is well documented. There are many studies that report increased adverse cardiovascular events, such as heart failure, and mortality. A study of 6132 participants, showed that SDB in middle-aged and older individuals was independently associated with systemic hypertension and was evident in both sexes and in different ethnicities (185). It has been reported that adults with SDB are 2-3 times more likely to suffer a stroke compared to community controls (186). A recent meta-analysis suggests that aortic aneurysm and aortic dissections are also higher in adults with OSA (187). Furthermore, an independent association between OSA and increased prevalence of aortic and high-risk carotid atheroma prevalence has also been established in adults with SDB (188). An association was also found between central aortic stiffening and OSA in participants with hypertension independent of obesity and intra-abdominal adiposity. Heart failure is commonly reported in adult patients with OSA and encompasses a range of dysfunction from asymptomatic cardiovascular disease, to left ventricular failure. Changes in diastolic function are also common in OSA and a major risk factor for cardiac associated mortality (28). There is evidence of damage to the minor arteries as Ip et al., showed impaired endothelium-dependent flow-mediated dilatation in peripheral vessels (brachial artery) compared to health aged match controls. In addition, they demonstrated that endothelial function normalized with just 4 weeks of compliance with CPAP treatment (189).

1.9.2 Cardiovascular Dysfunction in Children

A consistent pattern of changes in cardiovascular function and vascular tone is emerging from the limited studies conducted in children with SDB. Cardiovascular measurement in children is limited to non-invasive tests which include, (i) echocardiography to determine both

functional (M mode) and structural changes (2-dimentional) of heart and blood vessels has been used in the majority of cardiovascular studies in children with SDB (190); (ii) blood pressure taken before and after PSG and over 24 hours using an ambulatory system; and (iii) the use of flow mediated dilatation (FMD) a functional measure of arterial endothelial integrity using Doppler Ultrasound.

1.9.2.1 Cardiac Muscle Changes

Early studies of cardiac changes in children with OSA, using electrocardiography, reported varying degrees of right ventricular hypertrophy and cor pulmonale (135). In 1988, Tal and co-workers, using radionucleotide ventriculography, found that 37% of children aged between 9 months and 7.5 years (27 children) with OSA had a significant reduction in right ventricular ejection fraction and that 67% of treatment group had wall motion abnormalities. Both abnormalities improved within 2 months of surgery (191). Gorur et al., using echocardiography (both M-mode and 2D imaging) and overnight oximetry to determine severity, showed structural cardiac anomalies including increased right ventricle mass and left ventricular end-diastolic dimension and decreased left ventricular compliance in 33 children aged between 3 – 10 years with SDB and adenotonsillar hypertrophy. Cardiac structural changes improved significantly in most children after T&A (192). More recently, Ugur et al., using echocardiography and tissue Doppler imaging, showed that the early phase diastolic velocity and the ratio of early to late phase diastolic velocities recorded both at the mitral and tricuspid annulus moving towards the base, away from the apex, were all significantly decreased in children with OSA compared to controls. All parameters normalized 6 months post T&A surgery (193).

Amin et al., were first to show increased left ventricular mass was positively associated with SDB severity (AHI) (194). Further work by this group showed changes in left ventricular

diastolic function (left ventricular filling) in children with SDB, with a dose-dependent decrease in left ventricular systolic performance with increasing severity of SDB (195). Kaditis et al., also showed lower left ventricular systolic function in children with moderate to severe SDB compared to primary snorers (196). Brain natriuretic peptide (BNP) is a cardiac hormone secreted by the ventricular myocardium in response to pressure overload and ventricular volume expansion. Increased expression of this peptide is an indicator of both left and right ventricular overload and ventricular hypertrophy. Brain natriuretic peptide (BNP) is increased in adults with OSA and has recently been shown to be increased in children with SDB with enlarged tonsils/adenoids, compared to control children (197). Kaditis et al., showed that BNP levels were increased in children with OSA compared to primary snorers. The increase in BNP was inversely related to SpO_2 nadir and oxygen desaturation ($\geq$ 4%) (198). Oran et al., also showed a 33% increase in BNP and increased pulmonary arterial pressure in children with severe SDB. Both variables normalized after T & A treatment (197).

1.9.2.2 Blood Pressure

There are now many studies reporting abnormal blood pressure in children with SDB compared to controls or primary snorers. (199-202). (Table 1.4) Amin et al., demonstrated higher 24-hr blood pressure and faster heart rate in children with SDB, compared to matched controls. Changes in blood pressure were associated with left ventricular remodeling. Importantly, they found that the blood pressure changes (elevated morning systolic blood pressure) were evident in children classified as mild SDB (203). Leung et al., reported increased daytime systolic blood pressure and found both systolic and diastolic pressure in children with OSA were also increased during sleep (204). In support of these previous studies, The Tuscon Children's Assessment of Sleep Apnoea study also found elevated resting systolic blood pressure. More recently, postoperative assessment of blood pressure in children with SDB have shown that blood pressure variables normalize in non-obese children after treatment (199, 205). Naiboglu

el al., using Doppler echocardiographic demonstrated that pulmonary arterial pressure increased in children with OSA (severity determined by questionnaire and ENT adenoid/tonsil size assessment) compared to non-snoring controls. All but two children with OSA returned to normal pulmonary arterial pressures after T&As (206). This recent study mirrors the results of a similar study by Yilmaz et al., which demonstrated increased pulmonary arterial pressure in children with OSA that normalized once children had surgery (207). Further supporting the positive cardiac effects of T&A as treatment for SDB in children was the recent meta-analysis conducted by Weber et al., which showed improvement in both pulmonary arterial pressure and cardiac structure 6 months after surgery (208). The studies included in the meta-analysis did not report on the potential confound effects of comorbidities such as obesity and echocardiographic variables, however, both the Tuscon Children's study and a large study, conducted in Taipei, by Kuo et al, showed that blood pressure improvement was limited in obese participants (205).

Pulse transit time (PTT), is a measure of vascular compliance (arterial distensibility) which has been shown to be reduced in adults with SDB. Decreased arterial distensibility is considered a strong predictor of cardiovascular risk in hypertensive adults. Vascular compliance changes are also noted in children. Kwok et al., reported increased daytime systemic blood pressure and reduced arterial distendibility in children with primary snoring compared to healthy age matched control (148). Nisbet et al., more recently measured PTT during different sleep stages and found that children aged 3 - 5 years with moderate to severe OSA had lower PTT during REM sleep compared to primary snorers and mild SDB. The results of the study are somewhat confusing as there was no difference in PTT between normal control children and those with severe OSA (209).

Table 1.4: Listed are blood pressure studies conducted in children with SDB. Some studies have used non-snoring controls, however many have used children with primary snoring. In

addition, blood pressure was measured using different techniques which may account for the differences observed between different research groups.

Blood Pressure Parameters	Reference	Subjects	Measurement
Increased diastolic blood pressure	Marcus et al., 1998 (202)	PS (26), OSA (41) Age not balanced	Oscillometric
Increased systolic blood pressure (morning)	Bixler et al., 2008 (83)	Control (517), Mild SDB (175), M/Severe (8) Age 5 – 12	Oscillometric (triplicate)
Increased systolic and diastolic pressure (morning)	Li et al., 2008	Control (127), Mild (133), M/Severe (46), Age 6 – 13	24h Ambulatory BP
	Li et al., 2009 (210)	Control (56), PS, (46), OSA Mild (88), Age 6 – 13	24h Ambulatory BP
	Kohyama et al., 2003 (211)	AHI < 10 (16) AHI > 10 (7), Age 4 - 11	Oscillometric (triplicate)
	Kwok et al., 2003 (148)	Control (30), PS (30), Age 7 – 13	Oscillometric (triplicate)
	Amin et al., 2004 (212)	PS, (21), Mild (17), M/Severe (22) Age 5 – 17	24h Ambulatory BP
	Guilleminault et al., 2004 (213)	Control (30) SDB (271) Age 7 – 12	Not Stated
	Amin et al., 2008 (203)	Control, Mild, Severe (140 total) Age 7 – 13	24h Ambulatory BP
	Horne et al., 2011 (214) 2013 (215)	Control (36), PS (61), Mild (133), M/Severe (21), Age 7 – 13	Finometer
	Xu et al., 2012 (200)	PS/Mild SDB (38), M/Severe (107)	24h Ambulatory
Increased systolic and diastolic pressure (sleep)	Leung et al., 2006 (204)	Mild (79) M/Severe (17)	24h Ambulatory
	Amin et al., 2008 (203)	Control, Mild, Severe (140 total) Age 7 - 13	24h Ambulatory BP
	Horne et al., 2011 (214) 2013 (215)	Control (36), PS (61), Mild (133), M/Severe (21), Age 7 – 13	Finometer

1.9.2.3 Vascular Endothelial Damage

The endothelium consists of the single layer of cells that line all blood vessels and is the source and target of many growth factors and vasoactive mediators and a key regulator of vascular function (216). Alterations in endothelial function are reported to occur as the first detectable change in the development of atherosclerosis (217) and are associated with a higher incidence of cardiovascular events and increased progression of atherosclerotic disease.

Endothelial dysfunction and arterial wall thickening, the earliest stages of atherosclerosis, begin in childhood. Children with diabetes, (218) obesity (219) or hypercholesterolaemia, (220) who are at significantly greater risk of developing atherosclerotic disease as adults, have detectable endothelial dysfunction and arterial wall thickening, before overt signs of atherosclerosis are apparent (220). In adults with OSA, endothelial dysfunction is a precursor of atherosclerosis and hypertension (217).

Endothelial dysfunction has been reported both in adults and children with SDB (216, 221). Gozal et al. using a modified hyperemic test with a laser Doppler sensor to measure cutaneous microvasculature reperfusion blood flow in the metacarpals demonstrated a delayed return to baseline blood flow (took 40% longer) in children with OSA compared to non-snorers (222). In this same group of children, sCD40L, a marker of inflammation and increased risk of CVD, was elevated in the OSA group. This marker was weakly associated with AHI and SpO_2 nadir. Both SCD40L and vascular function returned to almost normal levels after treatment with T&A. Further to these studies, Kheirandish-Gozal et al. using a similar hyperemic test showed that the time it took to reach peak flow (Tmax) was delayed in children with OSA compared to non-obese non-snoring control children (223). They also noted an association between that inflammatory marker, high-sensitivity CRP and Tmax, implying a relationship between

delayed arterial dilation (cell-to-cell signaling) and inflammation. A modest association between Tmax and circulating endothelial progenitor cells (EPCs), a marker of vascular injury was also demonstrated. Unfortunately, EPCs counts, although lower in the OSA group, were not significantly different compared with control children values. The authors offered the opinion that vascular response in children with the OSA was variable as there was heterogeneity within the group response. They also suggested that the underlying inflammatory and possibly paracrine processes may play a pivotal role in maintaining vascular integrity, suggesting an interaction between the vascular response, ANS function and immune systems. They also proposed that children with delayed vascular results may have a blunted chemokine recruitment response. Also, adding to the current controversy with severity measures derived from the PSG and their inability to predict vascular morbidity, none of the PSG derived variables were associated with any of the vascular markers outlined above to be present in the OSA group.

1.9.2.4 Flow Mediated Dilatation (FMD)

The most widely used non-invasive test of endothelial function using high resolution ultrasound is flow mediated dilatation (FMD) (224). FMD measures brachial artery responses to a mechanical increase in blood flow, which induces an *endothelium-dependent* increase in vessel diameter through the release of endothelial derived nitric oxide. The most basic form of the test requires the use of a linear ultrasound Doppler transducer to image the brachial artery, 10cm above the elbow during rest to determine a resting brachial artery diameter. This is then followed by a resting image in the B-mode, to determine resting blood flow velocity (peak systolic velocity and velocity time integral), and resting heart rate. The blood flow to the forearm, is occluded using a blood pressure cuff, so as to induce hypoxia in the tissue distal to the ischemic site. The occlusion remains for 4 minutes in children (5min in adults) causing vasodilation and decreases vascular resistance in the distal vessels. The cuff is then released

and the reinstated blood flow induces hyperemia and increased shear stress, which activates the mechanoreceptors on endothelial cells at the site of imaging. The activation of mechanoreceptors releases nitric oxide (NO), which instigates the dilation process. The dilation response is recorded and, traditionally the 60s time point post cuff deflation is used in comparative tests to determine differences in endothelial function (Figure 1.4). This suggests that the response is dependent on an intact endothelium, and the release of NO, however, more recent studies question the validity of this belief (225) (226, 227).

Traditionally, endothelial dysfunction is reflected by an impaired FMD response at 60s, however studies looking at the impact of different stimuli (increased and decreased time during ischemia; placement of the cuff) (227) suggest that the endothelial dependent mechanism (NO release) involved in the dilatation is only partially responsible for the recorded dilatation response. This opens the possibility that mechanisms other than NO endothelial release are differentially affected in varying diseases e.g. diabetes, hypertension (226).

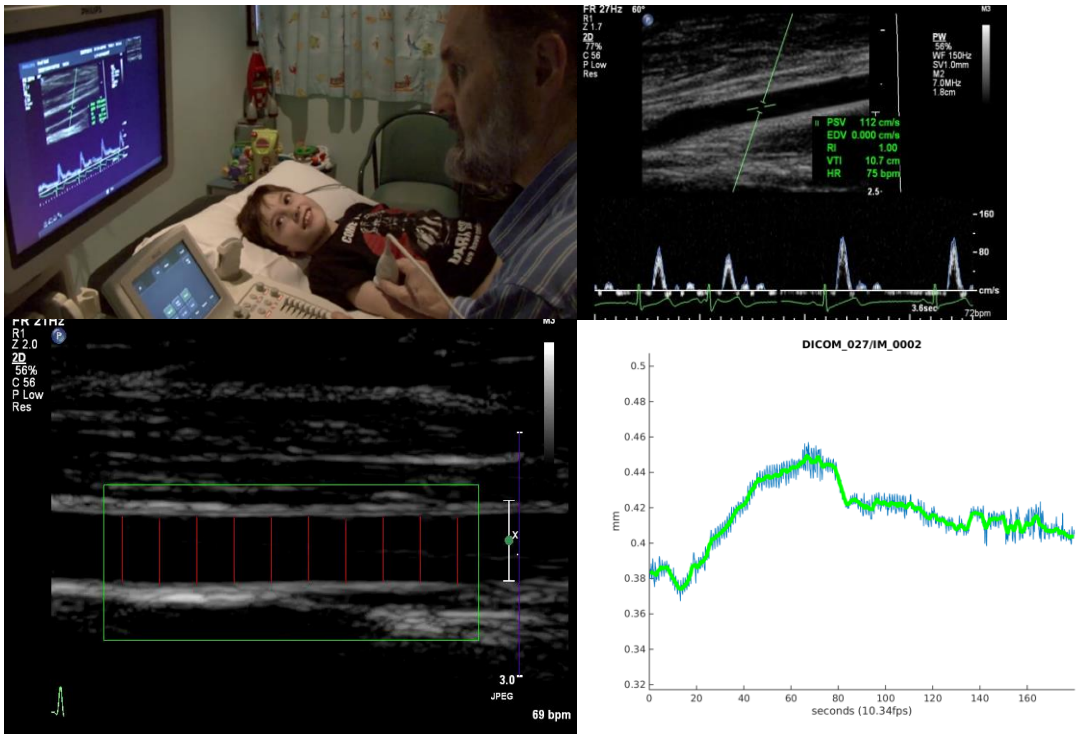


Figure 1.4: Top Left: FMD is a non-invasive procedure that uses Doppler ultrasound to measure changes in brachial artery diameter after a post-ischemic reperfusion. Top Right: Image of the brachial artery during B-mode functional evaluation of blood flow parameters which include peak systolic velocity (PSV), velocity time integral (VTi, area under the curve, peak systolic velocity x ejection time), heart rate (HR). Bottom left; the brachial artery, red lines measure the luminal diameter at ten sites per frame, over three minutes (15 seconds pre-cuff release and 165 seconds post). Bottom Right; shows the data vessel diameter over time (180s). Variables determined from the data include, time to maximal dilation (s), maximal dilation (largest dilation value – baseline value/baseline x 100 (%)) and FMD at 60 seconds, the percentage difference from baseline at 60 seconds (%).

Although the exact mechanisms of FMD are currently being debated, the consensus is that FMD reflects the arterial capacity to self-regulate blood vessel tone in response to localized stimuli such as the increase in a physical or chemical activator and can be used to determine differences in vascular dilatation response. A study in adults with OSA with no clinical evidence of cardiovascular disease, using *in vivo* vascular endothelial cell harvesting and FMD measurements reported significantly decreased expressions of proteins that regulate basal NO production, reduced FMD and endothelial progenitor cells (EPC), all markers for endothelial function. Conversely, nitrotyrosine and cyclooxygenase-2 (COX-2), measures of oxidative stress, were significantly greater in patients with increased respiratory events during sleep (228). This suggests that there is damage of the endothelium and NO release in adults with OSA even if there is no physical evidence of cardiovascular disease.

Recently, four groups, two from China and two from Italy, showed reduced dilation response using flow mediated dilation (FMD) in children with SDB. Chan et al., showed reduced dilatation in children aged between 6 – 18 years. The percentage diameter difference from baseline was 0.4%. This reduction normalized 6 months after T&A surgery (229). In normal children the average dilatation change from baseline diameter is 0.01mm (8%). A mean difference of only 0.4% would equate to a diameter difference between groups of only 0.005mm, which is within the margin of error for the test. In children with diabetes and/or obesity the difference from baseline is approximately 4%, which is considered evidence of endothelial pathology. In a recent study, Li et al., measured FMD in children ranging from 6 -18 years. They found significantly reduced dilation at hyperemia (dilation of vessel after just after cuff removal) and at 60 seconds, (the time point considered to be the maximal NO dependent dilation response) in primary snorers (230).

Loffredo et al., also recently showed reduced FMD in both children with OSA and primary snoring compared to non-snoring children (231). The reduction in FMD, approximately 4%, was similar to those shown by other groups measuring endothelial dysfunction in obese children (232). This group was also able to demonstrate an inverse relationship between reduced FMD measurement and enhanced NADPH oxidase (SNOX2dp), which is essential for modulating endothelial function and an indicator of oxidative stress (231). T&A in the children with SDB normalized these variables six months after surgery.

Collectively, these studies suggest that the cardiac structure and function and vascular function in children with SDB is altered and that the current treatment, T&A, may normalize these changes.

1.10 Arterial Compliance – Why Do The Arteries Stiffen? - Cardiovascular Autonomic Interactions

1.10.1 Vascular Physiology

Given the evidence of both cardiac and vascular function changes in both adults and children with SDB, understanding how the increased SNS activity modulates the decrease in vascular compliance and ventricular hypertrophy is the focus of the next section. A brief description of the vascular anatomy is supplied however a more in depth description can be found in Chapter 8, 'The Autonomic Nervous System and its Effectors', Brading 1999 (42).

The aorta and other arteries are made up of three distinct layers, the intima, media and the adventitia (Figure 1.5 & 1.6). The intima consists of the endothelial cells that line the lumen and the elastin layer (tunica) adjacent to these cells. It is surrounded by the medial layer which contains smooth muscle cells, little elastin and some collagen fibres. The outside layer, the adventitia is mainly composed of collagen, small amounts of elastin and precursor cells such

as fibroblasts and myofibroblasts. Also in this layer are immune cells such as monocytes and macrophages. Most arterioles contain a single layer of smooth muscle cells in the media, however some larger arterioles may have more layers of smooth muscle cells.

1.10.2 Vascular Properties, Windkessel Effect and Blood Flow

The large arteries proximal to the heart contain the largest amounts of elastic fibres with up to 40% composition of elastin contained in the thoracic aorta. As arteries radiate to the periphery they progressively reduce the composition of elastin and increase the contribution of smooth muscle cells and collagen fibres and, hence, are classified as muscular arteries. The elastic properties allow the vessels to distend during systole, when intraluminal pressure is increased, acting as an 'elastic buffering system' (233). As the left ventricle ejects approximately 60-100mls of blood into the aorta, 50% of the stroke volume is propelled through the arterial system, while the remaining 50% is absorbed/stored by the distention of the artery. During diastole no further blood is ejected and the aortic valves close, aortic pressure drops, the vessel recoils and its elastic properties forces the absorbed volume into the circulating blood flow, forming an almost complete continuous flow despite the rhythmic actions of the heart. This elastic function was named the Windkessel function by Weber in 1827. Another important factor in maintaining blood flow is the capacity of the vessel to transfer the pulse wave during systole, where it reflects with the peripheral circulation (at points of anastomoses) and returns to the aorta during diastole forming a dicrotic wave, which is dampened by the Windkessel function.

In the clinical situation, reduced vascular compliance and stiffening and, hence, a loss of Windkessel function results in an increase in systolic blood flow velocity and pressure, increased shear stress at the luminal surface, a decrease in diastole pressure and increased left ventricular afterload and drop in subendocardial blood supply during diastole. Reduced

vascular compliance also affects the pulse wave velocity, which increases and the reflective wave then shifts from early diastole to reverberate during late systole, further affecting systolic pressure and afterload (233). The heart has to then compensation for the increase in flow velocity by accelerating the blood mass of the stroke volume within the aorta and arterial system to match the returning venous blood flow. Hence a deviation from optimal vascular resistance and vascular capacitance, via vascular remodeling, has pathological consequence on the cardiovascular system as a whole (23, 233).

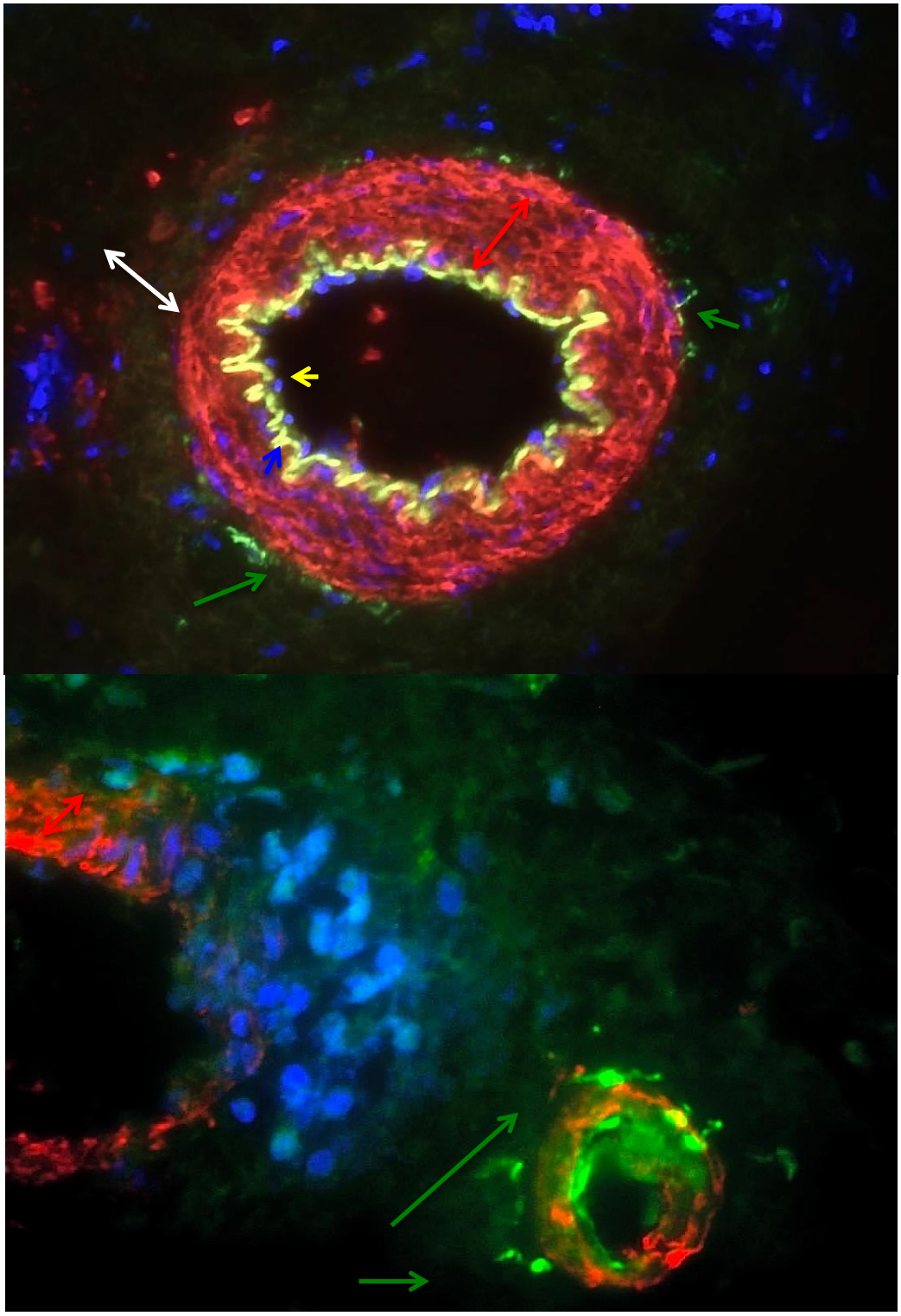


Figure 1.5: Top cross section of the dorsal lingual artery, shows nuclei of the endothelial cells (yellow arrow, blue dots) on the inside of the lumen, the intima layer (blue arrow) consisting mainly of elastin and shows fenestrate (convolutions in the layer), the media consists of smooth muscle cells, (red arrows), and adventitial layer (white arrows). Note the blue nuclear staining in the adventitial layers are a composite of different cells some are fibroblasts while others with duel staining with smooth muscle actin are myofibroblasts. Other cells in this layer include immune cells, including macrophages and monocytes. Green arrows indicate sympathetic nerves fibres which are immunoreactive to tyrosine hydroxylase. Note in the lower image that the small vessel cross section shows high TH immunoreactivity. Control of tissue perfusion occurs mainly via the activation of sympathetic nerves on smaller arteriole vessels.

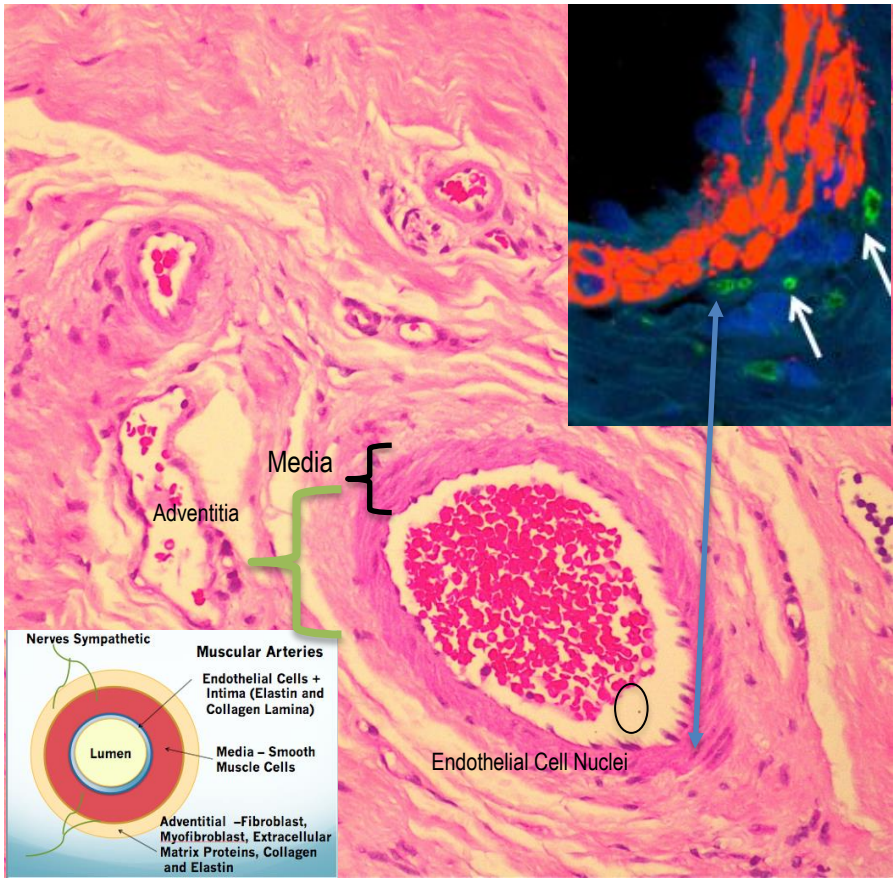


Figure 1.6: Shows H&E staining of the dorsal lingual artery of the left tonsil. Note the compact layer of smooth muscle cells (black bracket) aligning the endothelial cells (black circle). *Top right insert* shows the same vessel (next section stained with immunofluorescence for tyrosine hydroxylase, green (sympathetic nerves), nuclei stain, blue (precursor cells, fibroblasts and myofibroblast and immune cells) (white arrows), smooth muscle actin, red, medial layer. Blue arrows shows the same area in the previous section done in H&E staining. *Bottom left insert*: Schematic of an artery, note the three layers, intima, media and adventitia.

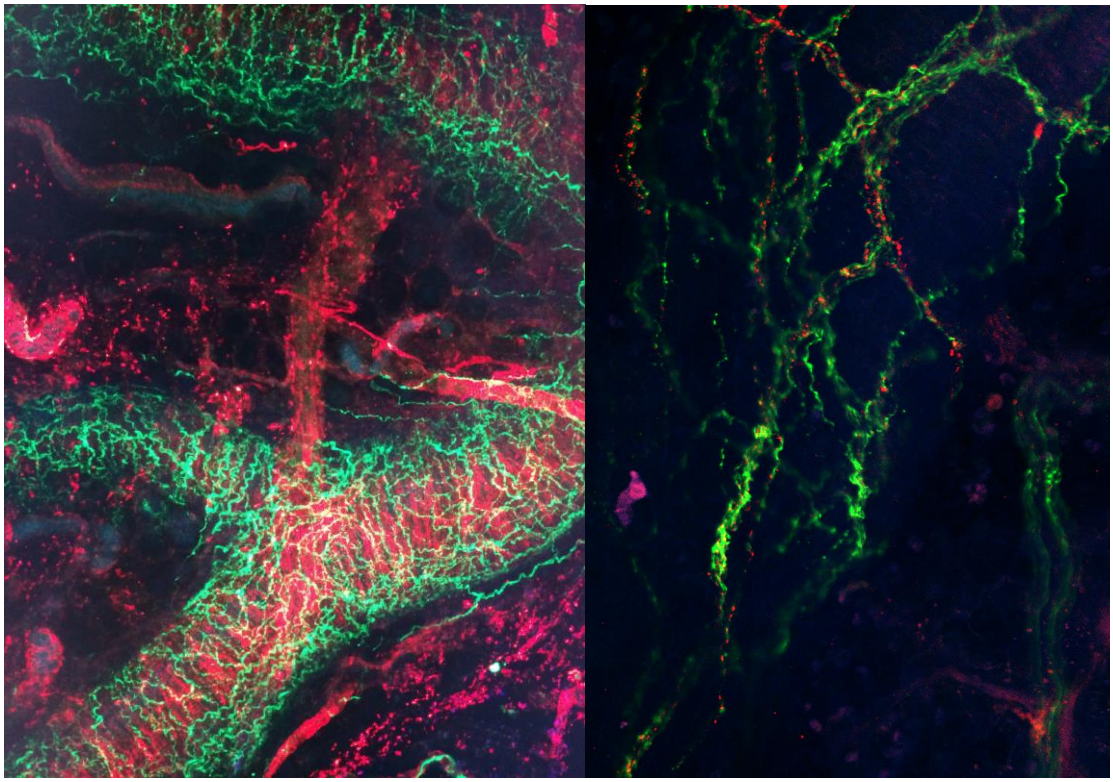


Figure 1.7: Left is the dorsal lingual artery, red stain is smooth muscle actin, green is tyrosine hydroxylase, (sympathetic nerve fibres) (50x). *Right* is a collateral of the dorsal lingual artery, green stain tyrosine hydroxylase, red stain is Calcitonin Gene Related Protein (CGRP) a potent vasodilator (200x).

1.10.3 Blood Vessels, Sympathetic Adrenergic Innervation and Vasoconstriction

Many factors influence the degree of vasomotor function of blood vessels. They include the intrinsic myogenic tone in the smooth muscle cells, the effects of locally produced and circulating vasoactive substances and the degree of activation of the nerves innervating the smooth muscle. The innervation to the vascular smooth muscle will be covered in the following paragraphs.

Almost all blood vessels are innervated by postganglionic sympathetic adrenergic nerves, however some vascular beds also have a preganglionic parasympathetic innervation. The

sympathetic nerves cell bodies are located in the paravertebral ganglia of the sympathetic trunks and the post ganglionic fibres exit either by (i) the sympathetic trunks into the spinal nerves, where they supply the blood vessels to the limbs, or (ii) sympathetic nerve fibres accrue also along the arteries where fibres emerge with the paravetrebral ganglia to form plexuses around the arteries and supply the branches to the vascular smooth muscle (42).

The sympathetic adrenergic nerves run along the adventitia layer and varicose terminal branches infiltrate the smooth muscle layers, with the majority of innervation occurring on the outer layer of smooth muscle cells (Figure 1.7) (42). These nerves release noradrenaline (NA), which activate post-synaptic receptors. The typical activation results in slow contraction of smooth muscle that spreads passively through interconnected gap junctions from the outer towards the inner smooth muscle cells. As well as NA, a number of peptides have been shown to be expressed by postganglionic neurons, including ATP, neuropeptide Y (NPY) and, in some cases, adrenaline. This suggests that the sympathetic nerves are comprised of different subgroups, which modulate the vasomotor function via co-transmitter release. The activation of these nerves can also be influenced by local factors, for example when circulating adrenaline levels are high, sympathetic nerves terminals can re-package the neurotransmitter and release it when it is required during prolonged stress response. Conversely, in some vascular beds acetylcholine released from inhibitory nerves can counter the effects of the sympathetically mediated vasoconstriction and promotes vasodilation.

Contraction of the smooth muscle alters vessel wall compliance more than narrowing of the lumen and hence vessels can have the same luminal diameter but have less distensibility (42). Vascular SNS activation plays a major role in controlling blood pressure and blood flow when other factors have caused alterations in flow to individual vascular beds, e.g. increased metabolite production. The largest influence on peripheral resistance occurs with the

sympathetic activation at the level of arterioles (Figure 1.6), and also at the level of veins to ensure correct venous return. Even at the capillary level, SNS activation has been shown to play a pivotal role in controlling blood flow in the brain (234), but it is unclear whether the same mechanism works in peripheral capillaries.

At rest, the post ganglionic vascular sympathetic nerves are tonically active, and cause periodic spontaneous vasoconstriction and reduced vascular compliance, but most contraction occurs in response to increased activation mediated as a baroreflex response (42). In some vascular beds activation of vascular sympathetic nerves results in vasodilation, such as the case in brown adipose tissue (42). This dichotomy in function arises because of (i) the combined activation of different adrenergic receptor types, some of which promote constriction ($\alpha 1$, and $\alpha 2$) while others ($\beta 2$ and $\beta 3$) promote dilation, (ii) presynaptic receptors that can exaggerate or dampen the post synaptic response by modifying neurotransmitter release (both in pre-ganglionic and post ganglionic synapses) (iii) the co-release of other peptide neurotransmitters, such as Calcitonin Gene Related Protein (CGRP) in post-ganglionic neurons (Figure 1.7) (42). Collectively, these factors modulate the effect of sympathetic activity in specific vascular beds and depending on the rate of activation may influence the composition of these receptor types and peptide production/release by terminals at these sites.

1.10.4 Vascular Remodeling

As research in both adults and children have highlighted the increased risk of cardiovascular issues and in particular hypertension associated with SDB, the following section will focus on vascular remodeling, which is a proposed mechanism linking hypertension with autonomic dysfunction.

Vascular remodeling, recognized for the past 40 years is an adaptive ubiquitous process which occurs in response to changes in blood flow dynamics, neuronal/endocrine function (sympathetic overactivity, renin-angiotensin system (RAS)) response and vascular injury (penetration, trauma – counter coup concussion etc.) (235). In hypertension there is evidence of remodeling in both large and small arteries (236). There are two types of remodeling, the first is '*inward eutropic remodeling*' where both the lumen and outer diameters of the small arteries are decreased but the media/lumen ratio is increased and the overall cross-sectional area of the media is unaltered. The second, '*hypertrophic remodeling*' occurs when there is increased media thickness that encroaches on the lumen and hence increases the media cross-sectional area and media/lumen ratio (normally observed in larger vessels) (236). In mild hypertension, there is evidence that structural alterations, by way of increased medial thickness to lumen diameter ratio, in the resistance vessels precedes endothelial dysfunction and cardiac remodeling (236, 237) and may be the first indicator of organ damage. All participants in a study looking at stage I hypertension exhibited small arterial remodeling, while only 60% had evidence of endothelial damage, and 45% left ventricular hypertrophy (236). In the follow-up study of these patients those with the highest media:lumen ratios reported increased incidences of cardiovascular events (237). This suggests that endothelial damage reported in hypertension may be secondary to small arterial remodeling and that the remodeling may have better prognostic significance.

In vitro studies have shown that prolonged vasoconstriction to small arteries results in inward eutrophic vascular remodeling (238). The proposed mechanisms that promotes inward eutrophic remodeling are not well defined, however, a combination of growth, fibrosis and peripheral apoptosis and/or 'vasoconstriction embedded in an expanded extracellular matrix are thought to be involved' (237, 239). Given that increased ongoing sympathetic activity has be reported in both adults and children with SDB, it may be the case that, especially in children,

early signs of cardiovascular disease is occurring in the minor arteries by way of inward eutrophic remodeling.

1.10.4.1 Growth, Fibrosis (Collagen), Peripheral Apoptosis and the Medial - Adventitial Layer

The role that the adventitial layer plays in vascular remodeling and hypertension has only now been recognized. The adventitial layer of arteries plays an integral part in arterial growth as cell differentiation of fibroblasts to smooth muscle cells (SMC) and the production of extracellular matrix proteins (ECM, (collagen and elastin)) occur here. Vascular motor tone is also mediated via interactions between the peripheral vascular SMC and sympathetic nerve terminals which infiltrate the adventitial and also interact with 'progenitor' cell types (fibroblasts and myofibroblasts) (240) and ECM (241).

Sympathetic activity promotes hypertrophy of SMCs in the medial layer, via the activation of α1-adrenoceptor (242) and its interaction with other cells in the adventitia. Angiotensin II (Ang II), the effector molecule of the renin-angiotensin system (243), is a potent vasoconstrictor and is also a strong promoter of smooth muscle cell proliferation. Ang II also increases smooth muscle cell size and ECM protein synthesis and is considered an indicator of vascular remodeling (244). Whether the Ang II's hypertrophic effect occurs directly with action to the smooth muscle cells or indirectly as a result of its action as a vasoconstrictor and potentiator of sympathetic activity is yet to be determined (244).

Also, activation of α1-adrenoceptor promotes adventitial fibroblast proliferation. In adventitial fibroblasts and myofibroblasts the production of ECM proteins is increased when exposed to noradrenaline (NA) (245) and influences both SMC and adventitial fibroblast migration and movement towards the inner vascular layers (246). Hence, increased sympathetic activity may

promote greater smooth muscle cell proliferation, more collagen and elastin production and compact the layers of the arteries, from the outside, eventually moving inward in the smaller arterioles. This over time causes the reorganization of the layers and effects the temporal ability to dilate SMCs during hyperemic stress, possibly by hindering the propagation process involving cell-to-cell communication through the layers of smooth muscle cells and promote reduced arterial dilation capacity. Increased fibrosis at the medial/adventitial layer may also be responsible for the peripheral apoptosis reported in inward eutrophic remodeling, by interrupting SMC connectivity.

The major component of arterial walls is collagen, making up 20-30% of vascular protein. Collagen is a fibrillar component that can change the passive pressure and diameter in arteries and results in stiffening of the vascular wall. In vascular injury, adventitial collagen can increase to in excess of 50% of the vessel wall and in hypertension vascular fibrosis via the deposition of extracellular matrix proteins, especially collagen which is a major contributing factor (239). There are five types of collagen found in blood vessels and an important aspect of collagen is its orientation and organization and the ratios of the subtypes, which are influenced by resident cells, vascular smooth muscle, and fibroblasts which can compact and organize collagen (247). In rat models of hypertension there are reports of increased collagen synthesis in the arterial wall (mesenteric arteries, cerebral microvessels, pial arteries and basilar arteries) and collagen was also increased in the subcutaneous resistance arteries of humans with essential hypertension (239).

Another important aspect of the outer vessel layer is the interaction between adventitial fibroblasts, Endothelin 1 (ED1) and collagen, which may also explain the arterial stiffness observed in hypertension. Adventitial fibroblasts are involved in the production and release of

ED1 when stimulated with Ang II (248). The release of ED1 invokes contraction in smooth muscle and also contracts adventitial collagen fibers (249), promoting collagen type I proliferation, the production and release of reactive oxygen species (ROS) in the adventitia and attracts immune cells into the outer vessel wall. Collagen may stiffen the arterial wall and hence increase total peripheral resistance by (i) encroaching the inner layers of the arteries and reducing the luminal diameter as it increases in volume or (ii) by reducing its compliance by contracting its fibres in response to ED1 or (iii) both.

The adventitia has been shown to be the primary site of ROS production in different animal models and ROS can affect endothelium-dependent relaxation by reducing the efficacy of NO and the number of endothelial cells (248). In addition, increased infiltration of inflammatory cells in the adventitia layer of both large and small arteries has been observed in hypertension. Together this suggests that in hypertension, oxidative stress and inflammation, possibly mediated via the actions of the sympathetic overactivity and its role in Ang II production, may promote vascular remodeling and stiffening in both large and small arterial vessels. Furthermore it links the association between increased sympathetic activity and the possible activation of inflammatory pathways.

1.10.4.2 The Effects of Increased Sympathetic Tone on Vascular Function

Increased vasoconstriction and/or reduced compliance results in an increase in haemodynamic pressure, shear stress and blood flow velocity, all of which associated with vascular remodeling. Blood flow velocity increase is considered the most powerful promoter of vascular remodeling as it influences the production of extracellular matrix proteins, cell proliferation, apoptosis and low grade inflammation in all three layers (236). Regulation of flow is maintained by endothelial derived dilation mechanisms including increased release of ATP or substance P to counteract the increase in luminal shear stress (250). This results in the release

of nitric oxide, a potent vasodilator and decrease in the release of endothelin-1 (ED1) a potent vasoconstrictor (251), relaxing the contraction in the smooth muscle cells and causing the arterial diameter to increase, thereby reducing blood flow velocity and dissipating luminal shear stress.

1.10.4.3 Downstream Effects of Increased Sympathetic Tone - Shear Stress

Prolonged systemic increases vasomotor tone, reduces vascular distensibiliy and also increases total peripheral resistance. This results in increased shear stress at the luminal surface. *In vivo* studies show that increased sheer stress from haemodynamic forces modifies endothelial cell structure and function (252). These induced changes include increased macromolecule permeability and expression (lipoprotein); leukocyte adhesion and recruitment near bifurcations and altered endothelial cell/nuclei shape (ellipsoidal) in the direction of flow in regions of laminar flow and a disrupted cell pattern at regions of disturbed flow. *In vitro* studies in cultured monolayers of endothelial cells have shown direct evidence of force and time (prolonged increased force exposure) induced shape changes in cell-shape and alignment, which are reversible when force is normalised (253). These changes have also been shown to influence actin-containing stress fibres and cytoskeletal components of endothelial cells. Biomechanical force applied to endothelial cultured cells has also been shown to up-regulate prostacyclin; growth factors, coagulation and fribrinolytic components and extracellular matrix proteins (EMC) and vasoactive mediators including Ang II (253). The interaction between haemodynamic pressure and endothelial cells plays an important part in vascular remodeling, angiogenesis, regulating growth, inflammation and the coagulation pathway (254). However, whether the changes to the endothelial function occurs secondary to changes in the medial and adventitial layers by way of the stiffening and reduced compliance (Windkessel effect) needs further evaluation.

Increased sympathetic overactivity has been described in all degrees of severity of SDB in childhood and may result in increased peripheral resistance and, thus, promote vascular remodeling in children with SDB, making them vulnerable to systemic hypertension over time. Chronic vasoconstriction precipitates low-grade inflammation and vascular fibrosis and promotes Ang II and growth factors to smooth muscle proliferation and extracellular matrix remodeling. These changes may alter cell attachment via the accumulation of extracellular fibrillar components and contribute to changes in both the cellular and vessel wall architecture which may lead to abnormal intracellular transduction to the smooth muscle cells, loss of gap junction connectivity and also contribute the restructure of the medial layer. It may also significantly alter the composition of the adventitial layer surrounding the vessel and lead to inward eutrophic remodeling. Over time these changes make the vessels more contracted, compact, and rigid, and less sensitive to required adaptive changes in pressure load, resulting in increased shear stress and damaging endothelial cells and compromising baroreflex sensitivity and the vessels ability to accommodate an increase in shear stress. What started as an adaptive process to meet the homeostatic requirements (O_2/CO_2 balance) may overtime develop into a maladaptive systemic phenotype that compromises vascular function and thereby effects the developing vessels and contributes to the cardiovascular disease state reported in adults with SDB.

1.11 Sources of Sympathetic Overactivity

As previously mentioned, SDB is associated with increased respiratory effort, sleep fragmentation and intermittent hypoxia, all of which cause surges in sympathetic nerve activity. The increase in sympathetic activity causes transient peripheral vasoconstriction, increasing peripheral vascular resistance and cardiac output leading to increase blood pressure (255, 256) (Figure 1.8). This next section will focus on the impact that respiratory effort, sleep

fragmentation and hypoxia have on vascular development and autonomic function, in particular the role they play in sympathetic overactivity.

1.11.1 Respiratory Effort

Obstruction to the airway results in an increased ventilatory response with the generation of large pleural pressure swings (19). It has been suggested that the afferents from mechanical receptors in the thorax and upper airway contribute to the increase in sympathetic activity and are responsible for the vascular changes observed in SDB (19). Interestingly, the respiratory rhythm pattern generator is centrally coupled to the activity of pre and postganglionic arterial sympathetic nerves, which exhibit intermittent bursts of varying amplitude and frequency (19, 257) (258). Therefore, increased effort generated by the negative intrathoracic pressure during inspiration required when the airway is obstructed, even partially, changes the sympathetic firing rate on pulmonary arterial vessels. Changes to the vascular sympathetic firing rate influences the contraction of the vascular smooth muscle cells and has been characterized in cardiovascular disease, including hypertension and heart failure.

In hypertensive rats exposed to increased sympathetic activity, there is evidence of amplified sympathetic respiratory coupling (259) and a 2-3 increase in action potential per respiratory burst. In recent modeling of this phenomenon, Briant et al., suggested that the repeated respiratory modulation in burst pattern produced a significant increase (10 fold) in contraction of vascular smooth muscle cells (258). This suggests that the changes in breathing pattern are directly associated with modulation of sympathetic activity on arterial vessels and that the level of vascular compliance is directly associated with changes in the way we breathe. Over time the ongoing activation results in long-term facilitation (LTF) induced in the phrenic motor neurons (sustained change) independent of changes in chemoreceptor activation (260). It has been suggested that respiratory LTF may thereby induce sympathetic LTF and contribute to

the development of hypertension. Repeated alterations to the respiratory generator pattern will over time change both the structure and, hence, function of the sympathetically mediated vessels and may account for the increase in pulmonary arterial pressure previously reported by researchers in adults and children with SDB. This supports the idea that snoring alone, which is evidence of resistant airflow and hence increased respiratory work, with no classically defined obstructive events, is enough to increased sympathetic activity at the vascular level. The resistance in airflow and increase in respiratory work also alters the cardiac rhythm, resulting in an increase in left ventricular afterload and a decrease in left ventricular preload. Together the net effect reduces stroke volume. Coupled with the intermittent vasoconstriction arising from vascular sympathetic activity, it further increases the afterload burden on the left ventricle. Unsurprisingly, left ventricular hypertrophy is commonly reported in patients with OSA (28).

The increase in respiratory effort also has flow on effects in the aorta where the increase results in increased aortic blood pressure that stretches the aortic wall, where blood pressure surges and shear stress on the vessel wall are the highest and, in response, promoting dilation (187). The increase in systolic pressure has been shown to accelerate the fragmentation of fibrin and collagen accumulation in the vessel wall, promoting aortic stiffening. The responding stretch (shear stress, and/or dilation) is thought to play an important role in the genesis of atherosclerosis.

Most, but not all, respiratory obstructive events result in a cortical arousal to restore pharyngeal patency and protect the body from prolonged hypoxemia (261). Whether the arousal results from hypoventilation or upper airway narrowing and occlusion is unclear. In dogs, arousal from

SWS is significantly delayed when the carotid bodies were removed, reaching desaturation levels of < 60%, (262). This does not explain the occurrence of arousals, which occur at the end of brief apnoeas or hypopneas that do not affect blood gas concentrations. One suggestion is that mechanoreceptors in or near the airway are activated by increased respiratory drive. This theory was test by Yasuma et al, in dogs. They tested the effects of respiratory load during isocapnic hypoxia (induced by rebreathing) and found that arousals also occurred at high SaO_2 levels in the loaded condition, suggesting mechanoreceptor activation also mediated arousals (263). This suggests that increased respiratory drive alone can cause surges in vascular sympathetic activity, independent of chemosensitivity and total obstructive events.

1.11.2 Hypoxia

Acute and chronic intermittent hypoxia (IH), as observed in post obstructive events, trigger peripheral chemoreceptors, which initiate an immediate cardiovascular and respiratory response that includes an increase in blood pressure and sympathetic activity, and persists after hypoxic events have ceased. This effect has been shown to still be present 60 minutes after hypoxic exposure and is independent of respiratory drive (62).

Osani et al, demonstrated reduced respiratory drive and chemosensitivity in adult participants with OSA in response to a hypoxic withdrawal test (264). Subject were asked to breathe air from a tube that was lower in O_2 and higher in CO_2 than normal atmospheric concentrations. This induced a mild hypoxic/hypercapnic state. When participants used domperidone, a dopamine antagonist, it resulted in an increase in hypercapnic ventilation (CO_2 increased) and hypoxic withdrawal response (O_2 decreased) response in OSA patients compared to controls. Dopamine is proposed to reduce chemoreceptor sensitivity and suggests that adults with OSA may have abnormal dopaminergic mechanisms (increase in ‘dopamine’ production and circulation/neural circuitry activation/receptors). They also showed a negative relationship between awake hypoxic ventilation response and severity of desaturations during sleep in OSA

patients. Researchers suspected that 'frequent asphyxia' during sleep may result in adaptation and hence blunting of chemoreceptors. Whether the reduction in chemosensitivity occurs as an adaptive response to preserve sleep continuity or is an anomaly of the chemoreceptors themselves remains to be determined.

Chemoreceptor activation also produces vascular changes by releasing a number of factors in to the blood. They include vasoconstrictors Ang II, circulating catecholamines and vasopressin. In addition, vasodilators such as NO are thought to be released to counter the effects of vasoconstrictors and hence decrease the effect on blood pressure. This has been demonstrated in rats, using telemetry, which shows that blood pressure initially increases to IH and is quickly followed by both hypotension and bradycardia. This also demonstrates the synergistic relationship between the chemoreflex and baroreflex (62).

Exposure to IH during early development in rodents has also demonstrated that the effects predispose animals to early hypertension as they mature even when the IH is limited to the early weeks of life. Chu et al., demonstrated the deleterious effect of intermittent hypoxia on mice pups exposed for the first four weeks of life (265). They noted stunted growth in the first 4 weeks of development but that after cessation of intermittent hypoxia weight gain increased to normal standards. After 14 weeks (10 weeks post intermittent hypoxia exposure) mean systolic blood pressure (24 hours) was higher in previously exposed mice but no differences in both diastolic and heart rate were observed compared to non-exposed animals. Baroreflex measured using the sequence analysis technique and heart rate variability (SD1 and SD2 from ECG) were also compromised while awake and during sleep in the exposed group. Laser Doppler post-ischemic reperfusion response was also reported to be altered at 12 weeks. Ang II protein and angiotensin-convertin enzyme mRNA where also increased suggesting the

dysregulation of the RAS. This is an important group of experiments as it suggests that the early exposure to hypoxia, at least in rodents, leads to sustained cardiovascular changes that do not normalize once exposure has ceased.

Lin et al., demonstrated significant structural remodeling in the Nucleus Ambiguus and its connections to primary neurons with a significant reduction in the cardiac ganglia after exposure to IH (266). In addition, they described reorganization of vagal efferent projections to the Nucleus Ambiguus with evidence of swollen axons, enlarged terminal varicosities, terminals which did not form close contact with primary neurons in the cardiac ganglia. These morphological changes are considered to be 'anatomical substrates' for ANS dysfunction. Authors proposed that these central changes may precipitate remodeling in peripheral ganglia including cardiac neurons and terminals in the heart.

1.11.3 Sleep Fragmentation

Whether the adverse cardiovascular changes noted in adult SDB are solely the result of the ongoing effects of bouts of oxygen deprivation and resulting changes in metabolite concentrations (e.g. increased lactate) throughout the night or whether it arises from autonomic dysfunction associated with increased arousals which in most cases (75%) (267) occur in tandem with respiratory obstructive events to restore airway patency has been debated. Guilleminault was first to speculate that habitual snorers, who showed little if no evidence of obstruction other than the snoring, may have cyclical increases in upper airway resistance culminating in arousals from sleep, thereby causing sleep fragmentation and daytime dysfunction (268, 269). Since then, research assessing the effects of sleep fragmentation without evidence of abnormal O_2 desaturation has suggested that the loss of sleep continuity has a deleterious effect on daytime functioning and may adversely impact the cardiovascular system (270).

Arousals during sleep are abrupt, transient, reflex responses in the CNS that are evident on the EEG and coincide with increases in autonomic parameters including heart rate, cardiac contractility, blood pressure, and ventilation (271). The American Association of Sleep Disorders (ASDA) definition of an arousal in the EEG trace is characterized when a sudden frequency change towards faster rhythms (alpha, beta and theta) occurs momentarily and replaces the previous sleep stage. In normal humans, the average duration of these events is 15s occurring often throughout total sleep time (272) and increasing in frequency with age (273). Arousal pathways are evoked by both external (acoustic) and internal (reduced oxygen saturation) stimuli. There are also 'autonomic arousals', where there are transient increases in autonomic parameters but no cortical changes on the EEG (274). These are often referred to as 'subcortical arousals'. Similar to the 'startle and orientating' response in the awake state (275), an arousal during sleep also involves a similar patterned response which includes vasodilation in skeletal muscle and vasoconstriction in the splanchnic area, kidneys and cutaneous vessels (271). The consequence of increased vasoconstriction to the peripheral vessels induced by an arousal, is an increase in arterial stiffening, which increases both blood flow velocity and the pulse wave travels faster and results in reduced pulse transit time (PTT) (276). Pitson and Stradling, explored the relationship between changes in autonomic parameters and markers of SDB, including AHI, micro arousals and oxygen desaturations. They showed an increase in blood pressure (PTT of > 15ms lasting for more than 5s and less than 45s) and rises in heart rate correlated with respiratory arousals, micro arousals, AHI and SaO_2 dips. The strongest correlation occurred between both autonomic parameters (PTT & heart rate rises) and SaO_2 dips ($r = 0.71$) and EEG micro arousals ($r = 0.65$) demonstrating the interrelationship between these physiological parameters.

1.11.3.1 Sleep fragmentation, effects on sleep, performance and subjective measures

Sleep fragmentation is a common complaint in clinical disorders including SDB (277), restless leg syndrome (278), post-traumatic stress disorder (279), narcolepsy (280) and mood disorders (281) (282). It has been proposed that sleep fragmentation, caused by external stimuli (noise, tones) or internal stimuli (apnoea, periodic leg movement), reduces the recuperative value of sleep even though total sleep time is not reduced (270) (283) and NREM sleep is unaffected. The quality and depth (increase in delta power), however, is significantly reduced both in human (284) and animal studies (282). The physiological effects of sleep fragmentation are the same as those experienced after partial and total sleep loss. All three result in reduced alertness, excessive daytime sleepiness, performance deficits and changes in sleep architecture associated with a rebound in sleep recovery (270) (285, 286). Sleep fragmentation effects autonomic balance and neuroendocrine function (287) and is associated with increased blood pressure (288) and disrupted lipid metabolism (289). The effect of sleep fragmentation on performance varies with age with greater impairment in both mood and cognitive performance in younger participants with just one night of fragmented sleep (286, 290).

Phillip et al., showed that different arousal threshold was evident between sleep stages and from the beginning of sleep compared to the last third of the night (291). They also demonstrated that there was an increase in arousal threshold during SWS (3 fold) compared to REM sleep and stage 2 NREM sleep in the latter stages of sleep. Guilleminault has found blunted arousal thresholds in patients with upper airway resistance syndrome and there was a significant impact to the defense mechanisms, with more repetition of fragmentation as the night progressed (291). He reported that end inspiratory negative esophageal pressure was more profound in SWS and arousals occurred more often in the presence of negative esophaeal pressure. In another study, Guilleminault et al., showed no relationship between

polysomnographic measures of sleep that are characteristic of oxygen deprivation, such as respiratory arousals, oxygen desaturation and objective measures of sleepiness (multiple sleep latency test) (292). They concluded that changes to sleep architecture and sleep disturbance was a better predictor of daytime sleepiness. Colt et al., came to the same conclusion when they looked at the effects of sleep disturbance compare to hypoxemia in OSA patients. Their study also showed that apnoea related variables did not reflect excessive daytime sleepiness (293).

The impact of sleep fragmentation, SDB and blood pressure was assessed in a population-based study and showed that sleep fragmentation was independently associated with increased morning systolic blood pressure (288). This study suggests that sleep fragmentation alone maybe sufficient to explain the cardiovascular deficits associated with milder forms of SDB that have been reported in children.

1.11.3.2 Sleep Fragmentation in Animal Models

Sleep fragmentation studies in animals have observed significant changes to the development of cardiovascular, nervous and immune systems. In the rodent, the hippocampal long-term potentiation associated with 'memory formation' and declarative memory was absent after only 24hr of sleep fragmentation. Furthermore, spatial learning (hippocampal-dependent water maze test) was impaired. Researchers speculated that sleep fragmentation may result in loss of N-methyl-D-aspartate (NMDA) receptor-dependent LTP in the hippocampal CA1 region (282). In mice, Baud et al., showed that sleep fragmentation impacted both brain specific and general metabolism. Food intake was increased without affecting body weight and an increase in sustained brain temperature during fragmented sleep was observed. At the end of a two week exposure, mice exhibited glucose intolerance and an increase in circadian peak level of glucocorticoids (294).

The underlying, physiological consequence of ongoing sleep fragmentation has been comprehensively examined by the Gozal group in Chicago, USA. Their development of a mouse model of sleep fragmentation has allowed for a better understanding of the consequences of interrupted sleep on the cardiovascular system. They demonstrated that chronic sleep fragmentation induced an increase in expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a marker of oxidative stress produced by most mammalian cells, including neurons (295). Mice subjected to sleep fragmentation (SF) took longer to locate hidden platforms compared to normal mice and NADPH null mice subjected to sleep fragmentation. SF mice also exhibited a decline in spatial learning and an increase of depressive-like behaviours and anxiety and also expressed increased P47phox and P67phox subunits of NADPH oxidase compared to the other groups. Cortical and hippocampal tissue showed an increase level of lipid peroxidation, an accepted marker of oxidative stress. Researchers proposed that chronic sleep fragmentation increases oxidative stress and that their results of increased NADPH-mediated oxidase and its relationship to impaired cognitive function (not seen in the NADPH null mice) was evidence of the detrimental effect of sleep fragmentation without obstructive events or infection. This group of researchers were also able to demonstrate a relationship between sleep fragmentation and a time-dependent increase in insulin resistance. They proposed that sleep fragmentation upregulates oxidative stress and inflammatory pathways which induces insulin resistance.

Following from these experiments this group of researchers observed an increase in hyperphagia in mice soon after exposure to sleep fragmentation (296). In addition, an endothelial function test, using a laser-doppler in the dorsal tail vein, showed that both peak flow and time to return to baseline perfusion values were increased in mice exposed to sleep

fragmentation from as early as 8 weeks and onwards. Both systolic and diastolic blood pressure increased at about the same time. Histological tests of the aorta in these same mice exposed to sleep fragmentation showed significant elastic fiber disruption and fiber disorganisation in both the arch and thoracic aortas, however there was no evidence of altered aortic wall thickness. The aortic walls showed 3.7 fold increased infiltration of foam cells and macrophages, particularly in the aortic root sections compared to controls. They propose that sleep fragmentation, a hallmark of SDB and other sleep disorders may be a harbinger of cardiovascular disease often reported in patients.

In larger mammals (dogs), Brooks et al., showed that long-term sleep fragmentation alone resulted in similar blood pressure changes observed in dogs that were subjected to a similar number of hypoxic/obstructive events (297). They concluded that the altered response to obstructive events observed in OSA are more likely to be attributed to the effects of the sleep fragmentation and arousal that is evoked by the event rather than the asphyxia, drop in oxygen and habituation to the obstructive events.

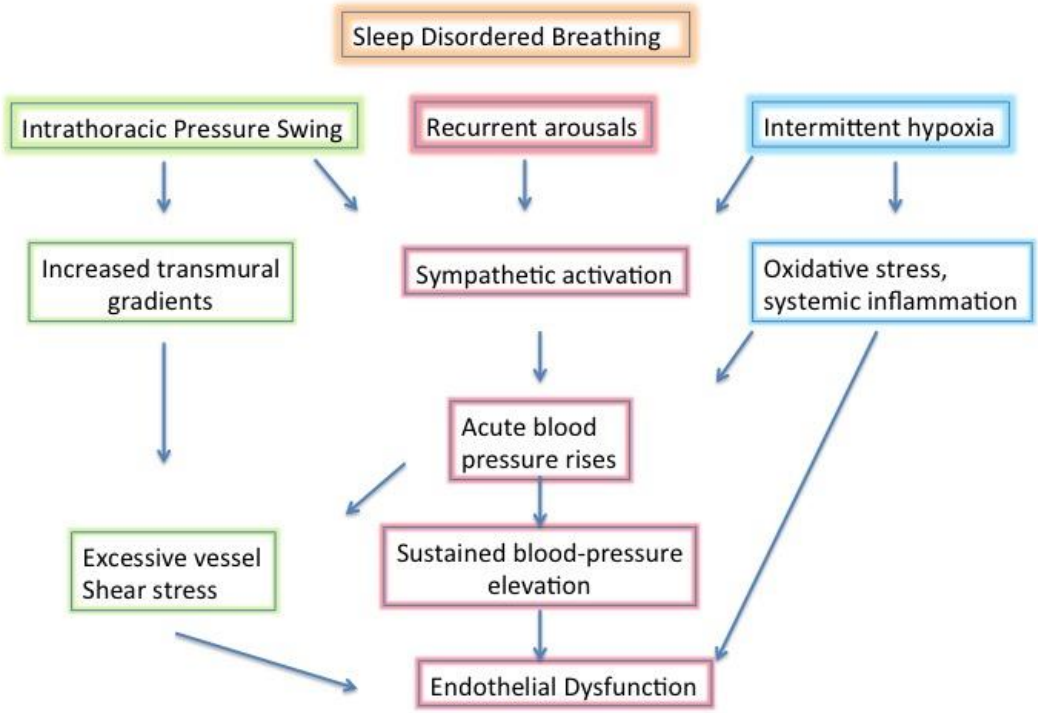


Figure 8: The mechanisms associated with endothelial dysfunction and vascular damage in obstructive sleep apnoea (298)

1.12 SDB and Autonomic Dysfunction – What is the Evidence?

Autonomic dysfunction has been proposed to be central to the cardiovascular changes observed both in adults and children with SDB. The next section will discuss current measures of autonomic function and look at the current literature supporting the hypothesis that autonomic dysfunction is central to cardiovascular changes observed in SDB.

1.12.1 Measuring Autonomic Function

Cardiovascular dysfunction precipitated by an imbalance in autonomic control can result as tachycardia, bradycardia, paroxysmal or sustained hypertension and orthostatic hypotension, which is often related to increased blood pressure in the supine position (299). In adults,

autonomic evaluation includes a thorough clinical history together with the autonomic symptoms profile questionnaire to provide a subjective composite score of autonomic function. The domains include orthostatic tolerance, reflex syncope, sexual failure (adult men), urinary and gastrointestinal complaints (diarrhea, constipation), excessive sweating, vision and vasomotor dysfunction and sleep disturbance (21). Objective measure of autonomic function however rely on the measurement of indirect reflex responses using physiological or pathological stimuli. These include cardiovascular tests that evoke a change in heart rate, blood pressure and respiratory rate. The Valsalva manoeuvre, handgrip, deep breathing, cold face test, pupillary light reflex and tilt table (head tilt) all assess heart rate and blood pressure changes in response to a specific manoeuvre. Using continuous measures of heart rate (ECG) and blood pressure, autonomic dysfunction of the cardiovascular system and hence the integrity of the parasympathetic and sympathetic drive can be assessed.

1.12.1.1 Catecholamine Concentration

The measurement of catecholamine levels is also used as a surrogate maker of autonomic function. Catecholamine, (L-DOPA, dopamine, noradrenaline and adrenaline) are the major neurotransmitters associated with ANS control and they can be easily measured using assays from urine, plasma and serum samples. The accuracy and reliability of catecholamine concentrations have been questioned as their release is not exclusive to sympathetic neurons. These measures rely on catchecholamine spillover and, hence, the rate of removal of transmitter (mediated by separate processes to release) does not allow for an accurate assessment of total SNS activity (22).

1.12.1.2 Muscle Sympathetic Nerve Activity

Sympathetic nerve activity measured in muscle (MSNA) and in skin nerve activity is considered the most direct measure of sympathetic outflow (300). Vasoconstriction evoked by sympathetic fibres to the arterial vessels is measured using microelectrodes inserted in the

cutaneous tissue of the peroneal or medial nerve. This allows for the assessment of baroreflex as it measures the reduction in burst firing evident during diastolic blood pressure. Activity to the muscle increases via inhibition of arterial baroreceptors, or activation of chemoreceptors or nociceptors (22). Hence, using both MSNA and continuous blood pressure monitoring together gives an optimal understanding of changes in ANS function (300). Unfortunately this technique is considered invasive (painful) and is not used in children.

1.12.1.3 Pupillometry (Pupil Light Reflex (PLR))

The ANS controls the pupil's response to light via the two iris muscles (radial and sphincter), which are composed primarily of smooth muscle cells. Light causes the constriction of the sphincter muscle via the activation of parasympathetic neurons located within the cephalic position of the oculomotor nucleus (Edinger Westphal (EW) or pupilloconstrictor neurons) (Figure 1.9). The PLR pathway begins when the stimulus activates the ganglion cells in the retina, which transverse to the optic nerve and synapses in the pretectal area. The EW cells are rapidly firing 'pacemaker cells', which increase their firing rate as light intensity increases. The EW cells also maintain the mid-position of the pupil in ambient light and are inhibited by the arousal centres of the brainstem (301).

There are two types of cholinergic synapses (muscarinic and nicotinic) controlling the pupillary sphincter muscles and both types are found in the ciliary ganglion (301, 302). The radial muscle (the weaker of the two muscles) is innervated by sympathetic neurons and activation causes dilation by pulling outward on pupillary margin, via α1-adrenergic receptors.

Although the PLR is a quick response, that is difficult to measure using non-digital methods, the recent development of portable infrared pupillometry is becoming increasingly used in research areas which include, psychiatry, emergency medicine, cognitive science and sleep

medicine (301, 303, 304). The technique requires the control of ambient light and the room needs to be free of distractions, e.g. monitors, televisions, etc. Age also alters the size of the pupil by 0.4mm per decade after the age of 16 years. Opoids, cognitive load and mood can also effect the size of the PLR (301). Variables of the PLR include the baseline pupil diameter, constricted diameter, constriction latency, constriction and dilation velocity and constriction duration. The automated and portable pupillometers have good interobserver agreement with desktop infrared pupillometers (305).

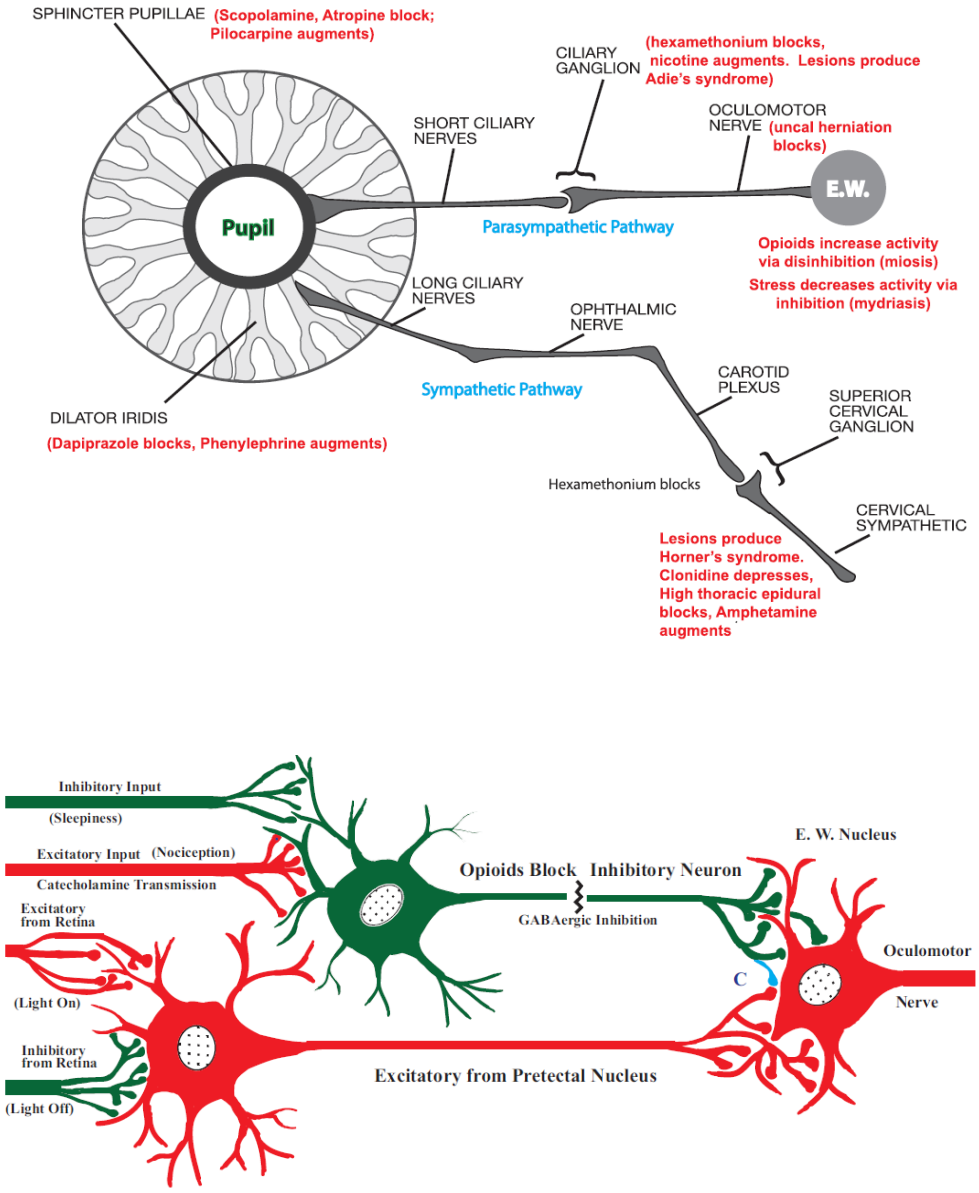


Figure 1.9: Top, Anatomy and pharmacology of the pupil light reflex. Bottom, illustrates the neuronal inputs that control the pupil (301).

1.12.1.3 Heart Rate Variability

Refer to section 1.3.2.3

1.12.2 Evidence of Autonomic Dysfunction – Adults

Somers et al., showed increased sympathetic burst frequency in adults with SDB while they were awake compared to controls and non-snore obese participants (306). They also showed that large burst of sympathetic activity synchronized with apnoeas and oscillations in blood pressure during sleep. When apnoeas ceased, sympathetic firing also ceased abruptly, however, blood pressure increased reaching 240/130 mm Hg in some participants. Muscle tone also returned and brief arousals were evident on the EEG. The overall sympathetic activity during sleep increased by 125% compared to the awake state in the SDB participants, which increased to almost 200% during apnoea events. Treatment with CPAP reduced sympathetic activity by 30%, during sleep, which was lower than the original waking activity in SDB participants and closer to that of non-snoring controls.

1.12.3 Evidence of Autonomic Dysfunction – Children

It has been proposed that the ongoing impact of SDB during childhood development may remodel both vasomotor tone and autonomic response mechanisms (307). Measuring meaningful changes to autonomic function is difficult, as it requires continuous monitoring of either heart rate and/or continuous blood pressure during a stress challenge. Hence there are limited studies in children with SDB.

1.12.3.1 Tilt-table, Valsava Ratio, Deep Breathing and PAT

Guilleminault et al., used the orthostatic challenge (tilt table test) to evaluate autonomic function in children with SDB aged 7 – 11 years compared to controls. The test measured continuous blood pressure and heart rate changes while being tilted. Generally there is an initial drop in blood pressure followed by an increase in heart rate and a normalization

occurring approximately 1 minute after tilt. They demonstrated reduced initial tachycardia and a greater drop in blood pressure in children with SDB compared to controls.

Using a battery of autonomic tests including the head up tilt table, the Valsalva maneuver (forced breathing test) and a deep breathing test (both reflect parasympathetic activity), Montesano et al., also demonstrated a greater change in blood pressure during initial tilt in children with SDB (308). They also showed an association between Valsalva ratio and increased AHI. Furthermore, heart rate changes during the deep breathing challenge were decreased in children with OSA and positively correlated with SpO_2 nadir.

O'Brien & Gozal, also used pulse arterial tonometry (PAT) to demonstrate increased attenuation of the PAT signal (sympathetic discharge-induced) and, hence, increased peripheral resistance during a breathing challenge (3 sighs) (15%) and also cold pressor test (9%) in children with SDB during wakefulness compared to controls (309).

1.12.3.2 Catecholamine Concentrations

Although considered an indirect marker of sympathetic activity, the reporting of morning urinary catecholamine is a non-invasive measure of sympathetic nerve activity. An increase in both plasma and urine noradrenaline has been shown in adults with OSA (310). Kaditis et al., showed a 40% increase in noradrenaline in children with OSA compared to non-snoring controls (311). In a larger cohort of primary snorers compared to children with OSA, Snow et al., showed a 22% increase in morning urinary noradrenaline concentration and 30% increase in adrenaline. Both studies showed that the increase in adrenaline was positively associated with an increase in OAHI/AHI but only the Kaditis group showed a positive association with SpO_2 nadir and ODI. Kelly et al., showed a strong association between Normetanephrine (metabolite of noradrenaline) and AHI, SpO_2 nadir and time spent with SpO_2 less than 90% (312).

Conflicting results have been observed in two recent studies using 24 hour urine collection methods. O'Driscoll et al., demonstrated increased noradrenaline (12%), adrenaline (80%) and dopamine (13%) in children with SDB aged 3-12 years compared to non-snoring controls (313). Similar to other groups, they reported positive associations with noradrenaline and adrenaline with OAHI, AHI and 4% ODI. Nisbet et al., however did not observe group differences in any catecholamine concentrations captured over 24 hours in preschool children with SDB compared to controls (314).

1.12.3.3 Baroreflex Sensitivity

McConnell and colleagues measured continuous blood pressure, just before and throughout sleep using photoplethysmography in children aged 7 – 13 years (315). They reported a dose-dependent increase in the magnitude of initial pressure change in baroreflex events and more variability with events compared to controls (primary snorers), who had more 'constant' pressure changes throughout the night. Also evident was an association with nighttime systolic blood pressure and left ventricular mass index. Consistent with adults with SBD, they also documented reduced heart rate variability and blood pressure variability (baroreflex sensitivity) in the children with OSA compared to controls. Researchers suggested that children with both mild and severe OSA have a reduced heart rate response to blood pressure changes and thus possible abnormal autonomic cardiovascular control.

1.12.3.4 Blood Flow Velocity

Cerebral blood flow velocity (CBFV) in both children and adult SDB participants has been shown to be increased compared to healthy controls. Urbano et al., demonstrated decreased recovery of blood pressure and CBFV and cerebrovascular conductance in adult SDB who underwent orthostatic and hypercapneic challenges to assess autoregulatory capacity (316). They suggested OSA patients had impaired compensatory responses to cerebral hypoperfusion and the possibility that cerebral autoregulation may also be compromised by SDB. In children,

Hill and associates (2006) reported increased mean CBFV (120 cm/second) in primary snorers age 3-7 years compared to healthy matched controls (84 cm/second) in the middle cerebral arteries using Transcranial Doppler (147). Their analysis also suggested a possible relationship between CBFV and indices of cognition and behavioural function.

1.12.3.5 Heart Rate Variability

HRV analyses have been performed in the framework of PSG, in both children and adults. Shiomi et al. (1996) found alterations in the *very low frequency* oscillations of HRV in adults with OSA, which might be caused by the abnormal, aperiodic breathing patterns that accompany obstructive sleep apnoea (317). Gula et al. (2003) found significant changes in the *low-to-high frequency* ratio suggesting an increased sympathetic activity in moderate OSA and a disturbed sympathetic-parasympathetic balance in severe OSA (318). Spicuzza et al. (2003) found changes in both *high* and *low frequency* bands which might also be interpreted as a shift in the sympathetic-parasympathetic balance towards sympathetic activation in patients with OSA (319). More recently, Walter and associates (2012) demonstrated reduced total power, low (LF) and high frequency (HF) during REM sleep in 7-12 year old children with SDB compared to matched controls. LF/HF ratios were also lower during SWS in all severities of SDB compared to control (320). In addition, treatment of adult OSA with the most effective current therapy (continuous positive airways pressure or CPAP) not only reduces hypoxaemia and arousals during sleep, but also attenuates cardiac sympathetic tone (255), suggesting that increased sympathetic outflow may underlie deficits associated with sleep disordered breathing.

1.13 SDB and Inflammation – What is the Evidence?

1.13.1 Inflammation and Autonomic Regulation

The interrelationship between the immune system and CNS has only been recently explored (321). In the past, the two areas of research were independently explored despite knowing that the lymph organs are innervated by sympathetic nerves for more than a century. Recent work has demonstrated integrative autonomic-immune physiological responses, with many signaling components from each of the systems, Immune, and CNS (particularly the ANS aspect) modulating the response of the other system. Central microinjections, electrophysiological studies, peripheral nerve recordings and other molecular techniques have been used to investigate the autonomic-immune systems interaction (322). For an extensive review of these interactions, the recent review by Kenney & Ganta, 'Autonomic Nervous System and Immune System Interactions' is recommended (322).

Many cytokines influence the regulation of various physiological processes, including sleep, neuroendocrine function, neural development, ANS responsiveness and temperature regulation. The effects of these cytokines on ANS function, in particular sympathetic nerve discharge and parasympathetic activity, has been demonstrated in animal studies. For example, micro-injections of interleukin 1β (IL-1β), has been demonstrated to alter SNS innervation to visceral organs and different brain regions, while vagotomy alters SNS regulation to the kidneys, spleen and lumber and also cytokine production. This suggests that a complex interaction occurs between inflammatory cytokines and SNS and PNS regulation. In both animals and human studies, various inflammatory cytokines have been shown to affect the cardiac sympathetic afferent reflex (323) and are associated with changes in heart rate variability (324). They include Interleukin 6 (IL6), tumor necrosis factor-α (TNFα), C-reactive protein (CRP) and interferon-γ (IFNγ).

The reciprocal response, where the ANS is activated, demonstrates the bi-directional relationship between these two systems. For example, heat stress has been shown to be associated with an increase in cytokine gene expression for both IL-1β and IL6 from the spleen, which is attenuated when the splenic nerve is denervated in the rat (325). Also, microinjections of Ang II, a regulator of cardiovascular function and sympathetic nerve outflow results in increased IL-1β, IL2, IL5 and TGF β1 (326).

1.13.1 Evidence of Inflammation - Adults

Systemic inflammation is a hallmark of both SDB and CVD. Many studies report increases in C-reactive protein (327), IL6, fibrinogen, Tumor Necrosis Factor-α (TNFα) intracellular adhesion molecule-1 (ICAM), vascular cell adhesion molecule-1 (VCAM-1), leptin (328), P-selectin, leukocyte superoxide and soluble circulating adhesion molecular in adults with SDB (216) (329) and are evidence of inflammation and endothelial dysfunction.

Some studies have shown adiponectin, a marker produced by mature adipocytes is reduced in adults with OSA both in the morning and evening while others have shown no difference or increased levels (330). Adiponectin inhibits glucose processing in the liver and increases oxidation of fatty acids. It also plays and important role as an anti-inflammatory as it inhibits NF-kB production, which in turn reduces IL6 production and promotes the production of IL10, an anti-inflammatory cytokine (330). IL6 is a mediator of atherogenesis which can induce chemokine and leukocyte recruitment during oxidative stress and both TNFα and IL6 inhibit adiponectin production (216). TNFα (331), NF-kB (332) and IL6 have all been shown to be increased in adults and children with SDB (333-336) suggesting a possible synergy between the inflammatory pathways and those involved in vascular repair (remodeling).

Whether the increased inflammatory response demonstrated in adults with SDB arises from 'infection' and, hence, activation of the immune response or from increased sympathetic activity associated with increased respiratory effort, sleep fragmentation and arousals have not been addressed in the literature. However, treatment with CPAP in adults has been shown to reduce both the number of respiratory events and also circulating CRP levels, suggesting the underlying inflammatory response may occur in response to increased sympathetic activity (337).

1.13.2 Evidence of Inflammation – Children

There are limited studies investigating the inflammatory response in children as they require blood samples to properly assess expression levels of markers. However, morning plasma TNFα levels have been shown to be increased and positively correlated to the frequency of respiratory arousals and level of sleepiness in children with SDB. Plasma levels reduced after T&A, matching the reduction in respiratory events and level of sleepiness assessed in children three months post-surgery (338). Levels of IFNγ, considered a key factor in the progression of atherosclerosis (339), were also found to be increased in children with OSA (331). Fibrinogen another inflammatory marker associated with cardiovascular disease, was demonstrated to be increased in snoring children but was not associated with any of the current PSG measures of severity (340).

Many studies in children have shown increased levels of C-reactive protein (CRP) (334, 341-343), but not all (198). A review by Gozal et al. has proposed that CRP may provide a potential biomarker for OSA (341). Their posit is supported by the meta-analysis conducted by Ingram and Matthews who demonstrated that collectively, the results of eight studies, which compared

pre-treatment to post-treatment CRP levels in children with OSA, showed that CRP levels significantly reduce after surgery (344).

Kheirandish-Gozal et al. demonstrated that children with delayed hyperemic dilation response (reduced vascular responsiveness) and OSA, were more likely to exhibit reduced $CD14^+$ monocytes and increased $CD16^+$ cells and hence reduced CD14:CD16 (ratio). In addition they found that $CD16^+$ cells expressed higher levels of the chemokine, Fractalkine (CX3CL1), which induces chemotaxis in monocytes and is associated with vascular disease (192, 345). Interestingly, they noted a strong inverse correlation between the CD14:CD16 and the time to maximal dilation during the hyperemia test (Tmax) and that CX3CR1 levels were inversely associated with nitric oxide production, demonstrating the interaction between the inflammatory response and vascular function.

More recently, Israel et al. showed increased expression from tonsillar and adenoid tissue of both NF-kB, a transcription factor and regulator of immune response, and IL-1α, a pro-inflammatory cytokine, in children with OSA compared to age matched control (346). In the rat model of SDB, intermittent hypoxia induces both NF-kB and TNFα expression in vascular tissue. Increased TNFα down-regulates endothelial nitric oxide production, which in turn impairs endothelium-dependent vasodilation (347). Together these studies suggest an inter-relationship between vascular changes and inflammation in children with SDB independent of infection.

1.14 Conclusion

Sleep is an important determinant of health and wellbeing and good sleep during childhood is vital for development and learning. There is evidence that SDB at the severe end of the spectrum is associated with reduced neurocognition and changes in cardiovascular function.

More recently, studies are suggesting that even children diagnosed as primary snorers using current AASM criteria are not protected from these changes. Given that more than 10% of children experience some level of SDB during the formative years and that most will not be diagnose or treated (less than 10%) the impact to their underlying physiology needs to be property understood so as to determine, (i) if and which children need treatment and (ii) whether the treatment is effective in reversing these changes. If the cardiovascular effects of childhood SDB persist, an important potential modifiable risk factor for adult cardiovascular disease may remain under-recognised and untreated and contribute to the early manifestation of cardiovascular deficits that can burden the individual and community at large. Therefore, understanding the underlying components that precipitate the changes in cardiovascular health, be they the impact of increased sympathetic activity and vasoconstriction of the peripheral vessels or the impact of an overactive inflammatory response or both, need to be better understood.

1.15 Aim of Thesis

The overall aim for this thesis is to compare vascular and inflammatory response in children with healthy age matched children who do not snore. PSG was used to determine SDB severity in all studies. FMD (brachial artery) and MRI (aorta) were used in separate studies to measure vascular responsiveness of both minor and major vessels. Flow cytometry of T-cell lymphocyte was used to examine the inflammatory response between the two groups.

Children with SDB in the study donated their tonsil tissue to be examined. Hence a second aim of the study was to look at markers of sympathetic activity on arteries from the tonsils (dorsal lingual artery) and determine if there is a relationship between changes in vascular response, platelet aggregation (endothelial damage) and pupil light reflex (autonomic function) which are reported to be affected in adults and children with SDB.

Hypothesis 1: Children with SDB will have impaired vascular function (brachial artery – FMD, aorta/pulmonary arteries – MRI) compared to healthy age-matched controls

Hypothesis 2: Children with SDB will have increased markers of inflammation compared with healthy aged-matched control. Children with increased markers of inflammation will have altered vascular response.

Hypothesis 3: Children with worst vascular responsiveness will have increased markers of sympathetic activity (sympathetic nerve fibre density measured using immunofluorescence for tyrosine hydroxylase, platelet aggregation (a marker of endothelial damage), and altered pupil light reflex (autonomic function).

Chapter 2

Flow mediated dilatation, using time course data shows maturation of the brachial artery from young children to mid-adolescents

2.1 Preface

The following chapter investigates the normal dilation response during hyperemic stress in the brachial artery, using flow-mediated dilatation (FMD) in children aged 6 – 15 years. Flow mediated dilatation has only recently been used in children, however there are no papers on the vessel temporal dilation dynamics in normal children under the age of 14yrs. Only one paper looks at the temporal differences in dilation response in FMD at brachial site and its relationship to age, gender and body habitus, however this study used a vast age range starting at 14 years until 75+ years.

See *Appendix 4* for the published journal article, in Clinical and Experimental Physiology and Pharmacology

Statement of Authorship

Title of Paper	**Flow mediated dilatation, using time course data shows maturation of the brachial artery from young children to mid-adolescents**
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Kontos, A., et al., *Flow-mediated dilatation, using time course data, shows maturation of the brachial artery from young children to mid-adolescents.* Clin Exp Pharmacol Physiol, 2015. 42(3): p. 240-5.

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Contribution to the Paper	Data Analysis, Interpretation of results, Preparation of manuscript:		
Overall percentage (%)			
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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2.2 Abstract

Flow mediated dilatation is a tool widely used to measure arterial responsiveness to sheer stress. However, there is scant literature to show how the peripheral arterial response changes as the vascular system mature. One reason for this is that the feasibility of measuring flow mediated dilatation in younger children has not been established. The aim of this study was to assess brachial artery function during flow mediated dilatation response after 4 minutes of ischemia of the forearm in children aged 6-15 years. Time to reach flow mediated dilatation maximum was found to correlate with age ($r = 0.4$, $p < 0.05$), resting brachial artery diameter ($r = 0.4$, $p < 0.05$), height ($r = 0.4$, $p < 0.05$), BMI ($r = 0.45$, $p < 0.05$), body surface area ($r = 0.44$, $p < 0.05$) and resting blood flow ($r = 0.37$, $p < 0.05$). However, no correlation between the traditional flow mediated dilatation response at 60s and for flow mediated dilatation maximal dilation was evident with age, resting brachial artery diameter, height, weight, BMI, body surface area, resting blood flow.

In conclusion, the time taken to reach maximal dilation response is related to age and brachial artery luminal diameter and body habitus but not the traditional measures: FMD response at 60s or the maximal dilatation percentage.

Key Words: flow mediated dilatation, brachial artery, children, blood flow.

2.3 Introduction

Early detection of impaired cardiovascular function is prerequisite for the prevention of future hypertension, atherosclerosis and cardiac disease. An integral component in that task is the assessment of vascular responsiveness before disease develops. A common non-invasive method for assessing vascular responsiveness is the measurement of flow-mediated dilatation (FMD). FMD measures endothelial response to an increase in sheer stress induced by increased blood flow in a peripheral conduit artery following the induction of ischemia, commonly in the brachial artery (348). The physiology underlying the FMD response has been well described (226, 349). The initial vascular dilation following forearm occlusion in FMD measurement is dependent on intact endothelial stretch receptors, which respond to the increased blood flow and the resultant sheer stress by the release of vasodilators, in particular nitric oxide. The latter induces relaxation of the smooth muscle cells surrounding the endothelial layer of the vessel thereby increasing luminal diameter. The magnitude of the vessel dilation (349) and time taken to maximal dilation (348, 350-352) are the two key parameters measured with the FMD technique.

Traditionally, maximal vessel dilation has been thought to occur at 60s following cuff deflation (FMD_{max}) and reflect the health of endothelial cells (EC). While the 60s time point has been used in many studies (230, 353-355) it may not adequately describe all of the important characteristics of the dilating vessel wall. Age related changes in blood vessel distensibility and therefore time to maximal blood vessel dilatation have been reported in adults (350, 352). Whether the same is true in young children is unknown. Only one study has assessed time to maximal brachial artery dilation, in healthy children but this was restricted to older children aged 9 – 16 years(356). It showed that most maximal dilations occurred at approximately 70s post cuff deflations. In order to comprehensively assess shear stress response throughout

childhood it is important that FMD responses be examined both over a longer time period than the currently accepted 60s and also over a wider age range. Specifically, the aim of this study was therefore to assess FMD responses over 180s in healthy young children aged 6 - 15 years.

Table 2.1: Blood Flow Dynamics recorded or derived during FMD measurements

VTi	Velocity time integral	Mean blood flow velocity during ejection time for one cardiac cycle (Peak systolic velocity x ejection time)
PSV	Peak systolic velocity	The fastest velocity during ejection time of one cardiac cycle
Q	Brachial Artery Blood flow	Amount of blood travelling through the brachial artery per second *Q = SV x HR, (Blood Flow = Stroke Volume x HR)* *SV = (Cross Section Area of the artery x VTi).*
SR	Shear Rate	(8 x VTI)/diameter of the artery is a measure of frictional force on the endothelial cells of blood vessels.

2.4 Results

2.4.1 Anthropometrics

Preliminary analyses revealed no gender differences in anthropometrics and therefore gender was not included in the analyses. The final group consisted of 12 males and 18 females aged 6.3 to 15.7 years.

2.4.2 Brachial Artery Dimension and Blood Flow Dynamics

As expected, brachial artery diameter was strongly correlated with age, height, weight, body surface area, BMI and moderately to resting blood flow. Also a moderate inverse relationship was evident between *rHR* and resting brachial artery diameter, and body surface area. Whole group data is reported in Table 2, while correlation results are presented in Table 2.3. Resting *HR* demonstrated a moderate inverse relationship with *hVTi*, resting blood flow, age, height, weight and body surface area. A moderate positive correlation was observed between *hVTi* and variables, age, height, weight, resting heart rate, and BMI. Resting blood flow was moderately correlated with resting brachial artery diameter, resting *VTi*, BMI, body surface area and body weight and weakly with age and height.

A moderate positive correlation between *rVTi*, weight, and BMI was reported but there was no association between *rVTi* and height. A strong negative relationship between *rHR* and *hVTi* was also observed.

2.4.3 FMD Response

The correlation between FMD and anthropometric parameters are reported in Table 2.3. The *time to reach* FMD_{max} was moderately correlated with age, BMI, resting brachial diameter and body surface area. Most of the younger children had reached peak dilation and were returning

to baseline by 45 seconds post-cuff deflation while the brachial artery of older children continued to dilate up to and beyond 60 seconds post-cuff deflation (Fig 2.1). No significant relationship was observed between both FMD_{60s} and FMD_{max} and age, brachial artery diameter, height, BMI, and body surface area.

Table 2.2: Anthropometric, Blood vessel dimensions and flow dynamics details for the combined sample (n = 30).

	Mean	Standard Deviation	Minimum	Maximum
Anthropometric values				
Age (years)	10.9	2.70	6.25	15.7
Height (cm)	145.5	16.0	118.5	177.0
BMI (cm/kg^2)	18.9	2.5	15.2	26.0
Weight (kg)	41.2	12.7	21.7	67.8
Waist/Hip Ratio	0.87	0.05	0.78	0.96
Body Surface Area (m^2)	140	57	583	272
Vascular parameters				
Vessel Resting Diameter (cm)	0.255	0.030	0.200	0.343
Resting PSV (m/s)	103.2	21.0	65.4	156.8
Resting VTi (m^2)	17.32	8.72	9.00	50.00
Resting HR (bpm)	72.8	9.3	57.0	95.0
Hyp PSV (m/s)	168.7	24.1	120.7	222.5
Hyp VTi (m^2)	67.76	16.18	26.40	97.70
Hyp HR (bpm)	76.2	10.4	57.8	100.5
Resting Blood Flow(Q) (ml/min)	66.6	39.4	23.8	170.4
Resting Shear Rate (rSR)	407	85	270	590
FMD_{60S} (%)	6.81	4.52	-0.40	17.11
FMD_{Max} (%)	8.39	4.04	0.81	17.11
Time to FMD_{Max} (s)	52.8	18.6	30.0	90.0

Table 2.3: Correlations between anthropometric and vascular parameters (n = 30).

	Age	Height	Weight	Body surface area	BMI
Resting brachial artery diameter	**0.70******	**0.75******	**0.72*****	**0.73******	**0.48****
Resting heart rate	**-0.57*****	**-0.56*****	**-0.51*****	**-0.46***	-0.18
Resting VTi	0.25	0.23	**0.42***	0.37*	**0.59*****
Resting blood flow[1]	**0.37***	**0.38***	**0.53*****	**0.49****	**0.64******
Hyperaemic heart rate	**-0.56****	**-0.53*****	**-0.46***	**-0.48****	-0.21
Hyperaemic VTi	**0.47****	**0.40***	**0.36***	0.35	0.27
Hyperaemic minus resting VTi	0.36	0.30	0.15	0.16	-0.06
Resting peak systolic velocity	0.31	0.34	0.32	**0.46***	**0.53*****
Hyperaemic peak systolic velocity	**0.42***	**0.37***	**0.49****	**0.41***	**0.47****
Resting shear rate	-0.15	-0.19	-0.19	-0.01	0.21
FMD_{Max}	-0.01	0.00	0.24	0.03	0.17
Time to FMD_{Max}	**0.40***	**0.41***	**0.46***	**0.44***	**0.45***
FMD_{60S}	0.06	0.08	0.05	0.14	0.28

NB: [1]Denotes n = 29; and *$p < 0.05$, **$p < 0.01$ and*** $p < 0.005$.

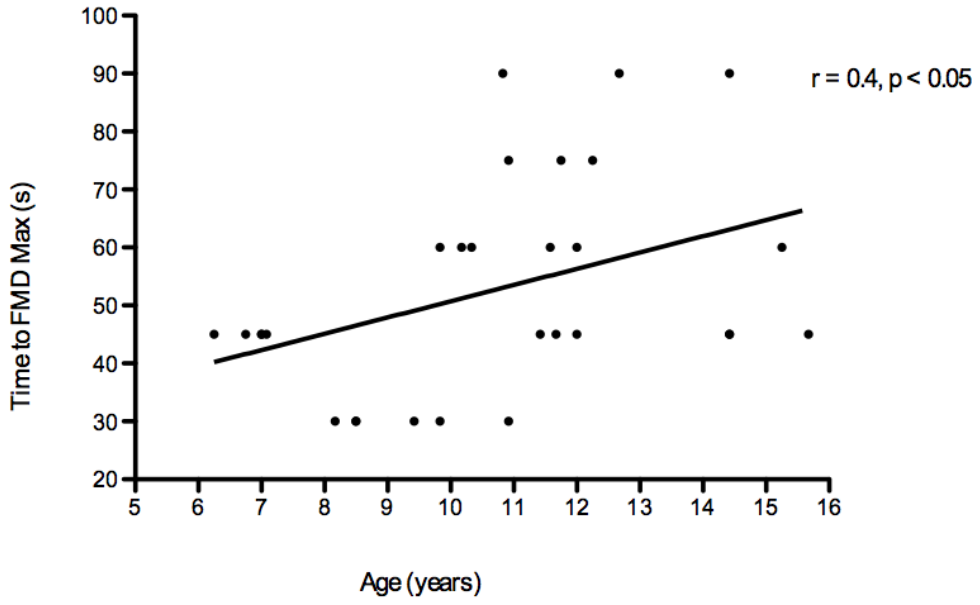

Figure 2.1: Scatterplot for age (years) versus time to FMD_{max}

2.5 Discussion

This study investigated the time to maximal FMD response in healthy children ranging from 6 – 15 years. Our results demonstrate that the time needed for the brachial artery to fully dilate in response to sheer stress and ischemia is related to age and body habitus (weight, height, and body surface area). This is consistent with the proposition that as children grow the vasodilation response of the peripheral arteries becomes slower over time and complements previous work by Herrington and associates (2001), who found the same relationship between *time to* FMD_{max} and age in large group of participants ranging from 14 to 80 years.

Arteries increase in size to keep up with the metabolic requirements associated with increased tissue mass, explaining the relationship that we found between both age and BMI and *time to* FMD_{max}. Arterial responsiveness is maintained through a series of complex interactions, some of which are short-term and activated through the central nervous system and/or direct cellular mechanisms (226). Other determinants are long-term, involving both the size of the lumen and wall of blood vessels, which are made up of vascular components such as extracellular matrix proteins, collagen and elastin, and smooth muscle cells. Together these elements alter the compliance of blood vessels, and compliance in turn is a critical determinant of vasomotor function and arterial capacity to dilate (254, 357). Jourdan et al., (2005) reported an increase intima-media thickness (IMT) of systemic arteries (e.g. carotid and femoral arteries) with age and height (358). They suggest the gain in IMT is due to increased smooth muscle cell and extracellular matrix protein formation.

As there are numerous long-term and short-term changes in the anatomical and physiological properties of blood vessels as children mature it is likely that the delay in reaching FMD maximal dilation we observed in older children is associated with changes in some, if not all, of these parameters and importantly the effect that a 4 minute hypoxic state has on the blood vessels distil to the cuff at different ages.

In the present study no significant linear correlations were observed between both FMD_{60S} and FMD_{max} and any of the anthropometric variables (i.e. age, weight, height, BMI and body surface area). The mean FMD_{60s} and FMD_{max} observed in this study are consistent with those of other studies showing the capacity for brachial artery dilation is between 8-11% in normal, non-obese subjects and independent of age (359). Consistent with other studies, we also observed a large variability in FMD_{max} (4-18%).

Over 50% of participants in this study reached peak dilation either before or after the 60 s time point, which traditionally other studies have used as the measurement end-point. This is consistent with the distribution of maximal dilation times reported by Black et al. (2008), Palinkas et al. (2002) and Järvisalo et al. (2002), with the majority of maximal dilation measurements occurring outside the standard window of assessment in normal adults, patients with coronary artery disease and healthy children (350, 351). Black and colleagues stressed that FMD taken at the traditional 60 s time point may result in difference outcomes reported when comparing groups (350). These studies did not examine the relationship between BMI or age and time to FMD maximal dilation. Contrary to previous reports (352, 360, 361) we did not find a relationship between FMD_{60s} and FMD_{max} and resting brachial artery diameter. The lack of association maybe to due to the small range in brachial artery diameter in our group. The diameter ranged between 2mm to 3.43 mm with a difference of only 1.4mm, where

as the Herrington et al., (2001) study the brachial artery diameter ranged from 2mm up to 8mm.

Our results support previous reports that heart rate decreases while the brachial artery diameter increases as children mature (362). At rest, the volume of blood passing through the resting brachial artery (resting brachial blood flow) increases with age and BMI, but not shear rate. Shear rate is a measure of frictional force on the luminal wall, which is dependent on the luminal diameter and the volume and rate of blood flowing through the vessel. We showed the brachial artery diameter and resting blood flow increases as children mature. As both variables increase by the same factor shear rate remained constant across ages. The consistent correlations between interrelated variables and brachial diameter adds to the accuracy of the measurement conducted by both the ultrasonographer and the echocardiologist in this study.

The temporal differences in FMD response and the relationship to age and body habitus may not seem important in normal children, but this may be critical in differentiating responses between normal children and those with disorders likely to affect vascular response. In conditions such as juvenile diabetes and obesity it is well established that vasculature is affected when measuring standard FMD response in older children. If *time to* FMD_{max}, age and body habitus are measured and accounted for, more subtle abnormal vascular responses may be found both earlier in those disorders with established changes and other disorders not currently associated with abnormal vascular response. Changes in affected groups, which show reduced dilation at 60s such as FMD studies involving children with type 1 diabetes mellitus (353); hypercholesterolemia or Kawasaki disease (355) does not mean the blood vessel does not fully dilate, it may be that the vessel dilates quickly and by 60s is returning to

normal baseline diameter or that the initial dilation is slow and the kinetic mechanisms between the smooth muscle is somehow interrupted. Again, these would have different causes and yet give the same values. Continuous measurements need to be performed to establish the nature of vascular response dynamics with time.

2.5.1 Limitation

One limitation to this study is that the estimated time to FMD maximal dilation does not accurately reflect the time of full dilation to the second. Although current recommendations specify the use of edge detection software to determine second by second dilation response rather than manual determination using caliper manipulation, the supersedence of the manual method does not invalidate the results of previous studies, of which the majority have used and reported the manual method. To our credit unlike most studies using the manual method that only report dilation at 60s to compare two distinct group of participants, this study used multiple time points per participant (maximum of 33 points), which produces a more accurate account of the progression of dilation of the brachial artery. Although we acknowledge that the small sample size maybe seen as a limitation we wish to reinterate that the study is not a comparative assessment but a rather an indication of how the brachial artery response to induced shear stress develops as healthy children grow.

2.5.2 Conclusion

The results of this study suggests that continuous FMD reporting over 3 minutes, would give a more accurate assessment of early changes to vascular function. We also demonstrated that FMD can be used in children as young as 6 years old and that the time taken to reach maximal vessel dilation increases with age and body habitus in

healthy children. As a research procedure FMD measured continuously maybe a more useful in determining early vascular changes between groups of healthy children and in those where vascular abnormalities might be expected.

2.6 Methods

Ethics approval for this study was granted by the Human Research Ethics Committee of the Women's and Children's Health Network. Healthy children aged between 6 - 15 years were recruited from the local community. Parents of participants completed a comprehensive child's health and behaviour questionnaire. Results of the questionnaire were used to screen children known to have genetic conditions, respiratory disorders (e.g. asthma) and developmental disorders (e.g. ADHD). The final sample consisted of 12 males aged 10.71 (2.02) years (Mean (standard deviation)) and 18 females aged 10.96 (2.86) years.

Children were weighed wearing minimal clothing using an electronic scale with a resolution of 0.1kg and height was measured using a wall mounted stadiometer. BMI and percentile was calculated using the Baylor College of Medicine website tool (http://www.bcm.edu/cnrc/bodycomp/bmiz2.html). Body surface area was calculated using the Du Bois formula:

$$\text{Body surface (cm2)} = \text{weight}^{(0.425)}\ \text{(kg)} \times \text{height}^{(0.725)}\ \text{(cm)} \times 71.84\ (363)$$

2.6.1 Vascular function ultrasound assessment

Children attended the Medical Imaging department between 7.30 and 8.30am. The ambient room temperature was maintained at 23°C throughout the FMD procedure.

The standard protocol adopted by the WCH imaging department over the past 12 years is to measure the diameter of the brachial artery using a two dimensional longitudinal ultrasound image of a section of the brachial artery at 2-15 cm above the elbow while the participant lies supine on an examination couch (353). The image is captured by a professional pediatric ultrasonographer of 20 years experience using a 10.0 MHz linear array transducer (Advanced Technology Laboratories (ATL), Bothel, WA), and an ATL HDI 3000 ultrasound system and recorded to a high quality VHS cassette. An electrocardiogram was also recorded simultaneously during the scan.

To ensure that the brachial diameter is measured in the same location during each scan, a reproducible site for vessel imaging is determined using standardized identifiers such as venous valves or vessel bifurcations. A resting scan of the vessel diameter was taken for 30 s. Subsequently, the blood flow to the lower forearm was occluded for four minutes using a sphygmomanometer to 250 mmHg. Deflation of the cuff then induces reactive hyperemia and the conduit arteries dilate in response to the increased blood flow. This physiological response is dependent on the presence of an intact endothelium. Thus the measurement of flow-mediated dilatation *in vivo* has been widely adopted as an assessment of endothelial function. A second scan (reactive hyperemia) was taken from 30 s after cuff deflation until 180 seconds. After 10 minutes a third recovery scan was made.

In addition, blood flow velocity was determined during resting scan and for the first 15 s post cuff deflation, using the pulse Doppler signal at less than 60° to the brachial artery (364). The ultrasound measuring parameters remained unchanged during the whole study.

A different second experienced echocardiographer used ultrasonic calipers to determine the arterial diameter during the R wave of the electrocardiogram. At each time point the average vessel diameter was derived from four cardiac cycles. This value was then used to calculate the percentage difference from resting vessel diameter from the first scan at 30 s and every subsequent 15 s intervals post cuff deflation (FMD_{30} - $FMD1_{80}$). Estimated maximal dilation (FMD_{max}) was calculated as the

> Difference between Maximal and resting baseline vessel diameter value divided by resting baseline diameter and expressed as a percentage.

The time to maximal dilation was estimated as the time taken to reach FMD_{max} after cuff deflation (350)). In cases where FMD_{max} values were constant for multiple time points the first point was considered FMD_{max}.

Brachial artery resting diameter (*rD)*, peak systolic velocity (*rPSV*), velocity time integral (*rVTi*) (the area under the curve value of the mean systolic velocity during cardiac ejection time) (365) and heart rate (*rHR*) was calculated during the resting scan. Hyperemic *PSV*, *VTi*, and *HR* was measured immediately after cuff deflation (*hPSV*, *hVTi*, *hHR*). The average of *PSV*, *VTi*, and *HR* for four cardiac cycles was determined for both at rest and during hyperemia.

Resting blood flow (*rQ*) was calculated as previously described in Black *et al*., (350), using *rD*, *rVTI* and *rHR:*

$$rQ = rSV \times rHR$$

$$rSV = \pi r^2 \times VTi,$$

Shear rate was a measure of the frictional force at the surface of the endothelium. Resting Shear rate (*rSR*) was also determined based on the following equation (366):

$$rSR = (8 \times rVTi) / rD$$

A summary of the FMD blood flow parameters is given in Table 1.

Data were analysed using SPSS (version 19). Values are presented as mean and standard deviations. Correlations were determined using the Pearson's correlation coefficient, *p*–values < 0.05 were considered statistically significant.

2.7 Acknowledgement

The authors thank Margaret Heddle, Fiona Futo, Narelle Adams and Cameron van den Heuvel for their assistance to this study.

2.8 Source of Funding

Financial support for this study was provided by the National Health and Medical Research Council, **project number 453637.**

Chapter 3

Delayed brachial artery dilation response and increased resting blood flow velocity in young children with mild sleep disordered breathing

3.1 Preface

This chapter compares FMD in young children with SDB to healthy age-matched control children. It also looks at the relationship between brachial blood flow velocity during baseline and hyperemia scans and PSG generated variables, including the OAHI, SaO_2 nadir and oxygen desaturation. This is the first study to look at FMD in children with SDB compared to healthy aged matched controls, exclusively between 5-9 years of age.

See *Appendix 5* for copy of published journal article in Sleep Medicine, 2015.

Statement of Authorship

Title of Paper	Delayed brachial artery dilation response and increased resting blood flow velocity in young children with mild sleep disordered breathing
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Kontos, A., et al., *Delayed brachial artery dilation response and increased resting blood flow velocity in young children with mild sleep-disordered breathing.* Sleep Med, 2015. 16(12): p. 1451-6.

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Overall percentage (%)			
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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3.2 Abstract

3.1 Rationale

To evaluate whether the vascular dysfunction detected in adults with sleep disordered breathing (SDB) is also evident in children with snoring referred for evaluation of clinically suspected SDB.

3.2 Objectives

This study compared flow mediated dilatation (FMD), measured at the brachial artery, at rest and during hyperemic stress between children who snore (n = 23; mean (SD) age = 7.51 (1.3) years) and healthy, non-snoring children (n = 11; age = 8.0 (1.3) years).

3.3 Methods

Children with suspected obstructive sleep apnoea (OSA) and healthy non-snoring controls underwent overnight polysomnography (PSG). Non-invasive FMD and brachial arterial blood flow velocity during rest and hyperemia were subsequently measured by ultrasound imaging using standard techniques.

3.4 Measurements

Resting and hyperemic velocity time integral (area under the curve of mean systolic velocity x ejection time), maximal dilation response (highest percentage difference from baseline diameter) and the time taken to reach maximal dilation were calculated.

3.5 Results

Children awaiting adenotonsillectomy compared to healthy non-snoring control children had higher velocity time integrals at rest (14±3m vs 20±8m, $p < 0.01$) and during hyperemic stress (56±6m vs 63±13m, $p < 0.01$) despite having only mild SDB

on polysomnographic assessment. Lower nadir oxygen saturation values during non-rapid eye movement sleep were negatively associated with higher resting (r = -0.58, p < 0.001) and hyperemic (r = -0.36, p < 0.05) velocity time integrals. Maximal FMD dilatation response was not significantly different between snoring and non-snoring groups but the estimated time to reach maximal dilation was significantly delayed in children that snored (60.7±28.4 vs 39.2±13.2 s, p < 0.05).

3.6 Conclusions

Children with mild SDB showed increased blood flow velocity at rest and during hyperemic stress suggesting altered cardiovascular and haemodynamic function. The delay in time to maximal vessel dilatation in snoring children also suggests possible reduced vascular compliance in response to hyperemic sheer stress. Mild SDB appears to alter the peripheral vascular response in young children. The long-term vascular implications of these changes in the growing child are unknown and warrant further investigation.

Keywords: Sleep apnoea, flow mediated dilatation, pediatric, cardiovascular, blood flow velocity.

3.3 Introduction

In adults, sleep disordered breathing (SDB) is an independent risk factor for cardiovascular disease (367), including left ventricular diastolic abnormalities (368), ischaemic cardiac events (369) and systemic hypertension (370-372). Cardiovascular abnormalities are also evident in children with SDB. These have been well documented at the severe end of SDB spectrum, i.e. obstructive sleep apnoea syndrome, and include right and left ventricular hypertrophy, pulmonary hypertension, cor-pulmonale, and cardiac failure (135, 191, 203). What is less well appreciated however is that cardiovascular abnormalities have also been shown in children with mild SDB, i.e. primary snoring, and include elevated sympathetic tone, raised blood pressure and increased cerebral blood flow velocity (147, 210, 309, 313). Children with mild SDB constitute the majority of SDB patients (84) and at present, knowledge of the potential impact of mild SDB on large blood vessel function is unknown.

Changes in large blood vessel function in children with SDB can be assessed using the non-invasive technique of flow-mediated dilation (FMD). FMD employs high-resolution ultrasound to measure dilatation of the artery in response to hyperemic shear stress induced by ischemia upon downstream vessels. Vascular dysfunction is reflected by an impaired FMD response in either the magnitude of vessel dilatation (373) or the time taken to reach maximal dilation post-cuff deflation (226). Two previous studies have reported that children with SDB have minor reductions in FMD response at the standard time-point of 60s (229, 230). However, recent literature has shown that measurements at 60s do not fully encapsulate the true overall functional dynamics of the vessels' response to shear stress and that extending the measurement time permits a more complete assessment of vascular compliance (348, 350).

This study evaluated blood flow and FMD parameters over an extended period of 180s and measured both the magnitude (i.e. FMD at 60s and FMD maximum) and temporal FMD response of the brachial artery (i.e. time to FMD maximal dilation) in children aged between 5 and 9 years with snoring referred for clinical ENT assessment compared to healthy non-snoring controls.

3.4 Methods

3.4.1 Subjects

Children with a history on parental report of snoring more than three nights per week, and who were referred by their primary physician for the evaluation of SDB to the Otolaryngology Clinic at the Women's and Children's Hospital Adelaide, Australia were recruited as participants (n=23). The control group (n=11) was comprised of healthy non-snoring children recruited from the community via advertisement and through friends of the children with SDB. The study was approved by the Child, Youth and Women's Health Services and University of South of Australia Human Research Ethic Committees.

Potential participants were limited to between 5-9 years of age to minimize the possible influence of pubertal developmental on sleep, neurovascular development and upper airway dynamics (131, 226, 374-377).

Exclusion criteria included a history of significant asthma, previous adenotonsillectomy, significant craniofacial abnormality, medications that affected sleep or respiration, developmental/psychiatric disorders and English as a second language. Given the evidence of an association between obesity and FMD, children with a body mass index > 95%ile were excluded (376).

3.4.2 Polysomnography

Overnight polysomnography (PSG) was conducted using the Compumedics E-Series Sleep System (Melbourne, Australia). The following standard parameters were measured: electroencephalogram (EEG; C3-A2, C4-A1, and F3-A2 F4-A1, O1-A2 and O2-A1), left and right electrooculogram (EOG), sub-mental, diaphragmatic and leg electromyogram (EMG), heart rate by electrocardiogram (ECG), oronasal airflow by thermistor and nasal pressure, respiratory movements of the chest and abdominal wall using uncalibrated respiratory inductive plethysmography (RIP), and arterial oxygen saturation (SpO_2) by pulse oximetry (Nellcor N-595; two second averaging time). Children were continuously monitored via infrared camera by a pediatric sleep technician who also documented observations of sleep behavior, which included the presence or absence of snoring.

3.4.3 Ultrasound Assessment of Endothelial and Artery Function

Once awoken from the PSG and while still fasting, children had a FMD assessment as previously described (378). Briefly, a resting scan measuring brachial artery diameter and blood flow dynamics was initially undertaken and, subsequently, blood flow to the lower forearm was occluded by inflation of a sphygmomanometer cuff to 250mmHg for 4min. In brief, the resultant ischaemia and post-cuff deflation induces reactive hyperaemia by lowering the vascular resistance within the vascular bed supplied by the brachial artery. The reactive hyperaemia, in turn, induces an increase in sheer stress within the brachial artery supplying the ischaemic area thereby causing dilatation. This physiological response is dependent of the presence of an intact endothelium where vasodilators, especially nitric oxide are released in response to the sheer stress. The

measurement of flow-mediated dilatation *in-vivo* has been widely adopted as an assessment of endothelial function (349, 379).

Using the pulse Doppler signal at 60° to the brachial artery, blood flow velocity was determined during the resting scan and for the first 15s post cuff deflation. The second scan (reactive hyperemia) was taken from 30s before until 180s after cuff deflation. A third recovery scan was made 10min after the cuff deflation scan.

3.4.4 Data Analysis

An experienced sleep technician blinded to subject status scored PSGs according to established sleep stage (10) and 2007 American Academy of Sleep Medicine (AASM) ventilatory criteria (11). All obstructive apnoeas ≥2 respiratory cycles in duration were scored even if not associated with cortical arousal or oxygen desaturation. All other respiratory events were scored if ≥2 respiratory cycles in duration and associated with a minimum 3% SpO_2 desaturation and/or an arousal or awakening within two breaths of event termination. Obstructive apnoeas were defined as a >90% reduction in airflow associated with continued chest and abdominal wall movement. Obstructive hypopneas were defined as a ≥50% reduction in airflow signal associated with paradoxical chest/abdominal wall movement or increase in RIP. Central apnoeas were scored if there was an absence of respiratory effort, as determined by RIP and diaphragmatic EMG, in association with an absence of airflow. Central apnoeas were also scored if the event lasted ≥20s. Central hypopneas were defined as a ≥50% reduction in airflow from baseline in association with a ≥50% reduction in respiratory effort from baseline. Apnoea events that included both central and obstructive components were scored as a mixed apnoea. The obstructive apnoea/hypopnea index (OAHI) was calculated as the total number of obstructive apnoeas, mixed apnoeas and obstructive hypopneas per

hour of total sleep time. The central apnoea/hypopnea index (CAHI) was calculated as the total number of central apnoeas and central hypopneas per hour of total sleep time. As described by Katz et al. (380), obstructive apnoea-hypopnea index (OAHI) was used to assess SDB severity.

Spontaneous and respiratory cortical arousals were scored according to the criteria of the American Sleep Disorders Task Force (272). Spontaneous arousal index (SAI) was expressed as the total number of spontaneous arousals per hour of total sleep time and respiratory arousal index (RAI) as the total number of respiratory arousals per hour of total sleep time.

An echocardiographer blind to subject group assessed arterial diameter during the R-wave of the electrocardiogram using ultrasonic calipers. The mean vessel diameter was calculated over four cardiac cycles. The percentage difference from the resting diameter was calculated at 30s post-cuff release and then every subsequent 15s until 180s. Estimated maximal dilation (FMD_{max}) was the greatest percentage of dilation in the individual's data set from the resting baseline diameter. The estimated time to maximal dilation ($FMD_{time\text{-}to\text{-}max}$) was determined as the time taken to reach FMD_{max} after cuff deflation (350).

Brachial artery resting diameter (rD), peak systolic velocity (rPSV), velocity time integral (rVTi) (the area under the curve value of the mean systolic velocity during cardiac ejection time) (365) and heart rate (rHR) were taken during the resting scan. Hyperemic VTi, PSV and HR were measured immediately after cuff deflation (hVTi, hPSV and hHR, respectively). The PSV, VTi and HR values were averaged over four cardiac cycles.

Kolmogorov-Smirnov test was used to assess the normality of data. In the case of normally distributed data, group differences were tested using Student t-tests and Mann-Whitney U tests for non-normally distributed data. Mean (SD) values are reported for normally distributed data and median (interquartile range) for non-normally distributed data. Pearson-r correlations were used to test the relationship between normally distributed data and significance was tested using r-z transformations. Spearman-Rho correlations were used to the test the relationship between non-normally distributed data.

3.5 Results

3.5.1 Anthropometric and Polysomnography

The SDB and control group were matched on anthropometric measures and had similar sleep stage percentages and sleep duration values, but consistent with expectations, children with SDB had significantly higher obstructive apnoea hypopnea index (OAHI) and lower NREM sleep oxygen saturation levels (Table 1). Sixteen children in the SDB group were classified as primary snorers (OAHI < 1) and seven as mild OSA (OAHI < 5: three children had an OAHI < 2 and three < 3). To test whether SDB severity influenced outcome variables we undertook additional analysis separately examining children with primary snoring compared to those with mild SDB. This analysis revealed no significant group differences in any variables apart from OAHI. Therefore both primary snoring and SDB groups were combined for analyses. All children in the control group had an OAHI < 1 and no history of habitual snoring (< 3 nights a week).

Table 3.1: Mean (SD) anthropometric and polysomnographic variables together with p-values from Student t-test comparisons (median and interquartile range are reported in parentheses for non- normally distributed data).

Variable	Controls (n=11)	Mild SDB (n=23)	*p*-value
Anthropometric			
Males (%)	57	60	ns (Chi-square)
Age (years)	8.0 (1.3)	7.5 (1.31)	ns
BMI z-score	0.61 (0.66)	0.59 (0.58)	ns
Height (cm)	128.8 (0.3)	125.7(8.4)	ns
Weight (kg)	28.7 (3.9)	27.2 (4.7)	ns
Waist/Hip Ratio	0.90 (0.04)	0.90 (0.04)	ns
BMI	17.2 (1.3)	17.0 (1.3)	ns
Polysomnographic			
Total Sleep Time (min)	418.4 (31.2)	416.2 (53.1)	ns
NREM 1 (%TST)	2.3 (0.8)	3.4 (2.0)	ns
NREM 2 (%TST)	37.5 (8.0)	46.6 (25.4)	ns
SWS (%TST)	43.2 (8.4)	38.3 (8.3)	ns
REM (%TST)	17.0 (2.6)	17.0 (3.7)	ns
Arousals/h sleep time	11.2 (2.0)	12.6 (3.0)	ns
Spontaneous Arousals/h sleep time	10.6 (2.1)	10.8 (2.5)	ns
Respiratory Arousal/h sleep time	1.00 (0.70)	1.77 (1.67)	ns
Central Apnoea Hypopnea Index	1.47 (0.40)	1.60 (1.20)	ns
Obstructive Apnoea Hypopnea Index	0.17 (0.30)	1.17 (1.50)	< 0.05
Nadir SpO_2 (%)	93.1 (0.7)	92.0 (2.5)	< 0.01
REM Nadir SpO_2 (%)	93.8 (1.2)	93.7 (2.5)	ns
NREM Nadir SpO_2 (%)	94.0 (0.8) *(94 (93-95)*	92.4 (2.3) *93(91-94))*	< 0.05 (Mann-Whitney U)
Average SpO_2desat (%TST)	3.00 (0.44) *(3 (3-3)*	2.78 (1.48) *3 (2-4))*	ns (Mann-Whitney U)

NB: ns = non-significant.

3.5.2 FMD

Blood velocity variables were comparable between groups apart from rVTI and hVTi, which were significantly higher in children with SDB (Table 3.2). Although no significant group difference were observed in FMD_{max}, the $FMD_{time\text{-}to\text{-}max}$ was significantly longer in children with SDB (Table 3.2). The mean percentage dilation time for the interval FMD_{30s} to FMD_{75s} was similar in both groups, however dilation was significantly greater at FMD_{90s} in the SDB group (Figure 3.1).

Table 3.2: Mean (SD) Flow mediated dilatation and blood flow variables together with p-values from Student t-test comparisons (median and interquartile range are reported in parentheses for non-normally distributed data).

Variable	Controls (n = 11)	Mild SDB (n = 23)	*p*-value
Blood flow dynamics			
Resting Diameter (cm)	0.233 (0.020)	0.229 (0.026)	ns
Resting PSV (m/s)	95.8 (9.1)	104.0 (20.8)	ns
Resting VTi (m)	13.9 (3.3)	19.7 (8.0)	< 0.01
Resting HR (b/m)	78.2 (8.8)	89.6 (9.8)	ns
Hyperemic PSV (m/s)	155.6 (12.3)	158.9 (22.5)	ns
Hyperemic VTi (m)	56.0 (6.2)	63.0 (12.9)	< 0.05
Hyperemic Heart Rate (b/min)	82.5 (7.8)	81.7(9.9)	ns
FMD_{Max} (%)	8.2 (4.7)	10.2 (4.8)	ns
$FMD_{Time\text{-}to\text{-}Max}$ (s)	39.5 (10.1) *(45 (30-60))*	60.7 (28.8) *45 (30-60))*	< 0.05 (Mann-Whitney U)

NB: ns = non-significant.

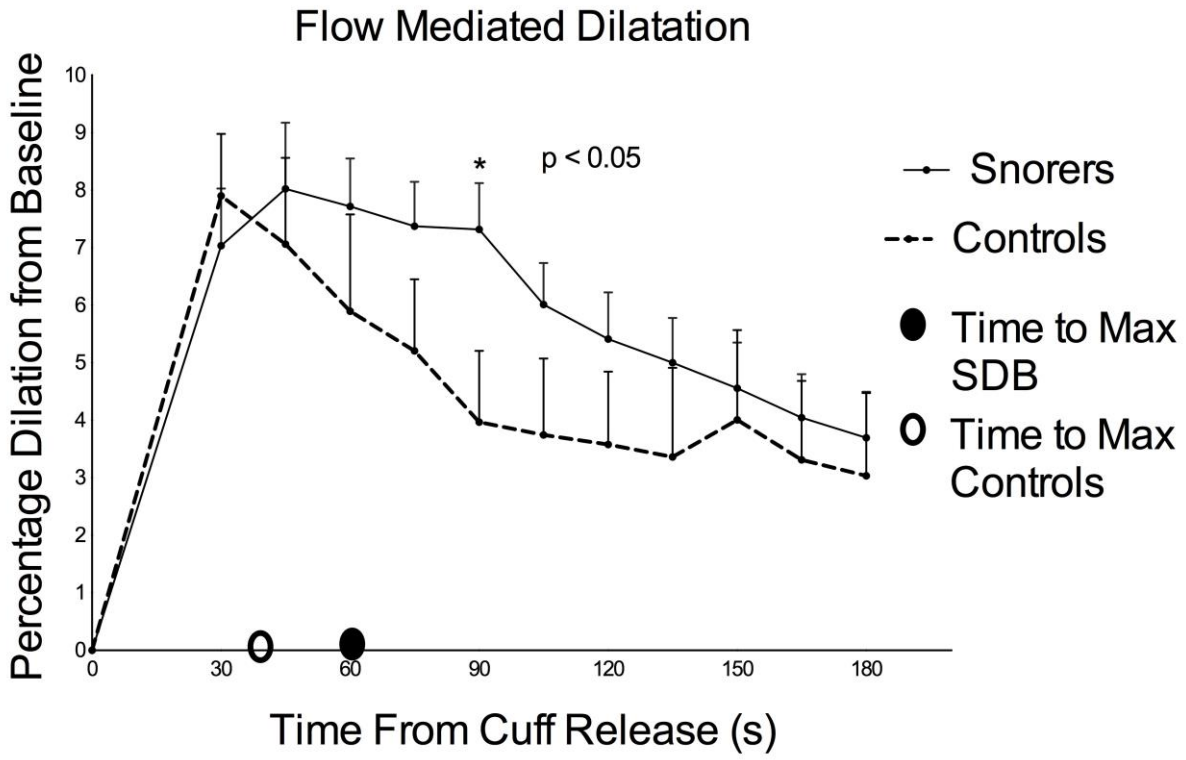

Figure 3.1: Mean (SE) temporal FMD response curve for 23 children with mild sleep disordered breathing (solid line) and 11 aged matched non-SDB children (dotted line). The mean time taken to reach mean maximal dilatation is shown by the corresponding circles on the x-axis. Asterisk denotes significant group differences at 90s post cuff release.

3.5.3 Correlation Between Polysomnographic and FMD Variables

A lower SaO_2 nadir during NREM sleep was associated with a higher rVTI and hVTi (r = -0.58, p < 0.01 and -0.36, p < 0.05; respectively). As well, a higher hVTi was associated with a greater $FMD_{time\text{-}to\text{-}max}$ (r = 0.42, p < 0.05). Also the average of the number of oxygen desaturation events ($SaO_2 \geq 3\% + SaO_2 \geq 4\% + SaO_2 \geq 5\%$) per total sleep time was positively correlated with both rVTi (r = 0.34, p <0.05) and $FMD_{time\text{-}to\text{-}max}$ (r = 0.42, p < 0.05).

3.6 Discussion

In this study young children with primary snoring and mild SDB demonstrated altered baseline vascular characteristics and FMD responses. They had higher brachial artery blood flow velocity both at rest and during hyperemic stress; and the brachial artery took approximately twenty seconds longer to fully dilate to maximum size after cuff release compared to controls. Lower nadir oxygen saturation during NREM sleep, evident in the SDB group, was correlated with increased blood flow velocity both at rest and during the hyperaemic FMD response. Children with a higher hyperaemic velocity time integral were also more likely to exhibit a delayed maximal dilation response.

The rVTi increase observed in the brachial artery of children with mild SDB is consistent with either increased blood viscosity or reduced blood vessel compliance or an increased inotropic response (381). The latter two are known to be augmented with increased sympathetic activity. As the resting brachial diameters were similar between children with SDB and controls, we hypothesize that the increase in resting blood flow velocity may be best explained by increased sympathetic activity and its subsequent impact on vascular resistance/compliance and cardiac contractibility. Noradrenaline (NA), a potent vasoconstrictor neurotransmitter and inotropic factor released by sympathetic nerves, has been shown to be increased in morning samples of plasma, urine and serum of both children and adults with SDB (311-313, 382). As the resting VTi was taken on waking, our results may reflect this previously reported increase in circulating morning NA levels in children with SDB (reflecting increased sympathetic activity). The increase in resting velocity also mirrors the findings of Hill and associates who reported increased cerebral blood flow velocity in young children with mild SDB

compared to non-SDB children using Transcranial Doppler (147). Similar pathophysiological mechanisms have been reported in other disorders. Increased blood flow velocity in both retinal arteries and veins has been observed in patients with diabetes mellitus (383) and hypothyroidism (384), both of these disorders are associated with cardiovascular dysfunction (385) and changes to peripheral resistance (386, 387).

It is unclear whether VTi is predictive of cardiovascular disease later in life. However, Lee and associates (388) using digital peripheral tonometry (PAT) have shown that a higher pulse volume amplitude (PVA) in the fingertip of adult participants is associated with a higher resting brachial artery VTi. Increased PVA as assessed by digital PAT is also predictive of cardiovascular disease in adults (389-391). Furthermore, Jarhult and associates have shown a significant and positive relationship between both systolic and diastolic hyperaemic mean blood flow velocity and ejection fraction in the brachial artery, and more importantly, increased mean blood flow velocity was evident in adults with 'concentric left ventricular remodeling' (392). Both of these independent studies in adults suggest that the increase in blood flow velocity measured at the brachial artery both at rest and during hyperemia in the present study of snoring children may be potentially predictive of cardiovascular remodeling in adult life and merits further evaluation.

Traditionally, studies in FMD report the percentage change in brachial vessel diameter from resting baseline to the diameter at 60s post-cuff deflation, with the value at 60s typically taken to be the time of maximum dilation. Attenuated FMD responses are associated with endothelial damage, atherosclerosis and coronary artery disease (379). Two studies in children have shown slightly reduced FMD_{60S} values of approximately 0.6% in primary snorers (230) and 0.4% reduction in moderate to severe SDB (OAHI

> 5/h) compared to controls (229, 230). However at the FMD_{90s} time point our results clearly demonstrate that the brachial arteries of children with SDB remains dilated, while in healthy children the vessels have started to contract. The $FMD_{time\text{-}to\text{-}max}$ values of the children with SDB in the present study are comparable to those previously reported by our group in older non-snoring healthy children (378). Gozal et al. (222) also reported a time delay in reaching maximal dilation in metacarpal blood flow of young children with moderate to severe OSA.

The present findings support the utility of examining FMD response time longer than the recommended 60s (349). Black and associates (2008) note that the majority of true maximum dilation occurs outside of these designated post-deflation diameter assessments. They advocated that best practice for endothelial function assessment requires prolonged continuous assessment of arterial diameter so as to determine the true time to peak and maximal dilation response (226). The present results indicate that a single measure at 60s may not be a good guide as to vascular health in children with mild SDB.

During NREM sleep, sympathetic activity decreases and parasympathetic vagal activity increases slowing both respiratory and heart rates. Furthermore cutaneous vessels are dilated in response to inhibition of the sympathetic efferents reducing vascular resistance with resultant reduction in global cerebral blood flow (77). As NREM sleep constitutes 75-80% of sleep, and slow wave sleep (SWS) constitutes up to 50% of NREM sleep, upper airway obstruction with resultant oxygen desaturation and increase in sympathetic tone during this period may have a greater impact on peripheral vascular physiology than previously thought. Reduced oxygen concentration activates central and peripheral chemo-reflex mechanisms resulting in increased heart rate, respiration

and constriction of resistance vessels (393). A greater level of sympathetic neural response is likely to be required to constrict vessels that are usually dilated during NREM compared to REM sleep where cutaneous vessels are already semi-constricted.

The finding in this study that children with mild SDB had the capacity to significantly lower their oxygen concentrations without arousing, indicated by their reduced nadir oxygen saturation compared to non-snoring children suggests attenuated chemosensitivity and specifically during NREM sleep. We recognize that the nadir is but a single moment and does not fully reflect the extent of the oxygen desaturation across the whole night, it does however, indicate a tendency to tolerate lower oxygen saturation levels before arousing and maybe a indirect marker of chemoreceptor sensitivity.

The positive association between average SpO_2 desaturation (%TST) and both rVTi and $FMD_{time\text{-}to\text{-}max}$ further suggests that there is an augmented sympathetic response with reduced blood vessel compliance secondary to altered chemoreceptor sensitivity. This potential increase in sympathetic tone we believe underlies the change in FMD response that we have demonstrated in children with mild SDB. We have recently shown significant differences in chest wall biomechanics during periods free of scored obstructive events in all sleep stages in children with sleep disordered breathing (394). Taken together these results suggest that children with SDB may be significantly more obstructed than the standard PSG scoring criteria would suggest and may have developed attenuated chemoreceptor sensitivity in response, particularly in NREM sleep.

In conclusion, our results suggest that the brachial vascular response is altered in young children with only primary snoring/mild SDB. We propose that this may be explained by a sustained increase in sympathetic tone, which alters downstream vascular compliance. It will also be important in future studies to examine this relationship in children with severe SDB. The unanswered and critical question is whether these changes increase cardiovascular morbidity as the child matures through adulthood.

3.7 Acknowledgement

The authors thank Margaret Heddle, Fiona Futo, Narelle Adams and for their assistance to this study.

3.8 Source of Funding

Financial support for this study was provided by the National Health and Medical Research Council, **project number 453637.**

Chapter 4

Increased aortic blood flow velocity and associated inflammatory response in children with primary snoring/mild sleep disordered breathing

Preface

Changes in ventricular thickness and cardiac function have been demonstrated in children with severe SDB, however there are no studies comparing children with primary snoring and healthy aged matched controls for cardiac structure and function using MRI. TNFα and IFNγ are markers known to be increased in children and adult with SDB and are also increased in cardiovascular disease. Hence, the purpose of this study was to examine the relationship between cardiovascular measures derived from cardiac MRI data and known markers of inflammation that are evident in cardiovascular disease. In addition this study looks at the relationship between PSG derived measures associated with SDB including OAHI, SaO_2 nadir and also the parental questionnaire, SDSC and cardiovascular and inflammatory markers.

Statement of Authorship

Title of Paper	**Increased aortic blood flow velocity and associated inflammatory response in children with primary snoring/mild sleep disordered breathing**
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Submitted to Journal of Vascular Research

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Contribution to the Paper	Data Analysis, Interpretation of data, preparation of manuscript.		
Overall percentage (%)			
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
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DISCLOSURE
This work was supported in part by the Faculty of Health Science, University of Adelaide. We would like to thank the staff of the MRI Unit of the Royal Adelaide Hospital. Ms Anna Kontos is supported by a Australian Post-graduate Scholarship through the University of Adelaide. Dr Scott Willoughby is supported by a Career Development Fellowship from the National Health and Medical Research Council (NHMRC) of Australia. A/Prof Matthew Worthley has a SA Health Early to Mid Career Practitioner Fellowship. The other authors have indicated no conflict of interest.

Authors' contribution: Study Design: CvdH, SW, GH, JM and DK. Data Collection: Cvd, YP, SW, MW, KT, AN, GH, A were all involved in data collection. Miss Kontos, Dr Willoughby, Dr Hodge, Dr van den Heuvel, Ms Chin Kaihui and Dr Lushington conducted the data analysis and interpretation of data. All authors were involved in the preparation of manuscript.

4.1 Abstract

Study Objectives: In children, sleep disordered breathing (SDB) results in a generalised inflammatory response and also adversely affects endothelial function of small and medium sized vessels. It remains to be established whether major blood vessels are similarly affected.

Methods and Results: Seven controls and twelve children with mild SDB (aged 5-14y) underwent overnight polysomnography, cardiac magnetic resonance imaging (cMRI) and intracellular cytokine analysis of T-cells by flow cytometry. Children with mild SDB exhibited increased ascending aortic peak systolic velocity and an increased percentage of T-cells producing interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) producing $CD8^+$ cells. The increased ascending aorta peak blood flow velocity was positively correlated with the increased $CD8^+$ cells TNFα and IFNγ productivity, and negatively with the oxygen saturation (SpO_2) nadir.

C**onclusion**: Children with mild SDB demonstrated increased ascending aortic peak systolic velocity which correlated with an increase in the proportion of CD8+ TNFα and IFNγ expressing cells compared to non-snoring children. The data suggests that mild SDB alters vascular function and the inflammatory response.

Keywords: sleep disordered breathing, inflammation, arterial, velocity, children

4.2 Introduction

Sleep disordered breathing (SDB) varies in severity from primary snoring to obstructive sleep apnoea syndrome (OSA) and is estimated to affect up to a quarter of all children. (84) At the severe end of the spectrum it adversely affects cardiovascular health resulting in pulmonary and systemic hypertension, left and right ventricular dysfunction and cor pulmonale (201, 203). In adults, the mechanisms proposed to explain the association between SDB and cardiac morbidity, endothelial dysfunction and arterial disease include intermittent hypoxia leading to increased oxidative stress, systemic inflammation and increased sympathetic activity and, furthermore, arousal induced up-regulation of the sympathetic nervous system (370).Similar mechanisms are likely to be involved in children particularly in the moderate to severe range of SDB. More recent evidence suggests that vascular changes are also present in children at the mild end of the SB spectrum with reported increases in daytime systemic blood pressure and reduced peripheral arterial distensibility (148, 334). In addition, children with mild SDB are reported to have increased cerebral blood flow velocity and increased brachial artery blood flow velocity both at rest and during the hyperaemic phase of flow mediated dilatation (147, 395). In the latter study significant hypoxia was absent suggesting that arousal induced up-regulation of the sympathetic nervous system is the more likely explanation for these reported vascular changes in small to medium vessels. Whether the compliance and function of major vessels such as the aorta are similarly impacted in children with primary snoring/mild SDB is unknown. In a recent study in adult mice, Carreras et al. (2014) found that sleep fragmentation without hypoxia resulted in the disorganization of elastic fibres in the aortic wall suggesting reduced vessel compliance, and an increase in the number of macrophages and foam cells in the aortic wall suggesting up-regulation of inflammatory pathways (296).

Inflammation also occurs in conjunction with cardiovascular disease in adults with SDB (396, 397). Increased inflammatory cytokine expression has also been reported in children with SDB (331) and evidence of alterations occurring in both vascular and inflammatory responses have been observed in smaller blood vessels. For example, Kheirandish-Gozal et al., (2014) reported in children with severe SDB that inflammatory up-regulation was associated with a delayed post-occlusion hyperaemic response in the microvasculature of the hand (345). Whether inflammatory up-regulation is associated with changes to endothelial function of larger vessels such as the aorta and, moreover, in children with primary snoring/mild SDB has yet to be established.

We hypothesise that children with primary snoring/mild SDB will demonstrate reduced aortic wall compliance which will be associated with evidence of increased inflammatory response.

4.3 Material and Methods

4.3.1 Participants

Twelve children aged 5 to 14 years referred to the Ears, Nose and Throat (ENT) Department of Women's and Children's Hospital, Adelaide, Australia for evaluation of SDB and who were subsequently scheduled for adenotonsillectomy, were invited to participate in this study. All child snored in excess of three times per week. Seven healthy age and body mass index (BMI) matched children from the community who on parental report did not snore or rarely snored were recruited as controls. Exclusion criteria included significant craniofacial abnormalities, current use of medications known to affect sleep or respiration, developmental/psychiatric disorders,

contraindications to cardiac Magnetic Resonance Imagining (cMRI) (e.g. internal metal object or claustrophobia) and inability to give informed consent.

This study was approved by the Human Research Ethics Committees of the Women's and Children's Hospital, the Royal Adelaide Hospital, and the University of Adelaide, Australia.

4.3.2 Apparatus

4.3.2.1 Sleep Disturbance Scale for Children,

The Sleep Disturbance Scale for Children (SDSC) was used to assess sleep in the prior six months (161). The SDSC contains 26 items and generates six subscales (Disorders of Sleep Breathing, Initiating and Maintaining Sleep, Disorders of Arousal, Sleep-Wake Transition, Excessive Daytime Somnolence and Sleep Hyperhydrosis) and a composite Total Sleep Problem score. The SDSC is reported to have good test-retest reliability and construct validity (160).

4.3.2.2 Polysomnography

A polysomnogram (PSG) was performed under infrared camera supervision by a sleep technician, who also documented sleep behaviour (165). The following montage was employed: electroencephalogram (EEG; C4-A1, C3-A2 and F3-A2 F4-A1 and O2-A1), electrocardiogram (ECG), electrooculogram, sub-mental and diaphragmatic and leg electromyogram (EMG), nasal pressure and oronasal flow by thermistor, respiratory movements of the chest and abdominal wall using uncalibrated respiratory inductive plethysmography (RIP). The data were collected using the Compumedics E-Series Sleep System (Melbourne, Australia). Oxygen saturation was monitored by pulse oximetry (Nellcor N-595). A sleep technician blinded to the subject's condition scored

the studies based on a standard sleep stage (398) and paediatric ventilatory criteria (11). The severity of SDB was determined using obstructive apnoea hypopnoea index (OAHI) (380) (11).

4.3.2.3 Cardiac and Arterial Function Analysis

A Siemens Sonata 1.5 Tesla MRI scanner (Siemens Medical Solutions, Erlangen, Germany) equipped with a phase array coil was used to acquire cMRI images. Long axis reference imaging was used to position 8 to 12 short axis imaging slices (Figure 1A). Images were acquired by a trained technician during 8-10 breath-holds, with prospective ECG-gated steady state free precision (SSFP) sequences. Qmass MR version 7.2 (Medis, Leiden, The Netherlands) was used to calculate the ejection fraction (EF). The arterial flow at the ascending aorta and pulmonary artery was determined by Argus software (Siemens Medical Solutions, Erlangen, Germany).

Based on the methodology described by Teo et al. (399), left ventricular ejection fraction (LVEF) was derived using manual traces of endocardial and epicardial contours from short axis images (Figure 1B) at the end diastolic and systolic. Due to inconsistencies in image acquisition, ejection fraction analysis was only available on 15 subjects (SDB = 10, control = 5).

To obtain measures of arterial blood flow velocity, phase contrast images consisting of phase and velocity encoded cine cross sectional images of the artery were recorded throughout the cardiac cycle (Figures 1A and B). Regions of interest (ROI) were then traced on phase encoded cross sectional images containing the artery of interest (Figure 1C). The ROI trace (in the phase encoded image) were then reflected on the velocity-encoded image to determine blood flow velocity, direction and volume (Figure 1D).

4.3.2.4 Leucocyte Stimulation

Blood aliquots in lithium heparin of 1ml volumes were diluted 1:2 with RPMI 1640 medium (Gibco, NY,USA) supplemented with 125 U/ml penicillin and 125 U/ml streptomycin (Gibco) in 10 ml sterile conical PVC tubes (Johns Professional Product, Sydney, Australia) Phorbol myristate (25 ng/ml) (Sigma, Sydney, Australia) and ionomycin (1 μg/ml) (Sigma, Sydney, Australia) were added for T-cell cytokine stimulation (400). Brefeldin A (Sigma, Sydney, Australia) (10 μg/ml) was added as a 'Golgi block' and the tubes re-incubated in a humidified 5% CO_2/95% air atmosphere at 37°C.

4.3.2.5 Cytokine determination

Cytokine determination was conducted on 250μL of blood sample following leucocyte stimulation. (400) 100μL 20mM EDTA/PBS was added to one of the whole blood culture tubes, which was vortexed vigorously for 20 seconds to remove adherent T-cells. To lyse red blood cells, 2mL of FACSlyse solution (BD, Biosciences, Sydney, Australia) was added and tubes incubated for 10 minutes at room temperature in the dark. After centrifugation at 500xg for 5 minutes and decanting, 0.5ml 1:10 diluted FACSPerm (BD, Biosciences, Sydney, Australia) was added to each tube, mixed and incubated a further 10min at room temperature in the dark. Two ml 0.5% bovine serum albumin (Sigma, Sydney, Australia) in Isoton II (Beckman Coulter, Sydney, Australia) was then added and the tubes centrifuged at 300xg for 5min. After decanting supernatant, Fc receptors were blocked with 10μL human immunoglobin (Intragam, CSL, Parkville, Australia) for 10 min in at room temperature. Five μL of appropriately diluted anti-CD8 and anti-CD3 PC5 (Beckman Coulter, Sydney, Australia) PE-conjugate anti-cytokine monoclonal antibodies to IFNγ and TNFα (BD) or isotype control monoclonal antibody (BD) was added for 15min in the dark at room

temperature. Two ml of 0.5% bovine serum albumin was then added and the tubes centrifuged at 300xg for 5min. After decanting, cells were analysed within 1h on a FACS Calibur flow cytometer using CellQuest software (BD, Biosciences, Sydney, Australia). Samples were analysed by live gating using FL3 staining versus side scatter (SSC). A minimum of 10,000 CD3 positive, low SSC events were acquired in list-mode format for analysis. Control staining of cells with anti-mouse IgGi-PE/IgG-PC5 was performed on each sample and background readings of < 2% were obtained. Unstimulated and unstained cells were also used to determine quadrant marker settings. Examples of the gating strategy and dot plots are shown in Figure 4.2A and B. Cytokine expression for IL2, IL4, IL6, IL8, IL10, IL12 and TGFβ were also determined.

4.3.3 Protocol

The SDSC and additional questions were completed by parents at screening. The overnight polysomnogram (PSG) was performed in a hospital sleep unit with fasting blood samples collected in the morning for inflammatory cytokines. The cMRI was completed within a week of PSG.

4.3.4 Data Analysis

Data were analysed using SPSS (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY), and GraphPad Prism version 5.00 (GraphPad Software, San Diego, USA). Unpaired Student's *t-test* (two tailed, 95% confidence interval) were used to determine group differences in continuous variables and Fisher's exact test for categorical variables. BMI values were adjusted for age and gender (https://www.bcm.edu/cnrc-apps/bodycomp/bmiz2.html). All data are represented as mean (SD) with a p-value < 0.05 considered statistically significant.

The relationship between SDB severity (PSG and SDSC) and cMRI variables were tested using Pearson-r correlations and significance was tested using r-z transformations. We restricted the correlational analyses to those SDB and cMRI variables, which were significantly different between children with SDB and controls. Pearson-r correlation was used as we anticipated an association between inflammatory up-regulation and aortic function. As previously, we also restricted the correlational analyses to those inflammatory and cMRI variables, which were significantly different between children with SDB and controls.

4.4 Results

4.4.1 Demographics, SDSC and PSG

No significant group differences were observed in demographic values between children with SDB and controls (Table 4.1). However, as expected children with SDB on recruitment had a significantly higher frequency of snoring on parental report. No significant group differences were observed in PSG variables apart for the SpO_2 nadir which was significantly lower in the children with SDB (Table 4.1). All SDSC scores were elevated in children with SDB with scores for Disorders of Sleep Breathing, Initiating and Maintaining Sleep and Excessive Somnolence and Total Sleep Problem reaching significance (Table 4.1).

4.4.2 Cardiac and Arterial Function

No significant group differences were observed with respect to both left and right ventricular function. However, the peak ascending aortic blood flow velocity was significantly higher (120%) in children with SDB compared to controls (Table 4.2).

4.3.3 Inflammatory Markers

Children with SDB had significantly higher percentage of $CD8^+$ T-cell expressing TNFα and IFNγ compared to controls but no increase in the percentage of $CD4^+$ T-cell expressing TNFα and IFNγ values (Table 4.3). There were no significant differences in expression of IL2, IL4, IL6, IL8, IL10, IL12 and TGFβ between groups.

4.3.4 Correlational Results

Examination of correlational values with a magnitude $r > 0.30$ revealed that a higher ascending aortic peak blood flow velocity was associated with a higher percentage of $CD8^+$ T-cells producing TNFα, IFNγ, Disorders of Initiating and Maintaining Sleep and Total Sleep problem score and a lower SpO_2 nadir (Table 4.4, Figure 4.3). A higher percentage of $CD8^+$ T-cells expressing TNFα value was associated with a higher percentage of $CD8^+$ T-cells expressing IFNγ, Disorders of Initiating and Maintaining Sleep and Total Sleep Problem score. Higher $CD8^+$ T-cell IFNγ values were associated with higher Disorders of Initiating and Maintaining Sleep and Total Sleep Problem score but a lower SpO_2 nadir. A lower SpO_2 nadir was associated with higher Sleep Breathing Disorder, Disorders of Initiating and Maintaining Sleep and Total Sleep Problem score (Table 4.4).

As expected there was a moderate but significant correlation between ascending aorta average blood flow per min and BMI ($r = 0.53$, $p < 0.05$) and BMI z-score ($r = 0.49$, $p < 0.05$). Also, pulmonary peak and mean velocity were both positively correlated to BMI and BMI *z*-score ($r = 0.51$, $p < 0.05$), ($r = 0.56$, $p < 0.05$), respectively.

4.4 Discussion

In children with primary snoring/mild SDB compared to non-snoring controls we observed alterations in cardiovascular function and inflammatory response. In particular, peak systolic blood flow velocity in the ascending aorta was significantly higher as were both the percentage of $CD8^+$ T-cell lymphocytes producing TNFα and IFNγ. Moreover these variables were inter-related with increased ascending aortic peak systolic blood flow velocity being associated with a higher percentage of $CD8^+$ T-cell lymphocytes producing TNFα and IFNγ values but not $CD4^+$ T-cells lymphocytes.

The present findings of increased blood flow velocity in the ascending aorta are consistent with those reported by Hill et al. (2006) who reported increased blood flow velocity in the middle cerebral artery (147) and previous findings by our group (395) demonstrating increased resting blood flow velocity in the brachial artery in children with primary snoring/mild SDB. In adults, increased brachial artery blood flow velocity has been associated with left ventricular hypertrophy and coronary heart disease (392) while increased blood flow parameters, including systolic velocity are considered the most powerful promoters of vascular remodelling (235). Nonetheless, in support of the hypothesis of reduced compliance as an explanation for the increased peak systolic blood flow velocity in the ascending aorta, evidence of reduced vessel wall compliance, measured as a delay in dilation response, have been reported in the brachial artery and metacarpal vessels in children with mild and severe SDB, respectively (395) (222). In the latter study, the delay in dilation response in the brachial artery was positively associated with increased blood flow velocity both at rest and during hyperaemia. Of note we did not find a significant difference in measures of left and right ventricular

function, which have been reported in children with severe SDB in some (192, 194) but not all studies (196).

The mechanism underlying increased ascending aortic peak systolic velocity is likely to be explained by increased periodic sympathetic activation, and its vasoconstrictive effect in the peripheral vessels. The vasoconstriction increases peripheral resistance, causing the central redistribution of blood into the major vessels, organs and muscle and increases shear stress on the endothelial cells, particularly on the peripheral and minor vessels, (401). Periodic changes to shear stress throughout sleep, via the activation of vascular sympathetic nerves, promotes vascular remodelling and pro-inflammatory signalling (402). Sympathetic over activity has been well documented in both adults and children with SDB (28, 307). The increased sympathetic activation can be attributed to either sleep fragmentation or hypoxia, with the former most likely to be the explanation in children with primary snoring/mild SDB such as those in the present study. This is consistent with the findings by Carreras et al (296) in adult mice without infection or nocturnal hypoxia where sleep fragmentation was associated with increased sympathetic activity, disruption of elastic fibres in the aortic wall, and an increase in the number of foam cells and macrophages in the aorta wall. We suspect that the increase in sympathetic activity over time, given that children may snore for years, may result in substantial vascular changes, increasing the elastic resistance of the aorta. The elastic properties of the aorta allow it to act as a buffering chamber, absorbing 50% of left ventricular stroke volume during systole (233). Increased aortic stiffness results in greater systolic blood pressure and reduced blood diastolic pressure, increased systolic blood flow velocity and left ventricular afterload (233). The potential long-term effects of such changes in the maturing childhood vasculature, even if mild, is all unknown.

Reduced vessel wall compliance and the associated increase in blood flow velocity increases shear stress on vascular endothelial cells which results in endothelial cell activation and up-regulation of adhesion molecules and chemoattractants for circulation leucocytes, leading to systemic inflammation (403). Altered endothelial cells and up-regulated inflammatory pathways are reported in adults with SDB (228) and emerging evidence suggests that inflammatory markers are also up-regulated in children with severe SDB (332, 338, 404). The potential impact of these inflammatory changes in the genesis of early atherosclerosis is unknown but given there is evidence of early atherosclerotic plaque formation reported in seminal studies of the coronary arteries of both children and young adults (405) the impact of chronic childhood SDB (which is often underdiagnosed) on cardiovascular health and the inflammatory process merits further evaluation..

We found that the percentage of $CD8^+$ T-cell lymphocytes expressing both TNFα and IFNγ but not CD4+ T-cell lymphocytes were elevated in children with primary snoring/mild SDB. Increased TNFα has been demonstrated in both children and adults with OSA, while IFNγ has been shown to be increased in children with OSA compared to healthy controls (331). TNFα and particularly IFNγ play an important role in host defense against infection as well as adaptive immune response (406). IFNγ may also play a role in cardiac remodeling, while IFNγ and TNFα are important signals required for macrophage activation and may be involved in macrophage foam formation in atherosclerosis (407). The strong positive correlation we observed between the inflammatory markers TNFα and IFNγ and the ascending aorta peak velocity, suggests that even primary snoring/mild SDB in young children may exacerbate the inflammatory response and alter vascular function simultaneously. Children with elevated TNFα and IFNγ values also had a greater number of sleep problems on parental

report and especially problems with initiating and maintaining sleep, further potentially supporting the role of sleep fragmentation in up-regulating inflammatory pathways. Increased fluid shear stress (blood flow velocity) promotes arteriogenesis and the increase in the formation of conducting vessels, (collateral arterioles) (408). The processes in collateral formation is complex requiring coordinated proliferation of the different vascular cell types including the endothelial cells, smooth muscle cells, fibroblasts, and myofibroblast, mediated in response to the increase in shear stress. Part of this process also requires the activation of $CD8^+$ T-cells, whether the increased number of $CD8^+$ T-cells expressing IFNγ and TNFα is part of this process is yet to be determined. However, the correlation between the ascending aorta systolic blood flow velocity and increase in the number of activated $CD8^+$ T-cells maybe evidence of this adaptive mechanisms of collateral formation.

More recently, Kheirandish-Gozal et al., demonstrated that children with delayed hyperemic dilation response and OSA, were more likely to exhibit reduced $CD14^+$ monocytes and increased $CD16^+$ cells and hence reduced CD14:CD16 (ratio). In addition they found that $CD16^+$ cells expressed higher levels of the chemokine, Fractalkine (CX3CL1) monocytes, which induces chemotaxis in monocytes in children with the delayed dilatation response (192, 345). Also noted was a strong inverse correlation between the CD14:CD16 and the time to maximal dilation during the hyperemia test (Tmax). What is interesting is that *in vitro* studies using isolated peripheral blood monocytes incubated with activated $CD8^+$ cells (409) or monocytes and macrophages exposed to high concentrations of IFNγ (410) show down-regulation of soluble CD14, highlight the interrelationship between these cell types and particular cytokine expression and possible vascular irregularities. Our data complements this previous work by Kheirandish-Gozal et al., and collectively suggests systemic vascular

irregularities both in minor and major vessels and an emerging pattern of inflammatory response that maybe central to SDB in children.

The increase in the percentage of cells expressing IFNγ was specific to $CD8^+$ T-cells and not $CD4^+$ T-cells. This may arise because of the effector function acquisition in these cell types require different processes. For example $CD4^+$ undergo a complex process to produce IFNγ requiring specific interaction with other cytokines with cell-signaling proteins, transcription factors and hence chromatin remodeling (411). As well $CD4^+$ cell differentiation requires IL12 production from antigen-presenting cells in response to Toll-like receptor stimulation with signaling and transcription factors including signal transducer and activator of transcription 4 (STAT4) and T-bet (the main transcription factor required for T helper cell differentiation) to induce a functional T helper 1 response for them to produce IFNγ. $CD8^+$ T-cells require only short-term primary stimulation to readily develop into effector cells that express IFNγ. T-bet, IL12 and IL4 have been shown to have little effect on the generation of IFNγ from $CD8^+$ T-cells. Also the stimulus required to activate differentiation has been shown to produce different profile for different stimuli in naïve $CD4^+$ and $CD8^+$ T-cells. Mice exposed to lymphocytic choriomeningitis virus (LCMV) (412), Sendau (413) and vaccinia (414) show increased number of $CD8^+$ T-cells producing IFNγ compared to $CD4^+$ IFNγ producing T-cells. In humans, Epstein-Barr virus exposure also results in an increase in $CD8^+$ IFNγ producing T-cells compared to $CD4^+$ T-cells (415). One possible reason for the difference other than the required substrate specific pathway processes previously mention, is the differences in replication between the two naïve cells types. $CD8^+$ T-cells have a faster rate of cell division than CD4+, (411) with $CD8^+$ T-cells replicate approximately 15 times within the first week of LCMV exposure

(412) hence the type of stimulus may alter the cell proliferation rate and cause the imbalance.

In the present study SpO_2 nadir was associated with increased ascending aortic peak systolic velocity. This is consistent with earlier findings by our group that SpO_2 nadir was associated with increased blood flow velocity in the brachial artery (Kontos et al., 2015) and supports the idea that blunted chemosensitivity, independent of OAHI values is associated with cardiovascular changes.

4.4.1 Limitation

A limitation of the present study was the technical requirement of undertaking a cMRI and blood tests in young children. Despite this limitation we obtained sufficient number of age and body mass matched children with a history of snoring and non-snoring controls. Also, this study looked at the percentage of cells that produced a particular cytokine, we recognise that it would have also been beneficial to have recorded concentration levels, however as previously mentioned, other studies have shown increased concentration of both TNFα and IFNγ in children with SDB. Previous studies have measured cytokine levels in children with SDB using ELISA quantification from serum and rtPCR of cytokine mRNA levels. These techniques have their limitations and in particular, do not indicate which cell types produce which cytokine. This study is novel as it uses flow cytometry to assess intracellular pro and anti-inflammatory T-cytokines from different T-cell types (400, 416, 417). This is important as cell specific cytokine production is becoming more evident in profiling different disorders (411).

4.4.2 Conclusion

In conclusion, although the pathophysiology of the development of cardiovascular sequelae in SDB is not as yet clearly defined, the balance of current evidence suggests that SDB affects multiple physiological pathways that are interdependent (145). The consensus in the literature is that the major pathways involved include the autonomic nervous system, in particular the sympathetic branch and inflammatory and metabolic pathways (lipid and insulin) (418). The results of this current study add to the growing evidence that these same pathways are affected in children with even the least severe form of SDB, i.e. primary snoring/mild SDB.

4.5 Acknowledgements

We would like to thank the staff of the MRI Unit of the Royal Adelaide Hospital and Sleep Disorders Unit, Women's and Children's Hospital, Adelaide.

Table 4.1: Mean (SD) Demographics, Polysomnographic and Sleep Disturbance Scale for Children scores and p-values from t-test results.

Variable	Controls (n=7)	SDB (n=12)	*p*-value
Anthropometric			
Males (%)	3:4	5:7	ns (Fisher exact analysis)
Age (M:F)	10.3 ± 2.4	9.3 ± 3.0	ns
Height (cm)	139.8 ± 14.5	135.8 ± 18.0	ns
Weight (kg)	35.7 ± 12.6	37.9 ± 14.4	ns
BMI	17.8 ± 2.6	19.6 ±5.3	ns
BMI Z-score	0.1 ± 0.6	0.7 ± 0.9	ns
Snoring severity[1]	**1.3 ± 0.5**	**3.8 ± 1.0**	**< 0.001**
Polysomnographic			
Total Sleep Time (min)	448.4 ± 29.9	433.3 ± 34.0	ns
Non Rapid Eye Movement Sleep (%TST)	81.4 ± 4.0	81.6 ± 3.1	ns
Slow Wave Sleep (%TST)	36.1 ± 6.2	37.2 ± 9.5	ns
Rapid Eye Movement Sleep (%TST)	18.6 ± 4.0	18.4 ± 3.1	ns
Arousals n/h	10.1 ± 3.05	12.5 ± 4.5	ns
Spontaneous Arousals n/h	9.0 ± 2.4	11.3 ± 4.2	ns
Respiratory Arousal n/h	0.9 ± 0.6	1.0 ± 1.3	ns
Obstructive Apnoea Hypopnea Index	0.3 ± 0.3	0.6 ± 1.5	ns
Nadir SpO_2 (%)	**94.4** ± **1.6**	**92.3** ± **2.7**	**< 0.05**
REM Nadir SpO_2 (%)	93.6 ± 2.1	93.6 ± 3.0	ns
NREM Nadir SpO_2 (%)	93.6 ± 1.1	92.7 ± 1.9	ns
Average SaO_2desat (%TST)	2.6 ± 1.1	2.9 ± 1.1	ns
Sleep Disturbance Scale for Children			
Total Sleep Problem Score	**37.0** ± **4.1**	**54.3** ± **10.3**	**< 0.005**
Disorders of Initiating and Maintaining Sleep	**12.2** ± **1.8**	**18.5** ± **4.2**	**< 0.005**
Sleep Disordered Breathing	**3.3** ± **0.5**	**7.2** ± **2.5**	**< 0.001**
Disorders of Arousal	3.2 ± 0.5	3.9 ± 1.0	ns (< 0.09)
Disorders of Sleep-Wake Transition	12.2 ± 1.8	18.5 ± 4.2	ns (< 0.08)
Disorders of Excessive Somnolence	**6.9** ± **1.1**	**10.7** ± **2.8**	**< 0.01**
Sleep Hyperhydrosis	2.3 ± 0.5	4.0 ± 2.7	ns (< 0.06)

NB: ns denotes non-significant. [1]Five point scales with 1 = Never to 5 = Always (daily).

Table 4.2: Mean (SD) cardiac and arterial function values together with results from t-test analyses.

Parameter	Control	SDB	*p*-value
	(n = 7)	(n = 12)	
Systolic Blood Pressure (mmHg)	107.4 ± 9.8	113.2 ± 17.0	ns
Diastolic Blood Pressure (mmHg)	69.1 ± 6.9	70.6 ± 8.7	ns
Ascending Aortic Peak Blood Flow Velocity (cm/sec)	**101.5 ± 16.3**	**120.0 ± 18.0**	**< 0.05**
Ascending Aortic Mean Blood Flow Velocity (cm/sec)	24.43 ± 3.9	22.7 ± 3.6	ns
Ascending Aortic Mean Blood Flow (l/min)	5.0 ± 1.5	4.75 ± 1.2	ns
Pulmonary Artery Peak Blood Flow Velocity (cm/sec)	76.5 ± 17.6	84.2 ± 7.4	ns
Pulmonary Artery Mean Blood Flow Velocity (cm/sec)	22.9 ± 6.3	21.3 ± 4.2	ns
	(n = 5)	(n = 10)	
Left Ventricular Ejection Fraction (%)	67.1 ± 4.4	64.9 ± 8.7	ns
Left Ventricular End Diastolic Volume (ml)	69.9 ± 14.2	61.1 ± 18.2	ns
Left Ventricular End Systolic Volume (ml)	22.7 ± 4.4	23.4 ± 6.2	ns
Left Ventricular Mass (g)	46.9 ± 4.2	47.0 ± 7.6	ns
Right Ventricular Ejection Fraction (%)	67.1 ± 4.4	63.4 ± 10.1	ns
Right Ventricular End Diastolic Volume (ml)	70.0 ± 14.2	43.6 ± 6.1	ns
Right Ventricular End Systolic Volume (ml)	47.1 ± 11.6	23.4 ± 6.2	ns
Right Ventricular Mass (g)	46.9 ± 4.2	44.6 ± 6.6	ns

NB: ns denotes non-significant

Table 4.3: Mean (SD) Inflammatory marker values and p-values from t-test results.

Inflammatory Markers	Controls (n = 7)	SDB (n = 11)	*p* - values
$CD8^+$ T-cell IFNγ (% of cells)	23 ± 10	42 ± 18	< 0.05
$CD8^+$ T-cell TNFα (% of cells)	20 ± 8	32± 12	< 0.05
$CD4^+$ T-cell IFNγ (% of cells)	22 ± 6	26.2 ± 5	ns
$CD4^+$ T-cell TNFα (% of cells)	32.9 ± 12	36.7 ± 8	ns

NB: ns denotes non-significant

Table 4.4: Correlations between, cardiovascular, inflammatory markers and Sleep Disturbance Scale for Children scores.

	Ascending Aortic Peak Blood Flow Velocity	$CD8^+$ T-cell TNFα	$CD8^+$ T-cell IFNγ	Nadir SpO_2
$CD8^+$ T-cell TNFα	**0.54****	-	**0.84*****	-0.05
$CD8^+$ T-cell IFNγ	**0.63*****	**0.84*****	-	-0.32
Nadir SpO_2	**-0.48***	-0.05	-0.32	-
SDSC: Sleep Disordered Breathing	0.14	0.15	0.26	**-0.53****
SDSC: Disorders of Initiating and Maintaining Sleep	0.31	**0.55****	**0.52****	-0.35
SDSC: Total Sleep Problem Score	0.48*	0.50*	**0.68*****	**-0.55****

NB: * denotes $p < 0.1$, $^{**} < 0.05$ and $^{***}p < 0.01$.

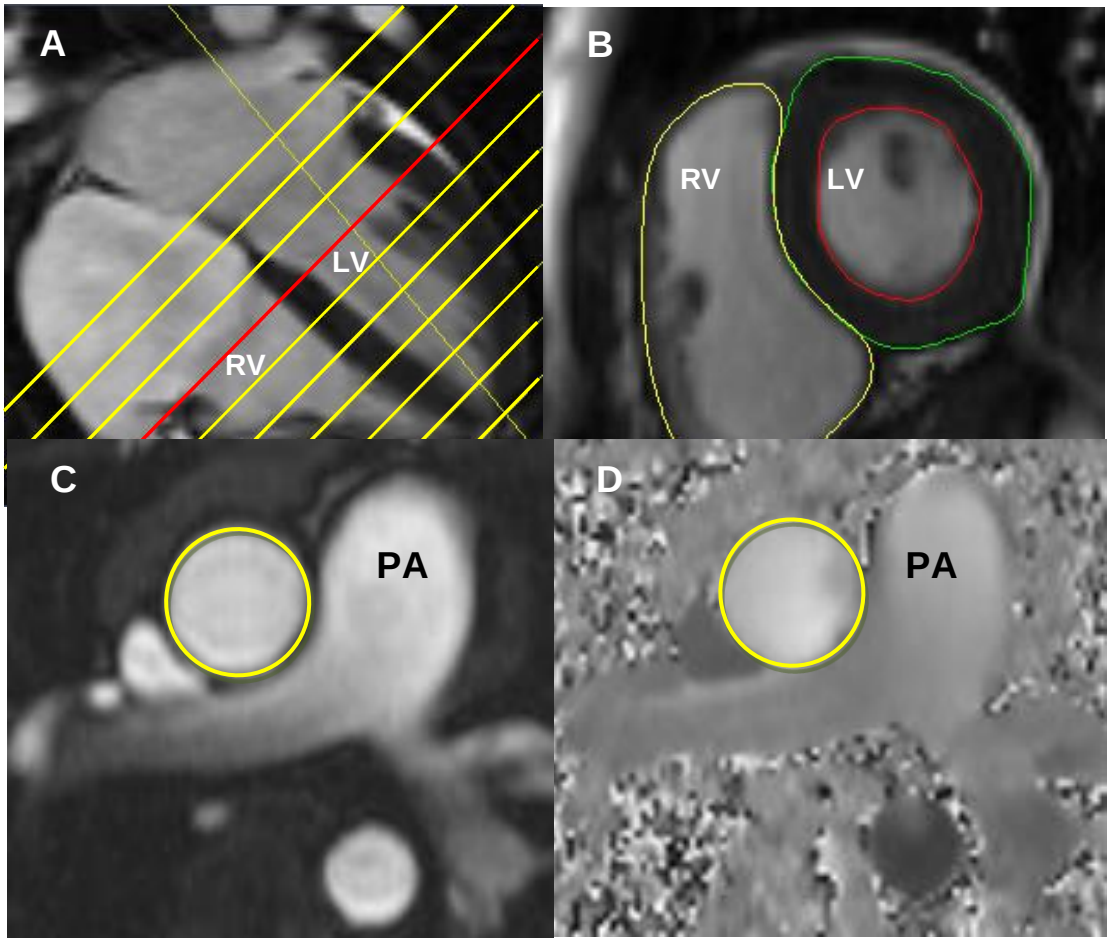

Figure 4.1: A) Representative long axis view (sagittal plane) in systole. RV and LV indicates right and left ventricle respectively whilst yellow lines illustrates cross sectional imaging slices acquired on the transverse plane. Red line indicates imaging slice of interest. B) Slice of interest depicted in short axis view. Green and red line illustrates left ventricular epicardial and endocardial contours respectively. Yellow line indicates right ventricular endocardial contour tracing. C) Phase encoded single image of the ascending aorta taken from a series of images acquired during the cardiac cycle. Yellow circle illustrates region of interest (ROI). PA indicates pulmonary artery. D) Velocity encoded image of the specific phase encoded image shown in Figure 1C. The respective ROI traced on the ascending aorta on the phase-encoded image was reflected on the velocity-encoded image seen here.

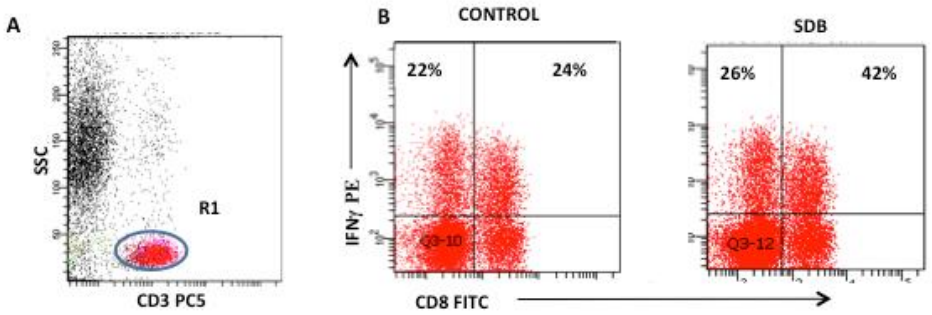

Figure 4.2: A) Representative dot plot showing CD3+ T-cell gating strategy. T-cells were identified as CD3PC5 positive low side scatter (SSC) events. B) Representative dot plots of CD3+ T-cells staining with CD8 FITC and intracellular cytokine IFNγ of a patient with SDB and control subject. There was an increase in CD8+ T-cells producing IFNγ in patients with SDB compared with control subject.

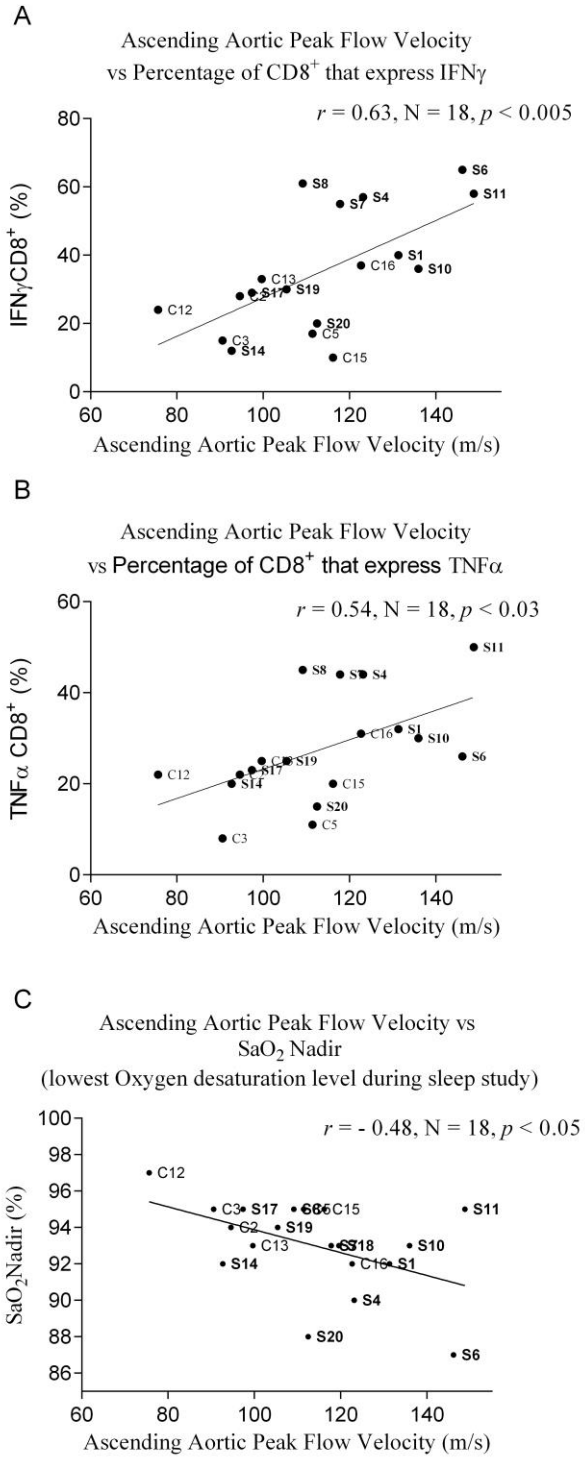

Figure 4.3: Scatterplots of the relationship between ascending aortic peak velocity measured using cMRI, and IFNγ (A) TNFα (B) expression from $CD8^+$ T-cells using flow cytometry and SpO_2 nadir (C) ('C' denote control participant, 'S' denote SDB participant results).

Chapter 5

Is sleep disordered breathing in children a precursor of cardiovascular disease?

5.1 Preface

This chapter looks at the relationship between the changes in blood flow velocity measured at the brachial artery using ultrasound Doppler and autonomic function measured using pupillary light reflex (digital pupillometry) and also endothelial integrity using platelet aggregation (whole blood impedance aggregation). In addition it compares the above parameters to sympathetic nerve fibre density (SNFD) of the dorsal lingual artery (confocal imaging, tyrosine hydroxylase antibody immunofluorescence staining) removed from the tonsillar tissue of children with SDB who have been treated with T&A.

Statement of Authorship

Title of Paper	**Is sleep disordered breathing in children a precursor of cardiovascular disease?**
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	This paper will be submitted to Hypertension.

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Name of Principal Author (Candidate)	Anna Kontos		
Contribution to the Paper	Study Design, Data Collection, Data Analysis, Interpretation of results, Preparation of manuscript.		
Overall percentage (%)			
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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5.3 Abstract

Background: Sleep disordered breathing (SDB) in children is associated with increased blood flow velocity and sympathetic overactivity. Sympathetic overactivity results in peripheral vasoconstriction and reduced systemic vascular compliance which increases blood flow velocity during systole. Augmented blood flow velocity is recognised to promote vascular remodeling. More importantly, increased vascular sympathetic nerve fibre density and innervation in early life plays a key role in the development of early onset hypertension. As yet whether these functional changes which include increased sympathetic activity, reduced vascular compliance and increase blood flow velocity are associated with changes to vascular structure in children are unknown. This can be addressed by an examination of the vasculature of the tonsillar tissue in children undergoing adenotonsillectomy for treatment of SDB.

Methods: Thirteen children (6.3 – 17.3 years; 6 males, 7 females) scheduled for adenotonsillectomy to treat SDB, underwent, pupillometry, polysomnography, flow-mediated dilation (FMD) (including resting brachial artery blood flow velocity (VTI)) and platelet aggregation studies. The dorsal lingual artery of the tonsil was dissected and stained for tyrosine hydroxylase using immunofluorescence techniques to identify sympathetic nerve fibres. The arterial sympathetic nerve density (SNFD) was determined using a ratio algorithm from confocal images.

Results: Children with increased SNFD had increased vascular resistance in the brachial artery and higher sympathetic tone on pupillometry. SNFD was correlated with increased resting VTI ($r = 0.63$, $p < 0.05$) and a lower Neuronal Pupillary Index^{TM} ($r = - 0.71$, $p < 0.01$), a smaller percentage change in pupillary diameter from baseline ($r = - 0.70$, $p < 0.05$), a slower mean and peak pupillary constriction velocity (Mean: $r = -$

0.64, $p < 0.05$; Peak $r = -0.63$, $p < 0.05$). A faster resting VTi was associated with a slower peak pupillary constriction velocity ($r = -0.77$, $p < 0.01$) and higher platelet aggregation to collagen antigen ($r = 0.64$, $p < 0.05$). A slower mean and peak pupillary constriction velocity were associated with higher platelet aggregation scores ($r = -0.60$, $p < 0.05$; $r = -0.77$, $p < 0.01$, respectively).

Conclusion: These results indicate that sympathetic overactivity is associated with changes in both the function (FMD and platelet aggregation) and structure (dorsal lingual artery) of vasculature in children with SDB.

5.4 Introduction

In adults, sympathetic tone is an important factor in vascular health with increased tone adversely affecting vascular function and leading over time to hypertension and vascular remodelling (419). Increased sympathetic tone is also a feature of several disorders (420, 421) including sleep disordered breathing (SDB) (306, 309, 422). SDB affects an estimated 10% of children, but is often underdiagnosed and is associated with hypertension which is thought to reflect the effect of a persistent increase in sympathetic tone (203). In adults with SDB there is evidence that the effects of the sympathetic overactivity on blood vessels is systemic (185). Our group and others have postulated that some children with SDB also develop systemic changes in vascular compliance, which arise in direct response to increased sympathetic activity (307, 395).

In SDB, partial or total collapse of the upper airway leads to increased respiratory effort, intra-thoracic pressure swings, intermittent hypoxia, sleep fragmentation and increased arousals (145). These factors lead to sympathetic over-activation with the release of noradrenaline from vascular sympathetic nerve fibre terminals and a consequent increase in heart rate, inotropy and peripheral vasoconstriction (423). In turn, the reduced vascular compliance and concomitant increase in total peripheral resistance (401) leads to an increase in blood flow velocity during systole and, thereby, also increases luminal shear stress (254, 357, 424, 425). A persistent increase in shear stress damages the vascular endothelial cells surrounding the lumen and increases platelet aggregation (426). Sympathetic overactivation is also known to promote vascular cell proliferation (smooth muscle cells) and increases the production of extracellular matrix proteins such as elastin and collagen (245, 427). Importantly, in rats, early exposure to sympathetic hyperactivity is associated with the development of early onset

hypertension (296) and increased recruitment of peripheral vascular sympathetic neuronal fibers (density) in early development (296, 428-430).

We have previously reported altered cardiovascular function in children with SDB including: an accelerated heart rate response to cortical arousals (431); and a delayed hyperemic dilatation response and increased blood flow velocity both at rest and during hyperemia in the brachial artery suggestive of reduced vascular compliance (395). Others have also reported abnormal vascular function in all severity levels of the disorder compared to non-snoring children including reduced dilation capacity, reduced vascular compliance and increased blood flow velocity (147, 210, 223, 231). But, to date, the relationship between sympathetic activity and its effects on vascular function (blood flow velocity, arterial dilation capacity and endothelium integrity) and vascular structure in children with SDB are unknown. This question may be answered by an examination of tonsillar blood vessels from children undergoing adenotonsillectomy for SDB, who have also previously undergone vascular functional testing (brachial artery blood flow velocity, flow-mediated dilation (FMD) and platelet aggregation). We hypothesize that the functional changes associated with sympathetic overactivation in children with SDB will be associated with changes in vascular structural— i.e. increased tonsillar artery sympathetic nerve fibre density. Adenotonsillectomy offers a unique opportunity to address this limitation by histologically evaluating tonsillar blood vessels for evidence of changes in sympathetic innervation and to match the histological findings with accepted functional markers of altered autonomic tone (432-434).

In summary, the aim of this study was to utilize vascular sympathetic nerve density as a structural measure of sympathetic innervation and to correlate this with a functional measure of autonomic tone (pupillometry) and the functional vascular response seen in

flow mediated dilatation and platelet aggregation in children who were scheduled for adenotonsillectomy for the treatment of SDB.

5.5 Methods

Children with a history on parental report of snoring more than three nights per week, and who were referred by their primary physician for the evaluation of potential SDB by experienced otolaryngologists at the Women's and Children's Hospital Adelaide, Australia and who were subsequently scheduled for adenotonsillectomy were recruited as participants (n=15). Children with a history of significant asthma, previous adenoidectomy, evidence of recent tonsillar infection, craniofacial abnormalities, medications that affect sleep or respiration, and English as a second language were excluded from the study. The study was approved by the Women's and Children's Hospital and University of Adelaide's Human Research Ethics Committees.

5.5.1 Anthropometrics

Children were weighed wearing minimal clothing using an electronic scale with a resolution of 0.1kg and height was measured using a wall-mounted stadiometer. BMI and percentile was calculated using the Baylor College of Medicine website tool (http://www.bcm.edu/cnrc/bodycomp/bmiz2.html).

5.5.2 Polysomnography

As previously reported, overnight polysomnography (PSG) was conducted using the Compumedics E-Series Sleep System (Melbourne, Australia (395). In brief, the following standard measures were collected: electroencephalogram (EEG; C3-A2, C4-A1, and F3-A2 F4-A1, O1-A2 and O2-A1), left and right electrooculogram (EOG),

sub-mental, diaphragmatic and leg electromyogram (EMG), heart rate by electrocardiogram (ECG), oronasal airflow by thermistor and nasal pressure, respiratory movements of the chest and abdominal wall using uncalibrated respiratory inductive plethysmography (RIP), and arterial oxygen saturation (SpO_2) by pulse oximetry (Nellcor N-595; two second averaging time). Children were continuously monitored via infrared camera by a pediatric sleep technician who also documented observations of sleep behavior, which included the presence or absence of snoring.

5.5.3 Pupillometry

The pupillary light reflex was used to evaluate autonomic tone (303, 434-437). Pupillary response was measured using the handheld NeurOptics device (NeuroOptics, Irvine, CA). The device consists of an infrared illumination source and a digital camera to capture pupillary images and an onboard processor with summary data output to a liquid crystal display. The device delivers a light pulse to stimulate the eye and then analyses subsequent changes in pupil diameter from a succession of digital images. On completion of the set-up for the overnight PSG the room light was dimmed to a standard lux (8-9 lux), and children were instructed to lie supine in their beds for 5 min. Children were then instructed to focus on a fixed point on the ceiling and their pupillary response to a 180 μW, 33ms light pulse was sampled at 30 frames per second (FPS) for three seconds in each eye consecutively (Figure 1). Children where then allowed to go to sleep. The pupillary light reflex was measured prior to sleep onset at a time when the sympathetic nervous system is quiescent and parasympathetic vagal control is dominant (434, 438).

The following pupillary parameters obtained from the NeurOptics device are outlined in Table 5.1. : (i) baseline pupillary diameter (Maximal Diameter) (ii) constricted diameter at peak of the light reflex, (Minimal Diameter) (iii) percentage change in pupil

diameter from baseline, (iv) constriction latency (time difference between initiation of retinal light stimulation and onset of pupillary constriction), (v) average constriction velocity (amount of constriction/duration of constriction mm/sec), (vi) maximal constriction velocity (peak value of velocity during constriction) and post constriction velocity (dilation velocity post after peak constriction). Using proprietal software the above variables were compared to a normative pupillary light reflex dataset to derive a Neurological Pupil Index$_{TM}$ (NPi) graded on 1-5 point scale with a score < 3 being indicative of an abnormal pupillary response (439).

5.5.4 Flow Mediated Dilatation

The measurement of flow-mediated dilatation (FMD) *in-vivo* has been widely used to assess endothelial function [27, 28]. A detailed explanation of the FMD protocol used in the present study can be found in our earlier study [26]. In brief, FMD was measured upon waking following overnight PSG and while still fasting. The brachial artery diameter was determined from a 30s resting scan measured perpendicular to the brachial artery and 10cm proximal to the elbow. The transducer was then set on B mode and positioned at a 60° angle to the vessel and subsequent blood flow dynamics were collected (resting peak systolic velocity (PSV), velocity time integral (VTi) and heart rate (HR)). A minimum of six image sets were taken and averaged values were used to determine resting blood flow dynamics. The lower forearm was then occluded by inflating a sphygmomanometer cuff to 200 mmHg for four minutes. A subsequent 3min scan was taken, begining 15s before cuff removal. This scan captured the changes in vascular diameter as diacom files.

Platelet Function Testing – Whole Blood Impedance Aggregation

Blood flow is an important mediator influencing the platelet aggregation process. It has been previously proposed that different shear conditions provide distinct patterns of aggregation. Whole blood impedance aggregometry is based on the principle that activated platelets stick via their surface receptors to artificial surfaces of two electrodes within the whole blood sample positioned at a determined distance between them (440). Hence, platelet aggregation was evaluated using Multiplate analyser (Dynabyte, Munich, Germany) within 45 minutes post venipuncture as previously described[441]. The device is considered a valid, rapid and complete measure of platelet function. Platelet aggregation was simultaneously measured using independent sensor units, for four different antagonists. The rate of aggregations was measured by detecting the increase in electrical impedance generated by the aggregation of the platelets upon those fixed to the electrodes. The degree of the increase in impedance was recorded in Ohms. (440)

15µl of $CaCl/NaCl_2$ (37°C) solution was used as a diluent for low dose adenosine diphosphate (ADP) (1:4 dilution), high dose ADP, thrombin receptor activating peptide (TRAP-6) and collagen (COL) aggregation, while, saline solution was used as a diluent for arachidonic acid (ARA) tests. All agonists were purchased from Multiplate Dynabyte, Munich, Germany). 150µL of whole blood was then added to the impedance channels containing diluent and incubation (2mins, 37°C) before addition of 20µL of respective platelet agonist to achieve the following final concentrations, 1.6µM low dose ADP, 6.5µM high dose ADP, 32µM TRAP-6 and 3.2µg COL aggregation and 0.5mM ARA test. Measuring time was 6 mins for all reactions, aggregation units (AU, Ohms) plotted against time (mins).

5.5.5 Vascular Histological Examination

Tonsils were resected using a cold steel procedure and the inferior lobes of both tonsils sutured to aid orientation. Dorsal lingual arteries were micro-dissected from tonsils within 30min of removal and immersed in modified Zambonis fixative for 24h (0.2% saturated picric acid in 2% paraformaldehyde in 0.1 olL-1 phosphate buffer). Vessels where then permeabilized by 3 x 10min washes with dimethylsulphoxide (DMSO) followed by 3 x 10min washes with phosphate-buffered saline (PBS) and then stored in PBS with 0.01% sodium azide at 4°C (442).

5.5.6 Immunohistochemisty

Vessels were blocked in PBS with 10% inactivated horse serum (Sigma) for 60 min at room temperature. Following removal of blocking solution, vessels were then placed into a solution of primary antibodies diluted in PBS with 0.1% Triton X and 5% horse serum for 60min at room temperature. Antibodies and dilutions included: anti-mouse alpha Smooth Muscle Actin (αSMA) conjugated to Alexa Fluor-555 (Abcam: 1:500) and anti-rabbit Tyrosine Hydroxylase (Millipore: 1:200). Samples were then washed three times with PBS prior to addition of donkey anti-rabbit Alexa Fluor-488 conjugated secondary antibodies (Thermo Fisher: 1:200) for 60min at room temperature. Whole vessels were subsequently mounted in NT_Prolong Gold anti-fade reagent containing DAPI (Thermo Fisher) on glass slides. All vessels were imaged using the same laser settings on Zeiss LSM 700 Confocal microscope at 5x magnification with Z-stacks set to a depth of 2.25-2.85 µm. Images were captured using Zen Black software (Zeiss) and converted to PNG files for image data processing. (Figures 5.4, 5.5, 5.6)

5.6 Data Analysis

5.6.1 Sleep Disordered Breathing

PSG records were scored by an experienced sleep technician blind to subject status and according to established sleep stage [29] and ventilatory criteria [30], details see Kontos et al. (2015) (395) (Chapter 3).

5.6.2 Flow Mediated Dilatation Analysis

A custom made program using Matlab (software details) was used to determine regions of interest (ROI) from the whole brachial vessel. The ROI window was manually manipulated for each individual vessel so that it remained within the ROI window for the duration of the scan (3 min/ 935 frames). The pixel to mm ratios were determined via the hardcoded dicom millimeter key (Figure 5.2). Ten perpendicular, evenly spaced interrogation points along the interior of the vessel were used for averaging the dilation response per frame. A pixel intensity threshold (40 from 8bit intensity values) was chosen via visual analysis of intensity gradients at the borders of the vessels (note: images have high spatial intensity homogeneity, and consistent intensity between individuals). At each of the 10 interrogation points the dilation of the vessel was measured by extending a line along the positive and negative y axis until the threshold value was reached, indicating the end of the vessel interior (Figure 2). Vessel dilation measurements in mm were calculated using the dicom and mm:pixel ratio. The interior of the vessel was then measured again from the same location in the following frame and compared to the previous measurement. To exclude values attributed to movement artifacts, measurements which exceeded the previous corresponding measurement by 10% were included as 'not a number' (NAN) in analysis until a subsequent measurement fell within the threshold of the last valid measurement, as they were

considered outliers. A two-dimensional matrix consisting of 10 rows, one per interrogation point, and columns determined by the number of frames was used to hold all the vessel measurements generated from one dicom image set. A mean dilation for each frame (10 vessel measurements/10) was then determined. These values were then plotted against a time axis, and used to determine the maximal diameter (FMD_{Max}), the diameter at 60s post cuff deflation (FMD_{60}) and time to maximal dilation ($FMD_{time\ to\ Max}$) (Figure 5.3).

5.6.3 Tyrosine Hydroxylase Nerve Fibre Density

5.6.3.1 Metrics Derivation

Raw microscope data (Figure 4) was exported in PNG format and then analysed in a custom algorithm (python script; Numpy for matrix analysis, Scipy for image support). Images were internally represented as three-dimensional matrices (x, y, rgb). The red and blue channels were discarded before immunofluorescence in the green channel was determined at three points along the vessel using circular interrogation regions (Figure 4) to avoid issues with rotational impartiality.

Interrogation regions were chosen using an heuristic approach and evenly spaced along clearly imaged portions of the vessel (Figure 5.5). The person picking the region of interest was unaware of the participant's results in their functional tests. Total green values were calculated using a simple sum function of the elements in the circular region of the matrix and the average ratio of green pixels per region of interest (ROI) of three circles was used as a measure of sympathetic nerve fibre density (SNFD).

5.6.4 Statistics Analysis

Data were analysed using SPSS (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY), and GraphPad Prism version 5.00 (GraphPad Software, San Diego, USA). BMI values were adjusted for age and gender (https://www.bcm.edu/cnrc-apps/bodycomp/bmiz2.html). All data are represented as mean (SD). The relationship between FMD, Pupillometry, SNFD, Platelet Aggregation and PSG variables were tested using Pearson-r correlations and significance was tested using r-z transformations, and p value of 0.05 was considered significant.

5.7 Results

Of the original 15 tonsil sets, the lingual arteries of 13 (6 females, 7 males) demonstrated immunoreactivity to TH antiserum (Examples are given in Figure 5.4, 5.5, 5.6). Preliminary Chi square and t-test analyses revealed no significant gender differences in any variable. The variable means, standard deviations and ranges are reported in Table 5.2.

5.7.1 Correlational Analyses

5.7.1.1 Sympathetic Nerve Fibre Density (SNFD)

A greater SNFD was moderately and significantly correlated with a higher resting VTi (Figure 5.6) (Figure 5.7A) and resting peak systolic velocity (Figure 5.7B). A greater SNFD was moderately and significantly correlated with a lower Neuronal Pupillary Index (Figure 5.9A), smaller percentage change from baseline pupil diameter (Figure 5.9D) and slower constriction latency, mean pupil constriction velocity (Figure 5.9C), peak pupil constriction velocity (Figure 5.9E) and post constriction dilation velocity (Figure 5.10C) (Table 2).

5.7.1.2 Flow Mediated Dilatation (FMD)

Apart for a strong positive relationship between resting VTi and $FMD_{Time\ to\ Max}$, (r = 0.71, p < 0.01). no significant relationship were observed between remaining FMD ($FMD_{maximal\ dilation}$, FMD_{60s}) and VTi and any of the other variables (Pupillometry, Platelet Aggregation, SNFD) (all r < 0.30).

5.7.1.3 Pupillometry

A faster maximal constriction velocity was significantly associated with a reduced rVTI and rPSV (Table 5.3) (Figure 5.8). A similar strong relationship was also evident between post constriction dilation velocity and rVTi (r = - 0.79, p < 0.01) (Figure 5.10A), rPSV (r = -0.78, p < 0.01) (Figure 9B) and also $FMD_{time\ to\ max}$ (r = 0.66, p < 0.05) (Figure 5.10D). There was no relationship between age, BMI, BMI (z-score), weight, height, and percentile with any of the pupillometry variables

5.7.1.4 Platelet Aggregation

No significant relationship was observed between FMD60, FMDmax with any of the platelet aggregation variables. However, resting VTI showed a significant positive association with COLauc and COLagg (r = 0.64, N = 12, p < 0.05; r = 0.64, N = 12, p < 0.05) and a trend to significance between COLauc and FMDttm, (r = 0.54, N = 12 p = 0.07). There were no relationships observed between any brachial artery vascular function test and the other four aggregation test results. SNFD was positively associated with increased platelet aggregation velocity variables (the rate at with aggregation occurred) with COL, and ADP (Table 5.4).

Significant correlations between pupillometry variables and platelet aggregation results are listed in Table 5.4. In brief, NPI, mean and peak pupillary constriction velocity were inversely associated with collagen; IADP; ASP and ADP aggregation. There were no significant relationship between any of the pupillometry variables and TRAP aggregation data. There was a positive relationship with constriction latency and all but TRAP aggregation variables (Table 5.4).

5.8 Discussion

This study is the first study to evaluate the relationship between functional and structural vascular parameters in children with SDB. We observed a significant relationship between sympathetic nerve fibre density (SNFD) of the dorsal lingual artery of children with SDB and resting blood flow velocity (rPSV/rVTI) measured at the brachial artery using FMD. We found that the SNFD was not related to anthropometric characteristics such as age, sex or BMI. Furthermore, we showed that increased SNFD was correlated with augmented autonomic function during the pupillary light reflex. This was measured using an automated pupillometry technique just before sleep onset and demonstrated an increased latency in pupillary constriction, reduced mean and peak constriction velocity and reduced constricted pupillary diameter. It is also the first study to show that increased resting blood flow velocity (rPSV/rVTI) measured at the brachial artery in children is associated with increased time to maximal dilation response using FMD. Taken as whole these results suggest that some children with SDB exhibit increased sympathetic activity. This activity is associated with the measured proliferation of autonomic nerve fibers of the dorsal lingual arteries of the lingual tonsil and an alteration in both the resting brachial blood flow velocity and dilatation response to hyperemia. Whether this proliferation in sympathetic nerve fibers that we have demonstrated affects the totality of the systemic

vascular system requires further investigation but our results do suggest that the effect is more widespread. The significant inter relationships between vascular functional parameters e.g. brachial artery resting VTI and peak systolic blood velocity and changes in autonomic tone as measured by the pupillary responses and the relationship of both to sympathetic nerve density of the dorsal lingual artery suggests that augmentation of sympathetic tone in this group of children with SDB is affecting blood vessel structure and function in a systemic response. The unanswered question is what are the long-term consequences of such an effect at a time when the child's vessels are rapidly developing?

5.8.1 Blood Flow Velocity

Our group has previously reported increased brachial artery blood flow velocity, both during rest and hyperemia in non-obese children with mild SDB compared to healthy non-snoring children. In addition, in a different cohort of children, we observed increased peak systolic velocity in the ascending aorta using cardiac magnetic resonance imaging also in children with SDB compared to controls. This adds to the findings of by Hill et al., who showed increased blood flow velocity in the middle cerebral artery in primary snorers compared to control children. Our group and others have postulated that children with SDB develop systemic changes in vascular compliance, which arise in direct response to increased sympathetic activity. The strong positive correlation between brachial artery resting VTi and the trend in $FMD_{\text{time to max}}$ with SNFD of the dorsal lingual artery in children with SDB provides supportive evidence that systemic changes are occurring in these children. These changes could be a significant precursor to future cardiovascular complications.

5.8.2 Blood flow velocity and Windkessel function

During systole the aorta and proximal larger vessels act as an elastic buffering chamber, storing approximately 50% of stroke volume, while in diastole elastic properties of the aortic wall push the excess volume to the peripheral circulation in a continuous circuit of peripheral blood flow (233). Known as the Windkessel function, it influences peripheral blood flow, and cardiac function as it reduces left ventricular afterload and relaxation and also improves coronary circulation. As resistance in the major vessels increases with aging i.e. become less elastic/less compliant, the reservoir associated with systolic distention decreases leading to increases in systolic blood flow velocity, left ventricular afterload and systemic blood pressure (233). Sympathetic nerves are continuously active and hence vessels are under a continuous and varying level of constricted (443) while a proportion of arterial sympathetic nerves are also auto-depolarizing (spontaneously active) requiring no stimulus to constrict the vessels. The variability observed in SNFD on the dorsal lingual arteries of children may dictate the level of vascular compliance at rest, with more SNFD associated with less compliance and hence increased VTi. The relationship that we have observed between the SNFD, arterial blood flow velocity and vascular kinetics ($FMD_{time\ to\ max}$) may represent a reduction in the Windkessel effect, systemically. This is potentially supported by the results of our previous work using cardiac MRI imaging in children with SDB compared to non-snoring controls which showed increased peak systolic velocity in the ascending aorta in the children with SDB.

5.8.3 Pupillary Light Reflex

The pupillary light reflex is considered a valid measure of autonomic nervous system function (436) and in particular reduced peak constriction velocity and delayed time to

minimum constriction are considered evidence of abnormal pupillary and hence autonomic nervous system function. (434, 438) Our results showed that the children with increased SNFD at the dorsal lingual artery were the ones who had delayed pupil constriction onset (constriction latency), reduced percentage diameter change to the light stimulus and indeed showed reduced capacity to constrict in response to the a light stimulus. This suggests that autonomic balance is altered in children with increased SNFD and the involvement of the eye and tonsillar vessel suggests the effect is systemic in nature. Further supporting systemic vascular effects is the demonstration of delayed dilation response to hyperemia in metacarpal 'microvasculature' in children with OSA (222).

5.8.4 Platelet Aggregation and Blood Flow

Blood flow is an important mediator influencing the platelet aggregation process. It has been previously proposed that different shear conditions provide distinct patterns of aggregation (426). Increased platelet aggregation is considered a marker of vascular dysfunction. Our results showed that the children with increased VTi also exhibited increased platelet aggregation in response to the collagen antigen. Platelets are crucial in the clotting process and also regulate angiogenesis. In short, collagen sensitive aggregation suggests that platelets have had contact with collagen. In health subjects collagen in the vessel wall is concealed by the endothelial cells that line the lumen. When there is injury to the vessel wall (possibly from increased shear stress) these collagens come in contact with circulating platelets and interact with the receptor on the platelets cell surface, thereby increasing their sensitivity to collagen. That there was such a strong association between the pupillary light reflex variables and the platelet aggregation, in particular to the collagen antigen, further suggests that the

underlying cardiovascular changes in children with SDB may stem from increased sympathetic activity.

As increased sympathetic tone is an integral factor in hypertension, both in adults and children and as most children with SDB remain underdiagnosed and untreated, we postulate that this may well be an unrecognised risk factor for hypertension in adult life. While we acknowledge that sympathetic activity has been shown to be regionalised, the high correlation between the altered FMD parameters, altered sympathetic nerve density on dorsal lingual arteries and sympathetic upregulation as measured on digital pupillometry in children with SDB suggests the effects of SDB are generalised. Our results may imply that SDB has a more deleterious effect on children's vascular health than previously recognised. Whether the chronicity of SDB or its severity are the more important contributors to increased vascular sympathetic innervation is unknown. Increase density of vascular sympathetic fibres has been shown in rats, exposed to hypoxia in utero. These rats went on to development early onset arterial hypertension as animals matured (430). In mice, sleep fragmentation has been shown to induce changes in peak blood flow and dilation response in the dorsal tail vein, and significant elastic fibre disruption and disorganization in both the aortic arch and thoracic aorta (296). Importantly we have demonstrated that techniques such as pupillometry and FMD are very helpful in identifying vascular changes in children both with SDB and may become important screening tests.

The range of brachial VTi both at rest and during hyperemia in our previous study varied from normal to high suggesting that not all children with SDB exhibited the increased VTi phenotype. This heterogeneity in vascular response has been noted in

studies looking at the effects of aging, disease and therapeutic interventions on vascular response and suggests the possibility of underlying genetic factors that may protect vascular integrity in some with SDB. Using VTi and pupillary light reflex could provide the basis of possible stratification of SDB subgroups into lesser of greater vascular changes in future exploratory genetic studies.

5.9 Limitations

The main limitation to this study is the low number of participants and the wide age range between youngest and the oldest participant (378). Further studies will be required to optimize these findings so that these techniques may be used as part of a clinical assessment.

5.10 Conclusion

This is the first time that arterial structural changes have been correlated with functional vascular changes (measures of resting blood flow velocity on FMD) and both shown to be significantly associated with increased sympathetic tone. Blood flow velocity variables using standard ultrasound techniques and pre-sleep pupillometry maybe a useful tools to measure vascular changes and increased sympathetic activity in children with SDB and other disorders which involve autonomic dysfunction. The long-term implications of increased resting blood flow velocity and potentially and increase in shear stress on a child's developing vasculature may be a harbinger of the accelerated cardiovascular disease in their middle years. Given that many children with SDB are not diagnosed and do not receive the appropriate treatment, the vascular changes described in this study are concerning.

Table 5.1: Index of Pupillary Light Reflex, provided by NeurOptics, (NeuroOptics, Irvine, CA), Information Guide.

Variable	Definition/Calculation	Units
Neurological Pupil Index ™ (NPI)	Algorithm that compares all variables to normative models – results in a composite score of pupillary response.	Scalar value 0 - 5
Baseline Diameter (Max)	Resting Pupillary Diameter	mm
Constricted Diameter (Min)	Pupillary diameter at peak constriction	mm
Diameter Percentage Difference	Percentage change from baseline (Max-Min/Max)	%
Constriction Latency	Latency = time difference between initial light stimulus presentation to onset of pupillary constriction	sec
Constriction Velocity	Average constriction velocity = (constriction/duration of time)	mm/sec
Maximum Constriction Velocity	Peak value of velocity during constriction	mm/sec
Post Constriction Dilation Velocity (post constriction return to baseline)	Amount of pupil size recovery (after constriction) divided by duration of recovery.	mm/sec

Table 5.2: Shows the means, standard deviation and range of anthropometrics, brachial artery blood flow, sympathetic nerve fibre density, pupillary light reflex and platelet aggregation.

Variable	Mean	Standard Deviation	Range (Min - Max)
Anthropometric			
Age (years)	11.13	4.10	6.25 – 17.25
Height (cm)	148.23	22.54	118.00 – 183.00
Weight (kg)	54.41	30.69	21.60 – 103.60
BMI	22.58	6.79	14.4 – 35.0
Percentile	76.67	31.61	16.6 – 99.3
Flow Mediated Dilation			
Brachial Artery Diameter (cm)	0.29	0.06	0.20 – 0.40
FMD_{60} (%)	5.5	5.9	0.0 – 16.6
FMD_{Max} (%)	9.22	5.90	2.3 – 20.0
$FMD_{\text{time to maximum}}$ (s)	68.4	30.6	38.1 – 144.5
Peak Systolic Velocity mm/s	113.4	34.1	83.0 – 197.0
Velocity Time integral (mm^2)	20.4	12.9	7.5 – 53.6
Heart Rate (bpm)	72.1	8.71	58.5 – 84.0
Pupillary Light Reflex			
Neuronal Pupillary Index ™	4.26	0.22	3.70 – 4.50
Baseline Pupillary Diameter	6.98	0.41	6.44 – 7.98
Constricted Diameter	4.41	0.43	3.82 – 5.18
Percentage change from baseline diameter	0.37	0.04	0.29 – 0.43
Constriction Latency	0.24	0.37	0.22 - 0.34
Mean Constriction Velocity	3.41	0.44	2.26 – 3.90
Peak Constriction Velocity	5.43	0.63	4.12 – 6.22
Dilation Velocity	1.48	0.13	1.20 – 1.73
Platelet Aggregation			
Collagen $_{\text{Area Under the Curve}}$	42.92	11.02	26.00 – 60.00
Collagen $_{\text{Aggregation}}$	97.13	16.81	67.90 – 123.40
Collagen $_{\text{Velocity}}$	14.25	3.43	8.00 – 21.80
IADP $_{\text{Area Under the Curve}}$	19.20	11.15	2.00 - 37.00
IADP $_{\text{Aggregation}}$	35.78	18.25	6.00 – 62.30
IADP $_{\text{Velocity}}$	5.23	1.92	2.40 – 8.10
ASP $_{\text{Area Under the Curve}}$	46.42	17.38	4.00 – 76.00
ASP $_{\text{Aggregation}}$	74.93	24.90	9.80 – 106.90
ASP $_{\text{Velocity}}$	13.35	5.22	3.00 – 24.10
$ADP_{\text{Area Under the Curve}}$	43.41	12.00	20.00 – 67.00
$ADP_{\text{aggregation}}$	71.82	18.38	38.60 – 102.00
ADP $_{\text{velocity}}$	11.69	3.17	5.30 – 19.30
Mean Tyrosine Hydroxylase density	2278	370	1803 – 3060

Table 5.3: Correlations results between brachial artery blood flow velocity and pupillary light reflex variables.

	NPi™	Latency (sec)	Constriction Velocity (mm/sec)	Peak Constriction Velocity (mm/sec)	Post Constriction Recovery
rVTi (mm^2)	r = - 0.31, p = 0.32	r = - 0.20, p < 0.60	r = - 0.40, p < 0.11	r = - 0.77**	*r = -0.79***
Resting Peak Systolic Velocity (mm/sec)	*r = -0.29 p = 0.40*	*r = 0.13, p = 0.7*	*r = - 0.47, p = 0.12*	*r = -0.64**	*r = -0.78***

NB * p < 0.05, ** p < 0.005

Table 5.4: Correlations results between platelet aggregation, sympathetic nerve fibre density and pupillary light reflex variables.

	SNFD	NPI ™	Latency (sec)	Constriction Velocity (mm/sec)	Peak Constriction Velocity (mm/sec)
Collagen Area Under the Curve	r = 0.43 $p < 0.17$	r = - 0.63*	r = 0.67*	r = - 0.63*	r = - 0.84**
Collagen Aggregation	r = 0.47 $p = 0.12$	r = - 0.60*	r = 0.65*	r = - 0.57, $p < 0.07$	r = - 0.77**
Collagen Velocity	r = 0.67*	r = - 0.67*	r = 0.70*	r = - 0.59, p < 0.06	r = - 0.66*
IADP Area Under the Curve	r = 0.48 $p < 0.12$	r = - 0.63*	r = 0.66*	r = - 0.43	r = - 0.54, *p< 0.1*
IADP Aggregation	r = 0.49 $p < 0.10$	r = - 0.57, $p < 0.07$	r = 0.63, *p < 0.06*	r = - 0.39	r = - 0.52
IADP Velocity	r = 0.33 $p < 0.29$	r = - 0.56, $p < 0.08$	r = 0.66*	r = - 0.38 *p < 0.3*	r = - 0.53 $p < 0.1$
ASP Area Under the Curve	r = 0.46 $p < 0.14$	r = -0.77**	r = 0.76*	r = - 0.69*	r = - 0.67*
ASP Aggregation	r = 0.34 $p < 0.28$	r = - 0.67*	r = 0.69*	r = - 0.61*	r = - 0.75**
ASP Velocity	r = 0.57 $p < 0.06$	r = -0.75**	r = 0.73*	r = - 0.63*	r = - 0.46
ADP Area Under the Curve	r = 0.52 p < 0.09	r = -0.75**	r = 0.83**	r = - 0.60 p < 0.06	r = - 0.64*
ADP aggregation	r = 0.43 $p < 0.17$	r = - 0.65*	r = 0.79**	r = - 0.54 p < 0.09	r = - 0.63*
ADP velocity	r = 0.65*	r = -0.79**	r = 0.83**	r = - 0.59 p < 0.07	r = - 0.51
SNFD		r = -0.72**	r = -0.65*	r = -0.65*	r = -0.63*

NB * $p < 0.05$, ** $p < 0.005$

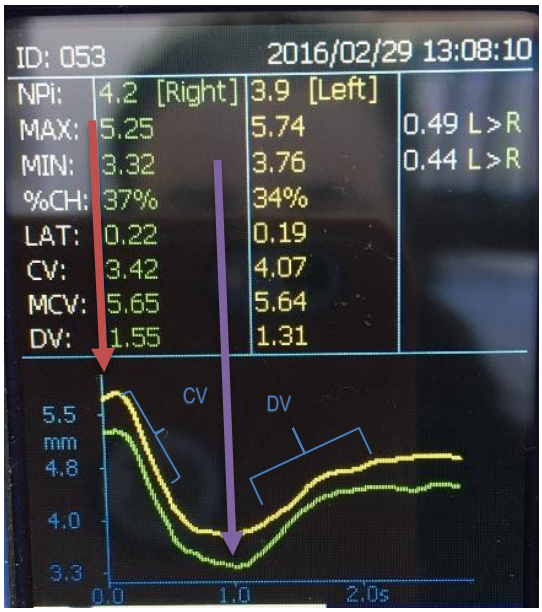


Figure 5.1: Shows the NeurOptic output variables from a participant, Max = baseline pupil diameter, Min = constricted pupil diameter, %CH = percentage difference of diameter from baseline, LAT = constriction latency, CV = mean constriction velocity, MCV = peak constriction velocity, DV = dilation velocity, post constriction.

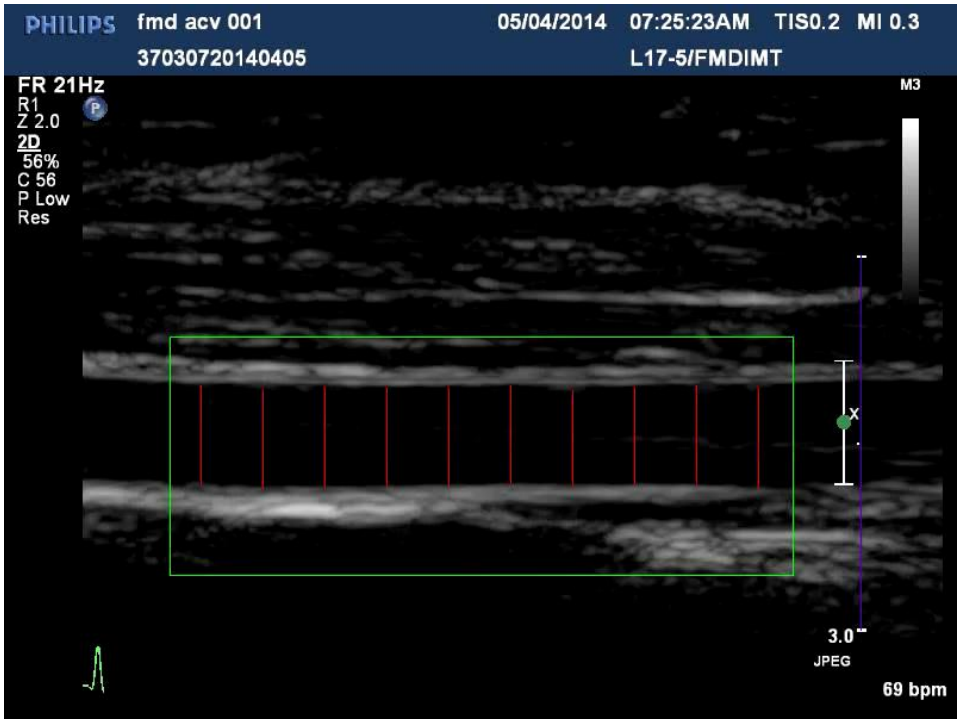


Figure 5.2: The sonogram of the brachial artery, 10cm proximal from the elbow, green box determines the region of interest, 10 equidistant red lines are used to determine the frame by frame diameter difference over 180sec (15sec pre-cuff release until 165sec).

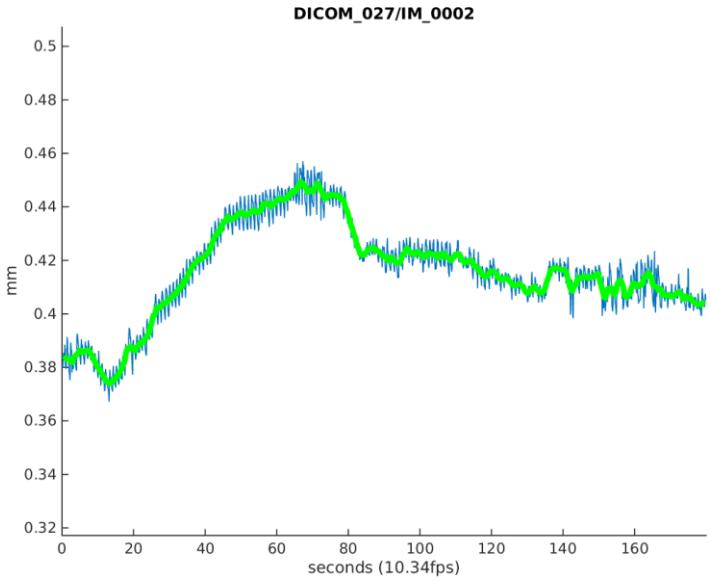


Figure 5.3: Example of the mean dilatation response from a participant. The computer program generates a graph with both the raw data is shown in blue and the averaged data in green.

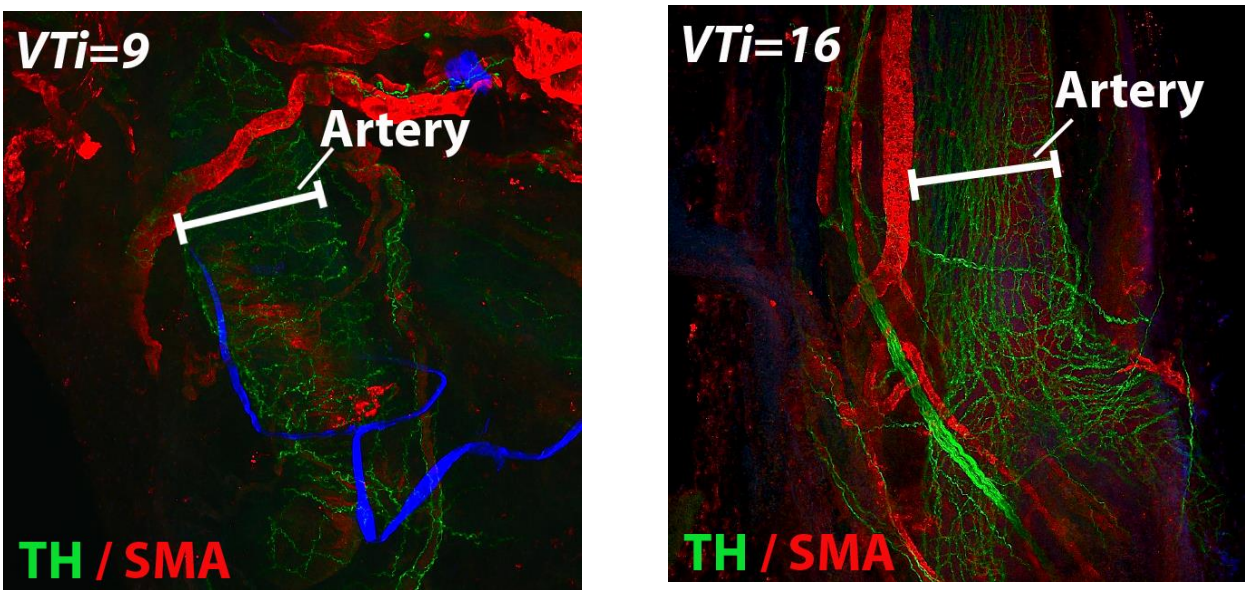


Figure 5.4: Shows two dorsal lingual arteries from different children. The red immunofluorescence indicated smooth muscle actin green is tyrosine hydroxylase, sympathetic nerve fibres. (ACV023, ACV022)

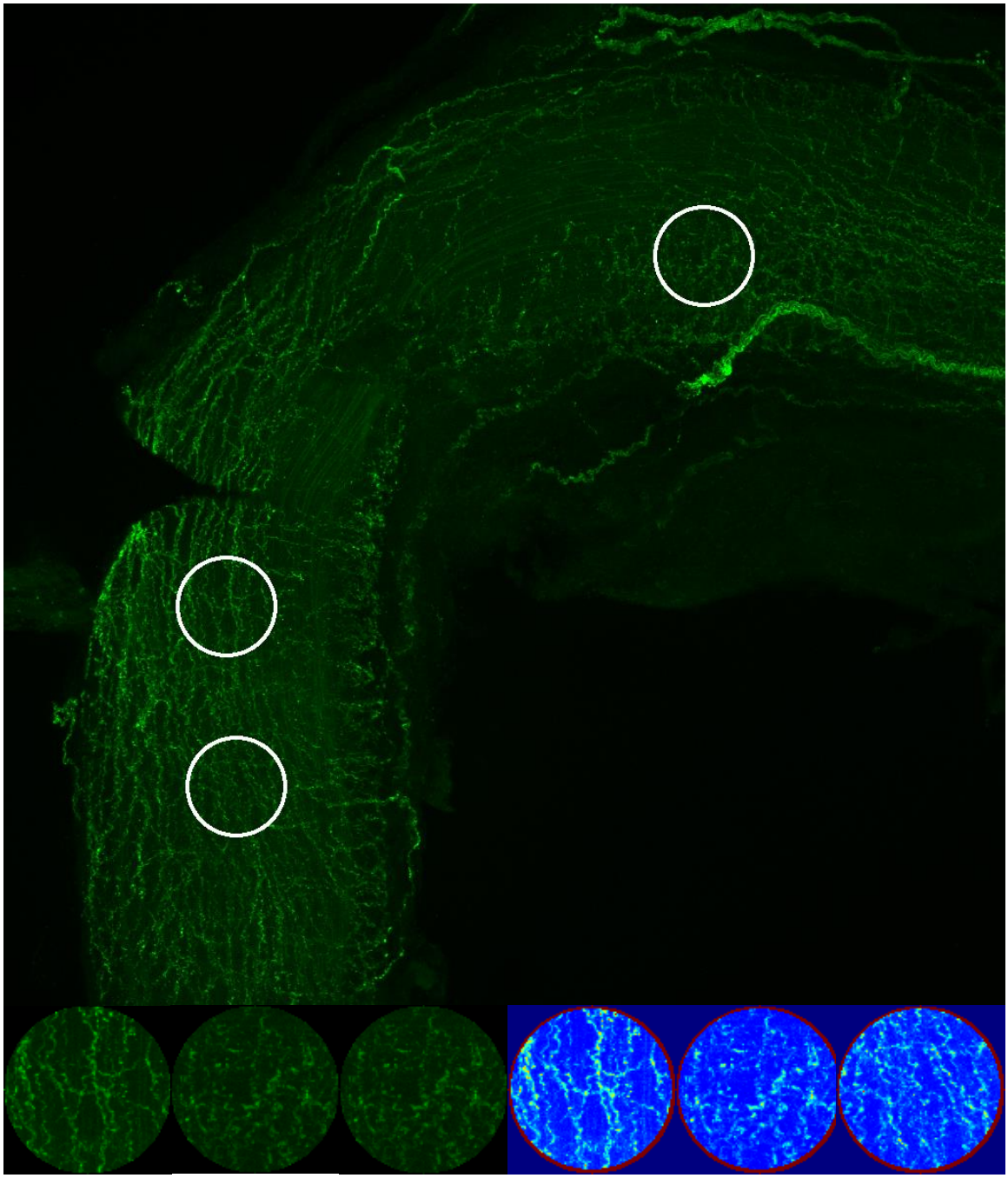
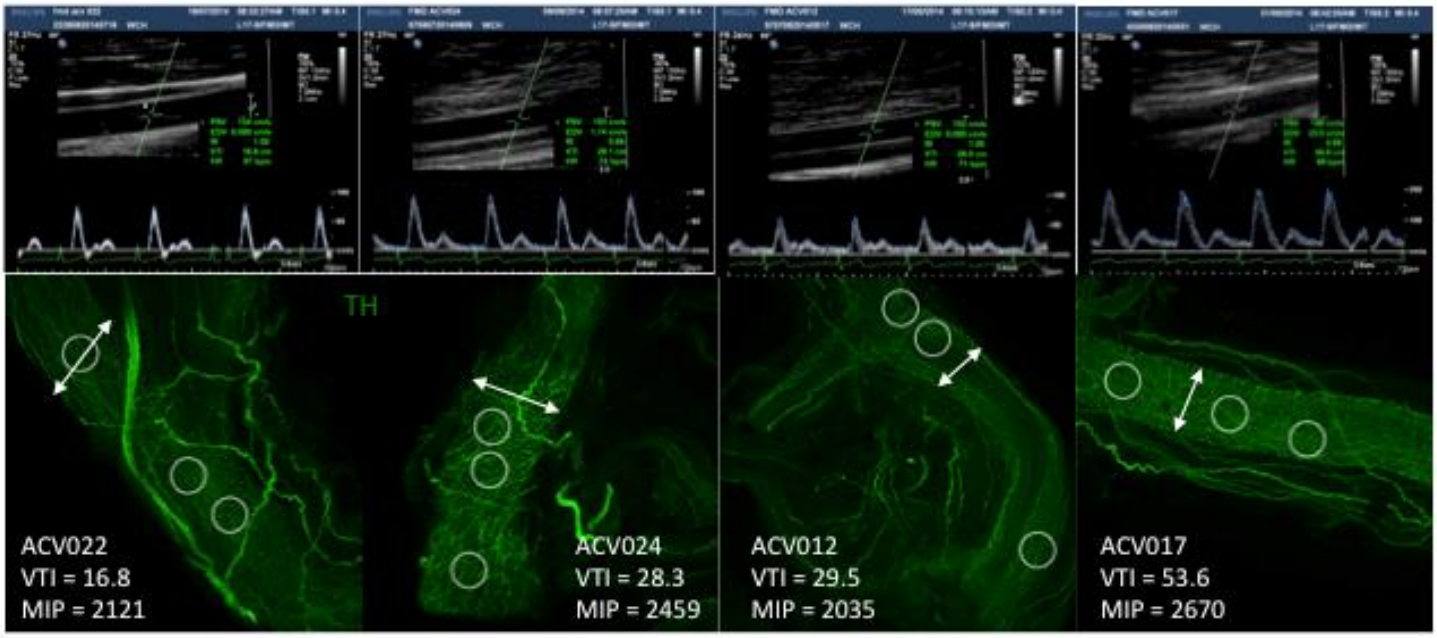


Figure 5.5: Immunofluorescence for tyrosine hydroxylase on the dorsal lingual artery, white circles indicate the three region of interest picked by the analyst for green pixel/region of interest, sympathetic nerve fibre density score. (ACV020)



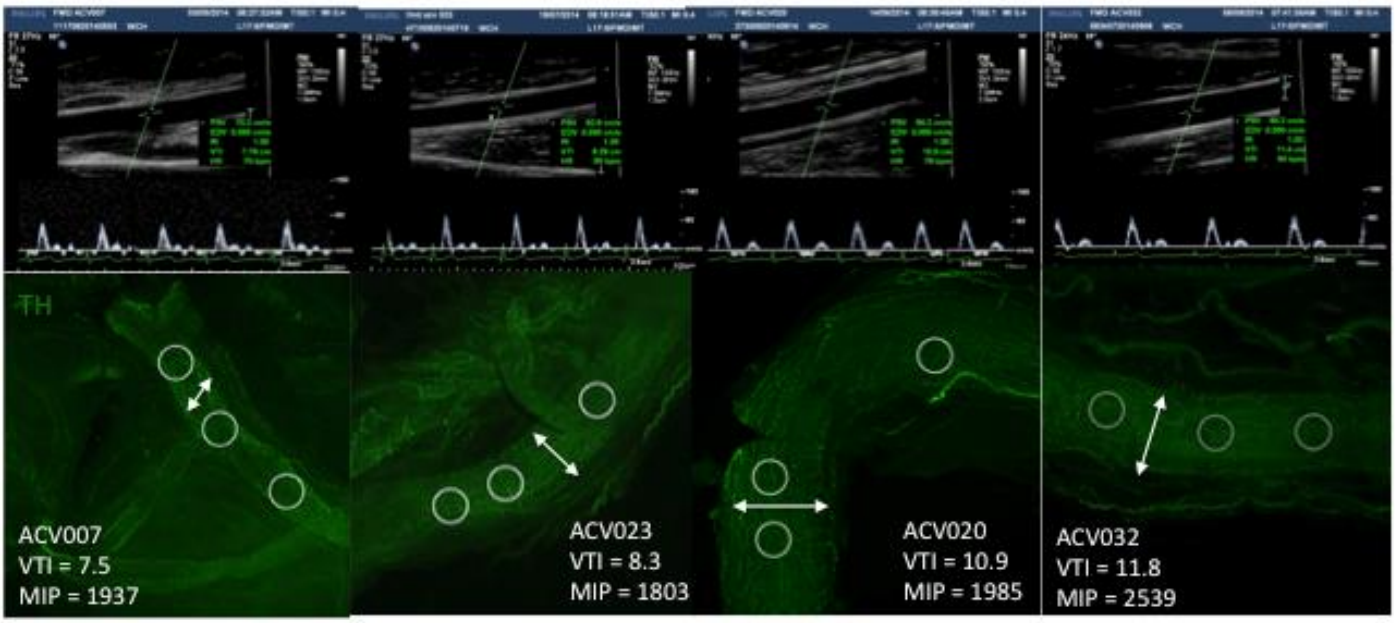


Figure 5.6: Shows the dorsal lingual arteries of 8 of the 13 participants and the brachial artery results.

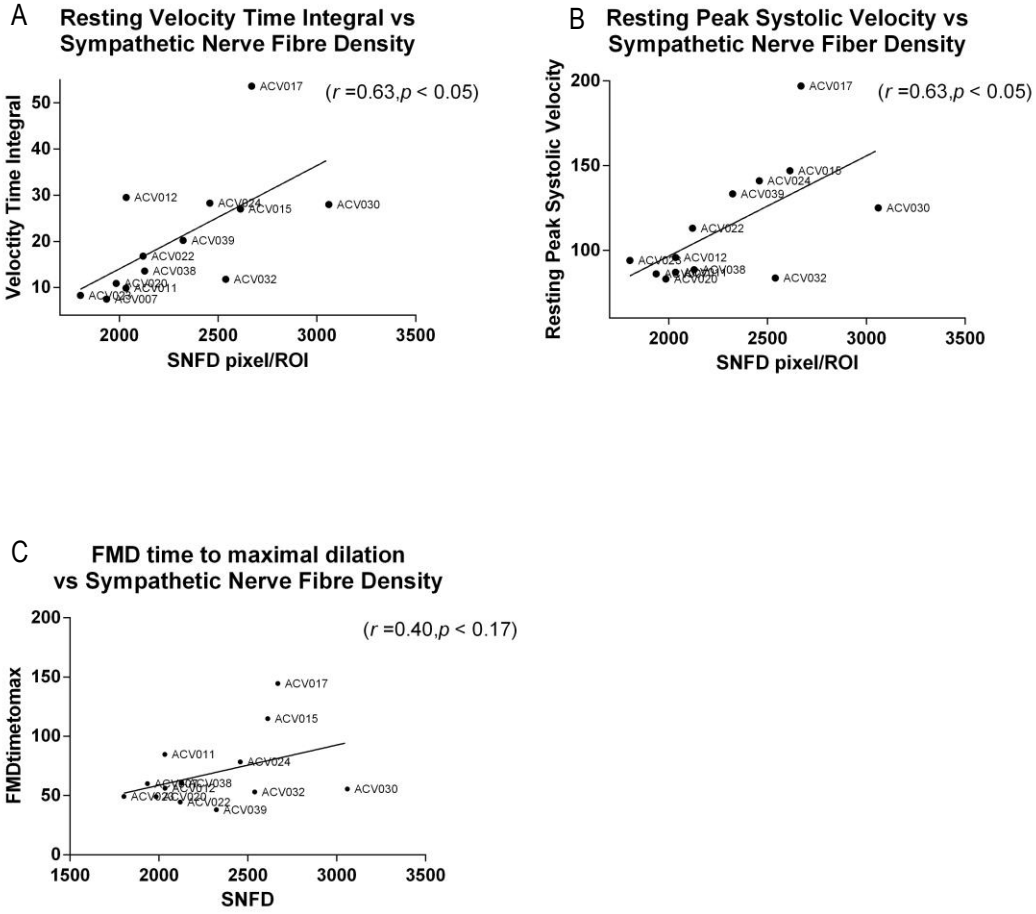


Figure 5.7: Shows the scatterplots of the brachial artery variables (velocity time integral, peak systolic velocity and flow-mediated dilatation - time to maximal dilation) versus sympathetic nerve fibre density measured from the dorsal lingual artery.

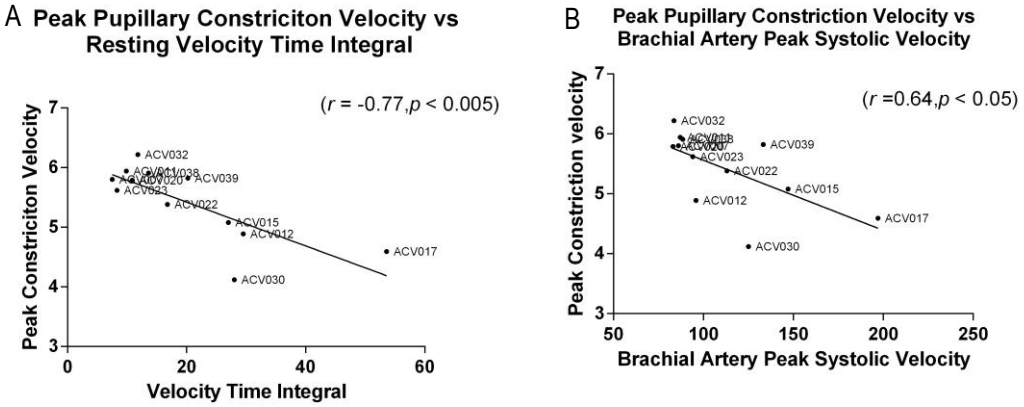


Figure 5.8: Shows the scatterplots of the brachial artery variables (velocity time integral, peak systolic velocity versus pupillary light reflex variables, peak constriction velocity.

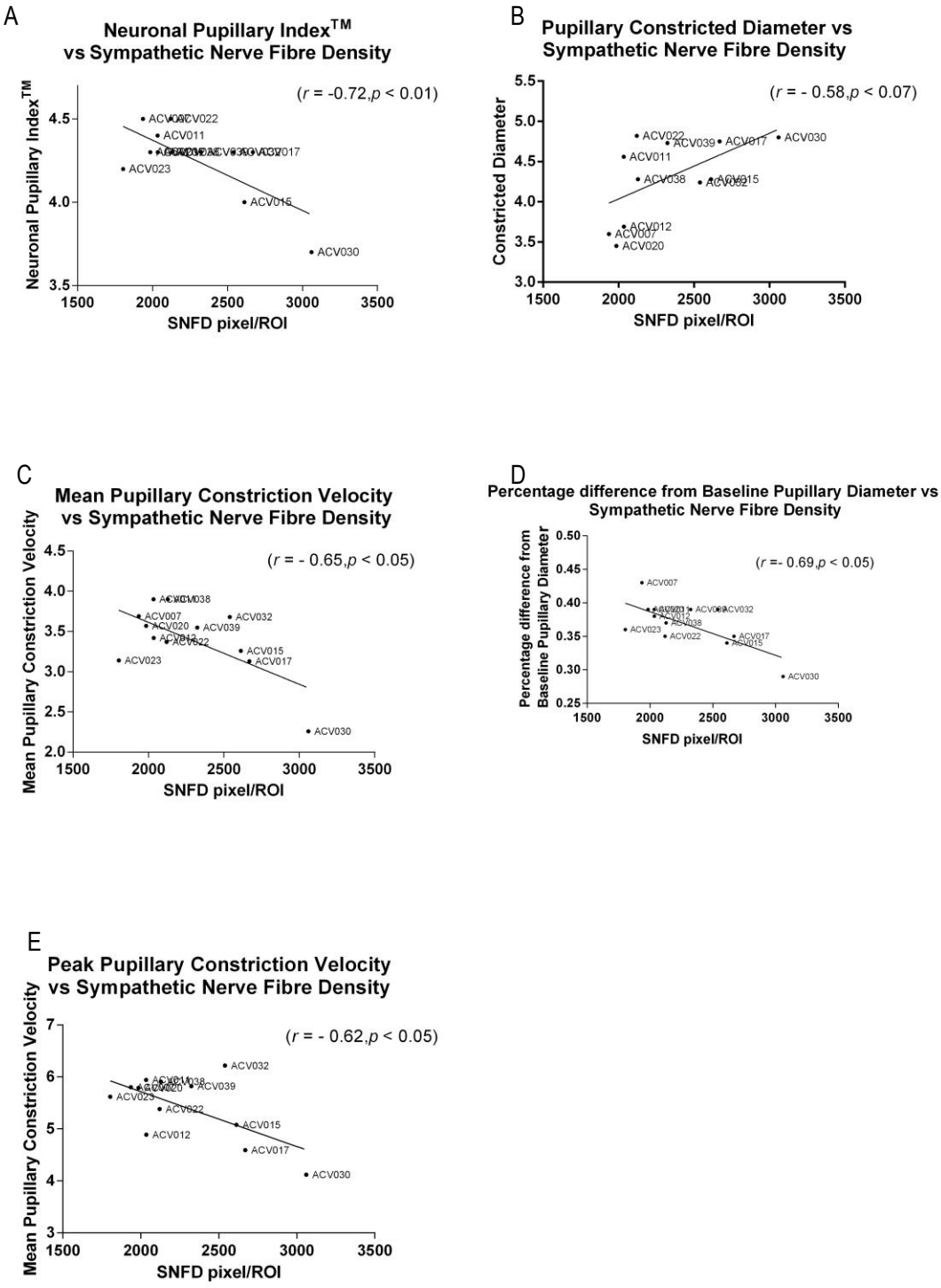


Figure 5.9: Shows the scatterplots of the pupillary light reflex variables versus sympathetic nerve fibre density measured from the dorsal lingual artery.

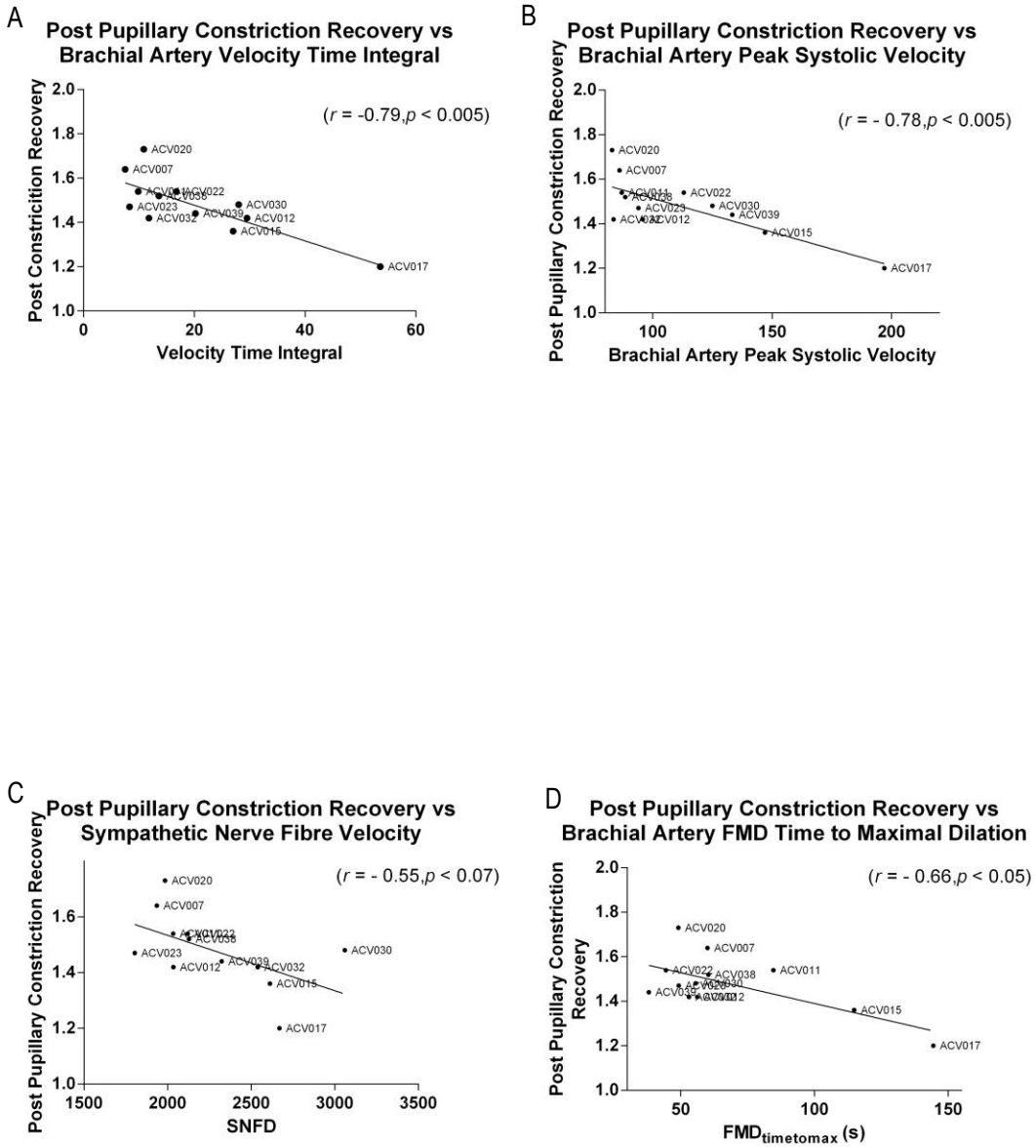


Figure 5.10: Shows the scatterplots of post pupillary constriction recovery (return to baseline) versus brachial artery blood flow variables (velocity time integral, peak systolic velocity and time to maximal dilatation and also sympathetic nerve fibre density.

Chapter 6

Discussion

6.1 Overview

The studies in this thesis suggest that there are significant changes in the underlying physiological systems in children with SDB, including changes to ANS function, vascular function and inflammation. These changes occur in children as young as six years old with evidence of reduced vascular compliance in both major and minor arteries as demonstrate by increased blood flow velocity at the brachial artery (Chapter 3) and ascending aorta (Chapter 4). The changes were coupled to changes in vascular dilation response (delayed dilation during FMD) (Chapter 3 and 5) and also in autonomic function (altered pupil light reflex) and structure (sympathetic nerve fibre density) (Chapter 5) and inflammatory response (increased $CD8^+$ T cells producing TNFα and IFNγ) (Chapter 4). The results support the hypothesis that the burden of SDB affects the development of the underlying integrated physiology and is evidence of established adaptive processes that result from allostatic overload. This new set-point will determine the trajectory of the underlying systems. Whether these changes are associated with the onset of early cardiovascular disease requires further longitudinal/cross-sectional studies to ascertain the long-term effects of untreated SDB associated vascular remodeling and its impact on the future health of the child as he/she matures.

This research supports previous limited reports, which suggest that even the mildest form of SDB (primary snoring), in children, is associated with remodeling of

cardiovascular response (148) (147) and vascular structure. This implies that the current gold standard measure to determine severity of SDB using PSG derived measures proposed in the AASM is severely limited in respect to predicting cardiovascular physiological changes, in children traditionally thought to not warrant treatment (primary snorers). Considering the majority (75%) of children with SDB present as primary snorers, it may be the case that the current dependence on a rigid OAHI as severity score obfuscates the potential alteration in the child's physiological response. Even if one accepts that a RDI/OAHI of 5 merits intervention, equally important from our data is the degree to which the obstructive events are associated with desaturations, e.g. a nadir of 85% compared to a desaturation to 95% which may indicate reduced chemosensitivity and a reduced capacity to arouse.

Although the use of parental questionnaires alone to diagnose SDB in children has been critised in the literature, the relationship between the SDSC questionnaire factors and the cardiovascular measures (increased blood flow velocity in the ascending aorta) and inflammatory markers (Chapter 4) suggests that parents are able to ascertain the degree of abnormal changes in their children's sleep behaviour that correspond to changes in their underlying physiology. Parental report using validated tests, such as the SDSC may be a valuable tool to aid in the identification of a specific pathological, behavioural profile that is possibly associated with cardiovascular disease risk. Our data suggests that all studies in paediatric SDB, in addition to the PSG measures may benefit from the use of parental questionnaires such as the SDSC as used in this study, especially when also assessing measures of autonomic and inflammatory function.

The widely adopted measure of FMD calculated at 60s has been reported in most studies as a measure of vascular endothelial integrity in adolescent and adult studies. More recent debate suggests that more parameters, other than this single time point be used to better understand the origin of changes in mechanisms driving the total dilation response (348, 444). These include observing the blood flow velocity and resting variables, such as heart rate, intima-media thickness, FMD time course data and arterial diameter. This thesis corroborates their recommendations. Chapter 2 highlights the relationship between the brachial artery dilation response and age and size (particularly height) in young healthy control children, with the time to maximal dilation increasing with both size and age but no relationship between FMD at the 60s time point. This has implications when we compare two groups with similar ages but with different degrees of vascular health, such as children with SDB (Chapter 3). Most healthy non-snoring children aged less than 10 years included in this study reached maximal dilation between 30-45s and were returning to baseline by 60s post cuff deflation. When we compared FMD at 60s between snoring and normal groups there was no significant difference, but when we looked at the 90s time point the two groups had diverged with most children in the control group returning to baseline, while children in the snoring group where still dilating. When we determined time to maximal dilation we demonstrated that children with SDB were only just reaching maximal dilation at 60s and beyond, while diameters in the control group where returning to baseline. This disparity would have been missed and no difference would have been reported between groups had we used the popular FMD at 60s. Both values would look the same but actually be measurement of different parts of the time course data. How non-endothelial mechanisms impact on vessel dilation is less well understood. By looking at the whole response curve after cuff release we may better understand and assessed the burden of

disease and hence develop targeted therapies. Continuous dilation response measurements are now considered the optimum evaluation in relation to the vascular response, both in adults and in children (348).

6.2 Systemic Changes to Vasculature and Loss of Windkessel Effect

The separate studies (Chapter 3 - FMD, Chapter 4 – MRI/Inflammation and Chapter 5 – FMD/Pupillometry/Platelet Aggregation/Histology) conducted in different cohorts of both healthy, non-snoring, control children versus children who were awaiting treatment for SDB provides evidence of physiological changes to vascular response. First, it was demonstrated that children with SDB exhibited delayed dilation response after a hyperemic stress at the brachial artery, 10cms proximal to the elbow. The children with SDB took approximately 20s longer than age-matched controls to reach their full dilation capacity. The initial dilation response was not different between groups suggesting that the mechanisms driving the dilation, (NO and vasodilators released from endothelial cells) are preserved but that as time progresses the cell-to-cell signaling is impaired in some way. In itself this decay in signaling suggests that structural changes have occurred in peripheral arteries in children with SDB and that these changes begin in the outer layers of the vessel, (medial/adventitia) where the interface between the smooth muscle cells and sympathetic nerve terminals occurs and is the site where extracellular matrix proteins (collagens) are primarily produced. A post endothelial NO release reduction in cell-to-cell connectivity would explain both the phenomenon observed in this study and would fit the model of reduced vascular compliance in these children. Furthermore it fits the model which is currently been suggested by some groups (248) (445, 446) that arterial pathology begins at the medial/adventitia interface, where the influence on precursor cells, such as fibroblasts

and myofibroblasts are significantly influenced by the activation of sympathetic nerves, and may lead to inward eutropic remodeling of the smaller arterial vessels (See Vascular Remodeling, Chapter 1).

We saw varying degrees of sympathetic nerve fibre density on the dorsal lingual artery of children with SDB which correlated with autonomic function measured during pupil light reflex and brachial artery blood flow velocity. Noradrenaline, the main vasoconstrictor neurotransmitter released by sympathetic nerves has been shown *in vitro* studies to promote extracellular matrix proteins secretion from both smooth muscle and fibroblasts (245) and the adventitia is only now being recognized to play an integral part of vasomotor function/tone, through its interaction with cells in the medial layer, and the involvement of precursor and immune cells. Increased sympathetic activity during sleep, a time when the sympathetic nervous system is more quiescent compared to the awake state may amplify the production of these proteins. This may result in an inward remodeling of the vessels and over time reduces their compliance if sympathetic overactivity persists. The reduction in compliance would reduce the Windkessel effect and the capacity of these vessels to expand during systole and hence reduce dilation in response to hyperemia. Reduced compliance would not alter luminal size measured at the top of the R wave, at the end of systole (as is the case for the baseline measurement of luminal diameter used in FMD Chapter 2 & 3), however the blood flow velocity and cardiac contractility (VTi = area under the curve (peak systolic velocity/ejection time)/2)) would increase and thereby increase shear stress on endothelial cells, without dramatically increasing heart rate. In severe OSA, children exhibit changes in ventricular thickness and left ventricular ejection fraction, which can result from the reduced vessel compliance and their limited capacity to act as a

buffering chamber during systole. This results in 'increased blood flow velocity' during systole, increased blood pressure and affects both the forward and back blood flow dynamics, putting increased pressure at the vessel wall but also to the heart which is trying to expel blood against a stronger reverberated wave. This requires the heart to pump with an increased inotropic force to extend left ventricular ejection fraction to maintain blood flow required to meet tissue perfusion. Over time, this leads to increased sympathetic activation to the heart to increase force of contraction and results in increased cell proliferation of myocardiocytes, and extracellular matrix proteins and the eventual thickening of the ventricular walls. At the vascular level however the flow on effect of ongoing, increased shear stress would include both inward eutrophic remodeling in small vessels, hypertrophic remodeling in the larger vessels and changes to endothelial NO production and release, making them less responsive, prone to leukocyte adhesion and hence unable to function properly.

Endothelial damage reported by other groups (230) (229) (231) maybe later stage changes that occur with more severe OSA and/or longer periods of exposure to sympathetic over-activity once vessels have remained under a constant increased shear stress, which eventually remodels the endothelial cells themselves. In support of this theory Loffredo et al., showed that children with primary snoring had 25% reduction FMD dilation response at 60s compared to healthy control children and that the reduction increased to 50% in children with OSA. This suggests that the children with primary snoring/mild SDB, may be an intermediate state of SDB or that members of this group may be underdiagnosed.

There are many possible molecular mechanisms that would change the vascular compliance at the medial/adventitial interface. In particular the interaction of Endothelin 1 (ED1) and its relationship to extracellular matrix proteins and cells types in media and adventitia layers may explain the delay in dilation response we observed in Chapter 3. ED1, a potent vasoconstrictor, and mediator of vascular remodeling (See Chapter 1.11.6.2) is produced by vascular endothelial cells and also adventitial fibroblasts (447). ED1 is also increased in plasma concentrations both in children and adults with SDB and concentrations have been shown to normalize after treatment for SDB both in adults and children. ED1 expression is influenced by adrenaline, TGF-b, Ang II and shear stress which are all influenced by sympathetic activity. In support of the theory that changes are occurring at the interface of the media and adventitia, *in vivo* studies in cerebral arteries of the feline and canine, have shown that the vasoconstrictive effect of ED1 occurs at the site of the adventitia and not at the lumen (endothelial cells), and that the constriction is long lasting (249). The effect of adventitial fibroblast ED1 expression may also explain the delay we observe in dilation response during FMD. ED1 has been shown to induce expression of proteins associated with a contractile phenotype, including smooth muscle actin, ezrin, moesin and paxillin in lung myofibroblasts and promotes the contraction of ECM, especially collagen 1 fibres (448). Whether adventitial fibroblasts/myofibroblasts produce more ED1 and hence cause a contraction and reconfiguration of adventitial collagen fibres, resulting in reduced vessel compliance, leading to increased resting blood flow velocity and delayed dilatation response would need further evaluation.

Increased blood flow velocity is an indicator of reduced vascular compliance and evidence of reduced Windkessel effect. Both the resting and hyperemic blood in the

brachial artery was increased in children with SDB compared to controls (Chapter 3). The increase in velocity was associated with delayed dilation response induced by hyperemia (Chapter 3). The results of the cMRI study (Chapter 4) further support this reduction in vascular compliance as it showed that resting peak systolic velocity measured in the ascending aorta, considered the most elastic of arteries, is also compromised. A sustained reduction in vascular compliance increases shear stress at the luminal surface and is reported to skew endothelial cell alignment and shape and promotes leucocyte adhesion at the luminal surface further hindering the function of the arteries. The site of the ascending aorta, requires the elastic properties and capacity to absorb the largest amount of blood propelled from the aortic valve during systole and has significant arching and branches which increases flow turbulence. Over time the effects of shear stress at this site may remodel and even weaken the aorta making it prone to 'inflammation' and 'aortic aneurysm'. The effects of prolonged sympathetic overactivity on peripheral vessels may have a more deleterious effect at the ascending aorta compared to the descending and pulmonary where blood flow is less turbulent. Hence, it is not surprising that the incident of aortic aneurysm is significantly increased in adults with OSA (187) and that we found increased peak systolic velocity consistent with decreased vascular compliance at the same site.

Further supporting the idea that reduced vascular compliance in children with SDB is demonstrated by the relationship between increased platelet aggregation in response to collagen antigen in children with higher brachial artery blood flow velocities (Chapter 5). This corroborates the deleterious effect of increased shear stress on the endothelial cells as it demonstrates that there is contact between the circulating platelets and the inner layers of the blood vessels, in children with the least compliant vessels. Increased

blood flow velocity would impact most at points of bifurcation and anastomosis, where flow is most turbulent. Over time the impact of the increased blood flow velocity would cause the greatest damage to the endothelial cells leading into the smaller arteries/arterioles and expose the medial layer to the circulating platelets and hence activate them to collagen. Increased platelet aggregation induced by collagen is the earliest 'outside-in' signals following injury to the vessel wall and is a predictor of acute coronary syndromes (449). Our results suggest that children with SDB are already experiencing significant endothelial damage.

6.3 Sympathetic Overactivity Vascular Changes

Sympathetic overactivity is considered a key driver of hypertension in adults and has been shown to be increased in adults and children with SDB (306, 307). The increase in sympathetic activity is proposed to arise from increased arousals/sleep fragmentation, increased respiratory drive and /or intermittent hypoxia associated with the disorder. To determine if the increased blood flow velocity we observed in the FMD study (Chapter 3) is associated with sympathetic overactivity we measured pupillometry just before sleep onset in children with SDB (Chapter 5). Sleep onset is a time where sympathetic activity is decreased while parasympathetic is increased. Hence any difference in autonomic nervous system control would be easily identifiable at this time compared to other times throughout the day where ultradian fluctuations of sympathetic nerve activity cannot be accounted for. In addition, we measured the sympathetic nerve fibre density on the dorsal lingual artery, removed from the participants' tonsil tissue. As predicted the blood flow velocity in the brachial artery was strongly associated to increased sympathetic nerve fibre density on the dorsal lingual artery. This was also correlated with variables of the pupillary light reflex,

which are measures considered to be primarily under autonomic nervous system control. Whether these extra sympathetic nerve fibres accrue because of sympathetic overactivity to the peripheral vessels in response to increased arousal, respiratory effort, hypoxia or vibration of snoring is yet to be examined but would require animal studies to ascertain the impact of each stimulus independently on vascular sympathetic nerve fibre density and the interaction between SNFD and other known contributors of vascular tone, namely Ang II and ED1. Studies that examine the role that these cytokines play in the changes in vascular compliance would be particularly beneficial as there are current pharmaceutical options that reduce their effect, especially in the event that current treatment in children with SDB, T&A does not reduce the vascular response.

In a series of elegant experiments using peripheral arterial tonometry (PAT) in adult patients with OSA, O'Donnell et al. showed reduced amplitude in PAT is associated with brief periods of airflow obstruction, a small reduction of arterial oxyhemoglobin saturation, with no arousal indicated in the EEG signal (450). Reduced PAT amplitude is an indicator of vasoconstriction in the minor vessels in the fingers. The reduction in amplitude was dependent on the degree of airflow obstruction with greater obstruction to flow associated with more reduced PAT amplitude. The presence of obstruction followed by an arousal in NREM sleep showed an even greater reduction in the PAT signal amplitude in the finger microvessels compared to reduced airflow without an arousal. Their data suggests that the effects of both reduced airflow with and without EEG recorded arousal is associated with sympathetic nervous system activation and peripheral vasoconstriction in the digital vascular bed, but that an arousal significantly potentiates the effect. Whether the vasoconstriction arises from the small reduction in

oxygen desaturation, micro-arousals not currently measured using EEG or other neural reflexes (pulmonary associated – stretch/chest wall mechanoreceptors) could not be clarified. Given that most children in our studies were classified at the mild end of the spectrum with mostly evidence of upper airway resistance and little to no obstruction, our data supports this previous work as it highlights the potential risk of sympathetic overactivity related peripheral vascular changes with upper airway resistance without evidence of a cortical arousal or obstructive event compared to 'non-snoring' controls.

6.4 Chemosensitivity and Vascular Compliance

Our results (Chapter 3 and 4) suggest that children with increased resting blood flow velocity, either at the brachial artery or in the ascending aorta are also the children that have the greatest capacity to reach lower oxygen saturations throughout the night regardless of how many obstructive events scored on the night of the PSG. Peripheral chemoreceptor activation is coupled with sympathetic nerve activity and chronic intermittent hypoxia has been demonstrated to be associated with systemic blood pressure increases (451). Increased intermittent hypoxia has also been shown to remodel the nucleus ambiguus projects to the cardiac principal neurons and alter the functional tonic parasympathetic inputs and vagal outputs. Considering chemosensitivity is at its peak during childhood, the children with SDB who reached lower SpO_2 nadirs may have developed adaptive peripheral chemoreceptor response so as to preserve the sleep state. This capacity to reach lower SpO_2 would increase the circulating CO_2 level, further dilating blood vessels and cause a greater global reduction in vascular resistance. An arousal from this state, particularly during NREM sleep would require an acute change to vascular compliance, via the activation of the peripheral vascular sympathetic nerves to push blood flow back to the core and brain to elicit a correction to posture and/or increase in upper airway patency to normalize

blood gas concentrations. Berthon-Jones and Sullivan, using a CO_2 rebreathing test to evoke an arousal, noted that (i) during NREM there were no changes during test sleep state (as CO_2 concentrations increase) until an arousal was evoked which was 'always abrupt and clear-cut', with associated changes occurring in the EMG and EEG (disappearance of SWS) and in ventilation (68). However they observed that during REM sleep the process was more complex. During REM sleep as the CO_2 concentration increased so did theta power and sawtooth waves (EEG), phasic burst in EMG and rapid eye movements. Also a 2-4s return of alpha activity occurring after 10-30s of rebreathing, loss of rapid eye movement with a transient increase in EMG and movement arousal were also evident. This suggests that (i) chemosensitivity during NREM sleep is less sensitive, but that when a tipping point is reached, a full transition from the dormant state occurs and (ii) during REM sleep chemosensitivity is acute (possibly similar to the awake state) and that the transition is possibly a low level change. Hence at the vascular level an arousal from NREM sleep has a more substantial activation required from the brain stem nuclei involved in the sympathetically mediated vasoconstriction response than during REM sleep.

6.5 Thermogenesis, Arousal and Autonomic Function and Cardiovascular Changes

In Chapter 4 we observed that the children who are worse effected, on top of snoring, also have evidence of changes to their sleep continuity as reported by their parents on the SDSC questionnaire. Children with an increased inflammatory response profile were also children whose parent's reported higher levels of sleep disruption and issues in initiating sleep.

Ultradian changes in temperature occur throughout sleep and are also required to initiate sleep, for example, sleep onset only occurs during somatic cooling periods, and NREM sleep is associated with a reduction in brain temperature, while the reverse has been demonstrated during REM sleep. The cooling arises from sympathetic withdraw and vasodilation of peripheral vessels, dissipating heat from the body's core and brain. In the rat, arousal response is coupled with an increase in hippocampal theta power signaling, vasoconstriction in the arterial flow to the tail, a rise in sympathetically activated brown adipose tissue (BAT) thermogenesis and a consequential rise in brain temperature (75). Whether these changes occur in humans in response to upper airway resistance activation of sympathetic efferents during sleep is yet to be determined. However, given that children have an increased propensity to arouse, and also have large amounts of BAT tissue, the relationship this has to their inability to go back to sleep or maintain continuous sleep needs to be further evaluated. The effects of ongoing upper airway resistance, arousals and obstructive events may hence elevate body and brain temperatures to levels experience during wakefulness, causing them to wake from sleep and disturbing sleep continuity. The body would then need to cool again to activate the associated processes required to return to sleep.

Our flow cytometry work in Chapter 4 implicated a specific inflammatory profile which included an increase in the number of $CD8^+$ T-cell producing IFNγ. Interestingly this specific profile has been associated with antigens that produce low grade fevers (412), (413) (414) and *in vitro* studies exposing $CD8^+$-T cells to higher temperatures, increases their expression of IFNγ (452). More importantly, sympathetic activity alone, without infection has been shown to increase the activation and proliferation of $CD8^+$ T-cells. Whether the children who had an increase in the number of $CD8^+$ T-cells producing

IFNγ are experiencing sympathetically mediated changes in thermoregulation during sleep also needs further investigation. If this is indeed the case then it may change the way we diagnose and treat the disorder, as it may be a specific profile not experience by all children who snore but by children who are attending clinic services.

6.6 Implications and Recommendations from this Research.

In adults increased blood flow velocity is a positive predictor of cardiovascular disease and autonomic dysfunction, especially sympathetic overactivity, is often reported in studies of chronic disorders. This thesis suggests that children with SDB may be predisposed to early vascular remodeling, which makes them susceptible to early onset systemic hypertension and/or cardiovascular event. The association we showed between the blood flow velocity and platelet aggregation to collagen suggests that there is early endothelial damage, a precursor state to cardiovascular disease, occurring in many of the children with SDB.

Our results in children classified as primary snorers or mild sleep disordered breathers, who exhibited little to no obstruction on the night of testing but were reported to snore by both the technician/scorer and parent/caregiver suggests that primary snoring/mild SDB may not be a benign sleep process and may be as detrimental to health as the more severe categories that are recognized to have cardiovascular implication. Our results suggest that the morbidity of mild SDB is underestimated and that studies which utilize primary snorers, as assessed by current AASM criteria, as control groups to compare children with moderate to severe OSA risk diluting their results, making it difficult to determine the real impact of their findings.

The association between the vascular changes (increased blood flow velocity and pupil light reflex) also has implications on the newly discovered glymphatic system in the brain that is proposed to be responsible for the neurotoxin clearance during sleep. The children with SDB and increased sympathetic activity may have reduced glymphatic clearance (which is increased during sympathetic inhibition and sleep) and hence may be prone to neurotoxin accumulation, which may further compromise brain function and their health in the future.

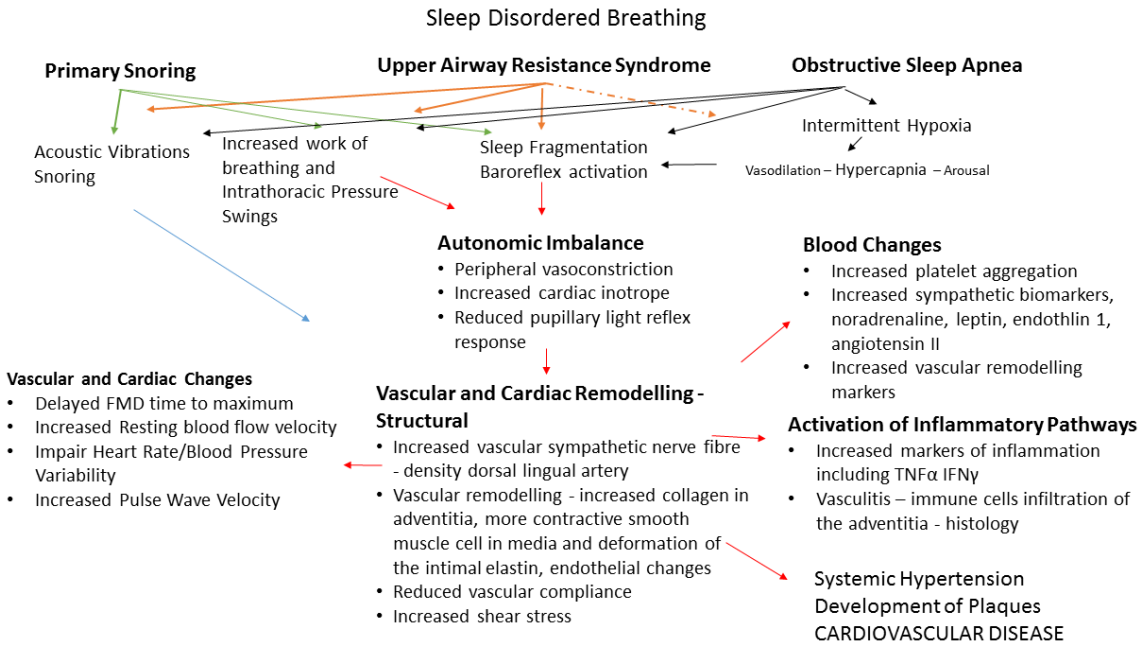


Figure 6.1: Schematic of the effects of SDB on the cardiovascular system by the over-activation of the sympathetic nervous system, via increased work of breathing, sleep fragmentation and intermittent hypoxia.

We, as have others (453) observed heterogeneity amongst the responses in the vascular tests in children with SDB. Why some children demonstrated increased blood flow velocity and delayed dilation response may be addressed by their underlying genetics. The APOE II and IV alleles have been demonstrated to be increased in adult patients with SDB in some studies (454, 455) but not all (456) and we know that not all adults and children who snore develop the neurocognitive and/or cardiovascular deficits. The APOE IV allele is over represented in Alzheimer's Disease (457) and children with SDB and the corrupted allele (IV) score lower in IQ tests compared to children with the normal alleles (III) and SDB (455). APOE has been shown to suppress the effects of sympathetic overactivity on vascular remodeling (458). The children in this study who exhibited higher SNFD, blood flow velocity, autonomic dysfunction and platelet aggregation may not necessary be the worst sleep disordered breathers, but may not have the capacity to repair the damage caused by the continuous vasoconstriction as they have acquired a copy of the corrupted gene. Genetic studies would be required to further evaluate this possibility. It may however just be the case that children are at different levels of disease with some children at early onset with others at the longer term end of the spectrum.

Our results also suggest that either current AASM guidelines and/or a single night PSG report is insufficient to determine the 'severity' at the pathophysiological level in many of these children. Our results highlight the hit and miss approach provided by the PSG assessment which, other than HRV and PTT, (both of which are not used as part of the child's SDB assessment) do not provided a clinical risk assessment in 75% (primary snorers and mild SDB) of the patients who undergo a PSG. Though the PSG is good at identifying hypoxic events and hence children at the severe end of the scale, its

inability to determine any tangible physiological changes in children even in those deemed severe suggests that there is either something wrong with the way we test or that there are aspects of the disorder and hence measurements that we have not accounted for, e.g. the loudness of snoring, open mouth breathing and/or the respiratory asynchrony which our group has shown to be increased in children with SDB. It may be the case that the PSG and AASM current lines alone are not good enough to determine risk (223). Perhaps future recommendations will include an assessment of blood flow velocity and cardiovascular measures. There are current technologies available, that are simple to use, non-invasive, time efficient and cost effective that can measure changes in blood flow velocity from the ascending aorta and aortic valve. The Ultrasonic Cardiac Output Monitor (USCOM) is a relatively cheap (approximately $30,000 per unit) Ultrasound Doppler device that can be used in all age groups to measure a range of haemodynamic variables in real time, that is reproducible. Also the new Neuroptics Pupillometer, which has only been available for clinical use since 2013 in Australia could be used in addition to current PSG guidelines just prior to sleep onset to ascertain if there is evidence of sympathetic over-activity in patients. However, a baseline activity range of variables in non-snoring, healthy children would need to be established prior to the clinical use of such measurements.

Also, given the correlations between the SDSC, and both measures blood flow velocity and inflammation, it may be the case that a simple questionnaire, a quick blood flow velocity measurement, clinical evaluation (ENT) and an overnight oximetry (which could be done in a short visit) may be sufficient to determine which children require surgery, especially when there is no PSG facility available.

6.7 Conclusion

Children with SDB present with a range of problems to the general practitioner. These problems range from failure to thrive, bad behaviour, poor academic performance, speech delay, bed-wetting, obesity, tiredness and a general lack of energy. Given that less than 10% of children with SDB are treated, what happens to the rest is a cause of great concern. At 10% of all children worldwide, considered a conservative estimate the number of children affected in Australia alone equates to more than 250,000. They are the children who are attending the health service providers in greater numbers and also utilising the speech therapist, occupational therapist, psychologist and teachers aids in classrooms. Furthermore they are the children who are prescribed an assortment of drug therapies and have more days off of school, when in most cases, a quick low risk overnight surgery, adenotonsillectomy, could greatly alleviate most of the problems.

Some studies looking at the efficacy of T&A treatment on IQ in children compared to untreated children (watch and wait) (see CHAT study) have shown no differences with treatment however, these studies did not compare children to non-snoring controls, and may demonstrate that once the damage has occurred in the early stages of life, that these changes are not reversible. Given that studies in rodents have shown that chronic intermittent hypoxia exposure in the first few weeks of life is associated with early onset hypertension (265) and that exposure in the womb to chronic hypoxia leads to increased vascular sympathetic nerve fibre density in offspring and also the development of early hypertension, (430) it may well be the case the these changes are not reversible without medication and may require ongoing assessment.

Our research implies that SDB has a considerable deleterious effect on children's vascular health previously not recognised. Importantly, we have demonstrated that techniques such as pupillometry and flow mediated dilatation are very helpful in identifying vascular change in children both with SDB and other conditions where increased sympathetic tone may be present. Techniques such as pupillometry and FMD have the potential to highlight and monitor children with SDB who may be at future cardiovascular risk. It is therefore crucial that the benefit or otherwise of A/T on the changes is clarified. Importantly the combination of techniques described is readily translatable to other childhood conditions that accelerate the risk of cardiovascular disease.

Appendix 1: Risk Factors for Sleep Disordered Breathing

App 1.1: The Upper Airway – Muscles and Innervation and Airflow Dynamics

The upper airway is a passage that moves gases, liquids and solids and is required for respiration, feeding and speech in humans (131). At birth the newborns pharynx is similar to other primates and mammals. The airway is securely maintained through the close proximity of the uvula and epiglottis and allows for independent breathing and suckling (131). By 18 months however, the larynx descends to the 5^{th} cervical vertebrae (131), to accommodate the role of pharynx in phonation. The pharynx remains a flexible tube consisting of constrictor muscle that in collaboration with the tongue muscle force masticated food from the mouth to the esophagus.

Comprised of four regions, the nasopharynx (extends from the nasal turbinates to hard palate), velopharynx (posterior to the soft palate), oropharynx (extends from hard palate to the epiglottis) and hypopharynx (from base of the tongue to the larynx) (25).

The velopharynx is the most vulnerable to obstruction as it is the narrowest cross section of the upper airway. To its anterior is the soft palate, and tongue, posteriorly the constrictor muscles (superior, middle and inferior) and to the lateral the orophargyngeal muscles (hyoglossus, styloglossus, stylohyoid, stylopharyngeous, palatoglossus, palatopharyngeus, pharyngeal constrictor muscles). As previously discussed the palantine and lingual tonsils are located on either side of the oropharynx (25).

The static influences of upper airway patency included the surface adhesive forces, which cause the soft palate to remain away from the tongue when the mouth is close; neck and jaw posture, with neck extension further opening the airway and flexion closing off the airway; lung volume, increased lung volume results in caudal displacement of the thoracic notch and passively opening the pharynx and tracheal tug (25).

Dynamic changes that affect upper airway patency include resistance in the nasal passage and pharynx, the Bernoulli effect and the dynamic compliance of the airway. Nasal and pharynx resistance results in negative nasopharyngeal intraluminal pressure and predispose the airway to obstruction (25). The Bernoulli effect, which refers to the conversion of fluid potential energy to kinetic energy in higher resistance regions of airway and demonstrates that flow velocity increases when the lumen size decreases,

causing a drop in lateral wall pressure and reduces the airway circumferences. The degree of airway narrowing is also determined by airway compliance, as the airway becomes more compliant the intraluminal pressure increases and the airway narrows.

SDB is considered a state-dependent disorder, where the level of affect is prominent during sleep and more prominent during some stages of sleep than others. During sleep the supraglottic airway resistance increases from 1-2 to 5-10cmH2O/L/s and to 50 cmH_2O in heavy snorers. (25) Autonomic function and muscle tone, including upper airway patency cyclically alternates throughout the sleep cycle (every 90-110min). During REM sleep where muscle tone is reduced, compliance of the further airway decreases and increases the propensity for the airway to collapse.

The upper airway patency also requires feedback from chemosensors and proprioceptors to maintain/reset optimal patency. In particular, increased circulating CO_2 activates the phrenic nerves in the inspiratory muscles and diaphragm and motor neurons of the upper airway to counterbalance any increased resistance. If the threshold for the receptors is low then the compliance of the airway and ventilatory response may be blunted, causing the airway to obstruct (25).

App 1.2: Enlarged Soft Tissues

Tonsils and Adenoids

The soft tissue of the upper airway consists of the palantine tonsils, adenoid, fat pads (located in the parapharyngeal) and musculature. Although the size and composition of soft tissue is primarily determined by genetic factors, inflammation, infection and infiltration of various storage/metabolites can alter their size (131). It has been proposed

that the mechanical movement (vibration from snoring) of the tonsil may causes hypertrophy of the tissue and inflammation of mucosa, increasing the size of the tonsils and hence further obstruct the airway in children with SDB (132).

Located at the roof of the nasopharynx, the adenoids are a mass of lymphoid tissues, which secrete mucus as part of a protective mechanism, which traps inhaled infectious antigens via the nose and transfers them to the pharynx (134). In addition, the lymphatic tissues produce antibodies and act as an immune barrier. They are absent at birth but become noticeable by 6 months, reaching a maximal size between the ages of 2-10 years. The tissue progressively decreases as children reach adulthood. Ranging between 7 – 12mm adenoids larger than 12mm are considered abnormal and in children with SDB, the adenoids are statistically larger (mean = 13.46mm) compared to age matched controls. Enlarged adenoids in children can result in mouth breathing, nasal obstruction, ongoing nasal congestion, middle ear infection, snoring and fragmented sleep.

The palantine tonsils are soft tissues located in between the glossopalantine and pharyngopalantine arches. They are aggregated lymphoid tissue encased in mucus membrane, which also act as an immune protective barrier.

Adenoid and tonsillar hypertrophy remains the major cause of sleep disordered breathing in children (133) as they can narrow the pharynx. The adenoid and tonsils continue to grow throughout childhood and reach maximal size just before the onset of puberty, which is also considered the peak age at which SDB is most prevalence in childhood. In the clinical setting, children with OSA are more likely to have enlarged

palantine tonsils compare to children without OSA. Inflammation induced adenotonsillar hypertrophy leading to clinical OSA has been noted in children under 1 years (135) and chronic infections are thought to promote lymphoid hyperplasia, and increase the size of the adenoid and tonsillar tissues (28). Although the link between enlarged T&A and SDB is well accepted, as most children diagnosed with OSA enlarged tonsil and adenoid tissue treated with adenotonsillectomy reduces their SDB severity, what is less understood is the lack of association between the size of the T&As assessed both clinically and using imaging and severity of SDB. Several studies have demonstrated the efficacy of T&A to treat snoring and OSA using both parental report measures and follow-up polysomnography. (136) However why approximately 33% of treated children continue to snore has not been effectively addressed (137).

Complications of T&A surgery for paediatric SDB are as uncommon as for T&A surgery performed for other purposes. Delayed discharge has been reported by groups in 1.3% - 2.3% of SDB paediatric patients and 3.2% – 3.7 % have secondary complications requiring readmission (170). Non-respiratory complications are the least reported complications, they include dehydration, hemorrhage and fever. While respiratory complications are reported in 5% - 23% of patients post operatively.

App1.3: Reduced Neuromuscular Innervation of the Upper Airway and Neurological Disorders

When innervation to the upper airway muscles is decreased or absent the airway becomes more collapsible (131). As previously mentioned, during sleep there is a natural loss of both tonic premotor input and neuromuscular compensation and reflex-driven muscle activation leading to a large decrement in electromyogram and ultimately

an increase in airway resistance (more collapsible). There are various neuromuscular disorders, including those that effect intracranial pressure and brain stem function (compression) such as Arnold Chiari malformations, which predispose children with SDB (131).

App 1.4 Tongue Size

The tongue is the largest soft tissue in the upper airway and consists of the genioglossus, hyoglossus and styloglossus muscles. The muscles control the tongues position and shape, which can affect the size and shape of the airway. Macroglossia can reduce the size of the airway and predispose children with conditions that result in abnormally large tongue mass, such as Down Syndrome, mucopolysaccharidosis and Beckwith-Wiedemann syndrome. Children with these conditions have an increased risk of SDB (131). However a study measuring tongue volume in normal children with SDB compared to controls showed not differences in volume (459).

Glossoptosis is characterized as the ‘abnormal posterior motion of the tongue during sleep (134). More common in children with macroglossia and micrognathia, it is also prevalent in patients with decreased muscular tone, including those with cerebral palsy, Down Syndrome and Pierre Robin sequence. MRI studies have shown that during sleep, the tongue in patients with glossoptosis falls posteriorly onto the velum and the wall of the pharynx obstructing the airway. In severe cases total closure of the nasopharynx may occurs when the tongue pushes the soft palate closing off the airway. Treatment in patients with glossoptosis involves positive pressure airway devices and in severe cases may require surgical intervention, tongue volume reduction, jaw realignment and even tracheostomy may be required.

App 1.5 Craniofacial Features

There is an increase prevalence of SDB in disorders that results in changes in the craniofacial structures and compromise the size of the airway, such as Crouzon, Treacher-Collins, Pfeiffer, Apert and Robin syndromes. However in non-syndromic children the link between SDB craniofacial features is not as clear as there are inconsistencies in the current literature. These contradictions maybe explained by the use of different methods to assess the size of the jaws and their association the oropharyngeal soft tissue.

Early studies using cephalometrics show subtle craniofacial features related to OSA. Shintani et al., 2003, showed reduced maxillary protrusion compared to controls on children aged 5-9 years and reduced mandible protrusion in children aged 1-2 year and a lowering of the hyphoid bone in children aged 3-6 years compared to controls. Most children in this study had adenotonsillar hypertrophy suggesting a possible underlying genetic component, which may affect both soft tissue growth and upper airway anatomy (460). Supporting this theory, Kawashima et al., also demonstrated retrognathic mandibles with enlarged tonsils and OSA (461). Also, many paediatric disorders/syndromes where there is evidence of craniofacial anomalies have higher than normal rates of SDB. (See Table, App1.1)

Hyperdivergent growth patterns, with increased craniomandibular, intermaxillary goniac and mandibular plane angles were featured in children with OSA (462). Conversely, other MRI studies have also shown no difference between SDB and non snoring children in airway bony structures (459). Schiffman et al., found no difference

using a comprehensive 3-dimensional reconstruction from MRI and measuring 8 variable of both upper and lower jaw size, between mild to moderate OSA and controls children aged 3 – 7 years (463). Some studies have even shown reversal of cranial facial differences after surgery (464), suggesting the possibility that the reduced jaw size may be associated with failure to grow mechanism often reported in the OSA, rather than a causal agent for SDB. A recent study showed that children treated with adenotonsillectomy and who continued to snore were more likely to have smaller manidibles compared to the normal mandibular size in non-snoring Japanese children (465).

App 1.6 Premature Birth

Preterm children are twice as likely to snore habitually, 3-5 times more likely to have OSA and have increased neurocognitive impairment compared to full term babies (466-468). The prevalence of SDB in preterm infants decreases by the 43rd week postconceptional age (469). The SDB may result from a combination of anatomical features, which included reduced muscle innervation and CNS immaturity, increased nasal resistance and a highly compliant chest wall (131). One study showed that of the 76 preterm infants in the study, almost 70% had obstructive events (470). While another study observed that 50% of apneic events occurred with periodic breathing resulting from upper airway obstruction and closure of the glottis (471). Thach and Stark however showed that spontaneous neck flexion was associated with obstructive events especially ‘apneic spells’ (472)

Table App 1.1: List of craniofacial anomalies, neurological disorders and miscellaneous disorders which report higher prevalence of SDB in children (131)

Craniofacial Anomalies	**Neurological Disorders**	**Miscellaneous Disorders**
Apert syndrome	Cerebral Palsy	Obesity
Crouzon syndrome	Syringobulbia	Prader Willi syndrome
Pfeifer syndrome	Syringomyelia	Congential hypothryroidism
Treacher-Collins syndrome	Myasthenia gravis	Sickle cell disease
Robin sequence	Möbius syndrome	Laryngomalacia
Stickler syndrome	Arnold-Chiari malformation	Subglottic stenosis
Nager syndrome	Poliomyelitis	Airway papillomatosis
Hallerman-Streiffer syndrome		Face and neck burns
Goldenhar syndrome		Gastroesophageal reflux
Rubinstein-Taybi syndrome		
Down syndrome		
Beckwith-Wiedemann syndrome		
Achondroplasia		
Klippel-Feil syndrome		
Marfan syndrome		
Choanal stenosis		
Mucopolysaccharidoses (Hurler, Hunter)		

App 1.7 Gender

In adults with SDB, males are more prominently affected than females. This male predominance in the paediatric population is not as well define with some studies finding no differences between the genders (108, 117, 473), while other showed an increase in the number of male affected compared to females (83, 468), particularly in the adolescent age group (79).

App 1.8 Nasal Congestion

Nasal obstruction is considered an important mediator of SDB as it increases resistance to air flow and increases intrathoracic pressure. The nasal passage counts for half of the respiratory system resistance (474). Partial or total obstruction to the nasal passage during inspiration, where intrathoracic negative pressure is produced, causes drag on the soft tissues of the upper airway making it more prone to collapse. Ambient temperature and humidity, posture, mucus production and nasal vasoconstriction all affect nasal resistance (475). Chronic stimulation of the nasal mucosa with an irritant (476) and obstruction (477) to the nasal cavity results in recurring episodes of apnoea and hypopnea. This suggests the mucosa and nasal passage play an important part in modulating resistance in the upper airway.

Infants and young children are obligate nose breathers and hence obstruction in the nasal passage can have a detrimental impact on development. Obstruction leads to chronic mouth breathing which can affect the growth of facial features including a narrow, high arched palate and poor maxillary growth, narrow dental arches and anterior crossbite (478). Epidemiological studies have shown an association with nasal obstruction and habitual snoring in adults (477, 479) and children with mild SDB (83) (103).

App 1.9 Tobacco Use

Several studies have reported a link between SDB severity and tobacco smoke exposure. (480, 481) A dose-dependent relationship was demonstrated between serum cotinine a measure of tobacco smoke exposure and snoring and reported apnoea in children (482). Tobacco smoke may irritate the lining of the airway making it

vulnerable to inflammation. Second hand smoke has been associated with many respiratory related disorders, including, asthma and allergic rhinitis. The cigarette smoke exposesure to the nasal and respiratory mucosa with a significant amount of endotoxins can cause ciliary dysfunction, inflammation of the airway and increase both the number of goblet cells and production of mucus (483). As previously mentioned nasal congestion is a considerable risk factor for SDB in young children. The ongoing exposure to tobacco smoke may be a significant contributing factor that assaults the upper airway and reduces airflow dynamics in children.

App 1.10 Obesity

In adults obesity is reported to increase the risk of SDB by 10-14 times (132). SDB is more common in obese children than non-obese, (473, 484) (485) with some studies reporting as many as 59% of obese children affected with OSA (486). SDB is an independent risk factor for metabolic syndrome, including dyslipidemia and insulin resistance. A positive relationship between severity of SDB and metabolic variables has been reported. Recently, Bhushan et al., confirmed previous work by de la Eva (487) that fasting insulin levels were increased in young children with increased SDB severity, independent of BMI and suggest that OSA was associated with insulin resistance. (488) Obese children with SDB are at a higher risk of postoperative respiratory complications after adenotonsillectomy (489). Van Eyck demonstrated reduced pulmonary function using spirometry (vital capacity, forced expired volume in 1s), helium dilution and full body plethysmography (total lung capacity, airway resistance and residual volume) (490). Flow limitations and lower forced expired volume were evident in the obese children with SDB compared to non-obese. They suspect that upper airway inflammation may explain a key role in the reduced pulmonary function in these children.

Katz et al., recently demonstrated that higher BMI z-scores and neck to waist ratios is an independent predictor of OSA in overweight/obese in children older than 7 years compared to overweight/obese non-snorers (491). Body fat distribution may play a bigger part in OSA with obesity than obesity alone, suggesting the possibility of a genetic phenotype that is more prone to OSA and weight gain.

Of concern is the recent report from Nobili et al., who have shown that more than 65% of obese and 44% of non-obese children diagnosed with nonalcoholic fatty liver disease had OSA (492). They also observed that OSA severity was associated with more severe liver histology, independent of whole body/abdominal obesity, metabolic syndrome and insulin resistance. The duration of hypoxia also correlated with liver infiltration by leukocytes and activated macrophage/Kupffer cells and makers of hepatocyte apoptosis.

Many studies have shown a relationship between reduce sleep duration and obesity. Interestingly, the recent study by Moraleda-Cibrian & O'Brien showed that reduced total sleep time by > 1hr from the national recommendation for the age, was positive associated with obesity, independent of SDB. (493) The association with SDB and obesity was not as clearly demonstrated in a recent study, with a large cohort, (> 1000 participant) (480). Hyperphasia and increased visceral and subcutaneous adiposity has been observed in mice models of sleep fragmentation (494). Collectively the research is suggesting that the relationship between SDB is complex and may involve interactions between the sleep wake cycle and in particular arousal from sleep, metabolism (possibly through thermogenesis) and feeding.

App 1.11 Genetic Predisposition

Evidence of a genetic link to SDB in the literature is yet to be properly addressed, with only few studies suggesting a genomic underpinning. Most studies report on familial prevalence, (495, 496) an association which has been appreciated for more than 40 years (497). Risk for SDB is reported to increase with the increase of the number of affected family members. One report suggested risk increased by 30 – 58 % with one other family member affected and subjects with three or more affected members were up to 4 times more likely to have SDB (498). The most compelling evidence for a genetic predisposition for SDB arise from 'twin studies', which have shown a greater concordance within pairs for snoring in monzygotic twin compared to dizygotic twins (499) (500). Ethnic differences have also been reported, with great incidence of early onset of OSA affecting adult African Americans, Maori and Pacific Islanders (132), however, the effect of BMI could not be delineated from the assessment, as there is a higher prevalence of obesity in these communities. Redline et al., estimated that African American children < 18 years were 3.5 times more likely to have SDB compared to Caucasian children. More recently, Goldstein et al, reported that African American children were 2.5 times more likely to snore and Hispanic 2.3 times more likely compared to Caucasian children (501). Similarly, Côté et al also demonstrated increased severity of SDB in African American children aged less than 2yrs (502). Interestingly, Pinto et al., showed no difference between African American and Caucasian children in upper airway collapsibility and upper airway dynamics during sleep (503). Authors concluded that the increased prevalence in the children was not associated to upper airway physiological difference.

A variety of physiological factors that predispose individuals to SDB, have their underlying effects based in genomics. Specifically, obesity is now recognized to have a heritable component, with multiple candidate genes showing linked evidence to both obesity and SDB (132). Twin studies suggest that up to 70% of variance in obesity can be attributed to genetics (504). Leptin, a hormone involved in the regulation of body weight, is increased in obesity, and has also been shown to influence lung growth, respiratory control and also sleep architecture (28). Varying levels of leptin expression have been noted, as well as, polymorphs for both the leptin hormome and its binding receptors.

Other physiological determinants include, craniofacial morphology, neuromuscular innervation and tissue composition of the upper airway and also ventilatory control and chemoreceptor sensitivity. A twin study investigating chemo-response to blood oxygen saturation demonstrated similar response to hypoxia in monozygotic twins compared to dizygotic. Researchers implied inherited traits accounted for between 30-75% of the variance, suggesting that genetic factors play a major role in chemosensitivity (505). Endothelin-1 (ED1) is a vasoactive (constrictive) peptide that influences blood pressure but has also been shown to affect the control of ventilation. Levels of ED1 are increased in adult patients with SDB (28).

Upper airway anatomy, and in particular, cephalic index (ratio of head circumference to head length) is completely genetically determined, as is the size of the tongue and lateral pharyngeal wall volume with 33% of the variability explained by familial factors (261). Patency of the upper airway is controlled by muscle innervation and soft tissue structures. Serotonin (5-HT) is a requisite component of upper airway patency, via its

innervation both in the brain stem motor neurons and upper airway dilator motor neurons in adults. There have been a number of studies looking at polymorph and expression level difference, which have not availed any conclusive evidence of difference in adults SDB. However one study showed that males with SDB were more likely to have a copy of the S (short) allele compared to both male controls and females with SDB (506). As previously mentioned, males are 5-8 times more likely to have OSA compared to females.

App 1.12 Apolipoprotein E

Apolipoprotein E proteins are produced in the brain (astrocytes), liver and macrophages and are involved in the transport of cholesterol to neurons via APOE receptors. APOE is polymorphic with 3 alleles, E2, E3, and E4. The APOE4 allele produces less APOE protein and has been associated with increased CVD and Alzheimers's disease and is more prevalent in both adults SDB (507) and specifically in children with SDB who exhibit reduced neurocognition (454). A series of elegant experiments by Kothapalli et al. showed that a significant role of APOE is 'cardiovascular protection' as it was shown to mediate mechanosensitive extracellular matrix protein expression in vascular smooth muscle cells, reducing its production during periods of increased shear stress (458). As the APOE4 allele produces less of the protein, suffers of SDB, whose peripheral vessels are vulnerable to increased sympathetic innervation because of increased arousals and hypoxic events and hence may more rapidly develop vascular stiffening. This would explain the heterogeneity observed in studies where vascular changes are not observed in all participants with SDB.

In 2009, Thakre et al., published meta-analysis of 8 genotype studies representing more than 6500 participants (456). Researchers reported no link between APOE polymorphs

and OSA. Their work suggests that studies need to be more unified in their methodologies and that more work needs to be carried out to fully understand if the association between APOE and SDB really exists.

Identifying ‘causal’ genes has proven to be less effective than observed phenotypical similarities and familial linkage studies. Apart from studies in APOE and serotonin there are as yet no published full genomic studies and ‘candidate gene’ studies available to make any conclusive and consistent pattern of specific genetic anomalies in SDB (261). Many studies are underpowered and maybe future meta-analysis will show a clearer understanding of the genes involved.

Appendix 2 Medical Image Techniques used in Diagnosis

App 2.1 Medical Imaging

There are many radiological techniques that have been used to assess the structural anomalies associated with SDB and the upper airway. For a comprehensive evaluation of these techniques and their use in assessing SDB, the recent literature review by Slaats et al. is recommended (508). See Table App 2.1 for current techniques used to determine SDB severity.

Table App 2.1: Medical Imaging techniques currently used in the diagnosis of SDB in children (508).

Lateral Neck Radiography	• consists of a side X-Ray of the head and neck, • it clearly outlines the bones and soft tissues • simple, cheap and easily accessible • the tonsil and adenoid masses are easily identifiable.	• uses a small amount of radiation, • only depicts a two dimensional image that can result in loss of information.
Cephalometry	• similar to LNR shows the skeletal, soft tissues and upper airway tissues. • can measure makers such as distances from different tissues and ratios to be calculated.	• limitations to the information gained from this technique as the procedure is conducted while participants are awake and in the upright position.
Magnetic Resonance Imagine	• considered the most advantageous as it allows for cross section of the airway via multiple planes (axil, saggital and coronal planes) • can be used while patient is sleeping to create moving images and also three dimensional images. • requires no ionizing radiation.	• is expensive, and requires a longer examination time • a greater incidence of motion artifacts • may require the participant to be sedated as the experience exacerbate anxiety and promote claustrophobia.
Computerised Tomography	• can be used both while awake and asleep, • is fast and available in most hospitals. • A study in OSA children using CT found a high correlation between OSA severity and imaging parameters but not clinical scores of upper airway patency and OSA severity. Authors suggested that CT imaging may be more accurate at measuring patency than clinical measures.	• does expose the patient to radiation

Appendix 3: Non-cardiovascular deficits

App 3.1 Neurocognition

In adults the link between OSA and neurocognitive deficit is well recognized (509). Scientific evidence for reduced neurocognitive performance and impaired behaviour in children has also been growing over the past 20 years. Reduced attentional capacity, executive function, academic performance, verbal/language and global IQ have been reported (165, 510-512). Of concern are reports showing deficits in children with primary snoring that mirror those with OSA, and in some cases increased in this group (513-515). A comprehensive evaluation of studies which show neurocognitive impairment in children with SDB is provided by Marcus et al., 2012 (84).

Research in adult SDB has shown significant brain injury occurs in the cerebellar and limbic regions/cortex and interconnecting fibres (515-517) and that untreated patients show reduced mean diffusivity localized to the medullary and brain stem areas critical for cardiovascular and respiratory regulation (518). Loss of regional gray matter and fibre injury has also been reported in multiple brain sites in adults (518, 519).

Evidence of altered brain function in children was offered by Halbower et al., who demonstrated reduced N-acetyl aspartate and choline levels (brain metabolites) expressed both in the hippocampus and right frontal lobe, in children with neurocognitive deficits and OSA, using Magnestic Resonance Spectroscopy (MRI) (520). These brain regions are important in executive function, learning and memory. This is a similar pattern noted in adult morphological studies, which show structural change in these areas and suggests that changes in CNS integrity are affected from an early age.

Somewhat contradictory, Jackman et al., showed no neurocognitive deficits in all severity levels compared to control and reported difficult behaviour in children diagnosed with primary snoring and mild SDB but not severe SDB. However, children with severe SDB were more likely to internalize problems compared to controls but were otherwise well behaved (521).

App 3.2 Behavioural Disorders

There are many studies looking at the effect of SDB on behaviour (84). The most common behavioural abnormality reported is hyperactivity and attention deficit/hyperactivity disorder (ADHD) is over represented in this group. Hypersomnolence, somatization, depression, abnormal social behaviour and aggression is also more commonly reported by parents and teachers in these studies (162). The results of the recent CHAT study showed that behaviour and quality of life dramatically improves after treatment with adenotonsillectomy (522).

App 3.3 Growth

Failure to thrive/grow has been frequently reported in children with SDB from as far back as the 1980s (523, 524). Studies have shown that delayed growth is more common in younger children than older children with rates of 52% reported in one study in children < 18 months (525) and similar results in a separate study of children < 36 months (526). Accelerated growth, in particular in height has also been reported in normal size, overweight/obese, infants and failure to thrive children after adenotonsiilectomy in some studies but not all (527). Katz et al., recently demonstrated

no significant changes in height between treated children and those on a watch and wait list, however they did note a significant increase in weight in the treated group (527).

The association between SDB and reduced growth is yet to be comprehensively understood. There is some suggestion that the obstructive events reduce the amount of growth hormones secreted during sleep. Growth hormones (GH) are secreted mostly (70%) during the first few hours of sleep, where the bulk of slow wave sleep is experienced. Obstructive events change the sleep architecture and may alter the GH secretion pattern. Insulin-like growth factor (IGF-1) a mediator of GH, increases in children after adenotonsillectomy.

Appendix 4 HRV variables

Table App 4.1: Time and Frequency Domain HRV Measures (Reproduced from Stein & Pu, 2012, (52)

Time Domain		
AVNN (ms)	Average of NN intervals for period of interest	Can convert to average HR of NN intervals (HR 60,000/AVNN)
SDNN (ms)	Standard deviation of NN intervals for period of interest Average coefficient of variance (SD/Mean) for 5-min intervals for period of interest	Total HRV
SDANN (ms)	Standard deviation of AVNN for 5-min intervals for period of interest	Circadian HRV
SDNNIDX (ms)	Average of 5-min standard deviations of NeN intervals for period of interest	Short-term HRV, SNS vs PNS influences
pNN50 (%)	Percent of NN intervals > 50 ms different from previous (NN) for period of interest	Vagal activity
pNN625 (%)	Percent of NN intervals different from previous by 6.25% or more of local AVNN (NN) for period of interest	Vagal activity normalized by HR
rMSSD	Root mean square of successive differences of NN intervals for period of interest	Vagal activity
CV	Average coefficient of variance (SD/Mean) for 5-min intervals for period of interest	Average short-term HRV normalized by HR
Frequency Domain		
TP (ms^2	Total power over measured period Averaged over 5-min periods or less.	Reflects total HRV
ULF (ms^2)	Ultra low frequency power measures rhythms greater than every 5 min	Reflects circadian HRV
VLF (ms^2)	Very low frequency power measures rhythms between every 25 sec and every 5 min, i.e., 0.0033e0.04 Hz)	Reflects vagal and renin-angiotensin system effects on HR. Exaggerated by SDB
LF (ms^2)	LF measures HR rhythms from 2.5 to 9 cycles/min, i.e., 0.04e0.15 Hz). Averaged over 5-min or less.	Reflect combination of SNS and PNS influences. Captures baroreflex rhythms
HF (ms^2)	HF captures variations in HR due to respiratory sinus arrhythmia at 9e24 cycles/min, i.e., 0.15e0.4 Hz.	Under normal circumstances reflects vagal activity
LF/HF	LF/HF average over 5-min periods or less [].	Purported to reflect SNS/PNS balance
LFnu (%)	LF/(TP VLF)] for the measured period (5-min or less	Purported to reflect SNS activity
HFnu (%)	Some calculate LF/(LF þ HF) [HF/(TP VLF)] for the measured period (5-min or less). Some calculate HF/(LF þ HF)	Purported to reflect PNS activity

Appendix 5

Kontos, A., et al., *Flow mediated dilatation, using time course data shows maturation of the brachial artery from young children to mid-adolescents.* Clin Exp Pharmacol Physiol, 2014.

Clinical and Experimental Pharmacology and Physiology (2015) **42**, 240–245 doi: 10.1111/1440-1681.12349

ORIGINAL ARTICLE

Flow-mediated dilatation, using time course data, shows maturation of the brachial artery from young children to mid-adolescents

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SUMMARY

Flow-mediated dilatation (FMD) is a tool widely used to measure arterial responsiveness to sheer stress. However, there is scant literature to show how the peripheral arterial response changes as the vascular system matures. One reason for this is that the feasibility of measuring FMD in younger children has not been established. The aim of the present study was to assess brachial artery function at rest and during the FMD response after 4 min ischaemia of the forearm in children aged 6–15 years. Time to reach maximum FMD (FMD_{max}) was found to be correlated with age ($r = 0.4$, $P < 0.05$), resting brachial artery diameter ($r = 0.4$, $P < 0.05$), height ($r = 0.4$, $P < 0.05$), body mass index (BMI; $r = 0.45$, $P < 0.05$), body surface area ($r = 0.44$, $P < 0.05$) and resting blood flow ($r = 0.37$, $P < 0.05$). However, there was no correlation between the traditional FMD response at 60 s or FMD maximal dilation and age, resting brachial artery diameter, height, weight, BMI, body surface area and resting blood flow. In conclusion, the time taken to reach the maximal dilation response is related to age, brachial artery luminal diameter and body habitus, but not the traditional measure of FMD response at 60 s or the maximal dilatation percentage.

Key words: blood flow, brachial artery, children, flow-mediated dilatation.

INTRODUCTION

Early detection of impaired cardiovascular function is a prerequisite for the prevention of future hypertension, atherosclerosis and cardiac disease. An integral component in that task is the assessment of vascular responsiveness before disease develops. A common non-invasive method for assessing vascular responsiveness is the measurement of flow-mediated dilatation (FMD), which measures endothelial response to an increase in sheer stress induced by increased blood flow in a peripheral conduit artery following the induction of ischaemia, commonly in the brachial artery.[1] The physiology underlying the FMD response has been well described.[2,3] The initial vascular dilation following forearm occlusion in FMD measurement is dependent on intact endothelial stretch receptors, which respond to the increased blood flow and the resultant sheer stress by releasing vasodilators, in particular nitric oxide. The latter induces relaxation of the smooth muscle cells surrounding the endothelial layer of the vessel, thereby increasing luminal diameter. The magnitude of the vessel dilation[3] and time taken to maximal dilation[1,4–6] are the two key parameters measured with the FMD technique.

Traditionally, maximal vessel dilation (FMD_{max}) has been thought to occur at 60 s following cuff deflation ($FMD_{60\ s}$) and reflect the health of endothelial cells (EC). Although the 60 s time point has been used in many studies,[7–10] it may not adequately describe all the important characteristics of the dilating vessel wall. Age-related changes in blood vessel distensibility, and therefore time to maximal blood vessel dilatation, have been reported in adults.[4,6] Whether the same is true in young children is unknown. Only one study has assessed time to maximal brachial artery dilation in healthy children, but this was restricted to older children aged 9–16 years.[11] That study showed that most maximal dilations occurred at approximately 70 s after cuff deflations. In order to comprehensively assess shear stress responses throughout childhood, it is important that FMD responses be examined both over a longer time period than the currently accepted 60 s and over a wider age range. Specifically, the aim of the present study was to assess FMD responses over 180 s in healthy young children aged 6–15 years.

RESULTS

Anthropometrics

Preliminary analyses revealed no gender differences in anthropometrics and therefore gender was not included in the analyses. The

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Received 17 September 2014; accepted 29 November 2014.

final group consisted of 12 boys and 18 girls aged 6.3–15.7 years. Of the 18 girls, five had commenced menses. Because menstrual cycle has been shown to affect FMD,[12] all results were recalculated without these five girls. The resulting correlations did not differ after recalculation compared with the results reported below.

Brachial artery dimension and blood flow dynamics

As expected, brachial artery diameter was strongly correlated with age, height, weight, body surface area and body mass index (BMI), and moderately correlated with resting blood flow. In addition, a moderate inverse relationship was evident between resting heart rate (rHR) and resting brachial artery diameter and body surface area. Whole-group data are reported in Table 1, whereas correlation results are presented in Table 2. Resting HR demonstrated a moderate inverse relationship with hyperaemic velocity time integral (hVTi), resting blood flow, age, height, weight and body surface area. A moderate positive correlation was observed between hVTi and age, height, weight, rHR and BMI. Resting blood flow was moderately correlated with resting brachial artery diameter, resting velocity time integral (rVTi), BMI, body surface area and bodyweight, and weakly correlated with age and height.

A moderate positive correlation between the rVTi, weight and BMI was reported, but there was no association between rVTi and height. A strong negative relationship between rHR and hVTi was also observed.

Flow-mediated dilation response

The correlations between FMD and anthropometric parameters are reported in Table 2. The time to reach FMD_{max} was moderately correlated with age, BMI, resting brachial diameter and body surface area. Most of the younger children had reached peak dilation and were returning to baseline by 45 s after cuff deflation, whereas the brachial artery of older children continued to dilate up to and beyond 60 s after cuff deflation (Fig. 1). No significant relationship was observed between $FMD_{60\ s}$ or FMD_{max} and age, brachial artery diameter, height, BMI and body surface area.

DISCUSSION

The present study investigated the time to maximal FMD response in healthy children ranging in age from 6 to 15 years. The results demonstrate that the time needed for the brachial artery to fully dilate in response to sheer stress and ischaemia is related to age and body habitus (weight, height and body surface area). This is consistent with the proposition that, as children grow, the vasodilation response of the peripheral arteries becomes slower over time and complements previous work by Herrington *et al.*,[6] who found the same relationship between time to FMD_{max} and age in a large group of participants ranging in age from 14 to 80 years.

Arteries increase in size to keep up with the metabolic requirements associated with increased tissue mass, explaining the relationship that we found between both age and BMI and time to FMD_{max}. Arterial responsiveness is maintained through a series of complex interactions, some of which are short term and activated through the central nervous system and/or direct cellular mechanisms.[2] Other determinants are long term, involving both the size of the lumen and wall of blood vessels, which are made up of vascular components such as extracellular matrix proteins, collagen and elastin and smooth muscle

Table 1 Anthropometric, blood vessel dimensions and flow dynamics details for the combined sample ($n = 30$)

	Mean ± SD	Minimum	Maximum
Anthropometric values			
Age (years)	10.9 ± 2.70	6.25	15.7
Height (cm)	145.5 ± 16.0	118.5	177.0
BMI (cm/kg^2)	18.9 ± 2.5	15.2	26.0
Weight (kg)	41.2 ± 12.7	21.7	67.8
WHR	0.87 ± 0.05	0.78	0.96
Body surface area (m^2)	140 ± 57	272	583
Vascular parameters			
Vessel resting diameter (cm)	0.255 ± 0.030	0.200	0.343
rPSV (m/s)	103.2 ± 21.0	65.4	156.8
rVTi (m^2)	17.32 ± 8.72	9.00	50.00
rHR (b.p.m.)	72.8 ± 9.3	57.0	95.0
hPSV (m/s)	168.7 ± 24.1	120.7	222.5
hVTi (m^2)	67.76 ± 16.18	26.40	97.70
hHR (b.p.m.)	76.2 ± 10.4	57.8	100.5
Resting blood flow (mL/min)	66.6 ± 39.4	23.8	170.4
rSR	407 ± 85	270	590
$FMD_{60\ s}$ (%)	6.81 ± 4.52	−0.40	17.11
FMD_{max} (%)	8.39 ± 4.04	0.81	17.11
Time to FMD_{max} (s)	52.8 ± 18.6	30.0	90.0

BMI, body mass index; WHR, waist : hip ratio; rPSV, hPSV, resting and hyperaemic peak systolic velocity, respectively; rVTi, hVTI, resting and hyperaemic velocity time integral, respectively; rHR, hHR, resting and hyperaemic heart rate, respectively; rSR, resting shear rate; $FMD_{60\ s}$, flow-mediated dilatation at 60 s after cuff deflation; FMD_{max}, maximum flow-mediated dilatation after cuff deflation.

Table 2 Correlations between anthropometric and vascular parameters ($n = 30$)

	Age	Height	Weight	Body surface area	BMI
Resting brachial artery diameter	0.70****	0.75****	0.72***	0.73****	0.48**
Resting heart rate	−0.57***	−0.56***	−0.51***	−0.46*	−0.18
rVTi	0.25	0.23	0.42*	0.37*	0.59***
Resting blood flow ($n = 29$)	0.37*	0.38*	0.53***	0.49**	0.64****
Hyperaemic heart rate	−0.56**	−0.53***	−0.46*	−0.48**	−0.21
hVTi	0.47**	0.40*	0.36*	0.35	0.27
hVTi − rVTi	0.36	0.30	0.15	0.16	−0.06
rPSV	0.31	0.34	0.32	0.46*	0.53***
hPSV	0.42*	0.37*	0.49**	0.41*	0.47**
Resting shear rate	−0.15	−0.19	−0.19	−0.01	0.21
FMD_{max}	−0.01	0.00	0.24	0.03	0.17
Time to FMD_{max}	0.40*	0.41*	0.46*	0.44*	0.45*
$FMD_{60\ s}$	0.06	0.08	0.05	0.14	0.28

*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, ****$P < 0.0001$.

rPSV, hPSV, resting and hyperaemic peak systolic velocity, respectively: rVTi, hVTI, resting and hyperaemic velocity time integral, respectively: $FMD_{60\ s}$, flow-mediated dilatation at 60 s after cuff deflation: FMD_{max}, maximum flow-mediated dilatation after cuff deflation.

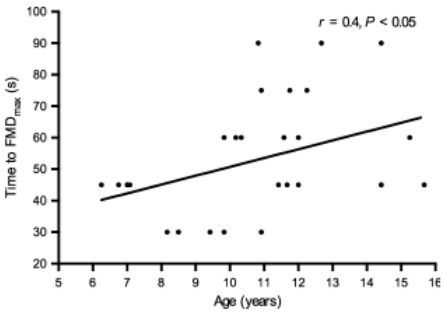

Fig. 1 Scatterplot for age (years) versus time to maximum flow-mediated dilatation (FMD_{max}).

cells. Together, these elements alter the compliance of blood vessels and compliance, in turn, is a critical determinant of vasomotor function and arterial capacity to dilate.[13,14] Jourdan *et al.*[15] reported an increase in the intima media thickness (IMT) of systemic arteries (e.g. carotid and femoral arteries) with age and height. They suggested the gain in IMT is due to increased smooth muscle cell and extracellular matrix protein formation.

Because there are numerous long- and short-term changes in the anatomical and physiological properties of blood vessels as children mature, it is likely that the delay in reaching PMD maximal dilation we observed in older children is associated with changes in some, if not all, these parameters and, importantly, the effect that a 4 min hypoxic state has on the blood vessels distils to the cuff at different ages.

In the present study, no significant linear correlations were observed between $PMD_{60\ s}$ or PMD_{max} and any of the anthropometric variables (i.e. age, weight, height, BMI and body surface area). The mean $PMD_{60\ s}$ and PMD_{max} observed in the present study are consistent with those of other studies showing the capacity for brachial artery dilation is between 8% and 11% in normal, non-obese subjects and independent of age.[16] Consistent with other studies,[4,11] we also observed a large variability in PMD_{max} (4–18%).

Over 50% of participants in the present study reached peak dilation either before or after the 60 s time point, which traditionally other studies have used as the measurement end-point. This is consistent with the distribution of maximal dilation times reported by Black *et al.*,[4] Palinkas *et al.*[5] and Järvisalo *et al.*,[11] with most maximal dilation measurements occurring outside the standard window of assessment in normal adults, patients with coronary artery disease and healthy children.[4,5] Black *et al.*[4] stressed that PMD taken at the traditional 60 s time point may result in different outcomes reported when comparing groups. These studies did not examine the relationship between BMI or age and time to PMD maximal dilation. Contrary to previous reports,[6,17,18] we did not find a relationship between $PMD_{60\ s}$ or PMD_{max} and resting brachial artery diameter. The lack of association may be to due to the small range in brachial artery diameter in our group. The diameter ranged between 2 and 3.43 mm, with a difference of only 1.4 mm, whereas in the study of Herrington *et al.* the brachial artery diameter ranged from 2 up to 8 mm.

The results of the present study support previous reports that heart rate decreases while brachial artery diameter increases as children mature.[19] At rest, the volume of blood passing through the resting brachial artery (resting brachial blood flow) increases with age and BMI, but not shear rate. Shear rate is a measure of frictional force on the luminal wall, which is dependent on the luminal diameter and the volume and rate of blood flowing through the vessel. We showed that brachial artery diameter and resting blood flow increase as children mature. Because both variables increase by the same factor, shear rate remained constant across ages. The consistent correlations between interrelated variables and brachial diameter adds to the accuracy of the measurement conducted by both the ultrasonographer and the echocardiologist in the present study.

Study limitations

One limitation of the present study is that the estimated time to FMD maximal dilation does not accurately reflect the time of full dilation to the second. Unfortunately, the technology used to record dilation requires manual measurement of the dilation response, which was outside the scope and funding of this project. However, unlike studies that only report dilation at 60 s, the present study used multiple time points per participant (maximum 33 points), which produces a more accurate account of the progression of dilation of the brachial artery. Another limitation of the present study is the small sample size.

Perspectives

The temporal differences in FMD response and the relationship to age and body habitus may not seem important in normal children, but this may be critical in differentiating responses between normal children and those with disorders likely to affect vascular response. In conditions such as juvenile diabetes and obesity, it is well established that vasculature is affected when measuring standard FMD response in older children. If time to FMD_{max}, age and body habitus are measured and accounted for, more subtle abnormal vascular responses may be found both earlier in those disorders with established changes and in other disorders not currently associated with abnormal vascular response. Changes in affected groups, which show reduced dilation at 60 s, such as FMD studies involving children with type 1 diabetes mellitus,[7] hypercholesterolaemia or Kawasaki disease,[9] does not mean the blood vessel does not fully dilate; it may be that the vessel dilates quickly and, by 60 s, is returning to normal baseline diameter or that the initial dilation is slow and the kinetic mechanisms between the smooth muscle are somehow interrupted. Again, these would have different causes and yet give the same values. Continuous measurements need to be performed to establish the nature of vascular response dynamics with time.

CONCLUSIONS

The present study shows that FMD can be used in children as young as 6 years old. The time taken to reach maximal vessel dilation increases with age and body habitus in healthy children. This is critical to understanding the relationship between groups of healthy children and those in whom vascular abnormalities may be expected. The results of the present study suggest that continuous FMD reporting over 3 min would give a more accurate assessment of early changes to vascular function.

METHODS

Ethics approval for this study was granted by the Human Research Ethics Committee of the Women's and Children's Health Network. Healthy children aged between 6 and 15 years were recruited from the local community. Parents of participants completed a comprehensive child's health and behaviour questionnaire. Results of the questionnaire were used to screen children known to have genetic conditions, respiratory disorders (e.g. asthma) and developmental disorders (e.g. attention deficit hyperactive disorder) and for medication use, including the contraceptive pill. The final sample consisted of 12 boys with a mean (±SD) age of 10.71 ± 2.02 years and 18 girls aged 10.96 ± 2.86 years. Fifty per cent of the group was over the group mean age of 10.9 years.

Children were weighed wearing minimal clothing using an electronic scale with a resolution of 0.1 kg and height was measured using a wall-mounted stadiometer. Body mass index and percentile were calculated using the Baylor College of Medicine website tool (http://www.bcm.edu/cnrc/bodycomp/bmi2.html, accessed 15 March 2013). Body surface area was calculated using the Du Bois formula[20] as follows:

$$\text{Body surface}\,(\text{cm}^2) = \text{weight}^{0.425}(\text{kg}) \times \text{height}^{0.725}(\text{cm}) \times 71.84$$

Vascular function ultrasound assessment

Children attended the Medical Imaging department of the Women's and Children's Hospital between 7.30 and 8.30 am. The ambient room temperature was maintained at 23°C throughout the FMD procedure.

The standard protocol adopted by the Women's and Children's Hospital Medical Imaging department over the past 12 years is to measure the diameter of the brachial artery using a two-dimensional longitudinal ultrasound image of a section of the brachial artery 2–15 cm above the elbow while the participant lies supine on an examination couch.[7] The image was captured by a professional paediatric ultrasonographer of 20 years experience using a 10.0 MHz linear array transducer (Advanced Technology Laboratories, Bothel, WA, USA) and an HDI 3000 ultrasound system (Advanced Technology Laboratories) and recorded to a high quality VHS cassette. An electrocardiogram was also recorded simultaneously during the scan.

To ensure that the brachial diameter is measured in the same location during each scan, a reproducible site for vessel imaging is determined using standardized identifiers, such as venous valves or vessel bifurcations. A resting scan of the vessel diameter was taken for 30 s. Subsequently, the blood flow to the lower forearm was occluded for 4 min using a sphygmomanometer

Table 3 Blood flow dynamics recorded or derived during flow-mediated dilatation measurements

VTi	Velocity time integral	Mean blood flow velocity during ejection time for one cardiac cycle (PSV × ejection time)
PSV	Peak systolic velocity	The fastest velocity during ejection time of one cardiac cycle
Q	Brachial artery blood flow	Amount of blood travelling through the brachial artery per second Q = SV × HR where stroke volume (SV) = CSA_{artery} × VTi
SR	Shear rate	(8 × VTi)/diameter of the artery is a measure of frictional force on the endothelial cells of blood vessels

HR, heart rate; CSA_{artery}, cross-sectional area of the artery.

inflated to 250 mmHg. Previous work in this age group has shown that 4 min is sufficient to induce reactive hyperaemia.[21] Deflation of the cuff then induces reactive hyperaemia and the conduit arteries dilate in response to the increased blood flow. This physiological response is dependent on the presence of an intact endothelium. Thus, the measurement of PMD *in vivo* has been widely adopted as an assessment of endothelial function. A second scan (reactive hyperaemia) was taken from 30 s after cuff deflation until 180 s. After 10 min, a third recovery scan was made.

In addition, blood flow velocity was determined during the resting scan and for the first 15 s after cuff deflation using the pulse Doppler signal at < 60° to the brachial artery.[22] The ultrasound measuring parameters remained unchanged during the whole study.

A different experienced echocardiographer used ultrasonic callipers to determine arterial diameter during the R wave of the electrocardiogram. At each time point, the average vessel diameter was derived from four cardiac cycles. This value was then used to calculate the percentage difference from resting vessel diameter from the first scan at 30 s and every subsequent 15 s interval after cuff deflation ($PMD_{30\ s}$ – $PMD_{180\ s}$). Estimated maximal dilation (PMD_{max}) was calculated as the difference between maximal and resting baseline vessel diameter values divided by resting baseline diameter and expressed as a percentage.

The time to maximal dilation was estimated as the time taken to reach PMD_{max} after cuff deflation.[4] In cases where PMD_{max} values were constant for multiple time points, the first point was considered PMD_{max}.

Brachial artery resting diameter (rD), peak systolic velocity (rPSV), rVti (the area under the curve value of the mean systolic velocity during cardiac ejection time)[23] and rHR were calculated during the resting scan. Hyperaemic PSV, VTi and HR were measured immediately after cuff deflation (hPSV, hVTi and hHR, respectively). The average of PSV, VTi and HR for four cardiac cycles was determined both at rest and during hyperaemia.

Resting blood flow (rQ) was calculated as described previously[4] using rD, rVTI and rHR:

$$rQ = rSV \times rHR$$

$$rSV = \pi r^2 \times VTi$$

Shear rate was a measure of the frictional force at the surface of the endothelium. Resting stroke volume (rSV) was also determined based on the following equation:[24]

$$rSR = (8 \times rVTi)/rD$$

A summary of PMD blood flow parameters is given in Table 3.

Data were analysed using SPSS version 19 (IBM, Armonk, NY, USA). Data are presented as the mean ± SD. Correlations were determined using Pearson's correlation coefficient and two-tailed $P < 0.05$ was considered significant.

ACKNOWLEDGEMENTS

The authors thank Margaret Heddle and Fiona Pulo (Women's and Children's Hospital, Adelaide, SA, Australia), Narelle Adams (Medical Imaging, Queen Elizabeth Hospital, Adelaide, SA, Australia) and Cameron van den Heuvel (University of Adelaide, Adelaide, SA, Australia) for their assistance with this study. Financial support for this study was provided by the National Health and Medical Research Council of Australia (project no. 453637).

DISCLOSURE

The authors declare no conflicts of interest.

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Appendix 6

Kontos, A., et al., *Delayed brachial artery dilation response and increased resting blood flow velocity in young children with mild sleep-disordered breathing.* Sleep Med, 2015. **16**(12): p. 1451-6.

Sleep Medicine 16 (2015) 1451–1456

Contents lists available at ScienceDirect

Sleep Medicine

journal homepage: www.elsevier.com/locate/sleep

Original Article

Delayed brachial artery dilation response and increased resting blood flow velocity in young children with mild sleep-disordered breathing

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ARTICLE INFO

Article history:
Received 29 April 2015
Received in revised form 4 August 2015
Accepted 7 August 2015
Available online 6 September 2015

Keywords:
Sleep apnoea
Flow-mediated dilatation
Paediatric
Cardiovascular
Blood flow velocity

ABSTRACT

Aim: This study aimed to evaluate whether the vascular dysfunction perceived in adults with sleep-disordered breathing (SDB) was also evident in children with snoring referred for evaluation of clinically suspected SDB.

Objectives: This study compared flow-mediated dilatation (FMD), measured at the brachial artery, at rest and during hyperaemic stress between children who snore [n = 23; mean standard deviation (SD) age = 7.5 1 (1.3) years] and healthy, non-snoring children [n = 11; age = 8.0 (1.3) years].

Methods: Children with suspected obstructive sleep apnoea (OSA) and healthy non-snoring controls underwent overnight polysomnography (PSG). Using standard techniques, non-invasive FMD and brachial arterial blood flow velocity during rest and hyperaemia were subsequently measured by ultrasound imaging

Measurements: Resting and hyperaemic velocity time integral (area under the curve of mean systolic velocity × ejection time), maximal dilation response (highest percentage difference from baseline diameter) and the time taken to reach maximal dilation were calculated.

Results: Children awaiting adenotonsillectomy compared to healthy non-snoring control children had higher velocity time integrals at rest (14 ± 3 m vs. 20 ± 8 m, $p < 0.01$) and during hyperaemic stress (56 ± 6m vs. 63 ± 13 m, $p < 0.01$) despite having only mild SDB on polysomnographic assessment. Lower nadir oxygen saturation values during non-rapid eye movement sleep were negatively associated with higher resting ($r = -0.58$, $p < 0.001$) and hyperaemic ($r = -0.36$, $p < 0.05$) velocity time integrals. Maximal FMD dilatation response was not significantly different between snoring and non-snoring groups, but the estimated time to reach maximal dilation was significantly delayed in children who snored (60.7 ± 28.4 vs. 39.2 ± 13.2 s, $p < 0.05$).

Conclusions: Children with mild SDB showed increased blood flow velocity at rest and during hyperaemic stress suggesting altered cardiovascular and haemodynamic function. The delay in time to maximal vessel dilatation in children who snored also suggests possible reduced vascular compliance in response to hyperaemic sheer stress. Mild SDB appears to alter the peripheral vascular response in young children. The long-term vascular implications of these changes in the growing child are unknown and warrant further investigation.

1. Introduction

Sleep-disordered breathing (SDB) in adults is an independent risk factor for cardiovascular disease [1], including left ventricular diastolic abnormalities [2], ischaemic cardiac events [3], and systemic hypertension [4–6]. Cardiovascular abnormalities are also evident in children with SDB. These have been well documented at the severe end of SDB spectrum, that is, obstructive sleep apnoea syndrome,

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http://dx.doi.org/10.1016/j.sleep.2015.08.004
1389-9457/

and include right and left ventricular hypertrophy, pulmonary hypertension, cor pulmonale and cardiac failure [7–9]. What is less well appreciated, however, is that cardiovascular abnormalities have also been shown in children with mild SDB, that is, primary snoring, and include elevated sympathetic tone, raised blood pressure and increased cerebral blood flow velocity [10–13]. Children with mild SDB constitute the majority of SDB patients [14] and, at present, knowledge of the potential impact of mild SDB on large blood vessel function is unknown.

The non-invasive technique of flow-mediated dilation (FMD) can be used to assess changes in large blood vessel function in children with SDB. FMD uses high-resolution ultrasound to measure dilatation of the artery in response to hyperaemic shear stress induced by ischaemia upon downstream vessels. Vascular dysfunction is reflected by an impaired FMD response in either the magnitude of vessel dilatation [15] or the time taken to reach maximal dilation post cuff deflation [16]. Two previous studies have reported that children with SDB have minor reductions in FMD response at the standard time point of 60 s [17,18]. However, recent literature has shown that measurements at 60 s do not fully encapsulate the true overall functional dynamics of the vessels' response to shear stress, and that extending the measurement time permits a more complete assessment of vascular compliance [19,20].

This study evaluated blood flow and FMD parameters over an extended period of 180 s and measured both the magnitude (ie, FMD at 60 s and maximum FMD) and temporal FMD response of the brachial artery (ie, time to FMD maximal dilation) in children aged between five and nine years with snoring referred for clinical ear, nose and throat (ENT) assessment compared to healthy non-snoring controls.

2. Methods

2.1. Subjects

Children with a history on parental report of snoring more than three nights per week, and who were referred by their primary physician for the evaluation of SDB to the Otolaryngology Clinic at the Women's and Children's Hospital Adelaide, Australia, were recruited as participants ($n = 23$). The control group ($n = 11$) comprised healthy non-snoring children recruited from the community via advertisement and through friends of the children with SDB. The study was approved by the Child, Youth and Women's Health Services and University of South of Australia Human Research Ethic Committees.

Potential participants were limited to between five and nine years of age to minimize the possible influence of pubertal development on sleep, neurovascular development and upper airway dynamics [16,21–25].

Exclusion criteria included a history of significant asthma, previous adenotonsillectomy, significant craniofacial abnormality, medications that affected sleep or respiration, developmental/psychiatric disorders and English as a second language. Given the evidence of an association between obesity and FMD, children with a body mass index >95%ile were excluded [24].

2.2. Polysomnography

Overnight polysomnography (PSG) was conducted using the Compumedics E-Series Sleep System (Melbourne, Australia). The following standard parameters were measured: electroencephalogram (EEG; C3–A2, C4–A1 and F3–A2 F4–A1, O1–A2 and O2–A1), left and right electro-oculogram (EOG), submental, diaphragmatic and leg electromyogram (EMG), heart rate by electrocardiogram (ECG), oronasal airflow by thermistor and nasal pressure, respiratory movements of the chest and abdominal wall using uncalibrated respiratory inductive plethysmography (RIP), and arterial oxygen saturation (SpO_2) by pulse oximetry (Nellcor N-595; 2-s averaging time). The children were continuously monitored through an infrared camera by a paediatric sleep technician who also documented observations of sleep behaviour, which included the presence or absence of snoring.

2.3. Ultrasound assessment of endothelial and artery function

Once awoken from the PSG, and while still fasting, children had an FMD assessment as previously described [26]. Briefly, a resting scan measuring brachial artery diameter and blood flow dynamics was initially undertaken and, subsequently, blood flow to the lower forearm was occluded by inflation of a sphygmomanometer cuff to 250 mmHg for 4 min. In brief, the resultant ischaemia and post-cuff deflation induce reactive hyperaemia by lowering the vascular resistance within the vascular bed supplied by the brachial artery. The reactive hyperaemia, in turn, induces an increase in sheer stress within the brachial artery supplying the ischaemic area, thereby causing dilatation. This physiological response is dependent on the presence of an intact endothelium where vasodilators, especially nitric oxide, are released in response to the sheer stress. The measurement of FMD in vivo has been widely adopted as an assessment of endothelial function [27,28].

Using the pulse Doppler signal at 60° to the brachial artery, blood flow velocity was determined during the resting scan at the first 15-s post cuff deflation. The second scan (reactive hyperaemia) was taken from 30s before until 180 s after cuff deflation. A third recovery scan was conducted 10 min after the cuff deflation scan.

2.4. Data analysis

An experienced sleep technician blinded to subject status scored PSGs according to established sleep stage [29] and 2007 American Academy of Sleep Medicine (AASM) ventilatory criteria [30]. All obstructive apnoeas ≥2 respiratory cycles in duration were scored even if it was not associated with cortical arousal or oxygen desaturation. All other respiratory events were scored at a duration of ≥2 respiratory cycles and associated with a minimum 3% SpO_2 desaturation and/or an arousal or awakening within two breaths of event termination. Obstructive apnoeas were defined as a >90% reduction in airflow associated with continued chest and abdominal wall movement. Obstructive hypopneas were defined as a ≥50% reduction in airflow signal associated with paradoxical chest/abdominal wall movement or increase in RIP. Central apnoeas were scored if there was an absence of respiratory effort, as determined by RIP and diaphragmatic EMG, in association with an absence of airflow. Central apnoeas were also scored if the event lasted ≥20 s. Central hypopneas were defined as a ≥50% reduction in airflow from baseline in association with a ≥50% reduction in respiratory effort from baseline. Apnoea events that included both central and obstructive components were scored as a mixed apnoea. The obstructive apnoea/hypopnea index (OAHI) was calculated as the total number of obstructive apnoeas, mixed apnoeas and obstructive hypopneas per hour of total sleep time. The central apnoea/hypopnea index (CAHI) was calculated as the total number of central apnoeas and central hypopneas per hour of total sleep time. As described by Katz et al. [31], OAHI was used to assess SDB severity.

Spontaneous and respiratory cortical arousals were scored according to the criteria of the American Sleep Disorders Task Force [32]. Spontaneous arousal index (SAI) was expressed as the total number of spontaneous arousals per hour of total sleep time and respiratory arousal index (RAI) as the total number of respiratory arousals per hour of total sleep time.

An echocardiographer blind to subject group assessed arterial diameter during the *R*-wave of the electrocardiogram using ultrasonic callipers. The mean vessel diameter was calculated over four

cardiac cycles. The percentage difference from the resting diameter was calculated at 30 s post cuff release and then every subsequent 15 s until 180 s. From the resting baseline diameter, estimated maximal dilation (FMD_{max}) was the greatest percentage of dilation in the individual's data set. The estimated time to maximal dilation ($FMD_{time\ to\ max}$) was determined as the time taken to reach FMD_{max} after cuff deflation [20].

Brachial artery resting diameter (rD), peak systolic velocity (rPSV), velocity time integral (rVTi; the area under the curve value of the mean systolic velocity during cardiac ejection time) [33], and heart rate (rHR) were taken during the resting scan. Hyperaemic VTi, PSV and HR were measured immediately after cuff deflation (hVTi, hPSV and hHR, respectively). The PSV, VTi, and HR values were averaged over four cardiac cycles.

Kolmogorov–Smirnov test was used to assess the normality of data. In the case of normally distributed data, group differences were tested using the Student's *t*-tests and Mann–Whitney U tests for non-normally distributed data. Mean (SD) values were reported for normally distributed data and median (interquartile range) for non-normally distributed data. Pearson-*r* correlations were used to test the relationship between normally distributed data and the significance was tested using *r*–*z* transformations. Spearman Rho correlations were used to test the relationship between non-normally distributed data.

3. Results

3.1. Anthropometric and polysomnography

The SDB and control group were matched on anthropometric measures and had similar sleep stage percentages and sleep duration values, but consistent with expectations, children with SDB had significantly higher OAHI and lower non-rapid eye movement (NREM) sleep oxygen saturation levels (Table 1). A total of 16 children in the SDB group were classified as primary snorers (OAHI <1) and seven as mild OSA (OAHI <5: three children had an OAHI <2 and three <3). To test whether SDB severity influenced outcome variables, we undertook additional analysis separately examining children with primary snoring compared to those with mild SDB. This analysis revealed no significant group differences in any variables apart from OAHI. Therefore, both primary snoring and SDB groups were combined for analyses. All children in the control group had an OAHI <1 and no history of habitual snoring (<3 nights a week).

3.2. Flow-mediated dilatation

Blood velocity variables were comparable between groups apart from rVTI and hVTi, which were significantly higher in children with SDB (Table 2). Although no significant group difference was observed in FMD_{max}, the $FMD_{time\ to\ max}$ was significantly longer in children with SDB (Table 2). The mean percentage dilation time for the interval FMD_{30s} to FMD_{75s} was similar in both groups; however, dilation was significantly greater at FMD_{90s} in the SDB group (Fig. 1).

3.3. Correlation between polysomnographic and FMD variables

A lower SaO_2 nadir during NREM sleep was associated with a higher rVTI and hVTi ($r = -0.58$, $p < 0.01$ and -0.36, $p < 0.05$; respectively). Furthermore, a higher hVTi was associated with a greater $FMD_{time\ to\ max}$ ($r = 0.42$, $p < 0.05$). In addition, the average of the number of oxygen desaturation events ($SaO_2 \geq 3\% + SaO_2 \geq 4\% + SaO_2 \geq 5\%$) per total sleep time was positively correlated with both rVTi ($r = 0.34$, $p < 0.05$) and $FMD_{time\ to\ max}$ ($r = 0.42$, $p < 0.05$).

Table 1
Mean (SD) anthropometric and polysomnographic variables together with *p*-values from Student's *t*-test comparisons (median and interquartile range are reported in parentheses for non-normally distributed data).

Variable	Controls ($n = 11$)	Mild SDB ($n = 23$)	*p*-value
Anthropometric			
Males (%)	57	60	ns (chi-squared)
Age (years)	8.0 (1.3)	7.5 (1.31)	ns
BMI *z*-score	0.61 (0.66)	0.59 (0.58)	ns
Height (cm)	128.8 (8.3)	125.7 (8.4)	ns
Weight (kg)	28.7 (3.9)	27.2 (4.7)	ns
Waist/hip ratio	0.90 (0.04)	0.90 (0.04)	ns
BMI	17.2 (1.3)	17.0 (1.3)	ns
Polysomnographic			
Total sleep time (min)	418.4 (31.2)	416.2 (53.1)	ns
NREM 1 (%TST)	2.3 (0.8)	3.4 (2.0)	ns
NREM 2 (%TST)	37.5 (8.0)	46.6 (25.4)	ns
SWS (%TST)	43.2 (8.4)	38.3 (8.3)	ns
REM (%TST)	17.0 (2.6)	17.0 (3.7)	ns
Arousals/h sleep time	11.2 (2.0)	12.6 (3.0)	ns
Spontaneous arousals/h sleep time	10.6 (2.1)	10.8 (2.5)	ns
Respiratory arousal/h sleep time	1.00 (0.70)	1.77 (1.67)	ns
Central Apnoea Hypopnea Index	1.47 (0.40)	1.60 (1.20)	ns
Obstructive Apnoea Hypopnea Index	0.17 (0.30)	1.17 (1.50)	<0.05
Nadir SpO_2 (%)	93.1 (0.7)	92.0 (2.5)	<0.01
REM Nadir SpO_2 (%)	93.8 (1.2)	93.7 (2.5)	ns
NREM Nadir SpO_2 (%)	94.0 (0.8) *(94 (93–95)*	92.4 (2.3) *53(91–94))*	<0.05 (Mann–Whitney U)
Average SpO_2 desat (%TST)	3.00 (0.44) *(3 (3–3)*	2.78 (1.48) *3 (2–4))*	ns (Mann–Whitney U)

NB: ns = non-significant.

Table 2
Mean (SD) Flow-mediated dilatation and blood flow variables together with *p*-values from Student's *t*-test comparisons (median and interquartile range are reported in parentheses for non-normally distributed data).

Variable	Controls ($n = 11$)	Mild SDB ($n = 23$)	*p*-value
Blood flow dynamics			
Resting Diameter (cm)	0.233 (0.020)	0.229 (0.026)	Ns
Resting PSV (m/s)	95.8 (9.1)	104.0 (20.8)	Ns
Resting VTi (m)	13.9 (3.3)	19.7 (8.0)	<0.01
Resting HR (b/m)	78.2 (8.8)	89.6 (9.8)	Ns
Hyperaemic PSV (m/s)	155.6 (12.3)	158.9 (22.5)	Ns
Hyperaemic VTi (m)	96.0 (6.2)	63.0 (12.9)	<0.05
Hyperaemic heart rate (b/min)	82.5 (7.8)	81.7(9.9)	Ns
FMD_{max} (%)	8.2 (4.7)	10.2 (4.8)	Ns
$FMD_{time\ to\ max}$ (s)	39.5 (10.1) *(45 (30–60))*	60.7 (28.8) *45 (30–60))*	<0.05 (Mann–Whitney U)

NB: ns = non-significant.

4. Discussion

In this study, young children with primary snoring and mild SDB demonstrated altered baseline vascular characteristics and FMD responses. They had higher brachial artery blood flow velocity both at rest and during hyperaemic stress; the brachial artery took approximately 20 s longer to fully dilate to maximum size after cuff release compared to controls. Lower nadir oxygen saturation during NREM sleep, evident in the SDB group, was correlated with increased blood flow velocity both at rest and during the hyperaemic FMD response. Children with a higher hyperaemic velocity time integral were also more likely to exhibit a delayed maximal dilation response.

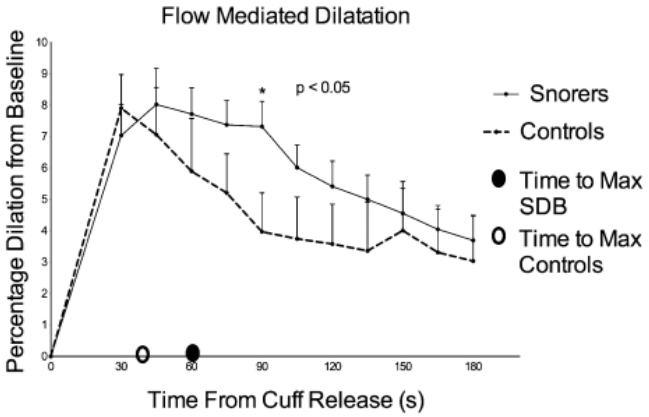

Fig. 1. Mean (SE) temporal FMD response curve for 23 children with mild sleep-disordered breathing (solid line) and 11 age-matched non-SDB children (dotted line). The mean time taken to reach mean maximal dilatation is shown by the corresponding circles on the X-axis. Asterisk denotes significant group differences at 90-s post cuff release.

The rVTi increase observed in the brachial artery of children with mild SDB is consistent with either increased blood viscosity or reduced blood vessel compliance or an increased inotropic response [34]. The latter two are known to be augmented with increased sympathetic activity. As the resting brachial diameters were similar between children with SDB and controls, we hypothesize that the increase in resting blood flow velocity may be best explained by increased sympathetic activity and its subsequent impact on vascular resistance/compliance and cardiac contractibility. Noradrenaline (NA), a potent vasoconstrictor neurotransmitter and inotropic factor released by sympathetic nerves, has been shown to be increased in morning samples of plasma, urine and serum of both children and adults with SDB [11,35–37]. As the resting VTi was taken on waking, the results may reflect this previously reported increase in circulating morning NA levels in children with SDB (reflecting increased sympathetic activity). The increase in resting velocity also mirrors the findings of Hill and associates who reported increased cerebral blood flow velocity in young children with mild SDB compared to non-SDB children using transcranial Doppler [13]. Similar pathophysiological mechanisms have been reported in other disorders. Increased blood flow velocity in both retinal arteries and veins has been observed in patients with diabetes mellitus [38] and hypothyroidism [39], both of these disorders are associated with cardiovascular dysfunction [40] and changes to peripheral resistance [41,42].

It is unclear whether VTi is predictive of cardiovascular disease later in life. However, Lee and associates [43], using digital peripheral tonometry (PAT), have shown that a higher pulse volume amplitude (PVA) in the fingertip of adult participants is associated with a higher resting brachial artery VTi. Increased PVA as assessed by digital PAT is also predictive of cardiovascular disease in adults [44–46]. Furthermore, Jarbult and associates have shown a significant and positive relationship between both systolic and diastolic hyperaemic mean blood flow velocity and ejection fraction in the brachial artery, and, more importantly, increased mean blood flow velocity was evident in adults with 'concentric left ventricular remodelling [47]. Both of these independent studies in adults suggest that the increase in blood flow velocity measured at the brachial artery both at rest and during hyperaemia in the present study of snoring children may be potentially predictive of cardiovascular remodelling in adult life and merits further evaluation.

Traditionally, studies in FMD report the percentage change in brachial vessel diameter from resting baseline to the diameter at 60 s post cuff deflation, with the value at 60 s typically taken to be the time of maximum dilation. Attenuated FMD responses are associated with endothelial damage, atherosclerosis and coronary artery disease [28]. Two studies in children have shown slightly reduced FMD_{60s} values of approximately 0.6% in primary snorers [18] and 0.4% reduction in moderate-to-severe SDB (OAHI > 5/h) compared to controls [17,18]. However, at the FMD_{90s} time point, the results clearly demonstrate that the brachial arteries of children with SDB remains dilated, while the vessels in healthy children have started to contract. The $FMD_{time\ to\ max}$ values of the children with SDB in the present study are comparable to those previously reported by our group in older non-snoring healthy children [26]. Gozal et al. [48] also reported a time delay in reaching maximal dilation in metacarpal blood flow of young children with moderate-to-severe OSAS.

The present findings support the utility of examining FMD response time longer than the recommended 60 s [27]. Black and associates (2008) note that the majority of true maximum dilation occurs outside of these designated post-deflation diameter assessments. They advocated that best practice for endothelial function assessment requires prolonged continuous assessment of arterial diameter so as to determine the true time to peak and maximal dilation response [16]. The present results indicate that a single measure at 60 s may not be a good guide as to vascular health in children with mild SDB.

During NREM sleep, sympathetic activity decreases and parasympathetic vagal activity increases slowing both respiratory and heart rates. Furthermore, cutaneous vessels are dilated in response to inhibition of the sympathetic efferents reducing vascular resistance with resultant reduction in global cerebral blood flow [49]. As NREM sleep constitutes 75–80% of sleep, and slow-wave sleep (SWS) constitutes up to 50% of NREM sleep, upper airway obstruction with resultant oxygen desaturation and increase in sympathetic tone during this period may have a greater impact on peripheral

vascular physiology than previously thought. Reduced oxygen concentration activates central and peripheral chemo-reflex mechanisms resulting in increased heart rate, respiration and constriction of resistance vessels [50]. A greater level of sympathetic neural response is likely necessary to constrict vessels that are usually dilated during NREM compared to REM sleep where cutaneous vessels are already semi-constricted.

The finding in this study that children with mild SDB had the capacity to significantly lower their oxygen concentrations without arousing, indicated by their reduced nadir oxygen saturation compared to non-snoring children suggests attenuated chemosensitivity and specifically during NREM sleep. We recognize that the nadir is but a single moment and does not fully reflect the extent of the oxygen desaturation across the whole night; it does, however, indicate a tendency to tolerate lower oxygen saturation levels before arousing and maybe an indirect marker of chemoreceptor sensitivity.

The positive association between average SpO_2 desaturation (%TST) and both rVTi and $FMD_{rest\ to\ max}$ further suggests that there is an augmented sympathetic response with reduced blood vessel compliance secondary to altered chemoreceptor sensitivity. This potential increase in sympathetic tone, we believe, underlies the change in FMD response that we have demonstrated in children with mild SDB. We have recently shown significant differences in chest wall biomechanics during periods free of scored obstructive events in all sleep stages in children with SDB [51]. Taken together, these results suggest that children with SDB may be significantly more obstructed than the standard PSG scoring criteria would suggest and may have developed attenuated chemoreceptor sensitivity in response, particularly in NREM sleep.

In conclusion, our results suggest that the brachial vascular response is altered in young children with only primary snoring/mild SDB. We propose that this may be explained by a sustained increase in sympathetic tone, which alters downstream vascular compliance. It will also be important in future studies to examine this relationship in children with severe SDB. The unanswered and critical question is whether these changes increase cardiovascular morbidity as the child matures through adulthood.

Authors' contribution

Study Design: CvdH, DK, JM, JC Data Collection: CvdH, YP, SW. Data Analysis: AK. Interpretation of results, AK, KL. Preparation of manuscript: AK, DK, KL, MB, YP, JM, JC, SW, CvdH.

Funding source

Financial support for this study was provided by the National Health and Medical Research Council, project number 453637.

Conflict of interest

None.

The ICMJE Uniform Disclosure Form for Potential Conflicts of Interest associated with this article can be viewed by clicking on the following link: http://dx.doi.org/10.1016/j.sleep.2015.08.004.

Acknowledgement

The authors thank Margaret Heddle, Fiona Puto, and Narelle Adams for their assistance in this study.

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