GLOBAL GENE EXPRESSION PROFILES AND PROTEOMIC ASSESSMENTS IN ADULT FEMALES WITH OBSTRUCTIVE SLEEP APNEA SYNDROME

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ABSTRACT

Obstructive sleep apnea syndrome (OSAS) is a complex disorder characterized by repetitive bouts of upper airway collapse during sleep, causing subsequent intermittent hypoxia, hypercapnia, and fragmented sleep. OSAS affects at least 4% of men and 2% of women; many of which remain undiagnosed. Both clinical characteristics and complex genetic and environmental interactions have made it difficult to understand OSAS disease etiology and identifying patients at risk is still elusive. A pattern of gene expression in cells or tissues related to a disease state for OSAS would provide beneficial information in screening or diagnosing this disease. **Objectives:** 1) map out the study design and bench assay strategies by which to investigate this issue; 2) find out if there are specific differences in the global gene expression profiles of adult females with OSAS; and 3) assess the protein expression differences in the presence and absence of OSAS. **Methods:** Subjects were overweight premenopausal Caucasian women with untreated OSAS (n=6), and control subjects (n=10) and were otherwise clinically similar. They were recruited from either Carilion Clinic Pulmonary/Sleep or Bariatric Surgery practices. The RNA was extracted from the monocytes, frozen and shipped to Dana-Farber Cancer Institute and hybridized to Affymetrix whole human genome chips. Two-step quantitative real time polymerase chain reaction (qPCR) was performed to verify the results from the microarray analysis. The laminin enzyme immunoassay (EIA), and cellular adhesion assays were also performed. **Results:** The array data showed three genes that were differentially expressed. qPCR verified a pattern in segregation between OSAS and controls subjects based on expression patterns of LAMC-1, CDC42, and TACSTD2 genes seen in the arrays. No differences were noted in the laminin EIA. There was a significant increase in PBMC cellular adhesion in OSAS patients versus control subjects ($p<0.05$) **Conclusions:** Cells isolated from women with moderate-severe OSAS show an abnormality in cellular adhesion, a process driven in part by the gene LAMC-1, which was also aberrantly expressed in these subjects. This pilot study has provided the framework and preliminary data needed to propose a larger study with extramural research funding

DEDICATION

To Don and Chase:

I wish to dedicate this dissertation to you both, first for all your love, support, and understanding as I worked through this project. I could not have made it through without it. And second, to what our future holds.

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CHAPTER ONE

INTRODUCTION

Obstructive sleep apnea syndrome (OSAS) is a condition characterized by repeated cessations of airflow at the nostrils and mouth for at least 10 seconds during sleep that results in oxygen levels falling in the blood. It has been estimated that 1 in 20 adults has this condition. It usually goes unrecognized and undiagnosed, resulting in behavioral changes (e.g., depression and irritability) and increased cardiovascular morbidity [1]. Undiagnosed OSAS has a wide spectrum of severity, with serious secondary comorbidities including but not limited to increased risk of hypertension [2, 3], congestive heart failure [4], cardiovascular disease [5], type II diabetes [6], and cerebrovascular disease [7]. Obstructive sleep apnea was clinically recognized more than 30 years ago [8], but awareness of this condition outside the field of sleep medicine has been slow to develop. The situation changed drastically in the early 1990's when population-based studies began to uncover an unexpectedly high prevalence of OSAS in adults. Findings from both clinical and community cross-sectional studies in the late 1990s examined the secondary consequences of OSAS such as hypertension, myocardial infarction, and other cardiovascular disease. These early reports indicated mixed results and, therefore, generated considerable controversy in the scientific and healthcare communities [1]. In the past, studies that attempted to distinguish between patients with and without OSAS focused on the clinical features, e.g., sleep topography, sleep fragmentation, and prevalence of intermittent hypoxia [9]. More recent research has increasingly centered on the underlying etiology. Being able to identify those who are affected by OSAS and its prevalence is critical to anticipating health care needs and allocating appropriate resources. Comparisons of prevalence by

demographic factors may help to yield etiological clues and identify subgroups at particularly high risk for targeted case findings. Thus far, prevalence studies conducted over the past decade have provided considerable data from diverse populations to estimate the health burden of OSAS and to explore important consequences of its occurrence. However, prevalence estimates are extremely vulnerable to the methodological issues such as the subject selection criteria (ethnicity, sex, and age) as well as the use of appropriate weighing techniques, similar measurement methods, and definitions of hypopnea and apnea-hypopnea index (AHI) cut points. With similar problems that plague the attempts to measure prevalence, there are special problems in identifying new occurrences of OSAS. Individuals have night-to-night variability in AHI and there are other measurement errors that can lead to difficulties in valid classification of OSAS status. This can cause systematic biases in estimating incidence, for example, due to regression to the mean across arbitrary disease-defining cut points [1] making the estimates of incidence challenging. Therefore, little is known about incidence (i.e., the occurrence of new cases over a given time interval) or progression (i.e., worsening over time) of OSAS.

It is thought that through public health education and initiatives with clinical support the reduction or elimination of causal or contributing factors of OSAS can be addressed. Data suggest that OSAS in females is associated with many factors including overweight and obesity, alcohol, smoking, nasal congestion, and estrogen depletion due to menopause, but at present, the only lifestyle intervention strategy supported with adequate evidence is weight loss [9]**.** The literature has recently identified that many of the common symptoms of this disorder are only present in ~50% of the diagnosed OSAS population [9]. Research suggests that at least some of these

differences could potentially be genetic in nature. Upon review of the current literature there have only been two studies that have investigated gene expression profiles of OSAS patients [10, 11]; one was a pediatric patient sample, in which the etiology and pathophysiology of the disorder are likely different than for adult OSAS [11]. The second study was one of a small sample of adult males with OSAS [10].

Both clinical characteristics and complex genetic and environmental interactions can make it difficult to understand OSAS disease etiology in adults and therefore identifying patients at risk is still elusive. A molecular signature, a pattern of expression in cells or tissues related to a disease state, for OSAS would provide beneficial information for physicians to be most effective in screening or diagnosing this disease. By combining both clinical and molecular features of this disease, predominantly intermittent hypoxia, and the examination of gene and protein expression differences, an enhancement of understanding of how this disease progresses may be possible. This dissertation research was a pilot investigation to assess the global gene and protein expression profiles that possibly relate to OSAS. This project is one of a very few attempts that have been made to characterize genome-wide gene expression profiling in OSAS. The aspiration was to work out the details of how best to design and execute a large-scale extramurally funded research project, as well as to generate a modest data set that supports the viability of the research hypotheses. The dissertation includes two sub-studies: 1) a transcriptional profile assessment of OSAS; and 2) a set of serum proteomic assessments, contrasting two small groups of clinically similar patients, with and without OSAS. The hypotheses and direction for guiding the second study has been generated from the transcriptional profiling from the first.

Research Aims

The three aims of this preliminary study were: 1) to establish a study design and bench assays by which to investigate this issue; 2) to determine presence or absence of different global gene expression profiles in adult females with and without OSAS, who were otherwise clinically similar; and 3) to assess and compare protein expression patterns in the presence and absence of OSAS.

Directional Hypotheses

While earlier studies of other chronic diseases have done cellular approaches, they often used all white blood cells to study changes [9], there are heterogeneity in responses of different cell types which could skew results and interpretations. Therefore, studies have started focusing on changes in specific subpopulations of leukocytes, such as monocytes. Recruitment of circulating monocytes into adipose tissue and inflamed intima [12] has been implicated in insulin resistance/type 2 diabetes as well as atherosclerosis, respectively, both of which are considered comorbidities of OSAS [13]. Monocytes have been affected specifically by OSAS, e.g., by NF-κB activation, by increased production of TNF-α, and by IL-6 [14]. A variation in monocyte functions may be one of the factors responsible for OSAS in certain patient subsets. From these variations, an analysis of monocyte complexity (the intricate arrangement of unique cellular behaviors) in these patients with OSAS may lead to an improved understanding of disease etiology. Thus, it is hypothesized that OSAS would lead to altered gene expression in circulating monocytes and that these results will develop a novel foundation for advancing the understanding of OSAS pathology. The expectation is that OSAS patients would have a unique global gene expression pattern that differentiates them from the clinically matched non-OSAS patients. It is also

hypothesized that a reasonably simple serum protein profile would be identified only in an OSAS patient as a function of his/her exposure to intermittent nocturnal hypoxia. The expectation is that these profiles are induced by aberrant regulation of specific genomic pathways.

Differences between those with OSAS and comorbidities and those without could also be genetic in origin. Descriptive reports of families with multiple affected members show that there is likely to be role for inheritance apart from familial influences related to obesity [15, 16]. Preliminary results from segregation analysis have further defined the likely magnitude of genetic influences [17]. Several studies have shown the possibility for inheritance of this disease. The Cleveland study found an association between sudden unexpected death in infancy and OSAS, suggesting that there are some families that may be predisposed to both syndromes [18]. Other researchers have looked at racial studies and chromosomal mapping by examining familial studies and twin studies. This has provided some evidence for the possible link between the OSAS phenotypes and genetic loci that could prove to be markers for further research, including obesity, fat distribution, snoring, and sleep regulation. There are a limited but growing number of studies that have addressed the question of gene variants and their pathophysiological effect in the OSAS population. Taken together, those investigations have provided compelling data linking sleep apnea to obesity, and chronic inflammation. To date, the implications of these comorbidities and overlapping pathogenetic pathways predominantly have been related to effects on risks for developing cardiovascular disease. Investigations of changes in gene expression in circulating cells will likely provide the clearest molecular signature to provide prognostic or diagnostic implications

specific to OSAS. The information gained from global gene expression profiling may help uncover the molecular mechanisms of OSAS etiology.

The investigation of potential genetic causes of OSAS has to initially start with assessing the global gene expression patterns in patients with and without OSAS. By examining the expression of patterns of up or down regulation of genes relevant to discrimination of patient classes a profile can be developed for OSAS. This would provide the framework for other more elaborate genetic analysis such as genetic variation and transcriptional control. Genetic variation is the ability to determine the inter-individual differences within the OSAS population may allow for the identification of which patients with OSAS are likely to develop specific consequences. It is then plausible that establishing this signature for OSAS will assist physicians identifying and assessing high risk patients by examining the transcriptional control (a major regulatory mechanism for differential control of protein synthesis in eukaryotic cells). The possibility for screening patients for OSAS before they become symptomatic may allow physicians to prescribe preventive interventions early enough to delay secondary health consequences associated with OSAS.

Capacity to Conduct the Study

Over the past 3 years, our laboratory has developed a partnership to conduct this research project with Carilion Clinic in Roanoke, VA, and the Functional Genomics laboratory at Dana Farber Cancer Institute at Harvard University, Boston, MA. Previous research conducted in our laboratories confirmed that we had the laboratory, personnel, and patient resources to successfully undertake this project. This dissertation is derived from the research project entitled “Monocyte transcription profiling risk markers of Obstructive Sleep Apnea Syndrome (OSAS)” and was part of a larger Carilion Clinic

study funded through their internal research grant program. Our investigator group was comprised of basic and applied scientists specialized in genomics, clinical physiology, sleep medicine, and bariatric surgery.

Delimitations

- Only Caucasian females were studied. Since the clinical features, etiology, and even pathophysiology of OSAS may vary according to gender and racial factors, we selected one race and one gender to minimize the influence of such factors in our study sample.
- The patients included were female non-smokers, between 20 and 45 yr of age.
    - Sleep studies were used to assess presence of OSAS and its severity in the study sample
    - A home somnography device (i.e., ApneaLink®) was used to qualify control group volunteer subjects who screened negative for sleep apnea based on the Epworth Sleepiness Scale (ESS) score (<10).
    - A clinical nocturnal PSG was used to determine the presence and severity of OSAS by full PSG study in which a subject had and ESS score ≥10.
- The goal was to have 16 subjects participate in this study, six subjects who were clinically positive for moderate to severe OSAS (AHI >15) and another ten, who served as controls (respiratory disturbance index or AHI <10). The subjects were compared and matched objectively to ensure that they had similar clinical characteristics (age, BMI, diabetes, cardiovascular disease status, medications, etc.).
- DNA was hybridized on 16 Affyrmetrix© GeneChip Human Genome U133A 2.0 Arrays, comprised of more than 22,000 probe sets corresponding to 14,500

genes with distinct oligonucleotide features, as oppose to other chips that have either more or less probe sets.

Limitations

- Since patients were Caucasian females between the ages of 20 and 45 yr., the results cannot be generalized to other gender or racial groups.
- Since this was a pilot study, a small number of subjects were tested. With the large amount of data from high through-put DNA microarray platforms for phenotypic classification this presented statistical concerns [19].
- The analytic methods were restricted to evaluation of gene expression changes within monocytes and may not reflect the response patterns within specific end-organs [major organs fed by the circulatory system (heart, kidneys, brain, etc.)], and therefore, provided only a non-specific cross-sectional picture of altered gene expression [11].

The remainder of this dissertation is organized into chapters, as follows: 2) a systematic literature review of OSAS including patient clinical features as well as genomic and proteomic analytic techniques; a manuscript chapter in which study results are presented, 3) Global Gene Expression Profiles and Proteomic Assessments in Adult Females with Obstructive Sleep Apnea Syndrome, organized as journal manuscripts with abstracts, introduction, methods, results, and discussion; and finally chapter 4) summary, and recommendations for future research.

Definitions

Adipokine – a hormone that produced by white adipose tissue that may modulate insulin resistance [20].

Angiogenesis – formation of new blood vessels [21].

Apnea – the cessation of airflow at the nostrils and mouth for at least 10 seconds [22].

Apnea/Hypopnea index (AHI) – the number of apneas and hypopneas per hour [22].

Arousal - abrupt change from sleep to wakefulness, or from a "deeper" stage of non-REM sleep to a "lighter" stage [23].

Berlin Questionnaire (BQ) - clinical questionnaire that can be used to identify individuals at risk of OSAS [24].

CPAP – Continuous Positive Airway Pressure - the device used to treat sleep apnea by sending positive airway pressure at a constant, continuous pressure to help keep an open airway, allowing the patient to breathe normally through his/her nose [22].

Cytokine – a chemical messenger that can induce or inhibit a variety of local and systemic physiological processes, e.g. inflammation [25].

Enzyme Linked Immunosorbent Assay (ELISA) – a method of measuring levels of antibodies in a plasma or serum [26].

Epworth Sleepiness Scale (ESS) – clinical questionnaire used to determine the level of daytime sleepiness [27].

Global Gene Expression Profile - is the pattern of up or down regulation of genes relevant to discrimination of patient classes [19].

Hepcidin – an iron-regulatory hormone produced by the liver which regulates extracellular iron concentrations [24].

Hypercapnia – increased levels of arterial blood carbon dioxide [22].

Hypopnea – significant reduction in airflow characterized by one of three criteria: 50% reduction in airflow; <50% reduction in airflow coupled with a >3% reduction in oxygen saturation; or <50% reduction in airflow coupled with EEG evidence of arousal [22].

Hypoxemia – below normal reduction in arterial blood oxygen concentration [28].

Interleukin-6 (IL-6) – a 28kDa pro-inflammatory adipokine that is associated with impairments in insulin signaling and glucose metabolism [29].

Molecular Signature - a pattern of genes or proteins, expressed in cells or tissues [9].

Monocytes –type of white blood cell intimately involved in body's immune response system valuable information in the production of proteins affecting vascular regulation [30].

Obstructive Sleep Apnea Syndrome - a complex disorder characterized by periodic complete or partial upper airway obstructions during sleep, leading to intermittent cessations of breathing, or reductions in airflow to the lungs despite ongoing respiratory effort [22].

Tumor Necrosis Factor-alpha (TNF-α) – a multifunctional 26kDa pro-inflammatory adipokine that affects lipid metabolism and upregulates the production of other proinflammatory cytokines [29].

Polysomnogram (PSG) – a test used to diagnose sleep disorders that involves continuous and simultaneous recording of physiological variables during sleep, i.e., EEG, EOG, EMG (the three basic stage scoring parameters), EKG, respiratory air flow, respiratory excursion, lower limb movement, and other electrophysiological variables [22].

Proteomic Assessment –examination of the expression and post-translational modifications of thousands of proteins simultaneously [31].

List of Abbreviations

AHI – apnea-hypopnea index

BMI – Body Mass Index

CPAP – Continuous Positive Airway Pressure

CM – Centimeter

CT – Computed Tomography

CV – Coefficient of Variation

EDTA – Ethylenediaminetetraacetic Acid

EIA – Enzyme Immunosorbent Assay

HIF-1α - hypoxia-inducible factor-1 alpha

ICAM-1 - intercellular adhesion molecule 1

IL-6 – Interleukin-6

IL-27 - Interleukin-27

ODI – Oxygen Desaturation Index

OSAS – Obstructive Sleep Apnea Syndrome

PAQ – Physical Activity Questionnaire

PSG – polysomnography

RDI – Respiratory Disturbance Index

SD – Standard Deviation

SST – Serum Separator Tube

TNF-α – Tumor Necrosis Factor-Alpha

References

1. Young, T., P.E. Peppard, and D.J. Gottlieb, *Epidemiology of obstructive sleep apnea: a population health perspective.* Am J Respir Crit Care Med, 2002. **165**(9): p. 1217-39.
2. Nieto, F.J., et al., *Association of sleep-disordered breathing, sleep apnea, and hypertension in a large community-based study. Sleep Heart Health Study.* JAMA, 2000. **283**(14): p. 1829-36.
3. Peppard, P.E., et al., *Prospective study of the association between sleep-disordered breathing and hypertension.* N Engl J Med, 2000. **342**(19): p. 1378-84.
4. Kaneko, Y., et al., *Cardiovascular effects of continuous positive airway pressure in patients with heart failure and obstructive sleep apnea.* N Engl J Med, 2003. **348**(13): p. 1233-41.
5. Peker, Y., et al., *Increased incidence of cardiovascular disease in middle-aged men with obstructive sleep apnea: a 7-year follow-up.* Am J Respir Crit Care Med, 2002. **166**(2): p. 159-65.
6. Babu, A.R., et al., *Type 2 diabetes, glycemic control, and continuous positive airway pressure in obstructive sleep apnea.* Arch Intern Med, 2005. **165**(4): p. 447-52.
7. Yaggi, H.K., et al., *Obstructive sleep apnea as a risk factor for stroke and death.* N Engl J Med, 2005. **353**(19): p. 2034-41.
8. Guilleminault, C., A. Tilkian, and W.C. Dement, *The sleep apnea syndromes.* Annu Rev Med, 1976. **27**: p. 465-84.
9. Arnardottir, E.S.M., M; Gislason, T; Teff, K.L.; Pack, A.I., *Molecular Signatures of Obstructive Sleep Apnea in Adults: A Review and Perspective.* Sleep, 2009. **32**(4): p. 447-470.
10. Hoffmann, M.S., et al., *Microarray studies of genomic oxidative stress and cell cycle responses in obstructive sleep apnea.* Antioxid Redox Signal, 2007. **9**(6): p. 661-9.
11. Khalyfa, A., et al., *Genome-wide gene expression profiling in children with non-obese obstructive sleep apnea.* Sleep Med, 2009. **10**(1): p. 75-86.
12. Hansson, G.K., *Inflammation, atherosclerosis, and coronary artery disease.* N Engl J Med, 2005. **352**(16): p. 1685-95.
13. Pack, A.I., *Advances in sleep-disordered breathing.* Am J Respir Crit Care Med, 2006. **173**(1): p. 7-15.
14. Yue, H.J., et al., *The roles of TNF-alpha and the soluble TNF receptor I on sleep architecture in OSA.* Sleep Breath, 2009. **13**(3): p. 263-9.
15. Redline, S., et al., *The familial aggregation of obstructive sleep apnea.* Am J Respir Crit Care Med, 1995. **151**(3 Pt 1): p. 682-7.
16. Strohl, K.P., et al., *Obstructive sleep apnea in family members.* N Engl J Med, 1978. **299**(18): p. 969-73.
17. Kaparianos, A., et al., *Obstructive sleep apnoea syndrome and genes.* Neth J Med, 2006. **64**(8): p. 280-9.
18. Palmer, L.J. and S. Redline, *Genomic approaches to understanding obstructive sleep apnea.* Respir Physiol Neurobiol, 2003. **135**(2-3): p. 187-205.

19. Chittenden, T.W., et al., *Transcriptional profiling in coronary artery disease: indications for novel markers of coronary collateralization.* Circulation, 2006. **114**(17): p. 1811-20.
20. Masserini, B., et al., *Reduced levels of adiponectin in sleep apnea syndrome.* J Endocrinol Invest, 2006. **29**(8): p. 700-5.
21. Chorostowska-Wynimko, J., et al., *Disturbed angiogenic activity in sera from obstructive sleep apnea patients.* J Physiol Pharmacol, 2005. **56 Suppl 4**: p. 71-7.
22. AASM, A.A.o.S.M.-. *Sleep-related breathing disorders in adults: recommendations for syndrome definition and measurement techniques in clinical research. The Report of an American Academy of Sleep Medicine Task Force.* Sleep, 1999. **22**(3): p. 667-89.
23. AASM, A.A.o.S.M.-. *Obstructive Sleep Apnea*. 2008; Available from: www.aasmnet.org.
24. Ganz, T., *Hepcidin in iron metabolism.* Curr Opin Hematol, 2004. **11**(4): p. 251-4.
25. Ciftci, T.U., et al., *The relationship between serum cytokine levels with obesity and obstructive sleep apnea syndrome.* Cytokine, 2004. **28**(2): p. 87-91.
26. Lai, Y., K.L. Feldman, and R.S. Clark, *Enzyme-linked immunosorbent assays (ELISAs).* Crit Care Med, 2005. **33**(12 Suppl): p. S433-4.
27. Johns, M.W., *A new method for measuring daytime sleepiness: the Epworth sleepiness scale.* Sleep, 1991. **14**(6): p. 540-5.
28. Levy, P., et al., *Intermittent hypoxia and sleep-disordered breathing: current concepts and perspectives.* Eur Respir J, 2008. **32**(4): p. 1082-95.
29. Imagawa, S., et al., *Interleukin-6 and tumor necrosis factor-alpha in patients with obstructive sleep apnea-hypopnea syndrome.* Respiration, 2004. **71**(1): p. 24-9.
30. Heil, M., et al., *Blood monocyte concentration is critical for enhancement of collateral artery growth.* Am J Physiol Heart Circ Physiol, 2002. **283**(6): p. H2411-9.
31. Gulcicek, E.E., et al., *Proteomics and the analysis of proteomic data: an overview of current protein-profiling technologies.* Curr Protoc Bioinformatics, 2005. **Chapter 13**: p. Unit 13 1.

CHAPTER TWO

LITERATURE REVIEW

To establish the foundations for this dissertation research, the following related literature areas were examined and the applicable papers reviewed with their implications being presented in this chapter: 1) the definition of OSAS, how it's assessed and treated; 2) impact of OSAS on the cardiovascular system; 3) the significant research in the area of gene expression profiling; 4) inflammatory biomarkers that may be unique to OSAS; and 5) technical performance considerations for measurement techniques including the monocyte isolation, quantitative polymerase chain reaction (qPCR), western blotting, and selected bioassays to relevant to conducting investigation in these two topic areas. This chapter concludes with an integrated summary of the related literature and how each section contributes essential background information and framework for the dissertation research.

Obstructive sleep apnea syndrome (OSAS) is one of the most common of sleep disorders and it often requires lifelong treatment. It manifests through a variety of signs and symptoms, many of which are witnessed by a sleep partner. As with many diseases, every patient presents with a slightly different symptomology. Snoring, long breathing pauses while sleeping, night sweats, multiple awakenings, or excess movements at night are classic indicators. Among the critical consequence during wakeful periods are excessive daytime sleepiness, problems with concentration, impaired memory, and depression. Apart from these more immediate and often observable consequences, OSAS is now understood to be a serious disorder that

increases longer-term risks of life-threatening diseases, such as hypertension, stroke or heart attack.

Using improved methodologies in recent years, epidemiologic studies have suggested that OSAS may affect as many as 1 in 20 adults in Western society. The nighttime respiratory events in OSAS are characterized in two ways: 1) by the repeated cessations of airflow at the nostrils and mouth for least 10 seconds [22]; and 2) by significant reductions in airflow. For a respiratory airflow reduction to qualify as an OSAS event, one of the following conditions must be satisfied: 50% reduction in airflow; <50% reduction in airflow coupled with a >3% reduction in oxygen saturation; or <50% reduction in airflow coupled with EEG evidence of arousal [22]. When an event occurs due to an episode of airflow cessation, this is defined as an apnea. When one of the qualifying conditions for reduced airflow has been met, the event is defined as a hypopnea. In general, apneas and hyopopneas precipitate dips in blood oxygen saturation (hypoxemia). Hypoxemia may, in turn, signify some period of reduced oxygen availability to vital body tissues (hypoxia). The more of these critical events that occur during sleep, the greater the potential for repetitive arousals, sleep fragmentation, as well as tissue hypoxia and distress to body organs and functions. Measurement of these events during sleep is done through the gold standard diagnostic test for sleep disorders, called polysomnography (PSG). The PSG test, therefore, provides the medical specialist with the means for diagnosis of OSAS, or other sleep-disorders, as well as a determination of severity for the particular disorder that is diagnosed.

The apnea-hypopnea index (AHI), obtained from the PSG, is the number of obstructive respiratory events that occur per hour. Mild OSAS is defined as 5-15

events/h, moderate is15-30 events/h, and severe is greater than 30 events/h. With the increase in severity of the disease comes an increase in secondary consequences, if left untreated. There are many varied sleep apnea symptoms and again, individuals may have one, some, or all of these symptoms and be diagnosed with sleep apnea. Unfortunately, due to the lack of awareness by the public and healthcare professionals, the vast majorities of patients remains undiagnosed and go untreated, despite its important health consequences. Current estimates indicate that at least 4% of men and 2% of women have OSAS; however, as many as 24% of men and 9% of women who are middle-aged and evaluated by a stringent diagnostic criteria may have mild OSAS or worse, even without manifesting the most common symptom, daytime sleepiness [32, 33].

Finding definitive means to identify patients at high risk for OSAS and sorting through the overlapping mechanisms has long been debated and the subject of considerable research. Generally, physicians use screening tools such as questionnaires (e.g., Epworth Sleepiness Scale, or Berlin Questionnaire), assessments of morphology of the upper airway, a history of snoring, witnessed apneas, gasping/choking episodes, clinical findings of excessive daytime sleepiness, obesity, retrognathia, and/or drug-resistant hypertension to identify these patients. Positive findings on any of these OSAS screens should lead to a more comprehensive sleep history and physical examination, including a sleep study (a polysomnography) to confirm apneic and/or hypoxic events. Currently, OSAS research has found that the common symptoms are only present in approximately half of the OSAS population, and there are some less common symptoms that have not been mentioned here, thereby making the identification of who is at risk for OSAS more of a guessing game [9] than a

scientific assessment. This would support the suggestion that the number of undiagnosed patients may actually be greater than 90% of adults [32].

This large untreated population is an increased risk for secondary consequences. The most notable consequence is a significant cause and contributor of increased inflammation leading to cardiovascular disease [17, 34, 35]. With the repetitive intermittent hypoxia during sleep, researchers have shown that systemic inflammation is a constitutive component and consequence of OSAS [36]. The chronic effects arising from repeated exposure to apneic events and intermittent hypoxia results not only in inflammation but an increased sympathetic tone and endothelial dysfunction, ultimately leading to vascular remodeling, vasoconstriction, and the development of vascular complications and cardiac dysfunction [37, 38]. These adverse effects of untreated OSAS are thought to arise from several overlapping mechanisms.

Since OSAS has many symptoms and mechanisms to consider, identifying patients who have OSAS with the clinical measure AHI has to be based on the gold standard, an overnight polysomnography (PSG) performed in a sleep clinic. With the substantial variation both in the nature of the breathing events during sleep and in the consequences of such events in different individuals accurate diagnoses are needed. Screening tools such as questionnaires [39] and morphometric [40] examinations are insufficient for accurate screening and produce a high rate of false negatives. Though it is accurate, the PSG is an expensive, laborious, and time consuming assessment for the diagnosis of OSAS. Therefore, more accurate and cost effective measures of risk are needed before giving sending patients for a PSG. It is thought that there may be, in

part, genetic variants between individuals and researching this area may provide valuable insight into the diagnosis.

Since there is not a clear understanding of the pathophysiological significance of molecular signatures and their relationship with OSAS comorbidities there is currently no other way to identify or diagnosis a person with OSAS. However, one of the newer techniques to assess diseases is to examine the molecular signatures. A molecular signature can describe a pattern of gene or protein expression in cells or tissues related to a given disease state [9]. Previously, this technique has been used to identify high risk patients with other diseases. OSAS is a disease that would be very amenable to this process due to the variety of individual responses to this particular disease. It may provide distinct endophenotypes that will help screen patients who are at an increased risk [9]. This approach may be able to provide both a diagnostic and prognostic assessment of OSAS to physicians.

Obstructive Sleep Apnea Syndrome (OSAS)

To understand the significance of OSAS outcomes, you must understand how it differs from other sleep-disordered breathing (SDB) problems. SDB describes a group of disorders comprised of a wide spectrum of sleep-related breathing abnormalities; central sleep apnea (CSA), obstructive sleep apnea syndrome (OSAS) and those related to increase upper airway resistance include snoring, and upper airway resistance syndrome (UARS). Snoring generally had been considered a social nuisance without consequences for the snorer, though it is common symptom of many SDB problems it may not always be present. CSA is a less common type of sleep apnea that occurs when the area of the brain that controls breathing doesn't send the correct

signals to muscles that are used to breathe. As a result, there is absolutely no effort to breathe for brief periods [22]; thereby, lowering your oxygen levels in the blood. This results in fatigue and possible heart rhythms. Compared to UARS and OSAS which are both characterized by repeated arousals, due to upper airway resistance, that lead to excessive daytime sleepiness [22]. In UARS, unlike in OSAS, there is no evidence of oxygen desaturation, and the events are noted to be typically short: one to three breaths in duration compared to OSAS events which can last up to two minutes [22]. Therefore OSAS is a unique SDB that can result in significant secondary health consequences.

The signs, symptoms, and consequences of OSAS are a direct result of the periodic complete or partial upper airway obstruction, leading to intermittent cessations of breathing (apnea), or reduction in airflow (hypopnea) despite ongoing inspiratory effort. These apneic and hypoxic events result a variety of issues including, sleep fragmentation, hypoxemia (decreased oxygen levels), hypercapnia (increased carbon dioxide levels), marked swings in intrathorasic pressure, and increased sympathetic activity [23]. It is not clear at this time whether the presenting symptoms (poor quality of health, memory problems, and decreased cognitive abilities) are from the intermittent hypoxia created from the obstructions themselves [41, 42] or from the sleep deprivation from the constant arousals to resume breathing [43-45]. Researchers are still debating this question and there are ample numbers of studies to support both sides. Given the potential interaction between sleep deprivation and OSAS, one could easily imagine a “self-feedback loop,” in which sleep deprivation worsens OSAS, which in turn has a negative impact on nocturnal sleep quality and sleep hours, worsening sleep deprivation even further [45]. Clinically, OSAS is defined by the occurrence of daytime sleepiness, and during sleep, loud snoring, witnessed breathing interruptions, or awakenings due to

gasping or choking in the presence of at least 5 obstructive respiratory events (apneas, hypopneas or respiratory effort related arousals) per hour [46]. The presence of moderate to severe disease (AHI ≥15) in the absence of sleep related symptoms is sufficient for the diagnosis of OSAS due to the greater association of this severity of obstruction with important consequences such as increased cardiovascular disease risk [46].

Patients with OSAS frequently are burdened by the presence of risk factors for major chronic diseases and these can constitute ways to improve identification of patients more likely to have this disorder. Therefore, variables that increase pre-test likelihood of OSAS (before decision to do a PSG test), include not only self-identification of EDS, advanced age, male gender [47], and in some cases unusual craniofacial features, but coexiting obesity, hypertension, or insulin resistance [33]. The most prevalent risk factors associated with OSAS are also affiliated with cardiovascular disease such as, obesity and age. Obesity is currently the most established and prominent concerning issue for OSAS, given that body mass index (BMI), visceral fat, and neck circumference are some of the major physical predictors seen in the clinical expression of OSAS [48, 49]. A large neck circumference (males > 43 cm; females >40 cm), suggests greater adiposity and tissue mass over the airway increasing an individual's risk for OSAS [35]. From the Wisconsin Sleep Cohort study [3], as little as a 10% increase in body weight increases risk for OSAS by as much as 6-fold. The association between obesity and OSAS has been well documented [50, 51], but the mechanisms responsible for this link remain unclear [47]. When examining age and OSAS, the mechanisms are a clearer. A greater prevalence of OSAS in older adults is due to the increase in pharyngeal resistance (negative pressure reflex is a major

mechanism humans maintain pharyngeal patency) in males [52, 53] and post-menopausal women are up to three times more likely to have OSAS compared to premenopausal women [54, 55]. The craniofacial morphology and OSAS risk has the clearest link, physiologically speaking. Anatomical abnormalities, such as maxillary and mandibular retrognathia, increase risk for the development of OSAS and often are present in those without the typical risk profile. Many OSAS patients' risk of upper airway collapsibility has been related to maxillofacial skeletal anomalies, but research has shown that many patients actually have soft tissue anomalies present that may be of equal importance [56]. There may be inherited factors which can increase the risk of OSAS [15, 16]. Ones' family history of OSAS may significantly increase the risk for OSAS, by as much as 2-4 fold [15, 57, 58]. This may be due to inheritance of such factors as craniofacial structure and body fat distribution, [57] or may reflect risk factors related to shared familial lifestyle [35].

Whatever the underlying factors for OSAS are, the importance of getting at risk patients diagnosed should be priority. Once OSAS has been diagnosed, there are medical, behavioral, and surgical options for the treatment of OSAS. The majority of patients with moderate to severe OSAS are encouraged to start treatment on a continuous positive airway pressure (CPAP) device. The CPAP therapy provides a constant airflow which holds the airway open so that uninterrupted breathing is maintained during sleep. This eliminates sleep apnea events and allows the patient to get restful sleep. Use of a CPAP device improved self-reported sleepiness, improved quality of life, and was an adjunctive therapy to lower blood pressure in hypertensive patients with mild OSAS [59] and significantly decreased and/or eliminated other secondary complications.

Factors Affecting Secondary Consequences of OSAS

As mentioned earlier, the long-term health consequences of OSAS are of considerable concern. These abnormal pathological consequences include but are not limited to hypertension, myocardial infarction, strokes, angina, palpitations, pulmonary-hypertension, and an increased risk of death due. Physiologically, the ability to maintain oxygen homeostasis is essential to the survival of all cells in an organism. In OSAS patients, homeostasis is difficult to maintain with episodic obstructions of airflow often more than 60 times per hour, and some with significant desaturations of oxyhemoglobin to levels as low as 50%. These events are not only associated with hypoxemia but significant hypercapnia and frequent arousals that lead to significant episodic increases in sympathetic nervous system activity [60] as well as cancer [61]. The physiological responses to hypoxia can occur acutely as well as chronically thereby allowing numerous and wide spread changes to transpire. Therefore, OSAS can have a major impact on multiple secondary systems throughout the body including the cardiovascular and central nervous systems. Predominately, hypoxemia creates a cascade of secondary consequences that have been associated with OSAS.

Hypoxia

With the chronic decrease or brief cessations in airflow associated with OSAS, the body as a whole (generalized hypoxia) or a region of the body (tissue hypoxia) is deprived of adequate oxygen supply. When this mismatch between the oxygen supply and its demand at the cellular level occurs, it may initiate a cascade of effects within the body. There are two significant but different areas of hypoxia that have been study,

chronic altitude adaptations and the secondary consequences of chronic intermittent hypoxia. As humans have ventured into higher altitudes, it has become apparent that our bodies can adapt to counter the chronic effects of sustained hypoxia by maximizing the efficient use of oxygen for a given metabolic demand. Exposure to high altitudes results in an effective stimulus for erythropoietin production [62, 63], which increases total red blood cells, and hemoglobin, and reduces the heart rate response to a given workload. The progressive increases in ventilation, and enhance oxygen delivery to tissues allowing for better extraction of oxygen under these conditions [64]. These unique adaptations are only in response to continuous chronic hypoxia, there are some important differences when exposure to intermittent hypoxia. Unfortunately, less is known about the effects of chronic intermittent hypoxia (CIH), one of the physiological markers of OSAS. CIH is characterized by transient periods of oxygen desaturation followed by reoxygenation, and this is a major cause of its systemic harmful consequences (oxidative stress, inflammation, sympathetic activity, vasculature remodeling and endothelial dysfunction) [28, 65-71]. The pathophysiology of this apnea syndrome has spurred a recent surge of interest in the physiological and genomic effects of these persistent intermittent episodes of hypoxemia that produce a variety of comorbid disorders.

Researchers have studied biologic effects of CIH. Some reports indicate that CIH can increases right ventricular heart mass, likely attributable to pulmonary vascular remodeling and pulmonary hypertension [72, 73]. Where others have examined how CIH contributes to increases in daytime blood pressures due to chronically enhanced daytime sympathetic activity [74, 75]. Numerous other alterations have been established such as changes in cerebral synaptosomal ATPase activities; impaired cerebral lipid

metabolism and regional changes in the activities of key metabolic enzymes in the brain and in the concentrations of brain stem methionine-enkephalin and serotonin [76]. These all of which are expected to impair neurotransmission. CIH has also been shown to enhance a host of growth factors, including vascular endothelial growth factor (VEGF) and it interacts with integrins, orchestrating the formation and maintenance of blood vessels. With hypoxic exposure there is a significant increase in the levels of VEGF in the brain that is correlated with the severity of the hypoxic stimulus [77]. These dynamic changes induce angiogenesis, and may be responsible for hypoxia-induced augmentation in vascular leakage following tissue hypoxia. Taken together, the influences of CIH on the body result in severe ramifications. This impairment to the vascular function could be one of the factors that promote vessel disease.

Hypertension

Hypertension is a complex disease, with a mosaic of potential interactive etiologies. Age, gender, nutrition, environment, stress, obesity, and genetics have been cited as causal factors contributing to this cardiovascular disease, as has been dysfunction in renal, endocrine, and neural circulatory control mechanisms [78]. Therefore, the primary cause of hypertension remains elusive. In recent years, OSAS has been demonstrated to be a secondary cause of hypertension, although the link between the two pathologies was long disputed due to the numerous confounding factors that complicated investigation [79, 80]. Currently, the estimated prevalence of hypertension in OSAS is nearly 50% [81]. The mechanisms that are potentially activated during nocturnal apneas and which could chronically raise blood pressure include increases in central sympathetic outflow, endothelial dysfunction, increased endothelin, and activation of systemic inflammation, among others [82].

The evidence supporting the association between OSAS and chronic, long-standing hypertension is compelling and is provided by several cross-sectional, longitudinal, and treatment studies. Perhaps the most convincing prospective data in support of a causal relation between OSAS and hypertension have been provided by the Wisconsin Sleep Cohort Study [3]. This study demonstrated an independent dose-response relation between sleep-disordered breathing at baseline and the development of new hypertension 4 years later. The odds ratios for the presence of hypertension at follow-up were 1.42, 2.03, and 2.89 for patients with an apnea-hypopnea index of <5, 5 to 15, and >15 events per hour at baseline, respectively. Effective treatment of OSAS with continuous positive airway pressure (CPAP) leads to a decrease in both daytime and nighttime blood pressure [83-85], further supporting the concept of a causal association between OSAS and chronic hypertension.

Similar results of chronic hypertension were reported in the in the Sleep Heart Health Study [2]. Neito et al. [2] examined baseline cross-sectional data from the Sleep Heart Health Study, a multicenter study of the cardiovascular consequences of sleep apnea in 6440 participants. In unadjusted analyses, the prevalence rates of hypertension according to severity of OSAS were 43% (AHI <1.5 per hour), 53% (AHI = 1.5-4.9 per hour), 59% (AHI = 5-14.9 per hour), 62% (AHI=15-29.9 per hour), and 67% (AHI > 30 per hour) [2]. After an adjustment for age and demographic characteristics, the odds ratio for hypertension in that same study increased with escalating AHI categories in a graded dose-response fashion. Thus, after these adjustments, the odds ratio for hypertension comparing participants with the highest AHI scores (30) to those with the lowest (<1.5) was 2.27 (95% confidence interval [CI], 1.76-2.92) [2].

With the resumption of breathing following an event, cardiac output to a severely constricted peripheral vasculature increase dramatically [86]. This creates surges in blood pressures, some even reaching as high as 250/110 mmHg. With these increases in blood pressure and the sustained sympathetic activation it is thought that they both may contribute in part to the baroreflex and chemoreflex dysfunction that has been seen in patients with sleep apnea [87]. As well, the endothelin system has been implicated in the chronic pressor effects of sleep apnea [88]. Endothelin-1 is a potent and long-acting vasoconstrictor with cell division properties and hypoxia is a powerful stimulus for its production. It has been suggested that the potent long-lasting pressor effects of endothelin may be implicated in the increased blood pressure in patients with sleep apnea [88]. Reduced nitric oxide production, sympathetic activation, and endothelin may elicit an imbalance in the regulation of vascular tone and function in patients with sleep apnea. Repetitive nocturnal episodes of hypoxemia may suppress endothelial nitric oxide synthase. Circulating nitric oxide was decreased and correlated inversely with severity of sleep apnea and oxygen desaturation time in patients with sleep apnea [89, 90], thereby increasing their blood pressure levels.

Thus, there are several factors inherent in OSAS that may predispose these patients to hypertension, but it is not possible to specify just a single mechanism of effect [81]. On the positive side, the presentation of hypertension to the primary care physicians may enhance early detection and referral to PSG in asymptomatic patients. Through effective treatment with continuous positive airway pressure (CPAP), improvements to the autonomic function and lower sympathetic tone have been seen [2]; this in turn has resulted in lowering blood pressure levels in patients with OSAS.

Inflammation

A growing body of evidence implicates inflammation and oxidative stress in the pathogenesis of OSAS in humans [91-93]. For instance, OSAS patients display increased circulating markers of oxidative stress and inflammation [94], though it is unclear at this time whether it's a result of sleep fragmentation or the hypoxic events. Predominately, the research has led to the hypothesis that repeated hypoxia/reoxygenation cycles, inherent in OSAS, have a greater impact on altering the oxidative balance through induction of excess free radicals in a similar fashion to that observed in ischemia/reperfusion injury [95]. OSAS is associated with multiple causal factors leading to endothelial damage, including inflammation [96], atherosclerosis [82], increased levels of plasma vascular endothelial growth factor (VEGF) [90, 97], increased levels of soluble adhesion molecules [98], and a production of reactive oxygen species [99]. Since obesity is highly prevalent in a majority of OSAS patients and has been shown independently to contribute to chronic inflammation [100], the interpretation of results of studies on inflammatory effects of OSAS has been made more complex.

Intercellular adhesion molecule 1 (ICAM-1) and interleukin (IL-8) are markers that are widely used in OSAS studies to investigate inflammation, but results of these studies have been mixed. Carpagnano et al. [99] found a significant increase in both plasma IL-8 and ICAM-1 concentrations in obese OSAS patients, non-obese OSAS patients, and obese non-OSAS subjects compared with healthy subjects. However, although these inflammatory markers followed an upward trend in obese OSAS patients, no difference was observed in these markers between non-obese OSAS patients and obese non-OSAS subjects [99]. Compared to the findings of Ursavas et al.

[101] who found that OSAS can independently increase circulating levels of adhesion molecules, there was a significant positive correlation between circulating level of ICAM-1 and AHI [101]. A multiple logistic regression analyses showed that OSAS was associated with high ICAM-1 levels independent of age, gender, BMI, smoking status and cardiovascular disease [101]. In addition, Lawati et al. [102] found no differences between patients with and without OSAS for IL-8, but did find OSAS severity was independently associated with serum levels of ICAM-1 and leptin. As previously mentioned, the data obtained continues to be elusive with respect to the occurrence of an ICAM-1 and IL-8-mediated airway inflammation in both OSAS and obese patients. The degree of inflammation, which seems to worsen in cases of comorbidity (OSAS and obesity), could be responsible for the increased risk of developing cardiovascular events observed in these subjects and deserves to further elucidated.

Serum VEGF, another important inflammatory biomarker, has been studied in efforts to further understand possible relations of OSAS to chronic inflammation with varying results. Circulating VEGF levels are elevated in OSAS patients, primarily due to nocturnal hypoxemia [103-105]. Even though VEGF levels were higher in obese patients than in lean controls [106], it is difficult to determine whether such elevations in VEGF are caused by or the result of OSAS, because of its interaction with other inflammatory markers such as TNF-α and IL-6, which are also elevated in OSAS patients. A recent study found that the elevation of serum VEGF in OSAS was not associated with the severity of the disease, but interestingly there was an association with patient age [107]. This was the first study to show a significant positive association between age and VEGF in a large group of patients with OSAS. One possibility of this change, is an age-dependent loss of prosurvival effects of VEGF on endothelial and

bone marrow progenitor cells [108] that may impact on VEGF release (increase) for any given level of hypoxia. With the large variability in VEGF expression, more research is needed to understand the pathogenesis of OSAS and how these markers interact.

Among the mixed findings of inflammatory and adhesion molecules, vascular remolding and arterial stiffness, there is an established association between OSAS and the development of atherosclerosis. This has been seen in multiple studies examining middle aged patients with severe OSAS and without overt cardiovascular diseases [109, 110]. This being said, the pathophysiology of atherosclerosis is more complex and is driven by multiple factors play a role in the development than just those presented here that is related to OSAS.

Cerebral Changes

Although the majority of work linking OSAS to oxidative stress has focused on the cardiovascular consequences of the disease, it is important to note that the brain is among the most sensitive organ to oxidative damage. This is a result of the large amounts of polyunsaturated fatty acids present (which are sensitive to oxidative changes), the high utilization of oxygen, and the relative paucity of antioxidant defense mechanisms [111, 112]. Given that oxidative stress has been implicated in the cognitive decline that occurs in both normal aging and neurodegenerative diseases [113], it is likely that such mechanisms play a major role in the neurocognitive morbidities associated with OSAS. Consistent with this hypothesis, OSAS patients develop regional alterations in brain morphology [114-116]. Researchers have discovered that patients suffering from OSAS show gray matter loss in brain areas that regulate breathing and speech. Macey et al. [115] determined that the morphology of brain areas in subjects

with OSAS and control subjects were dissimilar, with gray matter volume reductions up to 18% (in some regions of the brain) in patients with OSAS; the extent of decline increased with the severity of the syndrome. Such gray matter reduction may have been congenital or acquired. The nature of the volume loss in this latter scenario is speculative, but may originate from an initial brain insult or ischemic event, which leads to a cascade of neural damage resulting in ineffective capabilities to respond to otherwise minor respiratory challenges within sleep [115]. This early damage to the brain's speech center triggers problems in the muscles that control the airway and can lead to the development of central sleep apnea [115].

Central Adiposity and Insulin Resistance

Studies have shown that a pattern of central obesity was associated with OSAS severity [117-120]. There is a strong positive association specifically between OSAS severity and the indices of central obesity, i.e., body mass index and waist and hip circumferences [118]. This can be support not only with anthropometric measurements, but by utilizing alternative measures of fat mass such as computed tomography. Simpson et al. [120] suggest, in both men and women, that fat was centrally located rather than peripherally and that contributes to the pathogenesis and severity of OSAS. The role of abdominal fat in upper airway instability is increasingly recognized; recumbent abdominal obesity is likely associated with increased displacement of the diaphragm, decreasing longitudinal tracheal traction and increasing propensity for upper airway collapse [121]. Accumulation of fat in the chest wall (abdominal and thoracic) decreases functional residual capacity, particularly when the individual is recumbent and asleep, increasing intrathoracic pressure and thereby extramural tissue pressure at the thoracic inlet, further increasing upper airway collapsibility [122].

The link between central adiposity and OSAS has been well established and it becomes even greater when one considers additional effects of insulin resistance. There is an independent association between AHI levels and insulin resistance (HOMA-IR), which is generally believed to play a central role in the clustering phenomenon of cardiac risk factors known as the metabolic syndrome [118]. Akahoshi et al. [117] found that although Asians are generally less obese than Caucasians, their prevalence of the insulin resistance was high with OSAS.

These factors, hypoxia, hypertension, inflammation, cerebral changes, adiposity and insulin resistance can all be linked to each other and to OSAS creating a multitude of secondary issues for both the cardiovascular and central nervous systems. These secondary outcomes are usually an individuals' chief complaint when seeking medical attention. Therefore, understanding the pathogenesis of each of these factors can help better understand how OSAS can affect the body.

Global Gene Expression Profiles

OSAS is a complex disease that has both genetic and environmental factors involved and this makes the underlying pathophysiology multifaceted. This leads to considerable individual variation in the extent to which these factors contribute to the disorder [17]. Currently, to have a definitive clinical diagnosis of OSAS requires an overnight polysomnogram (PSG) evaluation [23], as well as an independent assessment of excessive daytime drowsiness. With the PSG procedures being very

labor intensive many individuals go untested; thereby, relying on research to develop more efficient and accessible screening evaluations.

The increased use of microarrays in research over the past decade has contributed valuable information allowing researchers to identify specific genes or protein expressions that are altered in a variety of diseases. This hypothesis generating approach to examining thousands of genes at once has provided insight into the changes in gene expression and to the discovery of unsuspected genes or proteins affected by disease status [9]. The use of high-throughput technologies has become a fundamental approach for identifying potential diagnostic and therapeutic targets for many diseases [11]. Other unique molecular signatures have already been identified for asthma [123], heart disease [19, 124], Huntington's disease [125], and arterial hypertension [126]. As well, similar gene expression profiling has been used to classify high-risk patients with a number human malignancies, such as breast [127] and ovarian cancers [128]. This well established methodology could be instrumental in developing a molecular signature for OSAS. Research in this area would allow for the discovery of novel and important genes underlying the clinical manifestation of OSAS.

The established methodology takes tissue samples from subjects, and age and gender matched controls, to isolate the RNA. From here the labeled cDNA or cRNA targets derived from the mRNA of an experimental sample are hybridized to nucleic acid probes attached to the solid support. This permits a wash and staining process to be applied so that the identified genes can be scanned and quantified. Borovecki et al. [125] utilized the exact methodology when comparing 62 patients with Huntington's disease (HD) to 53 age and gender matched control subjects. They found 12 genes that exhibited the most significant changes between the HD and control groups, and

validated these with qPCR at a 2 fold difference. These genes were later modified by treatment with sodium phenylbutyrate, a histone deacetylase inhibitor. Using QRT-PCR, Borovecki et al. [125] found a small but significant decrease in the expression of the 12-gene marker set in 10 of 12 patients after 4 weeks of treatment. When the marker genes were examined individually, 8 of 12 genes were decreased in response to 4 weeks of treatment with phenylbutyrate [125].

Timofeeva et al. [126] utilized a similar process when examining 58 patients with known thoracic aortic aneurysms (TAA). This study was able to identify a 41-gene classifier based on expression signature that can identify TAA patients with high accuracy. To further validate the TAA-status classifier genes, Timofeeva et al. [126] determined expression levels of the 41 TAA-status classifier genes using the more precise real-time PCR methodology on 82 samples for which enough RNA was available (50 samples from training set and 32 samples from test set). This can potentially lead to a diagnostic “point-of-care” assay, for identification of asymptomatic TAA disease with a simple blood-based test. The transcriptional programs in peripheral blood cells leading to the identification of these markers provide insights into the mechanism of development of aortic aneurysms, and highlight potential targets for therapeutic intervention [126].

The methodology is slightly different when examining cancer patients due to the complexity of the pathophysiology involved. Both Cheang et al. [127] and De Cecco et al. [128] studied breast and ovarian cancers, respectively, with a similar process. De Cecco et al. [128] utilized hierarchical clustering analysis to explore data obtained by gene expression profiling of all 81 samples of the study, using a cDNA microarray containing 4451 cancer-related genes. This analysis revealed the aggregation of cancer

specimens from the same patient, irrespective of the sampling site and consistent with the monoclonal origin of advanced disease. To generate the list of genes responsible for sample partitioning, which is not produced as an output by the ISIS software, a univariate F-test with a randomized variance model and false discovery rate correction using Biometric Research Branch Array Tools which resulted in 10 genes associated with the above-described partition selected to validate gene expression levels measured by microarray analysis and analyzed by quantitative real-time PCR [128].

The ability to identify patients who have or are at risk for certain diseases can play a crucial role in treating the disease or issue. Uniquely, the ability to identify patients who will develop protective mechanisms, such as collateral vessel growth of the heart, can affect the prognosis and the response to therapeutic interventions [19]. Chittenden et al. [19] separated monocytes from whole blood of 8 subjects who had well developed coronary collaterals to 8 subjects without and performed microarray analysis to suggest that differences in collateral vessel development may stem from genetic variation. These two groups showed two distinct global gene expression profiles allowing for the conclusion to be made that presence of collateral development may be independent of CAD as originally thought [19].

Although increased attention in sleep medicine has lead to increased research, the precise underpinning of etiological factors of OSAS has not yet been determined. The obstructive events accompanied by hypoxia could alter protein expression or gene regulation thereby providing a unique molecular signature specific to OSAS. The methodology to this form of research requires two areas to be considered. 1) distinct endophenotypes may be present and uniformed, and 2) that there will be a variety of interpersonal variations and how one adapts to the environment [9]. Genetic variation

between ethnicities as well as gender, can complicate the understanding of sleep disordered breathing [13]. Currently, there have been only two studies that have examined gene expression profiling in OSAS patients, one in adult males [10] and one in children [11]. In both studies researchers were able to show that there were abnormally expressed genes between OSAS and controls.

Hoffmann et al. [10] utilized microarray measures to compare eight male subjects (4 newly diagnosed severe OSAS and 4 BMI and age matched controls) before and after a night of normal sleep in controls and a night of untreated apnea in the OSAS. RNA was isolated from the blood samples collected before and after sleep, then hybridized onto the HG-U133 A and B Affymetrix arrays. These arrays were scanned using GeneChip scanner 3000 per manufactures instructions. Hoffmann et al. [10] hypothesized that the presence of OSAS would activate genomic adaptive responses specifically to reactive oxygen species (ROS), and induce changes that may attenuate oxidative stress in blood cells. In fact, the study produced results that were suggestive that the transcription of several genes related to encoding antioxidant enzymes may change acutely overnight during apneic sleep [10]. These differentially expressed genes are directly involved with in lowering ROS, such as catalase, an antioxidant enzyme that detoxifies hydrogen peroxide preventing cellular injury. Additionally, some proteins that are involved with cell cycle regulation, activation, and growth were different between groups [10]. Src-like adapter protein (SLAP), which participates in T cell signal transduction, a negative mitosis regulator, and cell division cycle 25 B (CDC25B), which activates CDC2 enabling the cell to enter the mitotic phase [10] changed dramatically overnight in OSAS patients. If oxidative stress occurs during the most vulnerable phases of cell cycling (mitosis and DNA synthesis), there is an increased risk of cellular

death. Therefore, for the cell to protect itself, it may attenuate growth during such conditions. Hoffmann et al. [10] concluded that overnight changes in OSAS patients were seen modulating and adapting to increases in ROS due to frequent episodes of intermittent hypoxia.

Khalyfa et al. [11] examined global changes in gene expression profiles as well. They examined children with OSAS and hypothesized that pediatric OSAS may lead to an altered gene expression in circulating leukocytes. They studied non-obese children between 4 and 9 years of age. Twenty habitually snoring children with an AHI > 2/h total sleep time were in the OSAS group, twenty matched children required the presence of an AHI ≤ 1 in the absence of a history of snoring served as the control group [11]. After the sleep test, fasting blood samples were collected and total RNA was extracted and hybridized onto the Agilent human array 60-mer (G4112A) containing 44,000 human probe sequences [11]. Of the genes that were highly expressed, 37 were classified in the cellular process, 43 in biological processes, and 46 in molecular functions [11]. A comparison with real time PCR was done on 6 of the genes differentially expressed, 2 up-regulated (Malonyl-CoA:ACP acyltransferase, Interleukin 27), 2 down-regulated (Multiple EGF-like-domains 11, Amino acid transporter), and 2 unchanged with OSAS (Diphthamide 4, and Zinc finger protein 430). The results from the PCR confirmed the changes in expression seen with the microarrays [11]. This study's analysis uncovered enrichment of genes annotated for important biological pathways including the up-regulation and modulation of inflammation modified by the presence of OSAS as well as cell survival, proliferation, and differentiation. Khalyfa et al. [11] concluded that with altered gene expression patterns in circulating leukocytes seen in OSAS children, and that the complex recruitment and interplay of genes involved lead to an inflammatory

response in children. Considering the prominent evidence accumulated linking pediatric OSAS to systemic inflammation, and the robust link between the magnitude of the systemic inflammatory response and end-organ mortality [11], further research needs to be done in this area.

The previous two gene expression profile studies were conducted on obese males as well as on non-obese children and that in their conclusions it is apparent that not only is the phenotypic expression of OSAS are multi-factorial so is the etiology. There are some putative candidate genes for OSAS that have a direct causal to the expression of the disorder or whether their roles in the OSAS disorder are mediated through other intermediate genes is still unclear. Additionally, there may still be multiple genetic and environmental factors as well as their interactions that have not yet been determined. These findings make it unrealistic to infer that the changes would be universal to all OSAS patients, specifically adult females.

Researchers have recently estimated that the prevalence of OSAS is 24% in men and 9% for women [32] thereby directing most of the research on men. Though the prevalence of OSAS is higher in males, females may actually be at higher risk of secondary consequences [129]. The vast majority of females are more predisposed to having longer and more severe oxygen desaturations due to the occurrence of apneic events during the REM stage of sleep [130]. This increases the severity of oxidative stress and its cascading effects may provide a greater insight into OSAS.

In order to assess the genomic expression in OSAS, the circulating peripheral blood monocytes may be the best reporter cells to study. Based on Rohde et al.'s [131] findings, they determined that blood monocytes act as reporters for endothelial cell function, essentially mimicking their activity. Further, Heil et al. [30] demonstrated that

there is a functional link between the monocyte concentration in the peripheral blood and the enhancement of arteriogenesis. Therefore, monocytes can provide the most valuable information in the production of proteins affecting vascular regulation. For instance, monocytes adhere to the collateral vascular endothelium when the adhesion molecule ICAM-1 is unregulated. The adhesion of monocytes at the shear stress-activated endothelium and the subsequent migration into the perivascular space is suggestive of subsequent cell proliferation, vessel remodeling processes, and growth of the collaterals. As the monocytes mature within the collateral vessel wall into tissue macrophages, they concurrently release growth factors, inflammatory cytokines, and metalloproteinases [30]. This activation may result in vascular remodeling seen in OSAS patients.

Vascular regulation is influenced by OSAS, specifically as a result of the intermittent hypoxia. Intermittent hypoxia increases sympathetic activation, which in turn leads to increases in inflammation. In vascular health, inflammation plays a key role, it can be altered in response to the up regulation of adhesion molecules as well as prothrombotic cytokines [132]. Therefore, it is reasonable to assume that monocytes would be the reporter cell of choice when studying the genomic expression between patients with and without increases in hypoxia and inflammation as seen in OSAS.

The Cleveland study examined the descriptive reports of families with multiple affected members. They [18] and others [17] hypothesize a role for inheritance in the development of OSAS, apart from familial influences related to obesity. Based on this study Palmer et al. [18] suggested that there are some families that may be predisposed, and actually found an association between sudden unexpected death in infancy and presence of OSAS in the family. Chromosomal mapping studies of different

racial groups and twin studies have provided evidence for the possible link between the OSAS phenotypes and genetic loci that could prove to be markers for further research, including obesity, fat distribution, snoring, and sleep regulation. Differences between those with OSAS and these comorbidities and those without OSAS but the comorbidities could also be genetic in origin. There are a limited but growing number of studies addressing the question of gene variants and their pathophysiological effect in the OSAS population. Recent research has provided compelling data linking sleep apnea, obesity, and inflammatory dysregulation [100], suggesting that these three have overlapping pathways to OSAS. To date, the implications of these comorbidities and overlapping pathogenetic pathways predominantly have been related to cardiovascular disease. Investigations genetic variation in circulating cells, as affected by exposure to intermittent hypoxia and/or inflammation, will likely provide the clearest and most accurate signature. Thought, the information gained from global gene expression profiling may help uncover the molecular mechanisms of OSAS etiology. It is plausible that establishing this signature for OSAS will assist to develop the framework needed for the next steps in the genetic analyses. This in the future could help physicians to assess high risk patients and the ability to determine the inter-individual differences within the OSAS population. The possibility for screening patients for OSAS, before they become symptomatic, may allow physicians to prescribe preventive interventions early enough to delay secondary health consequences associated with OSAS, and could possibly be a means to track treatment effectiveness as well.

Inflammation Biomarkers

Proteomics is a discovery strategy, similar to gene expression profiling, that examines expression of proteins and post-translational modifications of thousands of proteins simultaneously. The benefit of proteomics is to obtain information regarding changes in protein quantities, post-translational modifications, and protein-protein interactions [31]. This information is needed to understand the true molecular phenotype of a disease since knowledge on gene variants, and changes in gene expression levels, may not translate into actual changes in protein [31]. Proteomics is more complicated than genomics; currently the technology similar to microarrays for genomics has been developed but is constantly being reviewed for consistency. Major issues with proteomics are the sheer number of proteins compared to genes (100-fold increase) and mRNA (10-fold increase), the huge difference in protein concentrations of the dynamic range of 10^{10} , as well as the myriad post-translational modifications that alter the protein signatures of samples [9].

It has been well established that OSAS results in sleep fragmentation which increases sympathetic activity and inflammatory responses, where chronic intermittent hypoxia increases the previously mentioned as well as oxidative stress [28]. Therefore, it is not unreasonable to think that the ability to identify patients with OSAS will present with altered pathways in these three areas. Researchers have been finding evidence in these three areas but are still not able to fully understand this disease.

These next three sections provide details on the genes that were differentially expressed in the microarray data and their possible role in OSAS as to why they were differentially expressed.

Tumor-Associated Calcium Signal Transducer 2

Tumor-Associated Calcium Signal Transducer 2 (TACSTD2) is a signal exon of DNA that codes for a protein of 323 amino acids [133]. The physiological function of this protein has not been completely elucidated; however, it is hypothesized that this protein can act as a calcium signal transducer. Research of the TACSTD2 gene has estimated that there are 24 different mutations of this protein, predominately found in individuals from Asia [134, 135]. The TACSTD2 protein is a monomeric cell surface glycoprotein expressed in many organs such as cornea, placenta, lung, kidney, pancreas, prostate, and in trophoblasts, and at high levels in many carcinomas [136]. A specific mutation has resulted in a devastating corneal disease, gelatinous drop-like corneal dystrophy. Due to a loss of function of the TACSTD2 gene, tight-junction-related proteins cease to function, resulting in severe corneal epithelial barrier impairment. As a result, various proteins contained in tear fluid continuously penetrate into the corneal stroma, promoting the development of massive amyloid deposits [137]. Although the physiological functions of this protein are still obscure, several functional domains were proposed for this molecule from its amino acid sequence. This protein contains an epidermal growth factor-like repeat, a thyroglobulin repeat, a transmembrane region, and a phosphatidylinositol 4,5-bis phosphate-binding consensus sequence. It has been suggested that the TACSTD2 protein functions as a cell-to-cell adhesion receptor in cancer cells and as a calcium signal transducer [136].

Laminin

Laminin is an essential, abundant, and biologically active part of the basal lamina (basement membranes), influencing cell differentiation, proliferation, migration,

adhesion as well as phenotype and survival [138, 139]. Interestingly, laminin plays a unique role when exposed to reactive oxygen species [140]. Endothelial cells attached to oxidized laminin at a higher degree than native laminin, thereby increasing the ICAM-1 expression by the cells and increasing the recruitment capacity of monocytes [140]. Laminin may have a significant role in inflammation by influencing cell adhesion and reducing the ability of immune cells to penetrate the vessel wall if deficient [141]. Kenne et al. [141] were able to conclude that laminin is widely expressed in vascular endothelial basement membrane and plays a significant role in normal blood vessel maturation. In mice, a significant difference was found between laminin knockouts and wild types with regard to monocyte recruitment. This clearly indicated that laminin is required for effective monocyte extravasations and could play a key role during migration through the venules [141]. Laminin can influence its interactions with monocytes [140] and laminin oxidation may change its interactions with endothelial cells affecting their subsequent interactions with monocytes contributing in a way to the formation of atheromatic lesions. The monocyte attachment to, and the migration through the activated endothelium represents the first crucial step which leads to the initiation of atherosclerosis [140]. Kostidou et al. [140] were able to conclude that there is an increased monocyte attachment rate to surface of the endothelial cells that were attached to the oxidized laminin as compared to the same cells attached to native laminin. The increased affinity of endothelium to oxidized laminin as compared to the native molecule may reflect an in vivo tendency for faster coverage of lesions exposed to oxidative stress by endothelial cells. Oxidative stress is a common feature of atherosclerosis and therefore, extracellular matrix oxidation may be common in atherosclerosis [140].

With all the research in this area, no studies have been done to examine the epithelium and the epithelial-connective tissue boundary of the human OSAS patients. This boundary is a highly specialized interface formed by the cells of the epithelial and the lamina propria. Paulsen et al. [142] did investigate whether pathological changes in the epithelium or the epithelial-connective tissue interface are present in patients with OSAS. They found that utilizing the laminin antibody there was clear border between the laminia propria and the overlying epithelial in both patients and control subjects. However, no disruption or changes in integrity of the basement membranes where seen in patients with OSAS [142]. Though this does not exclude the possibility of changes in the basement membrane that could possibly be detectable only at the electron microscopic level but do not manifest under immunohistochemical analysis.

CDC42

Cell division control protein 42 (CDC42) is a small GTPase of the Rho-subfamily, which has been implicated in regulation of diverse biological activities involving various aspects of cellular growth and division [143]. On the basis of in vitro phosphorylation studies, it has been suggested that CDC42 may function in the signaling pathway of the epidermal growth factor receptor or related growth factor receptor protein kinases [144]. It has been suggested to play a role in transendothelial migration as well [145]. Honing et al. [145] examined the role of the small GTPase in monocyte transendothelial migration by using overexpress constitutively activated or dominant-negative form of CDC42. The findings suggest that CDC42 and other small GTPases have a distinct effect on monocyte adhesion and migration across endothelium and that inhibition of the these signals in monocytes may reduce monocytic recruitment into inflamed tissue and have beneficial effects in various condition of inflammation [145]. There is still a great

deal that needs to be researched since the precise mechanism by which this balance between all the GTPase-mediated signaling is regulated is still largely unknown. Moreover, although it is clear that a discrete 'on–off' switch is too simple a mechanism to account for the current experimental evidence, whether the regulated intracellular translocation of Rho family GTPases has a role remains to be addressed.

With the unique roles of laminin and CDC42 with monocyte adhesion, these are two very promising biomarkers that can help identify a signature for OSAS. The implication that an increase in these particular biomarkers will increase monocyte adhesion which can be linked back to the oxidative stress, inflammation, and vascular remodeling strengthens the research that chronic intermittent hypoxia is the key to understanding OSAS.

Measurement Techniques and Instruments

Genomic and proteomic assessments require a variety of different techniques to assess the presences or changes in DNA and RNA profiles in reporter cells. In this growing area of research, investigators have used and perfected a great variety of techniques, each having a specific rationale based on study aims. Below are the techniques utilized in this dissertation.

Monocyte Isolation

Monocytes in endothelial cell function can provide valuable information in the production of proteins affecting vascular regulation. Due to this unique role, selecting

these cells over any other could provide more specific information on the inflammatory responses seen in OSAS patients. The ideal method for monocyte isolation combining all factors of simplicity, cheapness, purity, and high yield does not currently exist [146, 147]. Initially, monocyte isolation was performed through adherence, density centrifugation, or elutriation with varying success rates. The most common procedure was adherence after Ficoll-Hypaque purification of peripheral blood mononuclear cells (PBMC) [146]. The monocyte isolation by adherence, although simple, has several disadvantages: high lymphocyte contamination, low flexibility, high manipulation and monocyte transient activation [146, 148]. Therefore, Flo et al. [149] determined human monocytes are best isolated by depletion of non-monocytes (negative selection) using magnetic polymer particles as an efficient method for the separation of monocytes with intact morphology and function as measured by chemiluminescence. Monocyte isolation is now quickly and consistently being applied to functional studies in which effects due to antibody crosslinking of cell surface proteins should be avoided, or in studies where monocyte activation, differentiation, and cytokine secretion are being examined. Specifically, utilizing the Miltenyi Monocyte Isolation Kit II®, the non-monocytes are indirectly magnetically labeled through incubation with a cocktail of biotin-conjugated monoclonal antibodies against CD3, CD7, CD16, CD19, CD56, CD123 and Glycophorin A as the primary labeling reagents. This technique permits the recovery rate for the monocytes upwards of 90% with very little contamination of lymphoid cells. Immediate stabilization of RNA in the monocyte samples is necessary to prevent changes in the gene expression pattern from occurring due to specific and nonspecific RNA degradation as well as to transcriptional induction.

RNA Extraction

The RNA molecule plays a critical role in transferring information encoded in the genome (DNA) to the many different forms of proteins. In order to examine the gene expression profile sample RNA must be extracted from the stored PBMC. The single stranded pieces of mRNA are needed to bind to known entities on gene array chips or to verify amounts by increasing a target from an undetectable amount of starting material. The isolation and purification of RNA samples can be perform effectively utilizing the Qiagen RNeasy Kits®, which provide enrichment for mRNA since other RNAs less than 200 nucleotides are excluded. Purity and integrity of RNA are critical elements for the overall success of RNA-based analyses, including gene expression profiling methods to assess the expression levels of thousands of genes in a single assay. It is universally accepted that RNA purity and integrity are of foremost importance to ensure reliability and reproducibility of downstream applications. Starting with low quality RNA may compromise the results of downstream applications which are often labor-intensive, time-consuming and expensive [150]. No currently available purification method can guarantee that RNA is completely free of DNA. The RNeasy kits will remove the vast majority of cellular DNA, but trace amounts may still remain. For amalysis of very low abundance targets and to prevent any interferences, DNase digestion is strongly recommended, thereby efficiently removing the DNA during the RNA purification [151]. By utilizing both the Experion™ automated gel electrophoresis system and the ND-1000, (NanoDrop Technologies), all RNA samples for good purity should have A260/280 ratios between 1.8 and 2.0 and then the concentration can be quantified by measuring A260 nm on a UV/vis spectrophotometer [152]. RNA extraction,

purification, and quantity are critical steps for genetic profiling such as microarray analysis.

Microarray Analysis

The basic concept behind the use of GeneChip arrays for gene expression is simple: labeled cDNA or cRNA targets derived from the mRNA of an experimental sample are hybridized to nucleic acid probes attached to the solid support. By monitoring the amount of label associated with each DNA location, it is possible to infer the abundance of each mRNA species represented of RNA sample quality control. Though cDNA is not 100% accurate when you reverse transcribe from RNA, this form provides a more stable protein to be able to work with. A variety of GeneChips are on the market; however, the Affymetrix GeneChip® Human Genome U133 A 2.0 Array is a comprehensive whole human genome expression array. This platform permits the monitoring of the transcription of thousands of genes simultaneously. This GeneChip has 22,000 probe sets corresponding to 14,500 genes [153]. The labeled cDNA targets derived from the mRNA of an experimental sample can be hybridized to nucleic acid probes attached to the solid support. By monitoring the amount of label associated with each DNA location, it is possible to infer the abundance of each mRNA species represented. With the large volumes of data generated by this type of experiment, a computer software program RMA (robust microarray analysis) in the Bioconductor project (which is open statistical software for the analysis of genomic data) can be used to normalize the array data [154]. This normalization step is required so that downstream analyses can be preformed. Further comparisons of the data analysis can allow for differentiation between subject groups with the detection of up or down gene regulation. The differentially expressed genes may not result in proteomic changes

within the individuals but can be hypothesis generating of areas to perform more research.

Assays

Based on findings from global gene expression profiles some genes or pathways may be expressed differently; therefore, potentially the expression of the specific proteins would also be altered. To understand the true molecular phenotype of a disease since knowledge on gene variants, and changes in gene expression levels may not translate into actual changes in protein, information obtained through proteomic assays regarding changes in protein quantities, post-translational modifications, and protein-protein interactions is necessary [31].

An enzyme immunoassay kit (EIA) can be used for the quantitative determination of a given protein. The laminin EIA kit is a solid phase EIA based on a sandwich method that utilizes two mouse monoclonal anti-laminin antibodies to detect laminin by a two-step procedure. Non-specific binding is blocked by a buffer, and the second anti-laminin which is labeled is added. This two-step process allows laminin to be captured onto the solid support on one side and tagged on the other. The plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample. Both the intra- and inter- assay precisions both replicated three times produced a confidence interval between 95-98% according to the manufacturer.

A cell adhesion assay makes an important contribution of specifics on the maintenance of tissue structure, the promotion of cell migration, and the transduction of information about the cell microenvironment across the plasma membrane. An ability to assess cellular adhesion has proven to be extremely valuable for those researchers studying the molecular mechanisms underlying these processes [155]. It is important to

realize that cell adhesion is a complex process that involves many different molecular interactions, including receptor-ligand binding, changes in the fluxes through intracellular signaling pathways, and modulation of cytoskeletal assembly [156]. Consequently, adhesion assays not only measure the contacts between a cell and extracellular adhesion proteins, but provide information about other cellular events [155].

Real-Time Quantitative Polymerase Chain Reaction (qPCR)

The principle and aim of PCR technology is to specifically increase a target from an undetectable amount of starting material. The primers are designed to specifically bind to the extremities of the DNA fragment to be amplified. The Taq polymerase uses the target DNA added to the reaction as a template for primer extension. As each cycles, more DNA is synthesized, creating more template DNA. The reaction proceeds in an exponential manner, doubling the amount of target during each cycle, until one of the reagents becomes limiting and the reaction reaches a plateau. Detection methods are based on changes in fluorescence, which are proportional to the increase of target. The fluorescence is monitored during each PCR cycle providing a plot that shows two phases, the exponential phase and the plateau phase. The cycle number at which enough amplified product accumulates to yield a signal is the threshold cycle. Relative quantification is the most widely used technique. Researchers can examine the expression level between two different cells (e.g., cancerous and normal) to determine if there is a 'fold difference' [157]. This provides insight into changes that may be occurring in the body.

With a starting material of RNA, a reverse transcription step is needed before qPCR can be performed. The two-step process where the reverse transcription is done

in a separate tube to the qPCR provides greater efficient since random primers can be used as well to stock cDNA to quantify several targets. When comparing and analyzing results there are several uncontrolled variables which can lead to misinterpretations of the results; therefore, normalizing to one housekeeping gene is strongly preferred. This gene needs to be expressed constantly at the same level through the experiment and between samples [158]. Generally, it is a gene such as the small subunit 18S rRNA gene that is one of the most frequently used genes in phylogenetic studies and an important marker for random target PCR in environmental biodiversity screening [159]. qPCR re-affirms what was previously seen in gene expression assays and provides greater confirmation that labor intensive procedures produced reliable results.

Western Blotting

Another assessment of proteomic results is to perform a Western blot, a technique whose ultimate goal is the identification of a specific protein band within a sample containing a multitude of proteins. Western blotting identifies with specific antibodies that have been separated from one another according to their size by gel electrophoresis. The blot is a membrane, and the gel is placed next to the membrane and application of electrical current induces the proteins in the gel to move to the membrane where they adhere. The membrane is then a replica of the gel's protein pattern and is stained with an antibody. From these stains and against standards, one can discern the presences or absence of the protein of interest.

Statistical Analysis

Since microarrays can measure the expression of thousands of genes to identify changes in expression between different biological states. A supervised resampling

approach to assess of the differential gene expression (control vs. OSAS) was preformed with Significance Analysis of Microarrays (SAM) within Multiple Experiment Viewer (MeV). SAM is a method for identifying genes on a microarray with statistically significant changes in expression, developed in context of an actual biological experiment [160]. SAM assigns a score to each gene on the basis of change in gene expression relative to the standard deviation of repeated measurements [160]. Each gene is assigned a score on the basis of its change in gene expression relative to the standard deviation of repeated measurements for that gene. For genes with scores greater than an adjustable threshold, SAM uses permutations of the repeated measurements to estimate the percentage of genes identified by chance, the false discovery rate (FDR). Based on the data genes were selected if the FDR ≤ 30%. This will generate a gene list that can be subjected to additional meta-analyses to identify over-represented functional classes or biological pathways in the selected gene set using first EASE [161] and then nEASE (nested Expression Analysis Systematic Explorer). The data will be run with EASE/nEASE in MeV on this gene list to assess enrichment of gene ontology (GO) terms. EASE is useful for summarizing the predominant biological "theme" of a given gene list. Enrichment of GO terms is detected by methods such as those described above, and the uncharacterized genes are presumed involved in the same biological processes as the genes with which they are grouped. The genes are grouped together on the basis of some criteria such as similar gene expression or through a protein–protein interaction network. Given a list of genes resulting from a microarray or other genome-scale experiment, EASE can rapidly calculate over-representation statistics for every possible GO term with respect to all genes represented in the data set [161]. nEASE identifies overrepresented GO sub-

terms driving the results in the first-level EASE analysis [162]. For each enriched GO term identified in the upper-level EASE analysis, nEASE first replaces the entire background distribution of a given experimental design with that of the specific enriched GO term [162].

Summary of Literature Review

Overall, this literature review highlights the need for additional research in the area of OSAS, predominately for conducting genomic and proteomic studies that might lead to the means for easily and inexpensively identify individuals at high risk for the disorder. In part, this review also provides an overview of methods used in out assessment of OSAS. With the increase in prevalence of OSAS within the US adult population, more research is needed to understand the pathophysiology and the underpinning mechanisms of this complex disease. A number of secondary consequences associated with OSAS result in substantial adverse effects on health, cognition, and quality of life. Researchers are still trying to determine if hypoxia results in the inflammatory response, or if it is sleep deprivation induced by sleep apnea arousals, that is more detrimental to ones overall health. Regardless of the cause, the direct impact of this disease is great and the long-term secondary consequences can be fatal. Research in the area of global gene expression profiling could provide a clearer understanding of how the disorder manifests uniquely in patients with OSAS. With only two studies assessing gene expression patterns of OSAS, one in non-obese children [11], and one in male subjects [10], they both added validity to the success of this type of research. Both Khalyfa et al. [11] and Hoffmann et al. [10] were able to discern two unique expression differences; unfortunately, these results cannot be applied to the general public since the subjects were very specific subgroups (non-obese children and

males). Further analysis of multiple subgroups (e.g., gender, obesity levels, and ethnicity) and their unique differences in the expression profile, a more comprehensive assessment of the proteomic expression may be determined for OSAS. This could allow for specific treatment and possibly allow physicians to get patients treated more effectively. Providing treatment in an efficient manner would minimize the secondary consequences of OSAS.

In addition to profiling the genomic differences between OSAS and non-OSAS patients, valuable information can be gained from the proteomic research regarding the various pathways that influence this disease. To date, most research has been directed at understanding the links among sleep apnea, systemic inflammation, and cardiovascular responses. Based on findings from global gene expression profiles in adult females with and without OSAS, it is anticipated that some genes or pathways would be expressed differently. These differentially expressed genes may not translate into changed at the proteomic level, the information regarding changes in protein quantities, post-translational modifications, and protein-protein interactions is needed to understand the true molecular phenotype of a disease [31].

Therefore, based on the related literature review presented here, there are still gaps and need for additional research in the area of OSAS and gene expression profiling. The framework and measurement techniques for this dissertation research were used to allow following manuscript chapter to examine the global gene expression profiles and proteomic assessments in adult females with OSAS.

References

1. AASM, A.A.o.S.M.-. *Sleep-related breathing disorders in adults: recommendations for syndrome definition and measurement techniques in clinical research. The Report of an American Academy of Sleep Medicine Task Force.* Sleep, 1999. **22**(3): p. 667-89.
2. Caples, S.M., A. Garcia-Touchard, and V.K. Somers, *Sleep-disordered breathing and cardiovascular risk.* Sleep, 2007. **30**(3): p. 291-303.
3. Young, T., et al., *The occurrence of sleep-disordered breathing among middle-aged adults.* N Engl J Med, 1993. **328**(17): p. 1230-5.
4. Arnardottir, E.S.M., M; Gislason, T; Teff, K.L.; Pack, A.I., *Molecular Signatures of Obstructive Sleep Apnea in Adults: A Review and Perspective.* Sleep, 2009. **32**(4): p. 447-470.
5. Eckert, D.J. and A. Malhotra, *Pathophysiology of adult obstructive sleep apnea.* Proc Am Thorac Soc, 2008. **5**(2): p. 144-53.
6. Kaparianos, A., et al., *Obstructive sleep apnoea syndrome and genes.* Neth J Med, 2006. **64**(8): p. 280-9.
7. Young, T., J. Skatrud, and P.E. Peppard, *Risk factors for obstructive sleep apnea in adults.* JAMA, 2004. **291**(16): p. 2013-6.
8. Gozal, D., et al., *Systemic inflammation in non-obese children with obstructive sleep apnea.* Sleep Med, 2008. **9**(3): p. 254-9.
9. Patt, S., et al., *Upregulation of vascular endothelial growth factor in severe chronic brain hypoxia of the rat.* Neurosci Lett, 1998. **252**(3): p. 199-202.
10. Sinor, A.D., et al., *Hypoxic induction of vascular endothelial growth factor (VEGF) protein in astroglial cultures.* Brain Res, 1998. **812**(1-2): p. 289-91.
11. Ahmadi, N., et al., *The Berlin questionnaire for sleep apnea in a sleep clinic population: relationship to polysomnographic measurement of respiratory disturbance.* Sleep Breath, 2008. **12**(1): p. 39-45.
12. Ramachandran, S.K., et al., *Derivation and validation of a simple perioperative sleep apnea prediction score.* Anesth Analg, 2010. **110**(4): p. 1007-15.
13. AASM, A.A.o.S.M.-. *Obstructive Sleep Apnea*. 2008; Available from: www.aasmnet.org.
14. Levy, P., M.R. Bonsignore, and J. Eckel, *Sleep, sleep-disordered breathing and metabolic consequences.* Eur Respir J, 2009. **34**(1): p. 243-60.
15. Lugaresi, E., et al., *Breathing during sleep in man in normal and pathological conditions.* Adv Exp Med Biol, 1978. **99**: p. 35-45.
16. Leiter, J.C., S.L. Knuth, and D. Bartlett, Jr., *The effect of sleep deprivation on activity of the genioglossus muscle.* Am Rev Respir Dis, 1985. **132**(6): p. 1242-5.
17. White, D.P., et al., *Sleep deprivation and the control of ventilation.* Am Rev Respir Dis, 1983. **128**(6): p. 984-6.
18. Desai, A.V., G. Marks, and R. Grunstein, *Does sleep deprivation worsen mild obstructive sleep apnea?* Sleep, 2003. **26**(8): p. 1038-41.
19. AASM, A.A.o.S.M.-. *International classification of sleep disorders, 2nd Edition: Diagnostic and coding manual*. 2nd ed. 2005, Westchester, IL: American Academy of Sleep Medicine.
20. Deegan, P.C. and W.T. McNicholas, *Pathophysiology of obstructive sleep apnoea.* Eur Respir J, 1995. **8**(7): p. 1161-78.

21. Haines, K.L., et al., *Objective evidence that bariatric surgery improves obesity-related obstructive sleep apnea.* Surgery, 2007. **141**(3): p. 354-8.
22. Yeh, P.S., et al., *Clinical Predictors of Obstructive Sleep Apnea in Asian Bariatric Patients.* Obes Surg, 2009.
23. Peppard, P.E., et al., *Prospective study of the association between sleep-disordered breathing and hypertension.* N Engl J Med, 2000. **342**(19): p. 1378-84.
24. Grunstein, R., et al., *Snoring and sleep apnoea in men: association with central obesity and hypertension.* Int J Obes Relat Metab Disord, 1993. **17**(9): p. 533-40.
25. Strohl, K.P., et al., *Insulin levels, blood pressure and sleep apnea.* Sleep, 1994. **17**(7): p. 614-8.
26. Ancoli-Israel, S., et al., *Sleep-disordered breathing in community-dwelling elderly.* Sleep, 1991. **14**(6): p. 486-95.
27. White, D.P., et al., *Pharyngeal resistance in normal humans: influence of gender, age, and obesity.* J Appl Physiol, 1985. **58**(2): p. 365-71.
28. Bixler, E.O., et al., *Prevalence of sleep-disordered breathing in women: effects of gender.* Am J Respir Crit Care Med, 2001. **163**(3 Pt 1): p. 608-13.
29. Wesstrom, J., J. Ulfberg, and S. Nilsson, *Sleep apnea and hormone replacement therapy: a pilot study and a literature review.* Acta Obstet Gynecol Scand, 2005. **84**(1): p. 54-7.
30. Olszewska, E., et al., *A comparison of cephalometric analysis using radiographs and craniofacial computed tomography in patients with obstructive sleep apnea syndrome: preliminary report.* Eur Arch Otorhinolaryngol, 2009. **266**(4): p. 535-42.
31. Redline, S., et al., *The familial aggregation of obstructive sleep apnea.* Am J Respir Crit Care Med, 1995. **151**(3 Pt 1): p. 682-7.
32. Strohl, K.P., et al., *Obstructive sleep apnea in family members.* N Engl J Med, 1978. **299**(18): p. 969-73.
33. Redline, S. and P.V. Tishler, *The genetics of sleep apnea.* Sleep Med Rev, 2000. **4**(6): p. 583-602.
34. Skomro, R.P. and M.H. Kryger, *Clinical presentations of obstructive sleep apnea syndrome.* Prog Cardiovasc Dis, 1999. **41**(5): p. 331-40.
35. Epstein, L.J., et al., *Clinical guideline for the evaluation, management and long-term care of obstructive sleep apnea in adults.* J Clin Sleep Med, 2009. **5**(3): p. 263-76.
36. Wang, Y., S.X. Zhang, and D. Gozal, *Reactive oxygen species and the brain in sleep apnea.* Respir Physiol Neurobiol, 2010.
37. Abrams, B., *Cancer and sleep apnea--the hypoxia connection.* Med Hypotheses, 2007. **68**(1): p. 232.
38. Knaupp, W., et al., *Erythropoietin response to acute normobaric hypoxia in humans.* J Appl Physiol, 1992. **73**(3): p. 837-40.
39. Koistinen, P.O., et al., *EPO, red cells, and serum transferrin receptor in continuous and intermittent hypoxia.* Med Sci Sports Exerc, 2000. **32**(4): p. 800-4.
40. Neubauer, J.A., *Invited review: Physiological and pathophysiological responses to intermittent hypoxia.* J Appl Physiol, 2001. **90**(4): p. 1593-9.

41. Belaidi, E., et al., *Major role for hypoxia inducible factor-1 and the endothelin system in promoting myocardial infarction and hypertension in an animal model of obstructive sleep apnea.* J Am Coll Cardiol, 2009. **53**(15): p. 1309-17.
42. Chen, L., et al., *Oxidative stress and left ventricular function with chronic intermittent hypoxia in rats.* Am J Respir Crit Care Med, 2005. **172**(7): p. 915-20.
43. Garvey, J.F., C.T. Taylor, and W.T. McNicholas, *Cardiovascular disease in obstructive sleep apnoea syndrome: the role of intermittent hypoxia and inflammation.* Eur Respir J, 2009. **33**(5): p. 1195-205.
44. Joyeux-Faure, M., et al., *Chronic intermittent hypoxia increases infarction in the isolated rat heart.* J Appl Physiol, 2005. **98**(5): p. 1691-6.
45. Levy, P., et al., *Intermittent hypoxia and sleep-disordered breathing: current concepts and perspectives.* Eur Respir J, 2008. **32**(4): p. 1082-95.
46. Liu, J.N., et al., *The effect of oxidative stress in myocardial cell injury in mice exposed to chronic intermittent hypoxia.* Chin Med J (Engl), 2010. **123**(1): p. 74-8.
47. Savransky, V., et al., *Chronic intermittent hypoxia induces atherosclerosis.* Am J Respir Crit Care Med, 2007. **175**(12): p. 1290-7.
48. Schulz, R., et al., *[Sleep apnea and cardiovascular disease--results from animal studies].* Pneumologie, 2008. **62**(1): p. 18-22.
49. McGuire, M. and A. Bradford, *Chronic intermittent hypoxia increases haematocrit and causes right ventricular hypertrophy in the rat.* Respir Physiol, 1999. **117**(1): p. 53-8.
50. Irlbeck, M., et al., *Effects of angiotensin II receptor blockade on hypoxia-induced right ventricular hypertrophy in rats.* J Mol Cell Cardiol, 1997. **29**(11): p. 2931-9.
51. Bao, G., et al., *Blood pressure response to chronic episodic hypoxia: role of the sympathetic nervous system.* J Appl Physiol, 1997. **83**(1): p. 95-101.
52. Greenberg, H.E., et al., *Chronic intermittent hypoxia increases sympathetic responsiveness to hypoxia and hypercapnia.* J Appl Physiol, 1999. **86**(1): p. 298-305.
53. Benzi, G., et al., *Age-related alterations by chronic intermittent hypoxia on cerebral synaptosomal ATPase activities.* J Neural Transm Suppl, 1994. **44**: p. 159-71.
54. Schoch, H.J., S. Fischer, and H.H. Marti, *Hypoxia-induced vascular endothelial growth factor expression causes vascular leakage in the brain.* Brain, 2002. **125**(Pt 11): p. 2549-57.
55. Kuniyoshi, F.H. and V.K. Somers, *Sleep apnea in hypertension: when, how, and why should we treat?* Hypertension, 2006. **47**(5): p. 818-9.
56. Chobanian, A.V., et al., *The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report.* JAMA, 2003. **289**(19): p. 2560-72.
57. Mancia, G., et al., *2007 Guidelines for the Management of Arterial Hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC).* J Hypertens, 2007. **25**(6): p. 1105-87.
58. Hla, K.M., et al., *Sleep apnea and hypertension. A population-based study.* Ann Intern Med, 1994. **120**(5): p. 382-8.
59. Shamsuzzaman, A.S., B.J. Gersh, and V.K. Somers, *Obstructive sleep apnea: implications for cardiac and vascular disease.* JAMA, 2003. **290**(14): p. 1906-14.

60. Becker, H.F., et al., *Effect of nasal continuous positive airway pressure treatment on blood pressure in patients with obstructive sleep apnea.* Circulation, 2003. **107**(1): p. 68-73.
61. Faccenda, J.F., et al., *Randomized placebo-controlled trial of continuous positive airway pressure on blood pressure in the sleep apnea-hypopnea syndrome.* Am J Respir Crit Care Med, 2001. **163**(2): p. 344-8.
62. Pepperell, J.C., et al., *Ambulatory blood pressure after therapeutic and subtherapeutic nasal continuous positive airway pressure for obstructive sleep apnoea: a randomised parallel trial.* Lancet, 2002. **359**(9302): p. 204-10.
63. Nieto, F.J., et al., *Association of sleep-disordered breathing, sleep apnea, and hypertension in a large community-based study. Sleep Heart Health Study.* JAMA, 2000. **283**(14): p. 1829-36.
64. Somers, V.K., et al., *Sympathetic neural mechanisms in obstructive sleep apnea.* J Clin Invest, 1995. **96**(4): p. 1897-904.
65. Narkiewicz, K., et al., *Selective potentiation of peripheral chemoreflex sensitivity in obstructive sleep apnea.* Circulation, 1999. **99**(9): p. 1183-9.
66. Phillips, B.G. and V.K. Somers, *Hypertension and obstructive sleep apnea.* Curr Hypertens Rep, 2003. **5**(5): p. 380-5.
67. Ip, M.S., et al., *Circulating nitric oxide is suppressed in obstructive sleep apnea and is reversed by nasal continuous positive airway pressure.* Am J Respir Crit Care Med, 2000. **162**(6): p. 2166-71.
68. Schulz, R., et al., *Decreased plasma levels of nitric oxide derivatives in obstructive sleep apnoea: response to CPAP therapy.* Thorax, 2000. **55**(12): p. 1046-51.
69. Lavie, L., et al., *Heat-shock protein 70: expression in monocytes of patients with sleep apnoea and association with oxidative stress and tumour necrosis factor-alpha.* J Sleep Res, 2010. **19**(1 Pt 2): p. 139-47.
70. Schulz, R., et al., *Enhanced release of superoxide from polymorphonuclear neutrophils in obstructive sleep apnea. Impact of continuous positive airway pressure therapy.* Am J Respir Crit Care Med, 2000. **162**(2 Pt 1): p. 566-70.
71. Wali, S.O., et al., *Susceptibility of LDL to oxidative stress in obstructive sleep apnea.* Sleep, 1998. **21**(3): p. 290-6.
72. Lavie, L., *Oxidative stress--a unifying paradigm in obstructive sleep apnea and comorbidities.* Prog Cardiovasc Dis, 2009. **51**(4): p. 303-12.
73. Lavie, L., *Obstructive sleep apnoea syndrome--an oxidative stress disorder.* Sleep Med Rev, 2003. **7**(1): p. 35-51.
74. Shamsuzzaman, A.S., et al., *Elevated C-reactive protein in patients with obstructive sleep apnea.* Circulation, 2002. **105**(21): p. 2462-4.
75. Schulz, R., et al., *Serum levels of vascular endothelial growth factor are elevated in patients with obstructive sleep apnea and severe nighttime hypoxia.* Am J Respir Crit Care Med, 2002. **165**(1): p. 67-70.
76. Chin, K., et al., *Effects of nasal continuous positive airway pressure on soluble cell adhesion molecules in patients with obstructive sleep apnea syndrome.* Am J Med, 2000. **109**(7): p. 562-7.
77. Carpagnano, G.E., et al., *Systemic and airway inflammation in sleep apnea and obesity: the role of ICAM-1 and IL-8.* Transl Res, 2010. **155**(1): p. 35-43.
78. Mehra, R. and S. Redline, *Sleep apnea: a proinflammatory disorder that coaggregates with obesity.* J Allergy Clin Immunol, 2008. **121**(5): p. 1096-102.

79. Ursavas, A., et al., *Circulating ICAM-1 and VCAM-1 levels in patients with obstructive sleep apnea syndrome.* Respiration, 2007. **74**(5): p. 525-32.
80. Al Lawati, N., et al., *Pro-atherogenic cytokine profile of patients with suspected obstructive sleep apnea.* Sleep Breath, 2009. **13**(4): p. 391-5.
81. Teramoto, S., et al., *Effects of oxygen administration on the circulating vascular endothelial growth factor (VEGF) levels in patients with obstructive sleep apnea syndrome.* Intern Med, 2003. **42**(8): p. 681-5.
82. Wahlin-Larsson, B., et al., *The expression of vascular endothelial growth factor in skeletal muscle of patients with sleep disorders.* Muscle Nerve, 2009. **40**(4): p. 556-61.
83. Ma, J., et al., *Serum level of vascular endothelial growth factor in patients with obstructive sleep apnea hypopnea syndrome.* J Huazhong Univ Sci Technolog Med Sci, 2007. **27**(2): p. 157-60.
84. Garcia de la Torre, N., et al., *Effects of weight loss after bariatric surgery for morbid obesity on vascular endothelial growth factor-A, adipocytokines, and insulin.* J Clin Endocrinol Metab, 2008. **93**(11): p. 4276-81.
85. Peled, N., et al., *Association of elevated levels of vascular endothelial growth factor in obstructive sleep apnea syndrome with patient age rather than with obstructive sleep apnea syndrome severity.* Respiration, 2007. **74**(1): p. 50-5.
86. Rivard, A., et al., *Age-dependent impairment of angiogenesis.* Circulation, 1999. **99**(1): p. 111-20.
87. Drager, L.F., et al., *Early signs of atherosclerosis in obstructive sleep apnea.* Am J Respir Crit Care Med, 2005. **172**(5): p. 613-8.
88. Helin, P., C. Garbarsch, and I. Lorenzen, *Effects of intermittent and continuous hypoxia on the aortic wall in rabbits.* Atherosclerosis, 1975. **21**(3): p. 325-35.
89. Butterfield, D.A., et al., *Amyloid beta-peptide and amyloid pathology are central to the oxidative stress and inflammatory cascades under which Alzheimer's disease brain exists.* J Alzheimers Dis, 2002. **4**(3): p. 193-201.
90. Butterfield, D.A., et al., *Vitamin E and neurodegenerative disorders associated with oxidative stress.* Nutr Neurosci, 2002. **5**(4): p. 229-39.
91. Beal, M.F., *Aging, energy, and oxidative stress in neurodegenerative diseases.* Ann Neurol, 1995. **38**(3): p. 357-66.
92. Macey, P.M., et al., *Brain structural changes in obstructive sleep apnea.* Sleep, 2008. **31**(7): p. 967-77.
93. Macey, P.M., et al., *Brain morphology associated with obstructive sleep apnea.* Am J Respir Crit Care Med, 2002. **166**(10): p. 1382-7.
94. Rae, C., et al., *Dynamic changes in brain bioenergetics during obstructive sleep apnea.* J Cereb Blood Flow Metab, 2009. **29**(8): p. 1421-8.
95. Akahoshi, T., et al., *Obstructive sleep apnoea is associated with risk factors comprising the metabolic syndrome.* Respirology, 2010. **15**(7): p. 1122-6.
96. Angelico, F., et al., *Obstructive sleep apnoea syndrome and the metabolic syndrome in an internal medicine setting.* Eur J Intern Med, 2010. **21**(3): p. 191-5.
97. Pack, A.I. and G.W. Pien, *Update on sleep and its disorders.* Annu Rev Med, 2011. **62**: p. 447-60.
98. Simpson, L., et al., *Sex differences in the association of regional fat distribution with the severity of obstructive sleep apnea.* Sleep, 2010. **33**(4): p. 467-74.

99. Rowley, J.A., et al., *Effect of tracheal and tongue displacement on upper airway airflow dynamics.* J Appl Physiol, 1996. **80**(6): p. 2171-8.
100. Stanchina, M.L., et al., *The influence of lung volume on pharyngeal mechanics, collapsibility, and genioglossus muscle activation during sleep.* Sleep, 2003. **26**(7): p. 851-6.
101. Khalyfa, A., et al., *Genome-wide gene expression profiling in children with non-obese obstructive sleep apnea.* Sleep Med, 2009. **10**(1): p. 75-86.
102. Izuhara, K. and H. Saito, *Microarray-based identification of novel biomarkers in asthma.* Allergol Int, 2006. **55**(4): p. 361-7.
103. Nanni, L., et al., *Differential gene expression profiling in genetic and multifactorial cardiovascular diseases.* J Mol Cell Cardiol, 2006. **41**(6): p. 934-48.
104. Chittenden, T.W., et al., *Transcriptional profiling in coronary artery disease: indications for novel markers of coronary collateralization.* Circulation, 2006. **114**(17): p. 1811-20.
105. Borovecki, F., et al., *Genome-wide expression profiling of human blood reveals biomarkers for Huntington's disease.* Proc Natl Acad Sci U S A, 2005. **102**(31): p. 11023-8.
106. Timofeeva, A.V., et al., *Altered gene expression pattern in peripheral blood leukocytes from patients with arterial hypertension.* Ann N Y Acad Sci, 2006. **1091**: p. 319-35.
107. Cheang, M.C., M. van de Rijn, and T.O. Nielsen, *Gene expression profiling of breast cancer.* Annu Rev Pathol, 2008. **3**: p. 67-97.
108. De Cecco, L., et al., *Gene expression profiling of advanced ovarian cancer: characterization of a molecular signature involving fibroblast growth factor 2.* Oncogene, 2004. **23**(49): p. 8171-83.
109. Pack, A.I., *Advances in sleep-disordered breathing.* Am J Respir Crit Care Med, 2006. **173**(1): p. 7-15.
110. Hoffmann, M.S., et al., *Microarray studies of genomic oxidative stress and cell cycle responses in obstructive sleep apnea.* Antioxid Redox Signal, 2007. **9**(6): p. 661-9.
111. Koo, B.B., et al., *The effects of gender and age on REM-related sleep-disordered breathing.* Sleep Breath, 2008. **12**(3): p. 259-64.
112. Muraki, M., et al., *Apnoea-hypopnoea index during rapid eye movement and non-rapid eye movement sleep in obstructive sleep apnoea.* J Int Med Res, 2008. **36**(5): p. 906-13.
113. Rohde, E., et al., *Blood monocytes mimic endothelial progenitor cells.* Stem Cells, 2006. **24**(2): p. 357-67.
114. Heil, M., et al., *Blood monocyte concentration is critical for enhancement of collateral artery growth.* Am J Physiol Heart Circ Physiol, 2002. **283**(6): p. H2411-9.
115. Bolad, I. and P. Delafontaine, *Endothelial dysfunction: its role in hypertensive coronary disease.* Curr Opin Cardiol, 2005. **20**(4): p. 270-4.
116. Palmer, L.J. and S. Redline, *Genomic approaches to understanding obstructive sleep apnea.* Respir Physiol Neurobiol, 2003. **135**(2-3): p. 187-205.
117. Gulcicek, E.E., et al., *Proteomics and the analysis of proteomic data: an overview of current protein-profiling technologies.* Curr Protoc Bioinformatics, 2005. **Chapter 13**: p. Unit 13 1.

118. Jing, Y., C. Liu, and L. Wang, *A novel TACSTD2 mutation identified in two Chinese brothers with gelatinous drop-like corneal dystrophy.* Mol Vis, 2009. **15**: p. 1580-8.
119. Zhang, B., Y.F. Yao, and P. Zhou, *Two novel mutations identified in two Chinese gelatinous drop-like corneal dystrophy families.* Mol Vis, 2007. **13**: p. 988-92.
120. Alavi, A., et al., *Four mutations (three novel, one founder) in TACSTD2 among Iranian GDLD patients.* Invest Ophthalmol Vis Sci, 2007. **48**(10): p. 4490-7.
121. Nakatsukasa, M., et al., *Tumor-associated calcium signal transducer 2 is required for the proper subcellular localization of claudin 1 and 7: implications in the pathogenesis of gelatinous drop-like corneal dystrophy.* Am J Pathol, 2010. **177**(3): p. 1344-55.
122. Kinoshita, S., *[Research and development for treating devastating corneal diseases].* Nihon Ganka Gakkai Zasshi, 2010. **114**(3): p. 161-99; discussion 200-1.
123. Timpl, R., et al., *Laminin--a glycoprotein from basement membranes.* J Biol Chem, 1979. **254**(19): p. 9933-7.
124. De Arcangelis, A., et al., *Inhibition of laminin alpha 1-chain expression leads to alteration of basement membrane assembly and cell differentiation.* J Cell Biol, 1996. **133**(2): p. 417-30.
125. Kostidou, E., et al., *Oxidized laminin-1 induces increased monocyte attachment and expression of ICAM-1 in endothelial cells.* Int J Exp Pathol, 2009. **90**(6): p. 630-7.
126. Kenne, E., et al., *Immune cell recruitment to inflammatory loci is impaired in mice deficient in basement membrane protein laminin alpha4.* J Leukoc Biol, 2010. **88**(3): p. 523-8.
127. Paulsen, F.P., et al., *Upper airway epithelial structural changes in obstructive sleep-disordered breathing.* Am J Respir Crit Care Med, 2002. **166**(4): p. 501-9.
128. Hall, A., *The cellular functions of small GTP-binding proteins.* Science, 1990. **249**(4969): p. 635-40.
129. Adams, A.E., et al., *CDC42 and CDC43, two additional genes involved in budding and the establishment of cell polarity in the yeast Saccharomyces cerevisiae.* J Cell Biol, 1990. **111**(1): p. 131-42.
130. Honing, H., et al., *RhoA activation promotes transendothelial migration of monocytes via ROCK.* J Leukoc Biol, 2004. **75**(3): p. 523-8.
131. Bennett, S. and S.N. Breit, *Variables in the isolation and culture of human monocytes that are of particular relevance to studies of HIV.* J Leukoc Biol, 1994. **56**(3): p. 236-40.
132. de Almeida, M.C., et al., *A simple method for human peripheral blood monocyte isolation.* Mem Inst Oswaldo Cruz, 2000. **95**(2): p. 221-3.
133. Haskill, S., et al., *Adherence induces selective mRNA expression of monocyte mediators and proto-oncogenes.* J Immunol, 1988. **140**(5): p. 1690-4.
134. Flo, R.W., et al., *Negative selection of human monocytes using magnetic particles covered by anti-lymphocyte antibodies.* J Immunol Methods, 1991. **137**(1): p. 89-94.
135. Imbeaud, S., et al., *Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces.* Nucleic Acids Res, 2005. **33**(6): p. e56.
136. Qiagen, *RNeasy Mini Handbook*. 2006. p. 25-35.

137. Schroeder, A., et al., *The RIN: an RNA integrity number for assigning integrity values to RNA measurements.* BMC Mol Biol, 2006. **7**: p. 3.
138. Affymetrix. *Affymetrix*. 2009 [cited 2010 10/25/2010]; Manufactors website with package insert]. Available from: http://www.affymetrix.com/support/support_result.affx
139. Irizarry, R.A., et al., *Exploration, normalization, and summaries of high density oligonucleotide array probe level data.* Biostatistics, 2003. **4**(2): p. 249-64.
140. Humphries, M.J., *Cell adhesion assays.* Mol Biotechnol, 2001. **18**(1): p. 57-61.
141. Kueng, W., E. Silber, and U. Eppenberger, *Quantification of cells cultured on 96-well plates.* Anal Biochem, 1989. **182**(1): p. 16-9.
142. Sellars, M.J., et al., *Real-time RT-PCR quantification of Kuruma shrimp transcripts: a comparison of relative and absolute quantification procedures.* J Biotechnol, 2007. **129**(3): p. 391-9.
143. Cook, N.L., et al., *Validation of reference genes for normalization of real-time quantitative RT-PCR data in traumatic brain injury.* J Neurosci Res, 2009. **87**(1): p. 34-41.
144. Meyer, A., et al., *Fast evolving 18S rRNA sequences from Solenogastres (Mollusca) resist standard PCR amplification and give new insights into mollusk substitution rate heterogeneity.* BMC Evol Biol, 2010. **10**: p. 70.
145. Tusher, V.G., R. Tibshirani, and G. Chu, *Significance analysis of microarrays applied to the ionizing radiation response.* Proc Natl Acad Sci U S A, 2001. **98**(9): p. 5116-21.
146. Hosack, D.A., et al., *Identifying biological themes within lists of genes with EASE.* Genome Biol, 2003. **4**(10): p. R70.

CHAPTER THREE

GLOBAL GENE EXPRESSION PROFILES AND PROTEOMIC ASSESSMENTS IN ADULT FEMALES WITH OBSTRUCTIVE SLEEP APNEA SYNDROME

Abstract

Study Objectives: To determine if there are specific differences in global gene expression profiles of adult females with moderate-severe obstructive sleep apnea syndrome (OSAS) verses those without OSAS, under conditions in which subjects are otherwise clinically similar.

Design: Qualified volunteers were screened with a polysomnogram (PSG) or a home somnogram and assigned to OSAS (moderate-severe) or non-OSAS groups based on their sleep scores. Subjects provided a fasting blood sample; monocytes were isolated from total blood using the Monocyte Isolation Kit and stored. From the monocytes, RNA was extracted and shipped frozen to Dana-Farber and hybridized on 16 gene chips. Microarrays were normalized by robust microarray analysis and the differential gene expression profile was assessed with significance analysis of microarrays. Two-step quantitative real time polymerase chain reaction (qPCR), laminin enzyme immunoassay (EIA), and cellular adhesion assays were also performed.

Setting: The study was conducted at Carilion Community Hospital research lab, Roanoke, Virginia, and the Microarray Core Facility at the Dana-Farber Cancer Institute, Harvard University, Boston, Massachusetts.

Patients: 6 OSAS and 10 non-OSAS volunteers

Interventions: N/A

Measurements and Results: The array data showed three genes that were differentially expressed. qPCR verified a pattern in segregation between OSAS and controls subjects based on expression patterns of LAMC-1, CDC42, and TACSTD2 genes seen in the arrays. No differences were noted in the laminin EIA. There was a significant increase in PBMC cellular adhesion in OSAS patients versus control subjects ($p<0.05$)

Conclusions: A gene implicated in inflammation and monocytic regulation (LAMC-1), was found to be aberrantly expressed in women with moderate-severe OSAS. As well, cells isolated from these OSAS patients display an abnormality in cellular adhesion, a process driven in part by LAMC-1.

Introduction

Obstructive Sleep Apnea Syndrome (OSAS) is the most common of sleep related breathing disorders, with an estimated prevalence of 1 in 20 adults in the United States [1]. Among patients with a body mass index greater than 28, OSAS is present in 41% [2] and the prevalence of OSAS can be as high as 78% in morbidly obese patients who present for bariatric surgery [3]. OSAS is associated with major comorbidities including daytime somnolence, impaired cognition, poor quality of life, and increased risk of motor vehicle accidents [4]. OSAS is an independent risk factor for a variety of adverse cardiovascular outcomes [4-6]. Moreover, multiple genetic and environmental factors underlie the pathophysiology of this complex disease state [5].

High-throughput measurement of gene transcription provides the means to examine how organisms respond on a genome-wide scale to experimental perturbations or to the development of pathological conditions [7, 8]. The analysis of DNA microarrays has become a fundamental approach for identifying potential diagnostic and therapeutic targets for many diseases [9]. A molecular gene expression signature is a cluster of abnormally regulated genes that uniquely correlate to a specific disease state or given condition under study. Molecular signatures have been identified for asthma [10], heart disease [11, 12], Huntington's Disease [13], and arterial hypertension [14]. Molecular signatures have been used to classify human malignancies, such as breast [15] and ovarian cancers [16]. Therefore, assessment of global gene expression patterning in OSAS may afford fundamental insights into the etiological nature of this complex human disease state.

Although etiologic factors of OSAS have not been determined, two studies have evaluated gene expression patterning in OSAS patients. These studies uncovered

unique molecular gene expression profiles in adult males [17] and in children [9]. Hoffmann et al. [17] identified aberrantly expressed genes responsible for modulation of reactive oxygen species and cell cycle regulation. Khalyfa et al. [9] identified several genes related to the inflammatory responses. These gene expression findings lead to the hypothesis that OSAS is, in part, an inflammatory disorder. Therefore, the purpose of this current study was to determine, a disease-related gene expression profile in female OSAS patients.

Methods

Subjects

Overweight premenopausal Caucasian women with untreated OSAS (n=6), and control subjects (n=10) matched for age, BMI, and similar clinical characteristics (diabetes, cardiovascular disease status, medications, etc.) were recruited from either Carilion Clinic Pulmonary/Sleep Medicine or Carilion Clinic Bariatric Surgery practices in Roanoke, VA. Subjects were between 20 and 45 years of age and were classified as overweight according to body mass index criteria [18]. All subjects were volunteers who were receiving care in one of these two clinical facilities. Each volunteer completed an informed consent prior to pre-screening, which included questionnaires to identify any potential exclusion criteria and a basic health history. All methods and procedures, approved by the Institutional Review Board of Carilion Clinic, Roanoke, VA, were explained to the subjects, who then read and gave written informed consent. Clinical variables were assessed with the Statistical Package for the Social Sciences, version 16.0 (SPSS, Chicago, IL); a p-value <0.05 was considered statistically significant.

Sleep Evaluations

Subjects were initially screened for symptoms of OSAS using two established questionnaires [19, 20]. Each was invited to participate, based on the one of the following two criteria: 1) to limit the probability of OSAS occurrence, the control group subjects (No OSAS) were excluded if they had an ESS >10 and were categorized as high risk using the Berlin Questionnaire (BQ). These subjects were screened for sleep disordered breathing symptoms, and had to be classified as negative by the ApneaLink™ portable device (ResMed, Poway, CA), an overnight home somnogram. The ApneaLink™ data were interpreted by a sleep technician, with the results verified by the physician investigator who is a sleep specialist; and 2) the OSAS group subjects (OSAS) were recruited if their ESS was >10 or they were symptomatic, and had undergone an overnight polysomnogram resulting in an AHI score ≥ 15.

Blood draw and Monocyte Cell Separation

Human venous blood was collected in lithium heparin tubes and processed within 1–2 h after sampling. Monocytes were isolated from total blood using the Monocyte Isolation Kit II (Miltenyi Biotec, Cambridge, MA), Peripheral Blood Mononuclear Cells (PBMC) were magnetically labeled through incubation with a cocktail of biotin-conjugated monoclonal antibodies for CD3, CD7, CD16, CD19, CD56, CD123, and Glycophorin A. Antibiotin monoclonal antibodies conjugated to MicroBeads were used as the secondary reagent per manufactures' protocol; blood samples were diluted with 1-2 ml of MACS® buffer and centrifuged for 10 min, at 300xg RT. The supernatants were removed, and the cell pellets were resuspended in 500μl MACS® buffer. Samples were run on the AutoMACS™ system, and purified monocytes were isolated by negative selection. The magnetically labeled PBMC were depleted with MACS®

Columns Isolated monocytes were treated with RNAlater®, and kept at 4°C overnight. Cells were pelleted for 10 min, at 300xg RT, placed in RNAlater® 10x the volume and stored at -80°C.

Human Monocyte RNA Extraction, Target Processing, and Labeling

Messenger RNA (mRNA) was isolated with Qiagen RNeasy Kits® (Qiagen #74106, Valencia, CA) according to the manufacturer's recommendations. DNase digestion (Qiagen, #79254, Valencia, CA) was performed according to manufacturer's recommendations. Quality and concentration of the mRNA was assessed with the Experion™ automated gel electrophoresis system, stored at -80°C, and then shipped to Dana-Farber Cancer Institute at Harvard University. RNA was processed and hybridized to sixteen Affymetrix GeneChip® Human Genome U133A Arrays (Santa Clara, CA) using standard protocols at the Microarray Core Facility at the Dana-Farber Cancer Institute [7]. Affymetrix GeneChip® Human Genome U133A Arrays contain 22,000 probe sets corresponding to 14,500 genes. The labeled cDNA targets derived from the mRNA of an experimental sample were hybridized to nucleic acid probes attached to the solid support. By monitoring the amount of label associated with each DNA location, it was possible to infer the abundance of each mRNA species represented.

Microarray Data Normalization and Analysis

Raw data were imported into R and data were normalized with RMA (robust microarray analysis) using the Bioconductor package, *affy [7]*. Normalization of the raw data is needed to minimize errors and restore equality across a series of chips. Initial exploratory data analysis was performed with hierarchical clustering analysis (average-linkage and metric 1 – Person correlation coefficient distance) using the Bioconductor package, *made4* [7]. Statistical power analysis estimation of preliminary microarray data

from isolated monocytes in patients indicate a 50% coefficient of variation with a standard deviation of 0.68 (log to the base 2) in gene expression [12]. Therefore, to discriminate a two-fold difference in gene expression with an alpha level of 0.0001 and 95% power, the experimental design calls for approximately 33 patients per group [12]. Power Analysis was conducted with the R version 2.11.1 statistical software package [12]. Therefore, the estimated power with 16 patients would equal 0.40 with a standard deviation of 0.68, and a two-fold difference in gene expression with an alpha level of 0.0001.

Since microarrays can measure the expression of thousands of genes to identify changes in expression between different biological states an assessment of differential gene expression (control vs. OSAS) with Significance Analysis of Microarrays (SAM) implemented within the Multiple Experiment Viewer (MeV) [21] was used. SAM is a method for identifying genes on a microarray with statistically significant changes in expression developed in context of an actual biological experiment [22]. SAM assigns a score to each gene on the basis of change in gene expression relative to the standard deviation of repeated measurements [22]. Each gene is assigned a score on the basis of its change in gene expression relative to the standard deviation of repeated measurements for that gene. SAM uses permutations of the repeated measurements to estimate the percentage of genes identified by chance, for genes with scores greater than an adjustable threshold. This is the false discovery rate (FDR). The q-value was estimated by calculating the minimum estimated false discovery rate among all instances. Genes with an FDR ≤ 30% were further assessed as described below.

Quantitative PCR

Two-step Quantitative Real-Time PCR was performed with the BioRad IQ5 system. Gene expression of CDC-42 and LAMC-1 was assessed using specific TaqMan Expression Assays (Applied Biosystems, # 4331182, Hs00741586_mH and Hs00267056_m1 respectively). The 18s rRNA endogenous control (Applied Biosystems, # 4333760T) was selected as a reference gene to correct for mRNA sample variations. All experimental samples were assayed in triplicate. Delta Ct (ΔCt) were calculated by subtracting the average Ct of the Eukaryotic 18S rRNA endogenous controls from the average Ct of the target genes. The delta-delta Ct (ΔΔCt) were calculated by subtracting the delta Ct of the target gene of the OSAS controls from the delta Ct of the target gene of the OSAS positive samples. Fold change was calculated as 2 to the power of negative delta-delta Ct ($2^{-\Delta\Delta Ct}$) (BioRad Real-Time PCR Applications Guide, #1709799).

Laminin EIA

Serum laminin was measured using an enzyme immunoassay (EIA) kit (Takara Bio Inc, Shiga, Japan) according to manufactures recommendations. Briefly, 100 µl of the subject's serum was plated in duplicate and incubated for 1 hr. The sample was removed and washed 3 times with washing buffer, and then 100 µl of the antibody-POD solution was added and incubated. The plate was then washed, and 100 µl of the substrate solution was added, incubated, and finally 100 µl of stop solution was applied. The absorbance was measured at 450 nm with a plate reader.

Adhesion Assay

Monocyte depleted peripheral blood mononuclear cells (PBMC) from OSAS patients and control subjects were plated in 12-well tissue-culture plates in duplicate

with $5x10^4$ cells/well and allowed to attach for 1 h. The cells were plated on either plastic or in wells that were previously coated with 400 µl of BSA, or laminin and incubated overnight at 4°C. Adherent cells were fixed with 3.7% PFA (Paraformaldehyde) in PBS and then stained with Coomassie blue. Wells were imaged with an AxioObserver Z1 microscope and an AxioCam MRc 5 high resolution camera. Cell adhesion was determined relative to total cells plated as previously described [7]. Briefly, six random fields were counted for each well and were averaged. Cell adhesion was determined relative to total cells plated.

Figure 1 presents the sequential order of experimental evaluations carried out in this study. Exploratory data analysis of global gene expression patterning was performed to generate working hypotheses relative OSAS etiology. Next, qPCR and EIA were used to confirm the molecular profiling findings. Finally, inferred phenotypic assessment of PBMC cellular adhesion was performed, since all monocytes were used up for the microarrays.

Results

Tissue samples from 16 female patients (volunteer subjects) were evaluated in this study: six who met criteria for OSAS and 10 controls, matched for age and several body mass indices. As shown in Table 1, no significant differences in age, BMI, neck, hip, and waist circumference were observed between groups. Diagnostic sleep studies were conducted and showed that the OSAS group had moderate-severe obstructive sleep apnea (Mean ± SD: AHI = 27.3 ± 16.0).

Messenger RNA from tissue samples donated by five OSAS patients and eight control subjects was used for initial exploratory global gene expression. The initial exploratory unsupervised cluster analysis indicated that subjects did not group according to clinical designation based on global gene expression patterning (Figure 2). However, supervised assessment of differential gene expression identified nine aberrantly regulated genes at or below 30% FDR. Table 2 shows the nine genes identified by SAM analysis. The heat map in Figure 3 shows a trend in segregation between OSAS and controls subjects based on expression patterns of LAMC-1, CDC42, and TACSTD2. Moreover, qPCR indicated a 2.1 fold increase in LAMC-1 and a 1.1 fold increase CDC42 expression unique to the tissue samples of patients with OSAS.

Based on these findings, the serum laminin protein levels were independently assayed, using enzyme immunoassay (EIA). The EIA results indicated a trend towards increased serum laminin levels in OSAS patients; despite this, differences in circulating levels of this protein did not differ between the six OSAS vs. 10 control subjects. OSAS subjects had an average laminin concentration of 392.80 ± 123.8 ng/ml, a non-significant difference to the controls with 415.60 ± 75.2 ng/ml ($p=0.657$).

Finally, because LAMC1 is known to regulate lymphocyte cell adhesion during extravasation (arising from conditions that favor vascular leakage to surrounding tissues), and thus promotes inflammation [23], a cell adhesion assay was performed under static conditions. The OSAS and control PBMCs were plated in duplicated on laminin and BSA coated wells, as well as plastic wells with a volume that would allow for 5×10^4 cells/well and allowed to attach for 1 h. The adherent cells were than fixed, stained, and counted. The average cell counts were calculated for each subject under

the three conditions, laminin, and BSA coated wells, and plastic wells. A statistically significant increase in PBMC cellular adhesion in OSAS patients versus control subjects was found (Figure 4). The OSAS subjects had an average well cell count of 9.27 ± 1.54 vs. controls 5.75 ± 0.78 ($p < 0.05$), which is relative to the 103 cells/field that were plated.

Discussion

It is thought that this study represents the first characterization of genome-wide gene expression patterning in Caucasian females with OSAS. Though unable to unequivocally identify aberrant gene and/or protein regulation in female patients with diagnosed OSAS patients, the data indicates a trend of dysregulation for three genes involved in inflammation, cell adhesion, and cell migration. This trend supports previous reports indicating that OSAS is, in part, an inflammatory disorder [9]. However, a potential limitation of the study is that it was restricted to examining gene expression patterns of circulating monocytes. Although these reporter cells are intricately involved in inflammation, they may not fully reflect the overall response patterns of OSAS affected tissues. Therefore, the analysis may be relative to only a small fraction of dysregulated processes that contribute to the OSAS etiology.

The data showed aberrant expression of three genes at an FDR of ~30%, suggesting that they be involved in the development of OSAS. Cell division control protein 42 (CDC42) is a small GTPase of the Rho-subfamily, which regulates signaling pathways that control diverse cellular functions including cell morphology, migration, endocytosis, and cell cycle progression. Research by other investigators suggests that CDC42 and other small GTPases have a distinct effect on monocyte adhesion and

migration across the endothelium. Aberrant GTPase regulation in monocytes may reduce monocytic recruitment into inflamed tissue and thus lessen the adverse effects of inflammatory processes [24]. The results of this study indicate abnormal regulation of tumor-associated calcium signal transducer 2 (TACSTD2). The TACSTD2 protein is a monomeric cell surface glycoprotein expressed in many organs such as cornea, placenta, lung, kidney, pancreas, prostate, and in trophoblasts. Elevated protein levels have been detected in various carcinomas, including gelatinous drop-like dystrophy, pancreatic, and gastrointestinal [25]. Therefore, further evaluation of this gene may reveal novel mechanistic links among OSAS and tumorigenesis. Finally, a 2.1 fold increase in LAMC1 expression in OSAS patients was detected. Laminins are an essential, abundant, and biologically active part of the basal lamina, influencing cell differentiation, proliferation, migration, and adhesion [26, 27]. In an article by Kostidou et al. [23], monocytes showed increased adherence to Human Umbilical Vein Endothelial Cells (HUVECs) attached to oxidized vs. native laminin coated plates ($p = 0.005$). This response suggests that laminin oxidation changes its interactions with endothelial cells, those in-turn recruit blood monocytes to the vascular endothelium contributing to the formation of lesions [28]. Moreover, studies indicate that OSAS patients express increased levels of circulating markers for oxidative stress and inflammation, including reactive oxygen species and reactive nitrogen species [29]. This corresponds with recent evidence that the hypoxic events, caused by obstructions in breathing during sleep, increase oxidative stress resulting in the promotion of both inflammation and atherogenesis [23]. In agreement with these reports, PBMCs isolated from OSAS patients were found to exhibit a significant increase in cellular adhesion. Taken together, the findings suggest that OSAS correlates with increased monocytic laminin

gene expression and increased cellular adhesion, thereby implicating inflammation as a contributing factor to OSAS etiology.

Conclusion

This is the first investigation to evaluate global gene expression in tissue (monocytes) from female OSAS patients. It was determined that LAMC1, a gene implicated in inflammation and monocytic regulation, was aberrantly expressed in women with moderate-severe OSAS. This study also showed that cells isolated from these OSAS patients display an abnormality in cellular adhesion, a process driven in part by LAMC1. Taken together, these findings suggest that inflammation may be linked to the pathogenesis of OSAS. In addition, the findings are consistent with recent findings implicating OSAS as a promoter of cardiovascular co-morbidity (e.g. stroke, cardiovascular disease etc.) through pathological regulation of inflammatory processes [30-36]. The eventual discovery of a molecular signature of OSAS will further the understanding of the etiological factors associated with this complex disease process and that goal should be central to the design of the next follow up investigation in this research.

Tables

Table 1: Subject Characteristics

	OSAS Group (n=6)	Control Group (n=10)	P Value
Age (years)	40.7 ± 3.4	38.2 ± 7.6	0.47
Height (m)	1.67 ± 0.08	1.64 ± 0.08	0.62
Weight (kg)	137.4 ± 27.5	130.0 ± 21.9	0.56
BMI (kg/m^2)	49.04 ± 6.97	47.94 ± 6.15	0.75
Neck Circumference (cm)	41.5 ± 3.9	41.4 ± 3.6	0.97
Waist Circumference (cm)	139.8 ± 20.1	125.8 ± 12.2	0.10
Hip Circumference (cm)	158.7 ± 21.4	145.7 ± 12.8	0.15
ESS	11.0 ± 5.0	5.0 ± 1.0	<0.001
Berlin Score, high risk %	100	0	N/A
AHI	27.3 ± 16.02	<5	--

Data are presented as mean ± standard deviation. BMI refers to body mass index; ESS, Epworth Sleepiness Scale; AHI, apnea hypopnea index; * P<0.05

Table 2: List of top 9 differentially expressed genes in 5 OSAS patients compared to 8 matched control

Description	Gene Symbol	Ref Seq#	Fold change	q-value	Chromosome Location
Laminin	LAMC1	NM_002293	2.446966*	26.87	1q31
Tumor-associated calcium signal transducer 2	TACSTD2	NM_002353	4.715181*	26.87	1p32
Cell division control protein 42	CDC42	NM_001039802 NM_001791 NM_044472	1.780631*	26.87	1p36.1
SMAD family member 5	SMAD5	NM_001001419 NM_001001420 NM_005903	1.3427658	20.89	5
Short stature homeobox 2	SHOX2	NM_003030 NM_006884	1.2263887	20.89	3
Chromosome 15 open reading frame 28	C15orf28	NM_001040150	1.3745538	26.86	NA
Pseudouridylate synthase 3	PUS3	NM_031307	1.1761632	26.86	11
Zinc finger protein 37A Zinc finger protein 37B	ZNF37A ZNF37B	NM_001007094 NM_003421 XM_001125758 XM_001125788 XM_001127459 XM_001127472	1.3620069	26.86	10
Ankyrin repeat domain 6	ANKRD6	NM_014942	1.4109348	26.86	6

Table 2 presents the top 9 differentially expressed genes with the gene name, symbol, location and reference sequence. Data with an * had a fold change greater than 1.5, with given q-value. q-value indicates the minimum estimated false discovery rate among all thresholds at which the false discovery rate is called significant [37].

Figures

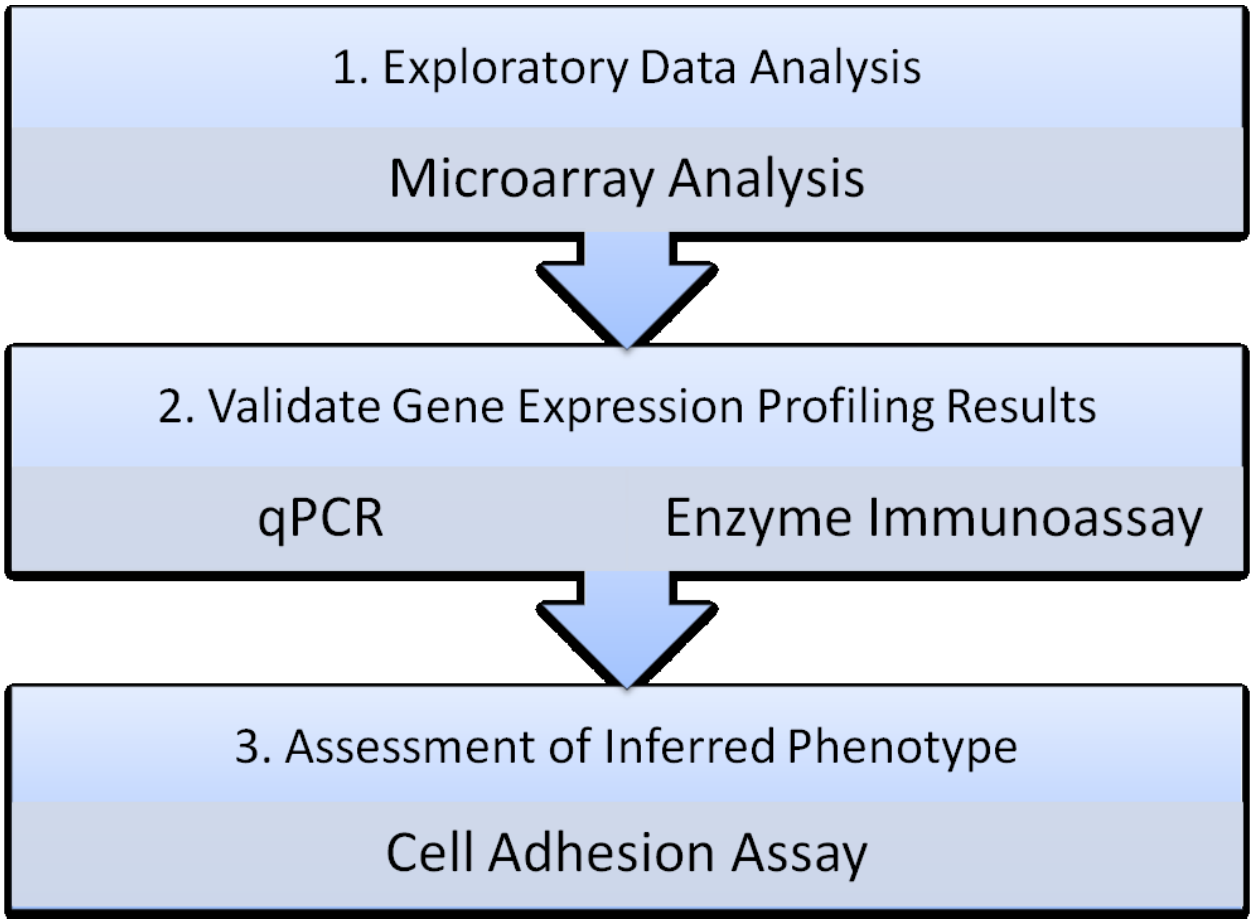

Figure 1: Flow Diagram. Sequence of testing microarray generated hypotheses.

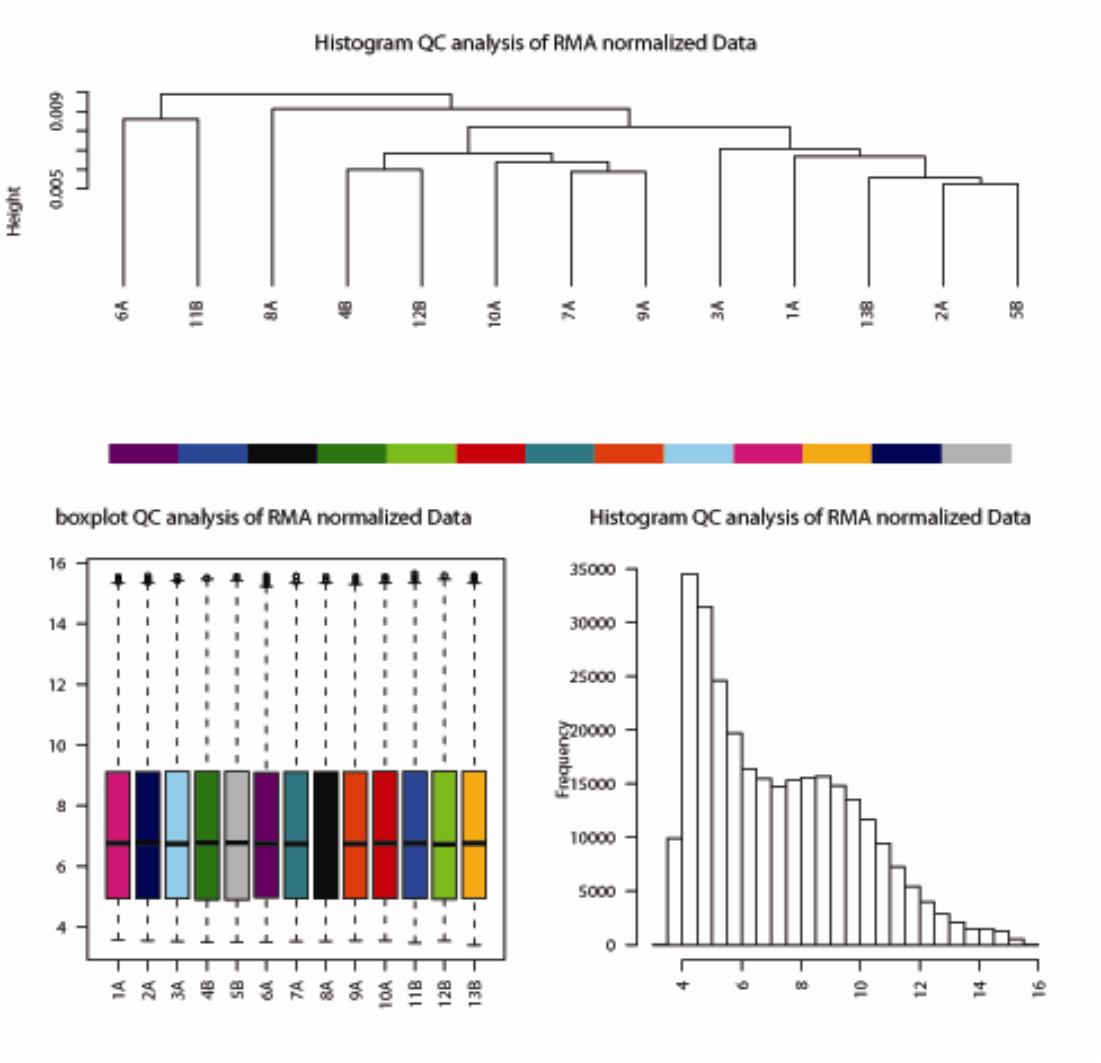

Figure 2: Quality Control Analysis of RMA Normalized Microarray Data. A. Hierarchical clustering of global gene expression patterns. B. Boxplots of the RMA normalized Data. C. Histogram of the RMA normalized gene expression. A designates OSAS patients and B represents control subjects.

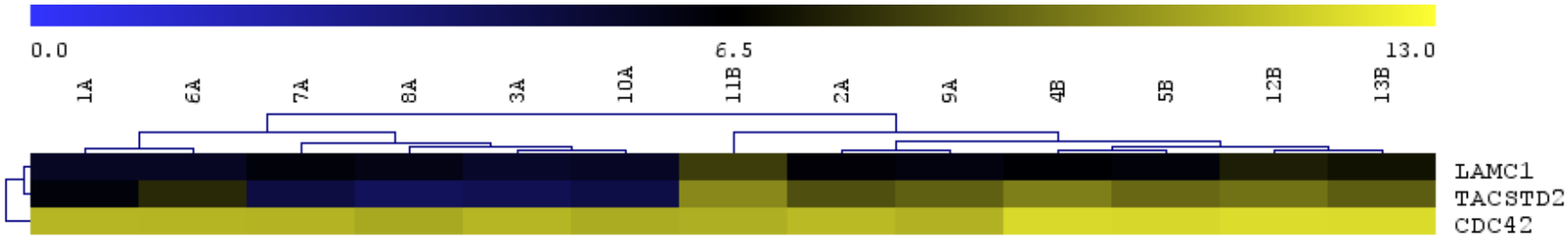

Figure 3: Hierarchical Clustering of the Top Three Differentially Expressed Genes from the SAM Analysis. The heat map indicates expression patterns for LAMC1, TACSTD2 among OSAS patients and control subjects. A designates OSAS patients and B represents control subjects. Yellow indicates high gene expression, whereas and blue represents low gene expression

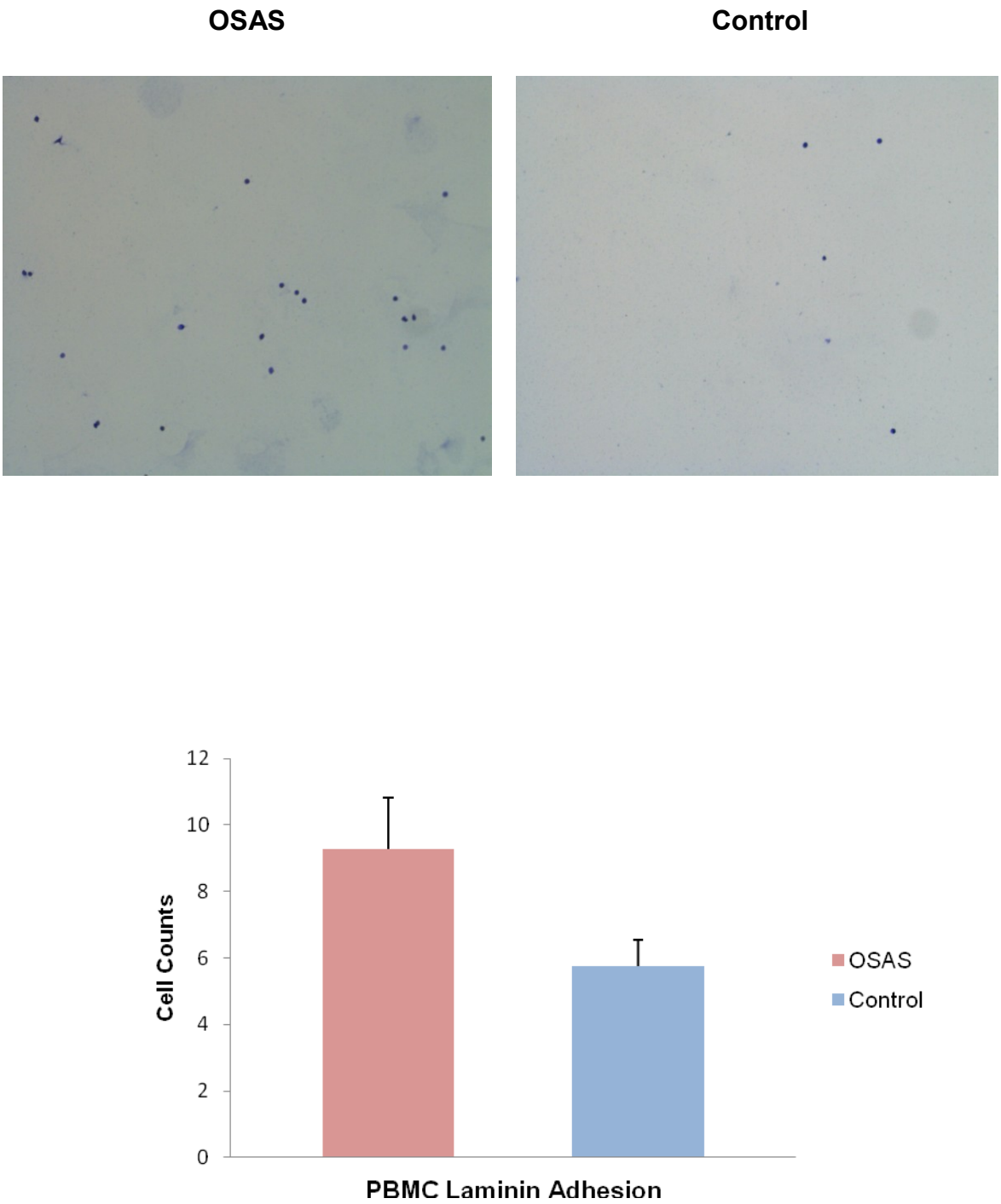

Figure 4: PBMC Laminin Adhesion Assay. Increased PBMC cell adhesion in OSAS patients vs. controls. (OSAS 9.27 cells ± 1.54 vs. Controls 5.75 cells ± 0.78; $p < 0.05$) relative to 103 cells/field

References

1. Young, T., et al., *The occurrence of sleep-disordered breathing among middle-aged adults.* N Engl J Med, 1993. **328**(17): p. 1230-5.
2. Vgontzas, A.N., et al., *Sleep apnea and sleep disruption in obese patients.* Arch Intern Med, 1994. **154**(15): p. 1705-11.
3. Lopez, P.P., et al., *Prevalence of sleep apnea in morbidly obese patients who presented for weight loss surgery evaluation: more evidence for routine screening for obstructive sleep apnea before weight loss surgery.* Am Surg, 2008. **74**(9): p. 834-8.
4. Eckert, D.J. and A. Malhotra, *Pathophysiology of adult obstructive sleep apnea.* Proc Am Thorac Soc, 2008. **5**(2): p. 144-53.
5. Kaparianos, A., et al., *Obstructive sleep apnoea syndrome and genes.* Neth J Med, 2006. **64**(8): p. 280-9.
6. Young, T., J. Skatrud, and P.E. Peppard, *Risk factors for obstructive sleep apnea in adults.* JAMA, 2004. **291**(16): p. 2013-6.
7. Chittenden, T.W., et al., *Therapeutic implications of GIPC1 silencing in cancer.* PLoS One, 2010. **5**(12): p. e15581.
8. Arnardottir, E.S.M., M; Gislason, T; Teff, K.L.; Pack, A.I., *Molecular Signatures of Obstructive Sleep Apnea in Adults: A Review and Perspective.* Sleep, 2009. **32**(4): p. 447-470.
9. Khalyfa, A., et al., *Genome-wide gene expression profiling in children with non-obese obstructive sleep apnea.* Sleep Med, 2009. **10**(1): p. 75-86.
10. Izuhara, K. and H. Saito, *Microarray-based identification of novel biomarkers in asthma.* Allergol Int, 2006. **55**(4): p. 361-7.
11. Nanni, L., et al., *Differential gene expression profiling in genetic and multifactorial cardiovascular diseases.* J Mol Cell Cardiol, 2006. **41**(6): p. 934-48.
12. Chittenden, T.W., et al., *Transcriptional profiling in coronary artery disease: indications for novel markers of coronary collateralization.* Circulation, 2006. **114**(17): p. 1811-20.
13. Borovecki, F., et al., *Genome-wide expression profiling of human blood reveals biomarkers for Huntington's disease.* Proc Natl Acad Sci U S A, 2005. **102**(31): p. 11023-8.
14. Timofeeva, A.V., et al., *Altered gene expression pattern in peripheral blood leukocytes from patients with arterial hypertension.* Ann N Y Acad Sci, 2006. **1091**: p. 319-35.
15. Cheang, M.C., M. van de Rijn, and T.O. Nielsen, *Gene expression profiling of breast cancer.* Annu Rev Pathol, 2008. **3**: p. 67-97.

16. De Cecco, L., et al., *Gene expression profiling of advanced ovarian cancer: characterization of a molecular signature involving fibroblast growth factor 2.* Oncogene, 2004. **23**(49): p. 8171-83.
17. Hoffmann, M.S., et al., *Microarray studies of genomic oxidative stress and cell cycle responses in obstructive sleep apnea.* Antioxid Redox Signal, 2007. **9**(6): p. 661-9.
18. *ACSM clinician profile.* Curr Sports Med Rep, 2010. **9**(1): p. 2.
19. Johns, M.W., *A new method for measuring daytime sleepiness: the Epworth sleepiness scale.* Sleep, 1991. **14**(6): p. 540-5.
20. Netzer, N.C., et al., *Using the Berlin Questionnaire to identify patients at risk for the sleep apnea syndrome.* Ann Intern Med, 1999. **131**(7): p. 485-91.
21. Saeed, A.I., et al., *TM4 microarray software suite.* Methods Enzymol, 2006. **411**: p. 134-93.
22. Tusher, V.G., R. Tibshirani, and G. Chu, *Significance analysis of microarrays applied to the ionizing radiation response.* Proc Natl Acad Sci U S A, 2001. **98**(9): p. 5116-21.
23. Kostidou, E., et al., *Oxidized laminin-1 induces increased monocyte attachment and expression of ICAM-1 in endothelial cells.* Int J Exp Pathol, 2009. **90**(6): p. 630-7.
24. Honing, H., et al., *RhoA activation promotes transendothelial migration of monocytes via ROCK.* J Leukoc Biol, 2004. **75**(3): p. 523-8.
25. Nakatsukasa, M., et al., *Tumor-associated calcium signal transducer 2 is required for the proper subcellular localization of claudin 1 and 7: implications in the pathogenesis of gelatinous drop-like corneal dystrophy.* Am J Pathol, 2010. **177**(3): p. 1344-55.
26. Timpl, R., et al., *Laminin--a glycoprotein from basement membranes.* J Biol Chem, 1979. **254**(19): p. 9933-7.
27. De Arcangelis, A., et al., *Inhibition of laminin alpha 1-chain expression leads to alteration of basement membrane assembly and cell differentiation.* J Cell Biol, 1996. **133**(2): p. 417-30.
28. Kenne, E., et al., *Immune cell recruitment to inflammatory loci is impaired in mice deficient in basement membrane protein laminin alpha4.* J Leukoc Biol, 2010. **88**(3): p. 523-8.
29. Lavie, L., *Oxidative stress--a unifying paradigm in obstructive sleep apnea and comorbidities.* Prog Cardiovasc Dis, 2009. **51**(4): p. 303-12.
30. Christou, K., et al., *Nasal continuous positive airway pressure treatment reduces systemic oxidative stress in patients with severe obstructive sleep apnea syndrome.* Sleep Med, 2009. **10**(1): p. 87-94.

31. Chung, S., et al., *Endothelial dysfunction and C-reactive protein in relation with the severity of obstructive sleep apnea syndrome.* Sleep, 2007. **30**(8): p. 997-1001.
32. Jung, H.H., H. Han, and J.H. Lee, *Sleep apnea, coronary artery disease, and antioxidant status in hemodialysis patients.* Am J Kidney Dis, 2005. **45**(5): p. 875-82.
33. Yamauchi, M., et al., *Oxidative stress in obstructive sleep apnea.* Chest, 2005. **127**(5): p. 1674-9.
34. Lin, C.L., et al., *Comparison of the indices of oxyhemoglobin saturation by pulse oximetry in obstructive sleep apnea hypopnea syndrome.* Chest, 2009. **135**(1): p. 86-93.
35. Punjabi, N.M., et al., *Sleep-disordered breathing and cardiovascular disease: an outcome-based definition of hypopneas.* Am J Respir Crit Care Med, 2008. **177**(10): p. 1150-5.
36. Sonka, K., et al., *Advanced oxidation protein products in obstructive sleep apnea.* Prague Med Rep, 2008. **109**(2-3): p. 159-65.
37. Kall, L., et al., *Posterior error probabilities and false discovery rates: two sides of the same coin.* J Proteome Res, 2008. **7**(1): p. 40-4.

CHAPTER FOUR

SUMMARY

Sleep disordered breathing (SDB) describes a group of disorders characterized by abnormalities of external respiration (pauses in breathing) during sleep. The National Commission on Sleep Disorders Research estimated that at least mild SDB affects 7-18 million people in the United States and that moderate-to-severe cases affect 1.8-4 million. SDB remains undiagnosed in approximately 92% of affected women and 80% of affected men.

Obstructive sleep apnea syndrome (OSAS) is the most common SDB, affecting up to 4% of the adult population [1] and characterized by the repetitive collapse or partial collapse of the pharyngeal airway during sleep and arousal in order to resume ventilation. OSAS is increasingly prevalent, due in large part to the widespread rise in obesity rates. Being overweight or obese is perhaps the major predisposing factor [2]. OSAS is a serious condition that severely affects health and quality of life. Since this sleep disorder involves cessation of airflow it is thought to have an inflammatory component due to the chronic intermittent hypoxia (IH). This can result in numerous adverse effects on multiple physiological functions including the cardiovascular, metabolic, and neurologic systems. Specifically, OSAS is associated with the activation of the oxidative stress, inflammation, and sympathetic pathways which can affect the expression of adipokines such as leptin and resistin, as well as other inflammatory markers present in plasma or cells (e.g., TNF-α, IL-6, ICAM-1 and HIF) [3]. Over time, untreated OSAS [4] may hasten death through increased risks for development of heart disease, hypertension, stroke, myocardial infarction, heart failure, cardiac arrhythmia,

diabetes, metabolic syndrome, or as a result of motor vehicle crashes due to sleepiness.

To date, most research had been directed at understanding the links among sleep apnea, systemic inflammation, and cardiovascular responses. Recently, disease progression is being given increased attention. Researchers have suggested that breathing disorders are continuum and that OSAS occupies a range of this continuum [4]. This range begins with trivial snoring, the initial presenting symptom, which increases in severity over time, resulting in a new hallmark symptom of sleepiness, and it seems to be synergistically related to certain other chronic diseases, such as obesity. This continuum predicts that over time a patient develops OSAS, if left untreated. Though the pathophysiology of OSAS still is unknown, researchers have examined both static and dynamic factors that may be involved. Such factors have included neck and jaw posture, tracheal tug, and gravity and any anatomic feature that decreases the size of the pharynx, as well as, nasal and pharyngeal airway resistance, gender, age, and ethnicity and the interplay of all of these [5]. All of which have lead to more questions than answers.

More recent studies have started to consider a genetic component to OSAS. Genetic studies in general have helped to explain the development certain diseases, as well as identifying patients who may be at increased risk for a given disease. Therefore, OSAS would lend naturally to research of this nature. Examining candidate genes most likely to influence OSAS through regulators of obesity and inflammation such as interleukin-6, may provide insight into a pivotal role causing OSAS [6]. Yet other researchers are using a broader approach. Global gene express profiling allows for

greater throughput of data and the analyses can be adjusted for body mass index, suggesting that the underlying genetic mechanism of sleep apnea is not dependent on obesity, a common comorbidity. Very recent genomic studies have revealed that the gene that encodes for oxidative stress uniquely contributes toward OSAS [7]. This suggests that the development of OSAS may be intimately associated with inflammation, but not necessarily mediated by oxidative stress, as was previously thought. Although most research of inflammation and OSAS has considered inflammation as a response to OSAS-related stresses, it is plausible that abnormalities in inflammatory pathways may contribute to OSAS severity by influencing pharyngeal patency through effects on mucosal edema, by contributing to pharyngeal neuropathic changes, or possibly through effects on central ventilatory control [7]. This molecular pathway may play a pivotal role by operating in a positive feedback loop, causing the OSAS to begin with and then triggering an inflammatory response that further narrows the upper airway, exacerbating the OSAS.

This study had three aims: 1) map out the study design and bench assay strategies by which to investigate this issue; in a future large-scale investigation; 2) determine if there are differences in global gene expression profiles of adult females, with OSAS vs. without OSAS, who were otherwise clinically similar; and 3) assess the presence or absence of protein expression differences that could potentially be linked via well-established molecular pathways for inflammation associated with any differences found in global gene expression profiles in the presence and absence of OSAS.

This was a small study designed to be hypothesis generating and provide preliminary results array analysis that could guide development of more definitive investigations. From there a systematic examination of both genetic and proteomic findings was performed. With the identification of three genes (TACSTD2, CDC42, and LAMC1) that were differentially expressed (up regulated) from the microarray analysis, a hypothesis that OSAS may in fact be an inflammatory disease and that CDC42 and LAMC1 may play a essential role in the development of OSAS was generated. Even with limited resources further investigations into these up-regulated genes and others are warranted. The qPCR results validated that both CDC42 and LAMC1 were in fact up-regulated in OSAS patients. With hopes that laminin may be a key protein marker that could identify patients with OSAS, a simple laminin EIA was run. Unfortunately, the results did not show a significant difference between the groups. Lastly, a cellular adhesion assay to examine the biological connections of the monocytic behavior; the results provided significant differences between OSAS and controls. This increase in monocytic laminin gene expression seen in OSAS patients is correlated with the increase in PBMC adhesion.

Though this was a small study (N=16), and only tested overweight females with moderate to severe OSAS, the results showed differences even with an FDR upwards of 30%. The data may not be strong enough to accept the hypothesis; it does not allow for it to be rejected either. The data suggests that there is a difference between patients with OSAS compared to those without and requires more rigorous testing.

Recommendations for Future Research

This pilot study has provided the framework and preliminary data needed to propose a larger study with extramural research funding. Based on the findings of the current study and the available research pertaining to a unique genetic signature for OSAS, the following recommendations are made:

1. Microarray analysis on OSAS patients may provide useful information for diagnosis and treatment of OSAS. The results from this study, though not statistically significant, showed that there were some variations in patients with OSAS. Further studies utilizing microarray analysis on an increased number of OSAS patients will provide a greater quantity of data that may be help to detect differences. A previous analysis of preliminary microarray data from isolated monocytes in patients indicate a 50% coefficient of variation with a standard deviation of 0.68 (log to the base 2) in gene expression. Therefore, to discriminate a two-fold difference in gene expression with an alpha level of 0.0001 and 95% power, the experimental design would call for approximately 33 patients per group.

2. Moving beyond sample size, the study reported in this research compared patients with moderate to severe OSAS (AHI ≥15) to non-OSAS (AHI <5). The OSAS subjects had sleep scores ranging from 15 to 51, with only two that were classified as severe (AHI ≥30). It would be ideal to look at patients with greater differences in severity of sleep apnea, severe OSAS to non-

OSAS. In order to do this, recruiting obese male patients from the sleep clinics would be beneficial since men tend to suffer from more severe apneas than women. With a greater difference in sleep apnea severity, the data may produce clearer and more definitive results, and possibly identify a clear group of genes.

3. By increasing sample size and comparison of severities, there may be more genes that are significantly differentially expressed between the two groups. This additional research would allow for the possibility to examine the abnormally expressed genes to see if there is a translational change at the proteomic level. These changes at the proteomic level could possibly lead to a protein marker than could be used to identify at risk individuals.

This future research will assist in the understanding in the pathophysiology of OSAS, and hopefully identify a molecular signature unique to this disease process. Through this process and testing the changes in the proteomic level it is hopeful that patients who are at a high risk can be identified through a simpler screening technique. By identifying those at greatest need for further diagnostic PSG testing, a greater number of individuals will potentially be diagnosed with OSAS and treated earlier. This could have a wide reaching impact on the healthcare system, by reducing OSAS related costs, reducing the risks for the development of, and costs related to, comorbid conditions, for which OSAS contributes.

References

1. Young, T., et al., *The occurrence of sleep-disordered breathing among middle-aged adults.* N Engl J Med, 1993. **328**(17): p. 1230-5.
2. AASM, A.A.o.S.M.-. *International classification of sleep disorders, 2nd Edition: Diagnostic and coding manual*. 2nd ed. 2005, Westchester, IL: American Academy of Sleep Medicine.
3. Larkin, E.K., et al., *Study of the relationship between the interleukin-6 gene and obstructive sleep apnea.* Clin Transl Sci, 2010. **3**(6): p. 337-9.
4. Downey, R., 3rd, R.M. Perkin, and J. MacQuarrie, *Upper airway resistance syndrome: sick, symptomatic but underrecognized.* Sleep, 1993. **16**(7): p. 620-3.
5. AASM, A.A.o.S.M.-. *Obstructive Sleep Apnea*. 2008; Available from: www.aasmnet.org.
6. Zhang, X., et al., *Genetic variants in interleukin-6 modified risk of obstructive sleep apnea syndrome.* Int J Mol Med, 2009. **23**(4): p. 485-93.
7. Larkin, E.K., et al., *A candidate gene study of obstructive sleep apnea in European Americans and African Americans.* Am J Respir Crit Care Med, 2010. **182**(7): p. 947-53.

APPENDIX A

DETAILED METHODOLOGY

Subject Recruitment

Primary recruitment for eligible subjects took place in Roanoke, VA. Eligibility was restricted to women (ages 20-45) accepted for elective bariatric surgery or for polysomnography evaluation at the Carilion Clinic Sleep Center for diagnosis of OSAS. All subjects were pre-menopausal and free from nicotine products for at least 3 months prior to study inclusion. The gender and race criteria are necessary to minimize variation in the subject population and enhance the likelihood of determining genetic profiles specific to OSAS patients. Patients were identified by the clinic staff and study personnel presented the possibility of joining the study in person. Subjects were divided into two study groups, based on the criteria listed below.

<u>OSAS Group</u>

Included those with moderate to severe sleep apnea

- Epworth Sleepiness Scale (ESS) of 11 or more (in accordance with the standard clinical pathway for bariatric patients, subjects >10 ESS were sent for an overnight PSG to screen for OSAS), or a patient of the Carilion Clinic Sleep Center
- Apnea-hypoxia index (AHI) score ≥ 15 (indicative of moderate to severe sleep apnea) determined by a polysomnogram (PSG)
- Or if ESS ≤ 10 and respiratory disturbance index (RDI) score > 10 determined by an at home somnography device (ApneaLink®) followed by a overnight PSG providing results of ≥ 15
- Neck circumference of 15 inches or more

<u>Non-OSAS Group</u>

- Included those with clinically negative results for sleep apnea
- ESS ≤ 10 (indicating not likely to have OSAS associated with excessive daytime sleepiness)
- Respiratory disturbance index (RDI) score ≤ 5 (indicative of mild sleep apnea) determined by an at home somnography device (e.g. ApneaLink®) RDI is an estimate of AHI determined by a simplified version of the polysomnography (PSG) device

Research Design and Methods:

The Institutional Review Board of Virginia Polytechnic Institute and State University approved the study and all subjects gave their informed consent to participate. Participants completed questionnaires (either electronically on a secure website hosted by Virginia Tech or paper copies) about physical activity history, sleep and health; as part of the initial screening questionnaires included on the study website. Those who required PSGs were then scheduled for their initial visit Carilion Clinic Sleep Center and the standard clinical protocol was followed. Patients identified with AHI ≥ 15 were scheduled for a visit during the morning hours to obtain a fasting blood sample following an 8 – 12 hour fast (detailed description presented below).

Subjects whose ESS was ≤ 10 were setup with the unattended, at-home sleep evaluation with the ApneaLink device (ResMed, Poway, CA). Patients were provided with verbal instruction by study staff on proper setup and activation of the ApneaLink, and were given written instructions to take with them. Phone numbers of study staff were also provided if the subject required further instruction at home. Detailed

instructions for the ApneaLink device are presented in Appendix F. Anthropometric data was also collected, i.e. height, weight, and neck, waist, and hip circumference from each subject. Subjects returned the ApneaLink device to the study personnel following the night of testing and the data were downloaded and evaluated using proprietary software (ResMed, Poway, CA) for the ApneaLink®. If the ApneaLink software, indicated an orange or red score on the risk level, ApneaLink data, ESS scores and Berlin scores were sent to the sleep physician investigator. The sleep physician determined, based on these three indices, whether patient was eligible for the non-OSAS group or was sent for in-lab diagnostic PSG testing and given nCPAP treatment. After completing the at-home sleep evaluation, subjects were negative for OSAS they scheduled a visit during the morning hours to obtain a fasting blood sample following an 8 – 12 hour fast (detailed description presented below).

Measurements

Anthropometric measures included height (meters), weight (kilograms), neck circumference (centimeters), waist circumference (centimeters), and hip circumference (centimeters). Body Mass Index (BMI) was calculated (kg/m^2) from measures of height and weight. A flexible tape measure was used to obtain neck, waist, and hip circumference measurements. Detailed instructions for the anthropometric measures are presented in Appendix E.

Fasting Blood Sample

Subjects were instructed to refrain from eating 8 hours prior to the blood draw, and this was confirmed immediately before the collection. Blood was drawn (120 mL (~8 Tbsp)) was drawn by certified personnel, e.g. Carilion Labs, Inc. The tubes containing the research samples was labeled with subject numbers and then immediately transported to the Carilion Community Research Lab. The samples were transported on ice immediately by individuals authorized by Carilion Clinic to do such transfers. Upon arrival at the lab the blood samples were divided into two portions. All blood samples were processed by study personnel, one portion was processed to separate and aliquots of 1.2mL each, plasma and serum samples (Appendix G), and stored at -80°C for later batch analysis of laminin levels. A detailed description of laminin enzyme immunoassay (EIA) presented in Appendix J. The second portion was processed to isolate monocytes and stored at -80°C by an experienced HNFE technician with Exposure Control Training, who has also been trained in the research procedures by Harvard study collaborators at the Dana Farber Computational Genomics Research Institute. A detailed description of monocyte isolation presented in Appendix H. The remaining PBMCs were stored -80°C for later batch cellular adhesion assay. A detailed description of cellular adhesion assay presented in Appendix K. Upon collection of the final sample from the last subject in the study, the frozen samples had the RNA extracted from subjects' monocytes using Qiagen RNeasy columns (a detailed description of RNA extraction presented in Appendix I) and sent to Harvard University for transcriptional profiling and final computational genomics analyses. Finally, all specific gene expression results were verified by quantitative polymerase chain reaction

(qPCR). A detailed description of quantitative polymerase chain reaction presented in Appendix L.

Details of the Genomics Analysis

The RNA was hybridized and profiled on Affymetrix U133 Plus2 arrays using standard protocols. The resulting CEL files from the core facility were sent to the Dana-Farber laboratory where they will be loaded into AMP, the Automated Microarray pipeline. AMP was perform to normalization using RMA or gcRMA, and subjected to a variety of quality control assessments, and raw and normalized data, together with detailed sample annotation, was loaded into a MIAME-compliant gene expression database for analysis and submission to public gene expression databases such as ArrayExpress or GEO. The resulting data was subjected to a variety of statistical tests including t-tests, ANOVA, or SAM to identify genes that correlate with clinical phenotype. Data was then subjected to additional meta-analyses to identify gene functional classes or biological pathways that are over-represented in the selected gene set using EASE.

APPENDIX B

INSTITUTIONAL REVIEW BOARD PROTECTION OF HUMAN SUBJECTS APPROVAL

CARILION CLINIC

July 14, 2010

T. A. Lucktong, MD
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Roanoke, VA 24016

RE: Preliminary Studies of Bariatric Surgery Patients: 1) Monocyte Transcription Profiling Risk Markers of Obstructive Sleep Apnea Syndrome (OSAS); and 2) Comparison of Roux-en-Y vs. Laparoscopic Adjustable Gastric Banding Procedures for Effects on Bone Geometry, Mass, and Density at 6 Months Post-surgery

Dear Dr. Lucktong:

I am pleased to inform you that the Institutional Review Board (IRB) of Carilion Clinic has reviewed the Protocol Revision, dated 6/11/10, for the above-mentioned protocol in an expedited manner. The research staff changes do not affect the scientific validity of the study or adversely alter the assessment of the risks and benefits of the study. The revisions have been approved.

I would like to remind you that the principal investigator must provide the Institutional Review Board with a report summarizing the status of the project every year. The principal investigator should submit a continuing review application thirty (30) days prior to the expiration date, providing a summary of the project to date and requesting permission for continuation of the original project. It is also your responsibility to report to the IRB serious adverse events or unanticipated problems, as outlined in the IRB Guidelines, which can be attributed to this study within seven (7) business days of notification. In addition, copies of reports from Data Monitoring Committees or auditing/monitoring reports from a sponsor must also be sent to the IRB Research Compliance Officer within seven (7) business days. Any changes to the research study must receive IRB approval before those changes can be implemented unless subject safety is directly affected. The IRB must be notified immediately about subject safety issues. The IRB must be notified within seven (7) business days if and when a project is discontinued.

The Institutional Review Board of Carilion Clinic would like to thank you for allowing us the opportunity to review this protocol. We look forward to learning of your results.

Sincerely,

Charles Hile, MA, CIP
Human Protections Administrator, Carilion Clinic IRB

cc: file

Institutional Review Board
2001 Crystal Spring Avenue, SW, Suite 202 Roanoke, VA 24014-2465 P.O. Box 13367 Roanoke, VA 24033-3367
540-853-0728 p 540-985-5323 f

APPENDIX C

INFORMED CONSENT

T. A. Lucktong, MD, FACS
Principal Investigator
3 Riverside Circle
Roanoke, VA 24016
Phone: 540-224-5170

TITLE: Preliminary Studies of Bariatric Surgery Patients: 1) Monocyte transcription profiling risk markers of Obstructive Sleep Apnea Syndrome (OSAS); and 2) Comparison of Roux-en-Y vs. Laparoscopic Adjustable Gastric Banding procedures for effects on bone geometry, mass, and density at 6 months post-surgery.

Sleep Apnea Study Consent

Co-INVESTIGATORS: William G. Herbert, PhD., Thomas W. Chittenden, MSc, PhD., Frank Biscardi, MD., Bruce Long, MD., Jonathan Dort, MD.

WHAT IS INFORMED CONSENT?

You are being asked to take part in a research study because you either have been accepted for elective bariatric surgery or referred for an overnight polysomnography sleep evaluation at the Carilion Clinic Sleep Center. Carilion Clinic sponsors the research. The person running this study locally is Tananchai Lucktong, MD. Before you can decide whether to take part in the research, you should be told about the possible risks and benefits with this study. This process is known as informed consent. This consent form will give you information about this study and your rights as a research subject. Please ask as many questions as you need to make sure that you know what will happen to you in this study and why you are being asked to be in it. Please take your time to make your decision about taking part. You may discuss your decision with your friends and family.

Be aware that the role of the research doctor is different from the role of your personal doctor. Your personal doctor decides how to treat your specific problem in order to help you. The research doctor treats all subjects under a specific protocol to obtain general knowledge that may or may not benefit you. Be sure to ask your doctors questions to help you know more about these different roles.

WHY IS THIS RESEARCH BEING DONE?

Bariatric surgery is an effective weight-loss treatment, yet 16% of patients undergoing surgery may suffer surgical complications. Obstructive Sleep Apnea Syndrome (OSAS), a nighttime breathing disorder, increases surgical risks. This research study will test

patients' blood for a special protein. This could allow patients to be identified who should be diagnosed and treated for OSAS to reduce surgical complications, as well as provide an additional means to help with early detection of those at risk for OSAS.

Locally there will be 18 subjects taking part in this study. The length of time you can expect to be in this research is six months following your bariatric surgery, or, if you are not a surgery patient, but coming to the study through the Carilion Sleep Center, you will only be involved so long as it takes for us to obtain your sleep study results and provide us with a blood sample.

WHAT WILL HAPPEN IN THIS RESEARCH STUDY?

You are being asked to take part in the study described below.

☐ **Carilion Clinic Sleep Center Patients**

For Carilion Clinic Sleep Center Patients: If you agree to take part and are eligible, you will be asked to schedule a visit to Carilion Consolidated Laboratory located on the 4th floor of the Roanoke Community Hospital where a qualified phlebotomist will collect a fasting blood sample from you by venipuncture. You will need to fast for 10 hours prior to coming to give this blood sample and the visit is not part of what the Sleep Center or your physician will require for your overnight sleep study.

You will be given questionnaires to determine your sleep habits, your health history, as well as your ability to perform certain tasks. Also, measurements of your neck, waist, and hip will be taken. You will have an overnight sleep evaluation at the Carilion Sleep Center. The study staff will have access to the results of your sleep evaluation in your medical record. The results of the sleep evaluation will show whether you are able to take part in this study. If your score does not meet the requirement, then you will not complete the study. If your score does meet the requirement, then you will be asked to take part in the blood drawing session (the donation will be about 8 tablespoons)

During this study, the tubes of blood you will have drawn for research purposes will be labeled with only a subject number. The blood samples will be processed at the Carilion Community Research Lab. Upon collection of the final sample from the last subject in the study, the blood samples will be transferred to Harvard University for final analysis.

Your blood drawn for this research study will not be used for any other purpose or research other than this one. Any results from the genetic portion of the study will not be

shown to anyone else outside the research group. All the blood samples obtained will be disposed of according to regulations of biohazards, and will not be held after the study is completed.

☐ **Carilion Bariatric Surgical Patients**

For Bariatric Surgical Patients: If you agree to take part, you will be asked to attend one testing session before your surgery and one testing session after your surgery. The first testing session will be included in one of your already scheduled clinic visits and the other testing session will require you to come to the clinic in addition to your already scheduled visits.

BARIATRIC SURGERY SESSION ONE:

You will be divided into one of two groups based on your Epworth Sleepiness Scale results. This will help the researchers determine who is eligible to take part in the study.

Group 1: During a routine blood draw, you will have an additional amount of blood drawn for this study. You will be sent to have an overnight sleep evaluation at the Carilion Sleep Center. The study staff will have access to the results of your sleep evaluation in your medical record. The results of the sleep evaluation will show whether you are able to take part in this study. If your score does not meet the requirement, then you will not complete the study. If your score does meet the requirement, then you will be asked to take part in session two after your surgery, as described on the next page.

Group 2: During a routine blood draw, you will have an additional amount of blood drawn for this study. You will be shown how to complete an at-home sleep test during a regularly scheduled appointment with your surgeon. One of the investigators or Virginia Tech graduate research assistants, who work with the investigators, will tell you and show you how to set-up and use an at-home sleep device. This testing session will take about 45 to 60 minutes of your time; however, you may take more time if needed. The at-home sleep test will last for one night. The length of time of the at-home sleep study will depend on how long you sleep during the night of the test.

The at-home sleep device that you will use is equipped with a strap, and a nasal cannula. You will wear the device for one entire night at home while you sleep. The device will measure your breathing activity. The at-home sleep device is a non-invasive (no needles) monitor sometimes used by sleep doctors to screen people who may need

more medical tests for possible nighttime breathing disorders. The investigators or investigators' students will make plans for you to take the device home, assist you by phone if needed to properly set it up for one night and make plans for you to return the device the next day.

For the test, you will first attach a flexible strap to chest to secure the recorder. You will also wear a string of tubing that fits around your ears and attaches to your nostrils to measure your breathing. You will return the at-home sleep device the next day to be evaluated by our sleep technician and sleep physician investigator. If you are found to have moderate to severe OSAS, you will be sent to the Carilion Sleep Clinic for further diagnostic testing and if indicated be given treatment (e.g. CPAP). Regardless of your OSAS result, you will participate in session two as described on the next page.

If your test results show you do not have sleep apnea, then you will also be asked to take part in another study looking at bone density in patients who have bariatric surgery.

SESSION TWO (POST SURGERY FOLLOW UP):

Three months after your surgery, you will be asked to repeat the at-home sleep test questionnaires. Also, during the routine blood draw that occurs at the three month follow up appointment, you will have an additional amount of blood drawn for this study. (about 8 tablespoons)

During this study, the tubes of blood you will have drawn for research purposes will be labeled with only a subject number. The blood samples will be processed at the Carilion Community Research Lab. Upon collection of the final sample from the last subject in the study, the blood samples will be transferred to Harvard University for final analysis.

Your blood drawn for this research study will not be used for any other purpose or research other than this one. Any results from the genetic portion of the study will not be shown to anyone else outside the research group. All the blood samples obtained will be disposed of according to regulations of biohazards, and will not be held after the study is completed.

WHAT ARE THE RISKS OF BEING IN THIS RESEARCH STUDY?

The investigators are not aware of any specific risks associated with the at-home sleep test.

There is minimal risk involved in blood draws. A bruise may result from blood collection procedures. You may rest for as long as needed after your blood is drawn.

WHAT ARE THE BENEFITS OF BEING IN THIS RESEARCH STUDY?

If you are a patient at the Carilion Clinic Sleep Center, participating in this research will provide no direct benefits to your sleep test or your doctor's evaluation of these results. If you are a bariatric surgery patient, there is no direct benefit of this research to your surgery. If you are a bariatric surgery patient and are not required to have a consult with a sleep physician, you will be screened for OSAS using an at-home sleep test. You will be referred to an appropriate health care professional, if necessary, based on your individual results. All costs related to follow-up appointments with these doctors, including further sleep evaluation, will be your responsibility and not the responsibility of Virginia Tech or Carilion. Although you may not personally benefit from taking part in this study, the knowledge gained may benefit others.

ARE THERE ANY OPTIONS TO BEING IN THIS RESEARCH STUDY?

You do not have to be in this research to receive bariatric surgery or, if you are doing a sleep study at Carilion Clinic Sleep enter, you do not have to be in this research to receive your sleep evaluation. You may choose to have bariatric surgery alone without taking part in the research. If you are a Sleep Center patient, you may do your sleep study without taking part in this research. Your doctors will discuss all of the choices with you.

WILL I RECEIVE NEW INFORMATION ABOUT THIS RESEARCH STUDY?

Sometimes new information comes out that may affect your health, welfare, or willingness to stay in a study. If that happens, the researchers will tell you about that information. They will also tell you about other options for your care. You may need to sign another form with your consent to continue in the study.

WHAT ABOUT CONFIDENTIALITY?

Individual subject data from this study will be kept strictly confidential. This means that all of your answers to questions that you are asked, measurement values, and blood test results will be kept confidential and shared only with you. Results will be kept in an electronic database which is password protected. A master list of subjects' code numbers will be kept in a locked filing cabinet separate from completed data, which will also be maintained in a locked filing cabinet. Paper files will be stored in a locked cabinet. All questionnaires, data collection sheets, data analysis sheets, blood collection and storage containers will be identified by code number and not by your name.

The investigators of this study and the investigators' students will be allowed access to data. During analyses and written reports of this research, the information provided will have names removed and only a three-digit code number will identify subjects. The Principal Investigator will keep a list of subjects' identifications, with corresponding coding to identify individual subject data.

HIPAA AUTHORIZATION

There is a federal law that protects the privacy of health information. This law is known as HIPAA. HIPAA stands for the "Health Insurance Portability and Accountability Act." Because of this law, your records cannot be looked at without your permission. The researchers for this study may need to look at and use information from your records. You need to give your permission for them to be able to do this. If you are giving permission for someone other than yourself, this permission applies to that person's health information.

Researchers may get new information about you from procedures, tests, surveys or interviews. In some cases, researchers may talk to your treating doctors about you.

The researchers will use the information they get about you. They may share it with others, including:

- researchers at other places who are taking part in the research
- other international, federal, state and local agencies that have authority over research
- Carilion Clinic Institutional Review Board (CC IRB), a committee that checks for quality and safety of research
- other organizations that also check for quality and safety

Some of the groups that receive information about you while you are in the research study could share it with others. If this happens, HIPAA may not apply. Other state and federal privacy regulations may protect your personal health information. However, absolute confidentiality cannot be guaranteed.

You must give your permission to access and use your health information in this study. Your permission will not expire unless you cancel it. Otherwise, your information will be used as long as it is needed for the study. You have the right to access your records about this research study but you may have to wait until the study is completed.

You may refuse to give permission to use your health information. This will not change your ability to get health care outside of the research study. If you do not give your permission to use and share your health information, you cannot be in the research study.

You may change your mind at any time and cancel your permission to use your personal health information. To cancel your permission, you must put it in writing and give it to your research doctor. However, any information the researchers got before you cancelled your permission may still be used. Also, any of your information that is being used for safety monitoring or certain other uses may still be used even after you withdraw.

WHAT WILL TAKING PART IN THIS RESEARCH STUDY COST OR PAY?

Taking part in this research study will not add any costs to your bariatric surgery or, if you are a patient at the Carilion Clinic Sleep Center there will not be any added cost to your OSAS evaluation. No procedures done just for research will be billed to you or your insurance company. You will not be paid to be in this research study.

WHAT WILL HAPPEN IF I HAVE COMPLICATIONS OR IF I AM INJURED BY THIS RESEARCH STUDY?

If you have a medical problem that happens because you are in this study, you will be able to get treatment. The treatment will be billed to you or your insurer at the usual charge. The study does not make any provisions for the payment of these costs. You will not receive any other financial compensation. However, you do not give up any legal rights to seek compensation for injury by signing this consent form.

WHAT IF I WANT TO STOP BEING IN THE STUDY BEFORE IT IS FINISHED?

Being in this research is voluntary. You may refuse to take part or you may withdraw at any time. Your decision not to take part or your decision to withdraw will not affect your ability to get care from your doctors or from Carilion Clinic. The researchers may take you out of the research study for any reason, without your consent, if they feel it is in your best interest. The reason for any exclusion will be explained to you.

ARE RESEARCHERS BEING PAID TO DO THIS STUDY?

This study is funded by a grant from Carilion Clinic to help cover basic costs involved in research. None of the investigators or research staff will receive money or other types of payment from this study.

WHO ARE THE CONTACT PERSONS?

If you encounter medical problems, complications or have any questions about the study, you may call the researcher, Tananchai Lucktong, MD, during the day at 540-342-6346 or at 540-521-4986 on evenings and weekends. If you have questions about your rights as a research subject, you may contact staff of the Carilion Clinic IRB at (540) 853-0728.

CONSENT SIGNATURES:

RESEARCH SUBJECT: The research study described in this consent form, including the risks and benefits, has been explained to me and all of my questions have been answered to my satisfaction. I consent to participate in this research study. My consent is given willingly and voluntarily. I may withdraw my consent at any time. I will receive a signed copy of this consent form.

_____

Printed Name of Research Subject

_____ _____

Subject's Signature Date

PERSON OBTAINING CONSENT: I certify I was present for the informed consent discussion and that the subject or legally authorized representative had an opportunity to ask questions about and appeared to understand the information presented and agreed to participate voluntarily in the research.

_____

Printed Name of Person Obtaining Consent

_____ _____

Signature of Person Obtaining Consent Date

APPENDIX D

SLEEP AND HEALTH QUESTIONNAIRES

BERLIN QUESTIONNAIRE

Category 1

1. Do you Snore?
- ☐ 1Yes
- ☐ 2No
- ☐ 3Don't know

If you snore

2. Your snoring is

- ☐ 1Slightly louder than talking
- ☐ 2As loud as talking
- ☐ 3Louder than talking
- ☐ 4Very loud-can be heard in adjacent rooms

3. How often do you snore?
- ☐ 1Nearly every day
- ☐ 23-4 times a week
- ☐ 31-2 times a week
- ☐ 41-2 times a month
- ☐ 5Never or nearly never

4. Has your snoring ever bothered other people?
- ☐ 1Yes
- ☐ 2No
- ☐ 3Don't know

5. Has anyone noticed that you quit breathing during your sleep
- ☐ 1Nearly every day
- ☐ 23-4 times a week
- ☐ 31-2 times a week
- ☐ 41-2 times a month
- ☐ 5Never or nearly never

6. Do you choke while you are sleeping?
- ☐ 1Nearly every day
- ☐ 23-4 times a week
- ☐ 31-2 times a week
- ☐ 41-2 times a month
- ☐ 5Never or nearly never

Category 2

1. How often do you feel tires or fatigued after you sleep?

□ 1Nearly every day
□ 23-4 times a week
□ 31-2 times a week
□ 41-2 times a month
□ 5Never or nearly never

2. During your waking time, do you feel tired, fatigued or not up to par?

□ 1Nearly every day
□ 23-4 times a week
□ 31-2 times a week
□ 41-2 times a month
□ 5Never or nearly never

3. Have you ever nodded off or fallen asleep while waiting in a line to meet your doctor?

□ 1Nearly in every visit
□ 23-4 visits
□ 31-2 visits
□ 5Never or nearly never

4. Have you ever fallen asleep while watching television at your home during the daytime?

□ 1Nearly every day
□ 23-4 times a week
□ 31-2 times a week
□ 41-2 times a month
□ 5Never or nearly never

5. Have you ever nodded off or fallen asleep while waiting in a line to pay your electricity or telephone bills?

□ 1Nearly in every visit
□ 23-4 visits
□ 31-2 visits
□ 5Never or nearly never

Category 3

Do you have high blood pressure?

□ 1Yes
□ 2No
□ 3Don't know

EPWORTH SLEEPINESS SCALE

The Epworth Sleepiness Scale is used to determine the level of daytime sleepiness.

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

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

Sitting and reading	_____
Watching TV	_____
Sitting inactive in a public place	_____
Being a passenger in a motor vehicle for an hour or more	_____
Lying down in the afternoon	_____
Sitting and talking to someone	_____
Sitting quietly after lunch (no alcohol)	_____
Stopped for a few minutes in traffic while driving	_____
Total score	_____

SLEEP HABITS EVALUATION

Do you have episodes of parasomnias (disorders such as sleep walking, sleep talking, night terrors, body rocking, bedwetting that will cause partial or full awakening?)

Yes _____ No _____

Do you show signs of sleep disturbances (such as insomnia, daytime sleepiness)?

Yes _____ No _____

Do you have difficulties falling asleep if a certain object or a certain situation is absent such as listening to the radio, watching the television, etc.?

Yes _____ No _____

Do you have difficulties falling asleep earlier or later than your usual bedtime?

Yes _____ No _____

Do you wake up at night to get a snack?

Yes _____ No _____

If “yes,” do you think that the snack helps you to go back to sleep?

Yes _____ No _____

Do you ever feel very tired or sleepy at school or work?

Yes _____ No _____

Do you have hallucinations (vivid images that look like dreams occurring when you sleep) or find yourself physically weak or paralyzed for a few seconds during sleep?

Yes _____ No _____

Tonsils and Adenoids Evaluation

Do you have a history of recurrent tonsillitis which is an inflammation of the tonsils (clusters of tissue that lie in bands on both sides of the back of the throat) caused by an infection?

Yes _____ No _____

Did you ever have inflammation of the adenoids (single clump of tissue in the back of the nose) causing a blockage of the back of the nose, chronic and recurrent fluid or infections of your ears, or chronic or recurrent sinus infections?

Yes _____ No _____

Have you had a tonsillectomy (tonsils removed) or an adenoidectomy (adenoids removed)?

Yes _____ No _____

Exercise Habits

Do you engage in regular exercise?

Yes _____ No _____

If "yes" please list:

Activity	**Frequency (times per week)**	**Duration (minutes)**
_____	_____	_____
_____	_____	_____
_____	_____	_____

Do you ever feel faint, short of breath, or chest discomfort with exertion?

Yes _____ No _____

If “yes,” please explain: _____

_____

_____

Are there any orthopedic limitations you have that may restrict your ability to exercise?

Yes ___ No ___

If “yes,” please explain: _____

_____

_____

HOME SLEEP STUDY QUESTIONNAIRE

(if ApneaLink was used)

Please answer the following questions after your sleep time

Bed Time_____ Wake Time _____

1. **The home sleep test was comfortable to sleep in?**

□ Strongly Disagree □ Disagree □ Undecided □ Agree □ Strongly Agree

2. **The home sleep test was easy to put on and remove?**

□ Strongly Disagree □ Disagree □ Undecided □ Agree □ Strongly Agree

3. **Last night my sleep was about the same as usual**

□ Strongly Disagree □ Disagree □ Undecided □ Agree □ Strongly Agree

4. **The quality of my sleep last night was**

□ Very Poor □ Poor □ Barely Acceptable □ Good □ Very Good

5. **I felt refreshed when I woke up this morning**

□ Strongly Disagree □ Disagree □ Undecided □ Agree □ Strongly Agree

SF-36™ V2 HEALTH SURVEY

This survey asks for your views about your health. This information will help you keep track of how you feel and how well you are able to do your usual activities. Answer every question by selecting the answer as indicated. If you are unsure about how to answer a question, please give the best answer you can.

---

1. In general, would you say your health is:

Excellent	Very good	Good	Fair	Poor
○	○	○	○	○

---

2. Compared to one year ago, how would you rate your health in general now?

Much better now than one year ago	Somewhat better now than one year ago	About the same as one year ago	Somewhat worse now than one year ago	Much worse now than one year ago
○	○	○	○	○

---

3. The following questions are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much?

	Yes, limited a lot	Yes, limited a little	No, not limited at all

a	Vigorous activities, such as running, lifting heavy objects, participating in strenuous sports	○	○	○
b	Moderate activities, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf	○	○	○
c	Lifting or carrying groceries	○	○	○
d	Climbing several flights of stairs	○	○	○
e	Climbing one flight of stairs	○	○	○
f	Bending, kneeling, or stooping	○	○	○
g	Walking more than a mile	○	○	○
h	Walking several hundred yards	○	○	○
i	Walking one hundred yards	○	○	○
j	Bathing or dressing yourself	○	○	○

4. During the past 4 weeks, how much of the time have you had any of the following

problems with your work or other regular daily activities as a result of your physical health?

	All of the time	Most of the time	Some of the time	A little of the time	None of the time
a Cut down on the amount of time you spent on work or other activities	○	○	○	○	○
b Accomplished less than you would like	○	○	○	○	○
c Were limited in the kind of work or other activities	○	○	○	○	○
d Had difficulty performing the work or other activities (for example, it took extra effort)	○	○	○	○	○

---

5. During the past 4 weeks, how much of the time have you had any of the following problems with your work or other regular daily activities as a result of any emotional problems (such as feeling depressed or anxious)?

	All of the time	Most of the time	Some of the time	A little of the time	None of the time
a Cut down on the amount of time you spent on work or other activities	○	○	○	○	○

b Accomplished less than you would like

c Did work or activities less carefully than usual

---

6. During the past 4 weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbors, or groups?

Not at all | Slightly | Moderately | Quite a bit | Extremely

---

7. How much bodily pain have you had during the past 4 weeks?

None | Very mild | Mild | Moderate | Severe | Very severe

8. During the past 4 weeks, how much did pain interfere with your normal work (including both work outside the home and housework)?

Not at all | A little bit | Moderately | Quite a bit | Extremely

---

9. These questions are about how you feel and how things have been with you during the past 4 weeks. For each question, please give the one answer that comes closest to the way you have been feeling.

How much of the time during the past 4 weeks...

	All of the time	Most of the time	Some of the time	A little of the time	None of the time
a Did you feel full of life?	○	○	○	○	○
b Have you been very nervous?	○	○	○	○	○
c Have you felt so down in the dumps that nothing could cheer you up?	○	○	○	○	○
d Have you felt calm and peaceful?	○	○	○	○	○
e Did you have a lot of energy?	○	○	○	○	○
f Have you felt downhearted and depressed?	○	○	○	○	○
g Did you feel worn out?	○	○	○	○	○
h Have you been happy?	○	○	○	○	○

i Did you feel tired?	○	○	○	○	○

---

10. During the past 4 weeks, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting friends, relatives, etc.)?

All of the time	Most of the time	Some of the time	A little of the time	None of the time
○	○	○	○	○

11. How TRUE or FALSE is each of the following statements for you?

	Definitely true	Mostly true	Don't know	Mostly false	Definitely false
A I seem to get sick a little easier than other people	○	○	○	○	○
B I am as healthy as anybody I know	○	○	○	○	○
C I expect my health to get worse	○	○	○	○	○
D My health is excellent	○	○	○	○	○

Thank you for completing these questions!

APPENDIX E

ANTHROPOMETRIC MEASUREMENTS

Data Collection Sheet

Screening and Measurement Data Sheet

Height _____ cm/_____ in **Weight _____ lbs. / _____ kg**

BMI _____ kg/m^2 **Resting BP _____ mmHg**

Anthropometric Protocol for VT/Carilion Research Project

A. Body Weight

1. Ask subject to remove any excess clothing, foot wear and anything from his/her pockets.
2. Zero the scale
3. Have subject step on scale, while standing with arms at sides, instruct subject to look straight ahead while relaxing the shoulders and neck.
4. Wait for a reading
5. Record weight in kg and lbs on sheet.
6. Ask subject to carefully step off the scale.

B. Height

1. Ask subject to stand with back against the wall (without shoes) facing toward you.
2. Position arm so it meets the top of the subject's head.
3. Record her height to the nearest centimeter and to nearest inches.
4. Ask subject to carefully step away from scale.

C. BMI

1. Using height and weight, subject's body mass index will be calculated using the unit's kg/m^2.

Nurses will then measure subject's waist, hip and neck circumference using a cloth tape measure and record values to the nearest centimeter. Measurements should be snug, but the tape measure should not be pushed tightly onto the skin. Each measurement will be done in succession and then repeated.

D. Neck Circumference

1. Ask subject to remove any clothing or accessories that obstruct access to the neck, i.e. collar, necklace, etc. While standing with arms at sides, instruct subject to look straight ahead while relaxing the shoulders and neck.
2. Place the tape measure around the midway of the neck, between the mid-cervical spine and the mid-anterior neck. In men with a laryngeal prominence (Adam's apple), place the tape measure just below the prominence.
3. Apply a slight tension to the tape measure; however, avoid compressing the subcutaneous adipose tissue. Record measurement to the nearest millimeter (mm).
4. Repeat steps 2-3 for second measurement. If second measurement IS within 5 mm of the original, calculate the average of the two and record. If duplicate is NOT within 5 mm of the original, obtain an additional 3rd measurement and calculate the average of all three measurements and record.
5. Any value that exceeds the difference of any other measured value by 10 mm or more should NOT be calculated into the average and should be replaced with another measurement.
   Reference: WHO

E. Waist Circumference

1. Ask subject to untie/loosen belt and pants and remove shirt. Have subject stand with feet shoulder-width apart with arms hanging on either side of the body at 30 degree angle. If this is uncomfortable, subject may cross arms on shoulders in a relaxed manner.
2. Use a pen to mark the bony landmarks of the distal points of the right and left last rib. Mark the bony landmarks of the right and left iliac crest. Mark the mid-distance between the last rib margin and the top of the iliac crest on both sides.
3. Instruct the subject to NOT contract the abdominal muscles. The measurement is taken at the end of a normal expiration.

4. Place the tape horizontally and directly on the skin with respect to both mid-distance landmarks**.** Ensure the tape measure is level around the circumference of the site. Apply a slight tension to the tape measure; however, avoid compressing the subcutaneous adipose tissue. Record measurement to the nearest mm.
5. Repeat steps 3-4 for second measurement. If second measurement IS within 5mm of the original, calculate the average of the two and record. If duplicate is not within 5mm of the original, obtain an additional 3rd measurement and calculate the average of all three measurements and record.
6. Any value that exceeds the difference of any other measured value by 10 mm or more should NOT be calculated into the average and should be replaced with another measurement.
   Reference: The Metabolic Syndrome Institute

F. Hip Circumference

1. Ask subject to raise bulky shirts and/or jackets to ensure the examiner has access to the hip region. Instruct subject to stand up straight with feet together.
2. Place the tape horizontally at the maximal circumference of the buttocks. Apply a slight tension to the tape measure. Record measurement to the nearest millimeter (mm).
3. Repeat step 2 for second measurement. If second measurement IS within 5mm of the original, calculate the average of the two and record. If duplicate is not within 5mm of the original, obtain an additional 3rd measurement and calculate the average of all three measurements and record.
4. Any value that exceeds the difference of any other measured value by 10 mm or more should NOT be calculated into the average and should be replaced with another measurement.
   Reference: ACSM Guidelines7th Edition

APPENDIX F

APNEALINK SETUP DETAILED PROCEDURES

RESMED

ApneaLink™

Patient Information

English

Intended Use

ApneaLink ™ is used for recording your respiratory nasal pressure during sleep. The device is intended for use as a screening device to determine the need for clinical diagnosis and evaluation by polysomnography based on the test score.

Caution: In the USA, federal law restricts this device to sale by or on the order of a physician.

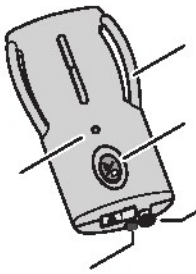
Important Notes

- ApneaLink and the accessories are to be used for the specified intended use only.
- Apply the nasal pressure cannula and the belt correctly to avoid the unlikely risk of strangulation.
- Keep all parts away from children.
- The ApneaLink breathing sensor is highly sensitive. For this reason, you should never blow directly into the nasal pressure cannula connection, and you should always screw on the vented protective cap supplied when the unit is not in use. Do not use any other cap.
- Wear ApneaLink over pajamas or nightshirts with long sleeves, in order to avoid any discomfort from the belt.
- Do not use the device if it is faulty or damaged.
- Only use the original nasal pressure cannula supplied in the original pack.
- The nasal pressure cannula must only be used once. Throw it away after use.
- Make sure that no fluids penetrate into the interior of the device.
- Cleaning and care of ApneaLink should be performed by the physician or his/her assistant.

Contraindications

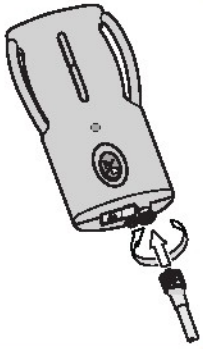
No contraindications known.

Overview



Get ready

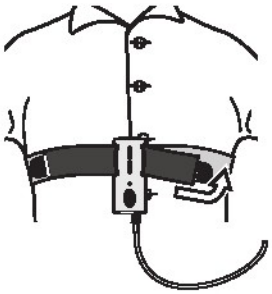
Nasal Cannula: Connect the nasal cannula to ApneaLink as shown.



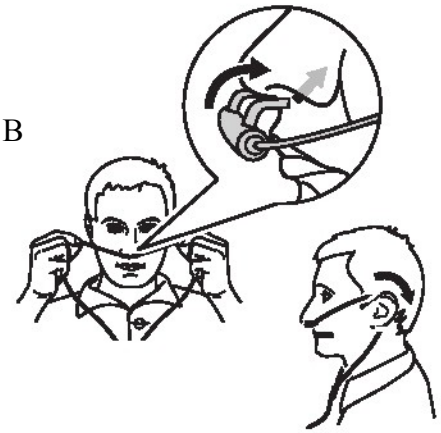
Get set

Attach the ApneaLink on the outside of your sleepwear using the adjustable belt as shown (A). Insert nasal cannula with the 2 prongs curved towards the face (B). Slide cannula adjustment ring to a comfortable position (C).

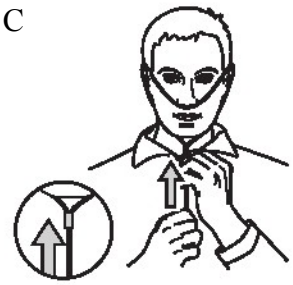
A



B

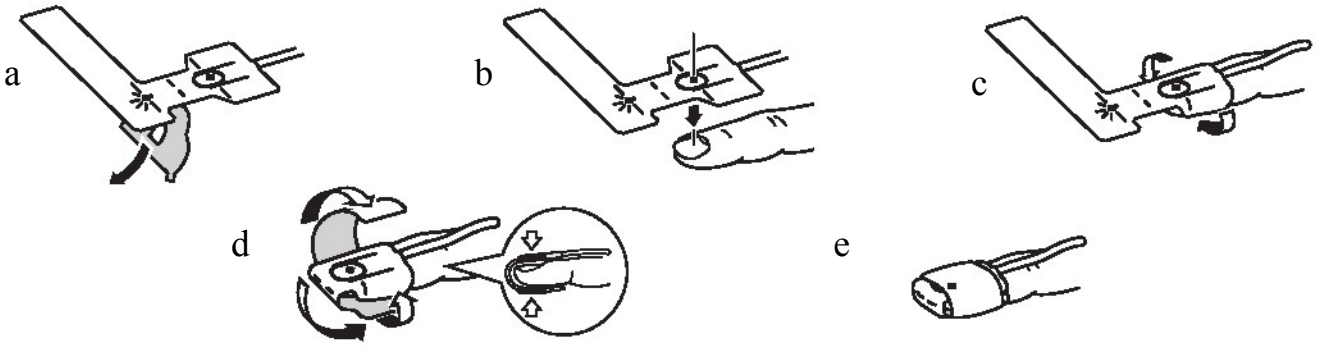


C

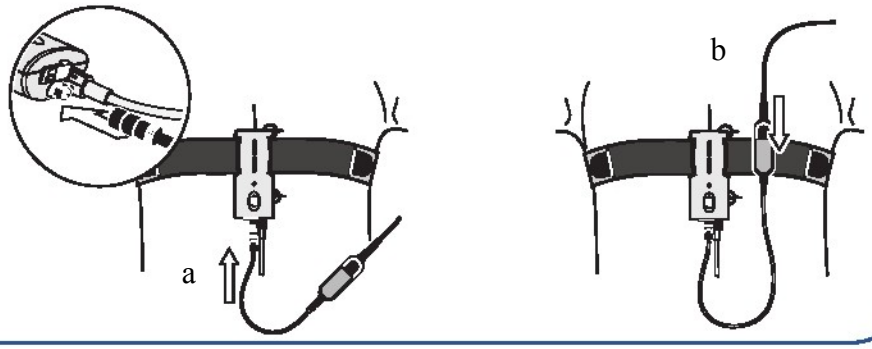


If tape or adhesive pads have been provided place them as indicated to help secure the cannula.

Finger Pulse Sensor: Attach the finger pulse sensor to a ring finger as shown (if provided).



Oximeter: Attach cable to the ApneaLink belt with the clip provided.



Go

START/STOP button: Press and hold the START/ STOP button until the green light **comes on**.

After the unit is on the small green light will flash occasionally. The light will dim after 10 minutes but ApneaLink continues to record.

Do not turn off your ApneaLink during the night.

Good morning

START/STOP button: Press START/STOP button 3 times in succession to turn ApneaLink OFF. The small green light will illuminate and turn off.

Return the ApneaLink, belt, finger pulse sensor, oximeter and clip fastener to your provider.

Cleaning the ApneaLink

Caution:

- Never use abrasive agents, alcohol, chlorine-containing substances, acetone, or other solvents to clean the recorder.
- Do not immerse the recorder, the pulse oximeter, or the finger pulse sensor in fluids, and ensure that no fluids penetrate into the products.
- Do not attempt to disinfect or sterilize the recorder, since this could cause unseen damage to the inside of the unit.

Cleaning instructions:

- Switch off the recorder.
- Remove and dispose of the nasal pressure cannula.
- Screw the ventilated cap onto the nasal pressure cannula connection.
- Detach the pulse oximeter from the recorder.
- Detach the finger pulse sensor from the pulse oximeter.

Note: Dispose of the single-use finger pulse sensor. If you are using other approved finger pulse sensors, follow the manufacturer's cleaning instructions.

- Press the protective cover for the USB cable in the housing opening.
- Clean the following parts with a damp cloth and a mild liquid soap: recorder and pulse oximeter housing, pulse oximeter cable, clip fastener.
- Leaved the cleaned parts to dry.

RESMED

ApneaLink - Report of 8/4/2009 5:44 PM

Treating physician

Referral to

Patient data

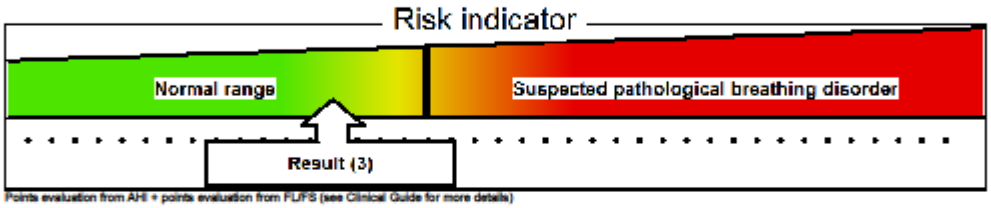
First name:	Mary Beth	Patient ID:	031
Name:	Kochie	DOB:	8/2/1975
Street:		Size:	5 ft 4 in
City, ST, Zip:		Weight:	222.96 lbs
Phone:		BMI:	38 kg/m²

Recording

Date:	7/24/2009
Start:	10:24 PM .
End:	5:10 PM .
Duration:	18 h 46 min

Evaluation

Start:	10:34 PM .
End:	12:11 PM .
Duration:	5 h 39 min



Points evaluation from AHI + points evaluation from FL/FS (see Clinical Guide for more details)

Analysis (Flow evaluation period: 5 h 39 min)

Indices		Normal	Result	
AHI*:	1	< 5 / h	Average breaths per minute [bpm]:	17.85
RI*:	3	< 5	Breaths:	6055
Apnea Index:	0	< 5 / h	Apneas:	2
Hypopnea Index:	0	< 5 / h	Hypopneas:	2
% Flow lim. Br. without Sn (FL):	33	< Approx. 60	Flow lim. Br. without Sn (FL):	2026
% Flow lim. Br. with Sn (FS):	0	< Approx. 40	Flow lim. Br. with Sn (FS):	1
			Snoring events:	139

Proportion of probable CS epochs: 0 0%

Analysis status: Analyzed automatically (Hypopneas based on flow only)

Analysis parameters used (Default)

Apnea [20%; 10s; 80s; 1.0s]; Hypopnea [50%; 10s; 100s; 1.0s]; Snoring [6.0%; 0.3s; 3.5s; 0.5s]; CSR [0.50]

Comments

* See Clinical Guide for abbreviations and ResMed standard parameters Firmware version: 03.4900000 Software version: 8.00

Figure 1: Sample of ApneaLink Report

APPENDIX G

BLOOD COLLECTION – DETAILED PROCEDURES

Important Pre-procedure Notes:

*** Tubes should be inverted ~8-10 times immediately after drawn before being transported!**

*All procedures using blood should be conducted in the Biosafety hood and blood should be treated as infectious (i.e., standard precautions in effect).

*All tubes should be sprayed with ethanol prior to entry into the hood. Tubes should be opened only inside the hood using multiple, folded kimwipes to absorb any blood from the top of the tube as the stopper is removed as this is a splatter hazard.

***Ensure LABELS have three identifiers: 1) patient ID code, 2) date, and 3) visit designation)**

***Consider the volumes you will need for additional testing when aliquotting serum and plasma to cryovials. Base volumes per tube on amounts required for these later assays; a maximum of 3 freeze/thaw cycles is generally acceptable for general downstream applications such as ELISAs.**

*** If processing must be delayed once blood is brought to the lab, place tubes on tube roller (back bench) at RT for up to 2 hours.**

*Use the pipet-aid on SLOW or MED speed for processing until you are comfortable with the control.

***Note: This procedure is to be done *individually* for each study participant although multiple samples may be processed simultaneously. Special care must be taken to record specimen ID, date and visit designation on each vessel used. Work carefully and label well to ensure that samples from two patients are not mixed at any point during the procedure.**

Procedure for K_2EDTA (purple top tube):

1. Balance tubes (opposite buckets) and spin tubes for 10 minutes at RT at 1200 x g in the (tissue culture) Legend RT+ benchtop centrifuge. [Program #1 on Legend cfg but ADD 5 more minutes]

2. Label appropriate number of cryovials with the **specimen/patient code ID and other identifiers and "EDTA-P" for plasma.** Repeat labeling for <u>each</u> unique patient ID. (note volume of plasma on the vial where warranted for convenience when thawing for subsequent use).

3. Carefully remove the tubes from the centrifuge, so as not to disturb the gel barrier, and place them in the hood.

4. **A. If more than one EDTA tube has been drawn per patient**, pool the plasma (yellow-ish, top layer above gel in tubes) from **all** tubes into a sterile 15 ml (or 50 ml) conical tube and mix gently, by pipetting up and down, to ensure homogeneity of plasma before aliquotting to freeze.

   B. Aliquot 500-1000 μl of EDTA plasma (top layer above gel) **into pre-labeled cryovials (**from step2) using 1 ml micropipettor and sterile tips. [As long as the tip remains sterile, you may use the same tip for all aliquots of **one** patient. Change tips if it touches anything besides the plasma/tubes OR when you begin a new patient sample.]

5. Place the serum vials in the designated box in the -80°C freezer. **Record the location of the vials on a box map , with complete identifiers, so that they may be entered into Cryotrack software by the lab staff.**

<u>Procedure for SST (tiger top tube):</u> [see "Tech Talk" Vol. 4, No. 2; Nov 2005 for additional info]

***Note: this tube should be inverted ~5 times immediately after drawn before being placed upright to clot!**

1. Allow the tube to clot for up to 30 minutes undisturbed, vertically at room temperature (RT) (in a rack on the bench).

2. Once clot has formed, spin tubes for 10 minutes at RT at 1200 x g in the (tissue culture) Legend RT+ benchtop centrifuge. [Program #1 on Legend cfg but ADD 5 more minutes]

3. Label appropriate number of cryovials with the **specimen/patient code ID and other identifiers** and **the letter "S" for serum.** Repeat labeling for each unique patient ID. (note volume of serum on the vial where warranted for convenience when thawing for subsequent use).

4. Carefully remove the tubes from the centrifuge, so as not to disturb the gel barrier, and place them in the hood.

5. Aliquot 500-1000 μl of serum (top layer above gel) **into pre-labeled cryovials (**from step3) using 1 ml micropipettor and sterile tips.

6. Place the serum vials in the designated box in the -80 °C freezer. **Record the location of the vials on a box map , with complete identifiers, so that they may be entered into Cryotrack software by the lab staff.**

APPENDIX H

MONOCYTE ISOLATION AND STORAGE – DETAILED PROCEDURES

Important Pre-procedure Notes:

***Bring all media and solutions to ROOM TEMP (RT)- if indicated- before you begin working**

***Set up a bleach bucket in the hood for neutralization of blood**

*All procedures using blood should be conducted in the Biosafety hood and blood should be treated as infectious (i.e., standard precautions in effect).

*All tubes should be sprayed with ethanol prior to entry into the hood. Tubes should be opened only inside the hood using multiple, folded kimwipes to absorb any blood from the top of the tube as the stopper is removed as this is a splatter hazard.

***Ensure LABELS have three identifiers: 1) patient ID code, 2) date, and 3) visit designation)**

***Consider the volumes you will need for additional testing when aliquotting serum and plasma to cryovials. Base volumes per tube on amounts required for these later assays; a maximum of 3 freeze/thaw cycles is generally acceptable for general downstream applications such as ELISAs.**

*** If processing must be delayed once blood is brought to the lab, place tubes on tube roller (back bench) at RT for up to 4-6 hours.**

*Use the pipet-aid on SLOW or MED speed for processing until you are comfortable with the control.

***Note: This procedure is to be done *individually* for each study participant although multiple samples may be processed simultaneously. Special care must be taken to record specimen ID, date and visit designation on each vessel used. Work carefully and label well to ensure that samples from two patients are not mixed at any point during the procedure.**

Procedure for processing Heparin tubes (green top tubes):

1. Empty green top tubes into a 100 ml sterile screw top receiver/bottle (or 50 ml conical) depending on volume drawn [for volumes ≥40 ml total, blood should be placed into a sterile100 ml receiver to mix]. The blood should be swirled **gently** in

the bottle or tube so as to homogenize the blood from all tubes. Do not create bubbles! The receiver/bottle should not be inverted.

2. Divide homogeneous blood from bottle into 50 ml conicals using a serological pipet [to conveniently balance the tubes, split the volume evenly into 2 tubes if possible]

3. Spin the 50 ml tubes at 400 x g for 5 minutes to separate plasma. [Program #2 on Legend cfg]

4. Transfer plasma from each 50ml conical to a 15 ml tube and spin at 1200 x g for 5 minutes. [Program #1 on Legend cfg]. (This will be aliquotted into vials in step 9 below).

5. Label appropriate number of cryovials with the **specimen/patient ID and other identifiers and the letter "P" for Plasma** for each patient ID. (note volume of plasma on the vial where warranted for convenience when thawing for subsequent use).

6. Resuspend the remaining blood components from **each** 50 ml conical in HBSS to bring the volume up to 35 ml total.

7. Slowly layer 13 ml of Ficoll underneath the blood/HBSS mixtures by placing the tip of the pipet containing Ficoll at the bottom of the 50ml tubes then carefully remove the pipet-aid from the top, leaving the pipet in the tubes. Take care not to mix layers! The Ficoll will slowly drip from the pipet and form a layer under the blood. When the meniscus hits the top level of the blood, you will need to **slowly** lift out the pipet and drag it up the back of the 50 ml tube. Securely cap tube and carefully transfer the tube to the centrifuge.

8. Centrifuge for 35 minutes, 400 x g, 18-20 °C [Program #3 on Legend cfg; NOTE very slow deceleration!].

9. During the long spin, transfer clarified plasma from step 4 to pre-labeled ('plasma') cryovials.

10. Place the plasma vials in the designated box in the -80 °C freezer. **Record the location of the vials, with complete identifiers, so that they may be entered into Cryotrack software by the lab staff.**

Continue to step 11 with the Ficoll gradient.

When the 35 minute spin is complete, use a sterile pipet to remove the upper layer that contains excess HBSS and platelets and discard it into the bleach bucket (see Figure 1 below). Do this for each tube.

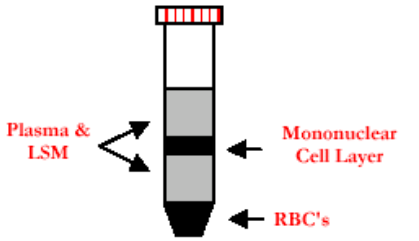


Figure 1. Separation of mononuclear cells from whole blood.

11. Using a new sterile pipet, transfer the mononuclear cell layer or "buffy coat" (see Fig.1) to 2 new 50 ml conical tubes. Make sure to get as much of the layer as possible to increase the cell yield. [Note: Use a 10 ml serological pipet to take off most of the cell layer and then use a micropipettor (200 μl or 1 ml works best for this) to get the last bit by tilting the tube to the side/angle]. Carefully discard the lower two layers from all tubes in the bleach bucket after all cell layers are removed.

12. Add HBSS to the new conical tubes to bring total volume of the cell layer to 50 ml. (Note: you are washing the cells in this step to remove all traces of Ficoll since it is toxic if left on cells; this should be done in a timely fashion). The buffy coat may be approximately 12 ml from a 70 ml blood draw.

13. Centrifuge the cells for 10 minutes, 400 x g, 18-20 °C [Program #4 on Legend cfg].

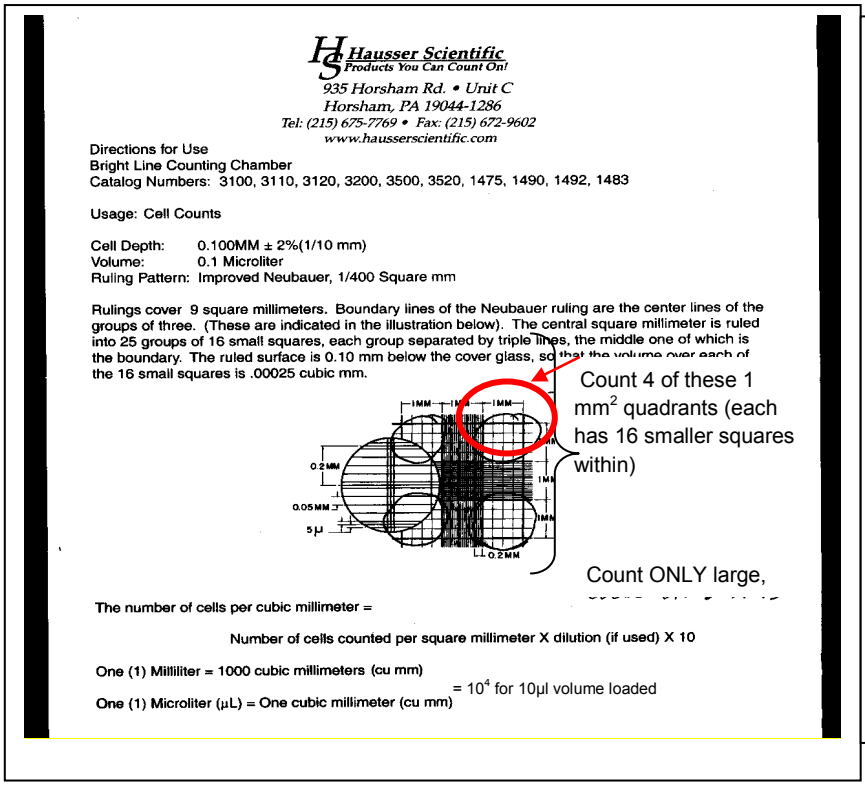
14. Observe pellet at bottom of tube. Using a 10 ml serological pipet, discard the supernatant from each tube into the bleach bucket. Stay above the pellet with your pipet so you don't disrupt it as you draw off the sup. *Maximizing cell number is crucial to the success of the isolation.

15. Resuspend and combine the cell pellets of the two 50 ml conicals into a 15 mL conical in 5 ml **total** of HBSS. It is recommended this be carried out as follows: Suspend one of the pellets in 3 mL of HBSS- mix well by pipetting slowly up and down a few times (avoid creating bubbles which put oxidative stress on the cells). Add this 3 ml to the tube containing the second pellet. To recover the maximum

number of cells, use a 1 ml micropipettor and tip to rinse the 50 mL conical (that you just removed the pellet from) with 1 mL of HBSS. Again, add this 1 ml rinse volume to the second pellet. *Make sure to deliver all the visible liquid/cells out of the first conical before discarding; you may need to use a smaller volume micropipettor and tip. Resuspend the second pellet in the 4 mL transferred into that tube by pipetting slowly to mix and transfer the entire volume to the new 15 mL conical. Follow by rinsing the second 50 mL conical with an additional 1 mL of HBSS using a 1 ml micropipettor and tip. Transfer this additional 1 ml of cells to the 15 mL conical for a final cell volume of 5 mL. NOTE: Careful transfer of these cells volumes using appropriate sized pipettors will improve PBMC and subsequent monocyte yields.

16. NOTE: From this point forward, cell solution should be placed on ice or in a chill rack to maintain viability and limit transcription. Ensure the cell solution is homogeneous and remove a 10 μl aliquot from the 5 ml PBMC suspension for cell counting (see step 18 below). Then, fill conical up to 15 ml with HBSS and place back on ice/in chiller rack**. Do not start the spin in #19 until you have counted cells because if you are delayed, the pellet may become dislodged while it sits with supernatant on top and you will lose cells.**

17. **Manual cell count under inverted microscope**: Add 30 µl of Trypan blue stain to 10 µl of cells and mix well by pipetting up and down (easily mixed in the counting plate well). Load 10 µl of these stained cells into the hemocytometer. Using the 10X objective (with 10X oculars; total 100X magnif.) on the inverted scope, count the total number of cells in the 'quadrant' (1 big box= 16 small boxes). Count all four quadrants and take the average. Multiply the average number of cells by the dilution factor of trypan blue (4 if you follow the 30 µl into 10 µl method above), then multiply by the hemocytometer dilution factor of 10^4. This will give you the number of **cells per ml**. Multiply this number by **the total volume** of the cell mixture you took the sample from (in this case = 5ml) and this gives you total number of cells. (See hemocytometer grid and equation at right). Record cell counts and equations.



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Directions for Use
Bright Line Counting Chamber
Catalog Numbers: 3100, 3110, 3120, 3200, 3500, 3520, 1475, 1490, 1492, 1483

Usage: Cell Counts

Cell Depth: 0.100MM ± 2%(1/10 mm)
Volume: 0.1 Microliter
Ruling Pattern: Improved Neubauer, 1/400 Square mm

Rulings cover 9 square millimeters. Boundary lines of the Neubauer ruling are the center lines of the groups of three. (These are indicated in the illustration below). The central square millimeter is ruled into 25 groups of 16 small squares, each group separated by triple lines, the middle one of which is the boundary. The ruled surface is 0.10 mm below the cover glass, so that the volume over each of the 16 small squares is .00025 cubic mm.

Count 4 of these 1 mm^2 quadrants (each has 16 smaller squares within)

Count ONLY large,

The number of cells per cubic millimeter =

Number of cells counted per square millimeter X dilution (if used) X 10

One (1) Milliliter = 1000 cubic millimeters (cu mm)
One (1) Microliter (µL) = One cubic millimeter (cu mm)

= 10^4 for 10µl volume loaded

Trypan Blue (TB) exclusion principle:

Nonviable cells, with a comprised membrane, will take up the dye; whereas live cells with an intact membrane will exclude the dye. Blue cells= dead; clear/"halo" cells = live.

Equations:

1) $\frac{\text{Cells counted in 4 quads}}{4}$ = average cell #

2) ave cell # **X** dilution in TB* **X** 10^4 = $\frac{\text{cells}}{\text{ml}}$

*[for dilution in TB it is most useful to use a 1:3 or 1:1 ratio; whereby: 10µl to 30µl TB or a 1:3 ratio = a dilution factor of "4", or 10µl to 10µl TB or a 1:1 ratio = a dilution factor of "2"]

18. Centrifuge cells for 10 minutes, 400 x g, 4 °C [Program #4 on Legend cfg but change temp]. (Temp. will not be at 4 °C during the spin, but the cfg should begin cooling to this temperature for future spins).

19. Again, observe pellet at bottom of tube. Using a 5 ml serological pipet, carefully decant the supernatant from the pellet and discard the sup into the bleach bucket. Remember to stay above the pellet with your pipet! Pull off remaining sup if needed with a micropipettor and tip. Then, briefly invert conical on doubled KimWipe to drain off final HBSS before MACS.

20. Based on your total cell number from your cell count, **resuspend** the pellet in a FACS tube using MACS buffer **at a final concentration of 1e7 cells per 30 µl of MACS buffer.** This will be a very thick suspension! Pipet up and down with the micropipette slowly to suspend.

21. Make the following calculations before opening Monocyte Isolation Kit II reagents. Keep these reagents on ice the entire time you are working with them.

Add your cell # here:	8.00E+07			
Volume per	standard unit	Component	Amount for your cell #:	
30	1.00E+07	MACS buffer	240.0	
10	1.00E+07	FcR blocking reagent	80.0	
10	1.00E+07	BIOTIN Antibody Cocktail	80.0	
30	1.00E+07	MACS buffer	240.0	
20	1.00E+07	anti-BIOTIN MicroBeads	160.0	

22. Add **10 µl of FcR Blocking Reagent per 1e7 cells** and **10 µl per 1e7 cells of Biotin-Antibody Cocktail**. Mix well by pipetting up and down slowly and incubate for **10 minutes at 4 °C**, using either ice or the Chill racks.

23. Refer to the calculations table: Add **30 µl per 1e7 cells of MACS buffer** and **20 µl per 1e7 cells of Anti-Biotin MicroBeads**. Mix well and incubate for an additional **15 minutes at 4 °C**.

24. Wash cells with buffer by adding 2 mL of MACS buffer and centrifuge at 300 xg for 10 minutes at 4 °C. Pipette off supernatant completely and resuspend cells in 500 µl of MACS buffer (regardless of cell count, unless it is over 10^8 cells, if so then scale up buffer accordingly).

25. Take the resuspended cells in the FACS tube and place it in "1-A" slot of the Chill5 rack. Place two new empty FACS tubes in 1-B (negative fraction) and 1-C (positive fraction) for the eluted volumes. See the AutoMACS Chiller rack diagrams posted at the MACS workstation.

26. On the AutoMACS Pro command screen, at the top menu press "**Separation**". Then press on the gray shaded area below that with **two "/" (backslashes)** and you will then have a selection area for what cell isolation method you would like to use, select "**depleteS**". Beside the selection method is your Rinse method, select **Sleep** if only 1 separation will be performed that day. [The AutoMACS should be turned off (power switch on right side) after it performs its shutdown procedure following the separation (you will be prompted to do so].

27. At the end of the AutoMACS program, collect cells/tubes from the POSITIVE and NEGATIVE ports (See the AutoMACS Chiller rack diagrams posted at the MACS workstation with positions of tubes to capture the correct eluates). **IT IS IMPERATIVE HERE THAT THESE TUBES BE WELL LABELLED SO THAT YOU KNOW WHICH TUBE CONTAINS THE POPULATION OF MONOCYTES!**

28. Count cells. **First count the non-selected/non-tagged population of MONOCYTES**. Use the minimum number/volume of cell suspension for counts (i.e., not more than 10 μl to mix with TB). The monocytes are now in 2.5 mL of MACS buffer - in the FACS tube you collected the eluate in.

29. Once cell number for all MONOCYTES in suspension is ascertained, pellet the cells by centrifugation for 10 minutes, 400 x g, 4 °C in the FACS tube.

30. Carefully remove MACS buffer from MONOCYTE pellet and add sterile PBS to the pellet (do this in the hood) so that the cells are at a **final concentration of 1e7 cells/ml**; pipetting slowly up and down with a 1000 μl tip to mix. **Note: this will be equal to 1e6 cells/ 100μl (if cell # is 1e7, one ml of PBS would be added here).**

31. Once cells are thoroughly resuspended in PBS, add **10 volumes of RNAlater** and pipet again to mix well with cell solution. This 10 volumes will be in reference to the amount of PBS added to resuspend the pellet—(if you added 0.5 ml PBS- you will add 5.0 ml RNAlater here). **Note:** The entire solution will need to be moved to a screw cap15 ml conical tube at this step. To do this most effectively, add 1 mL of RNAlater to the PBS monocyte suspension (in FACS tube), pipette up and down a few times and then transfer that entire suspension into the 15 mL conical. Rinsing the FACS tube here with a mL of RNAlater will improve monocyte transfer and therefore recovery. After tube is rinsed once, add the remaining volume of RNAlater (to make 10 volumes total RNAlater) and mix by pipetting.

32. Place the 15 ml conical containing monocytes in RNAlater at 4°C (pre-amp refrigerator) in a rack for overnight incubation to allow RNAlater to properly penetrate the cells for RNA preservation (as instructed by Ambion).

33. The following day, the monocyte cell solution should be mixed gently and divided between **well-labeled** Eppendorf BioPur 1.5 ml microcentrifuge tubes with locking caps/lids. Divide cells such that one tube gets **approx. 1e6 monocytes** to send to the lab at Harvard and at least two other tubes get the remainder of the cells (these are "back-ups" which will stay at -80˚ C in case Tom needs additional stocks). Additional tubes may be required depending on the total volume of RNAlater + monocytes. Spin these tubes at 5000 x g for 10 minutes at 7°C in the microfuge to pellet cells. **Note:** Pellets are very difficult to see and somewhat loose at this step! Carefully draw off the supernatant- staying away from the pellet- with a 1 mL pipette tip; use a smaller size tip to get close to the pellet. Keep all tubes in chiller rack when not in use.

34. Once all supernatants have been removed from all tubes, use parafilm to seal the lids of the tubes and insure tubes are well-labeled with cell numbers as they may be different per tube. All tubes containing the monocyte pellets, which have undergone RNAlater treatment, should now be placed at -80˚ C (Rack 1 Box 4- "Monocytes for Tom" box).

35. This step is least important and should not take place until monocytes are fully taken care of:

    Count cells from the selected (non-monocyte) population to freeze down for future use. This tube of cells should be kept in the chiller rack/on ice until this step. Once cell number for all non-monocyte PBMCs is ascertained, pellet the cells by centrifugation for 10 minutes, 400 x g, 4 °C. Carefully remove MACS buffer from pellet and resuspend in cell freezing medium (45 ml HI FBS + 5 ml DMSO) at a concentration of **1e7 cells/ml**; adding ≤1ml volume into each well-labeled cryovial. **These need to go into a MR. FROSTY overnight at -80˚ C. The following day, these cryovials should be placed into LN2 (VAPOR PHASE; rack 1 box 3)**.

APPENDIX I

RNA EXTRACTION – DETAILED PROCEDURES AND RESULTS

1. Harvest cells and pellet 1 x 10^6 in a centrifuge tube. Carefully remove aspiration
2. Disrupt the cells by adding Buffer RLT - 350µl Buffer RLT pipet to mix
3. Homogenize the lysate by pipetting the lysate directly into a QAIshredder spin column placed in a 2 ml collection tub and centrifuge for 2 min at full speed
4. Add 1 volume of 70% ethanol to the homogenized lysate, and mix well by pipetting
5. Transfer 700 µl of each sample to an RNeasy spin column placed in a 2 ml collection tube. Close lid gently and centrifuge for 15 sec ≥ 8000 x g. Discard the flow-through
6. Add 350 µl Buffer RW1 to the RNeasy spin column. Close lid gently and centrifuge for 15 sec ≥ 8000 x g to wash the spin column membrane. Discard the flow-through
7. Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube and centrifuge briefly to collect residual liquid from the sides of the tube
8. Add the DNase I incubation mix (80 µl) directly to the RNeasy spin column membrane and place on benchtop for 15 mins.
9. Add 350 µl Buffer RW1 to the RNeasy spin column. Close lid gently and centrifuge for 15 sec ≥ 8000 x g to wash the spin column membrane. Discard the flow-through.
10. Add 500 µl Buffer RPE to each RNeasy spin column Close lid gently and centrifuge for 15 sec ≥ 8000 x g to wash the spin column membrane. Discard the flow-through.
11. Add 500 µl Buffer RPE to each RNeasy spin column Close lid gently and centrifuge for 2 min ≥ 8000 x g to wash the spin column membrane.
12. Place the RNeasy spin column in a new 2 ml collection tube and discard the old collection tube with the flow-through. Close lid gently and centrifuge for 1 min at full speed
13. Place the RNeasy spin column in a new 1.5 ml collection tube Add 40 µl RNase-free water directly to the spin column membrane. Close the lid gently and centrifuge for 1 min ≥ 8000 x g to elute the RNA
14. Verify purity and concentration using Experion and Nanodrop

Table 1: RNA Extraction Concentration and Purity

Subject ID	Total Amount (uL)	Experion Ratio (18S/28S)	Experion [ ] (ng/ul)	Total RNA (ng)	NanoDrop Ratio (260/280)
NG008	**60**	1.36	35.04	2102.4	2.05
NG028	**50**	1.19	34.81	1740.5	1.98
NG020	**80**	1.38	43.48	3478.4	2.09
OG083	**60**	1.59	39.41	2364.6	1.99
OG085	**80**	1.61	27.64	2211.2	2.07
NG015	**80**	1.45	29.78	2382.4	2.02
NG029	**50**	1.56	33.72	1686	2.06
NG013	**50**	1.64	73.26	3663	2.05
NG016	**50**	1.69	53.03	2651.5	2.1
NG017	**50**	1.62	41.87	2093.5	2.04
OG080	**50**	1.59	60.52	3026	2.06
OG081	**50**	1.52	53.98	2699	2.1
OG084	**50**	1.48	20.31	1015.5	2.04

NG = non OSAS; OG = OSAS;

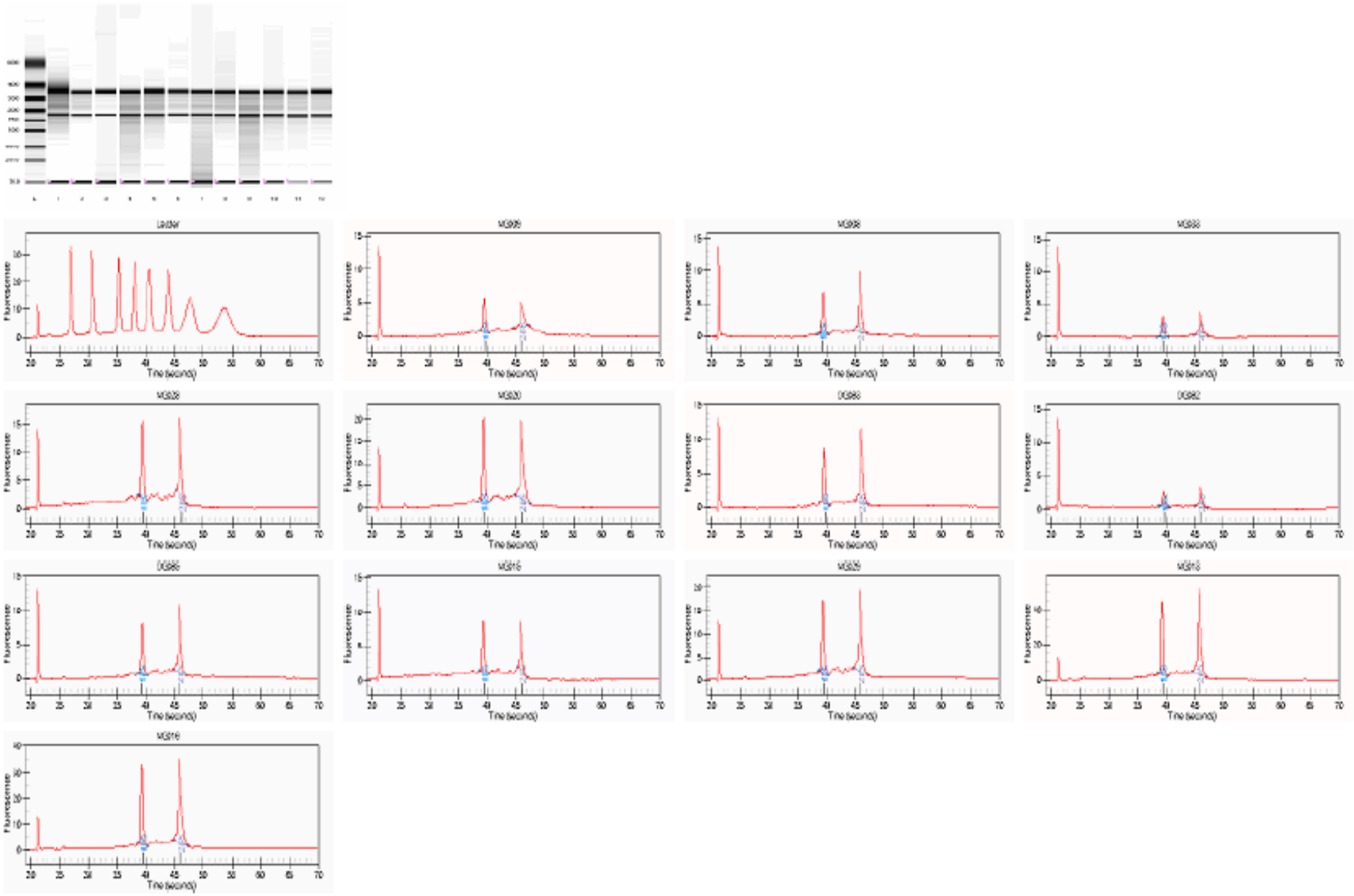


Figure 1: Eukaryote Total RNA Summary report from Experion, two peaks located at 18S and 28S to assess concentration and purity

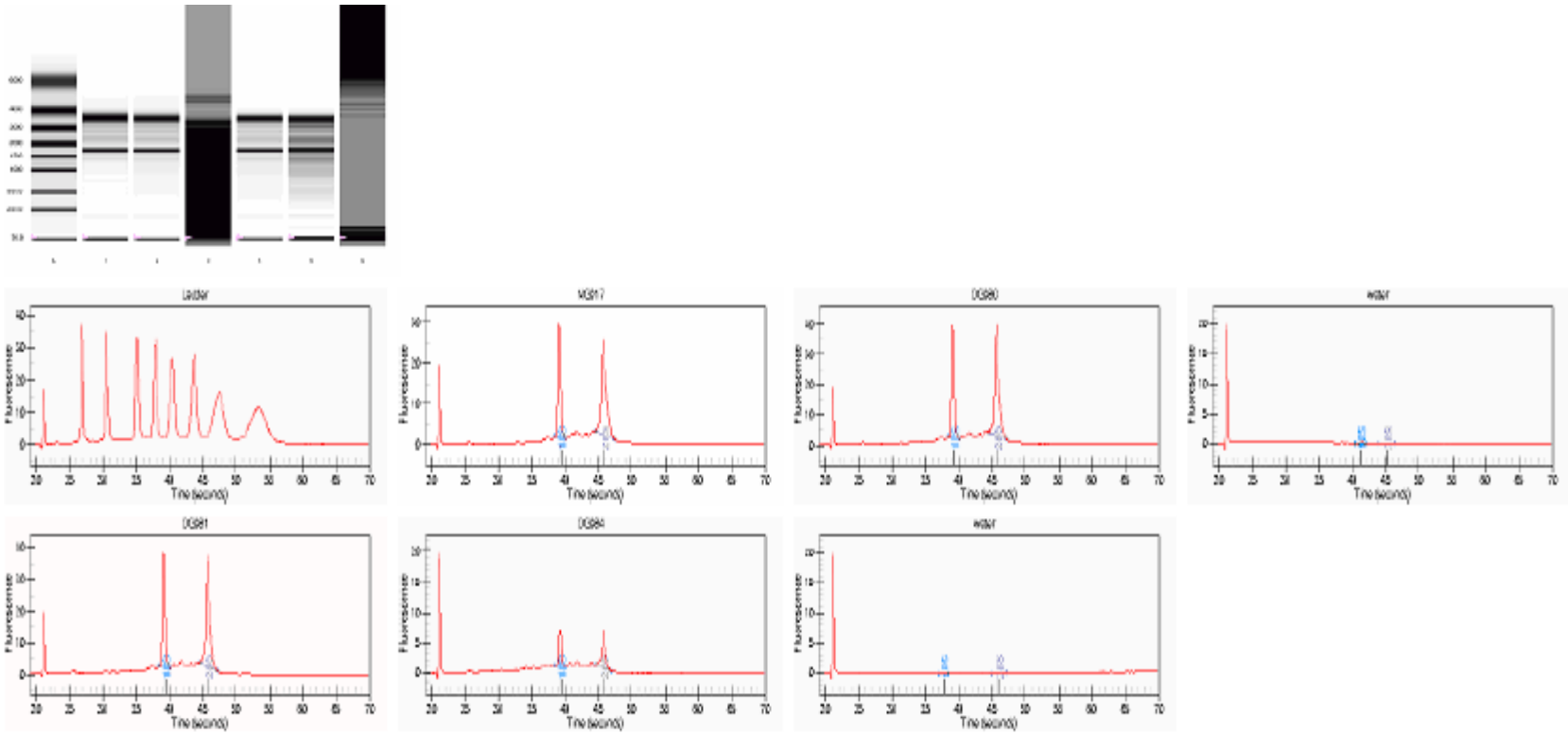


Figure 2: Eukaryote Total RNA Continued Summary report from Experion, two peaks located at 18S and 28S to assess concentration and purity

APPENDIX J

LAMININ EIA – DETAILED PROCEDURES AND RESULTS

Preparation of solutions

Solution 1. Antibody-POD Conjugate Solution
Dissolve the contents of Vial 2 in 11 ml of distilled water and mix gently followed by 10 min slowly rolling or occasional mixing, avoiding foam formation.

Solution 2. Standard Solution
Rehydrate Standard (Vial 3) with 1 ml of distilled water. Slowly roll for approximately 10 min or let vials stand and sporadically mix gently.

The Standard Solution contains 320 ng human Laminin/ml. Prepare dilution series of 160, 80, 40, 20, 10 and 5 ng/ml by diluting the Standard Solution with Sample Diluent (Vial 4).

Procedure
1. Prepare all reagents as directed in the Package Insert.
2. Bring all reagents to room temperature.
3. Add 100 μl of Standard or sample to appropriate wells, and incubate 1 hour at 25°C.
4. Remove Standard or sample and wash the wells 3 times with 400 μl of PBS + 0.1% Tween 20.
5. Add 100 μl of Antibody-POD Conjugate Solution into wells and incubate at 25°C for 1 hour.
6. Remove the solution from wells. Wash the wells 4 times with 400 μl of PBS + 0.1% Tween 20, aspirating thoroughly between washes.
7. Add 100 μl of Substrate Solution to each well. Incubate 15 minutes at room temperature.
8. Add 100 μl of Stop Solution to each well. Mix gently.
9. Read at 450 nm as soon as possible.

Results
1. Standard curve
- Record the absorbance at 450 nm for each standard well.
- Average the duplicate values and record the averages.
- Plot the absorbance (vertical axis) versus the LN concentration in ng/ml (horizontal axis) for the standards.

2. Samples
- Record the absorbance at 450 nm for each sample well.
- Average the duplicate values and record the averages.
- Locate the average absorbance value on the vertical axis and follow a horizontal line intersecting the standard curve. At the point of intersection, read the LN concentration (ng/ml) from the horizontal axis.
http://catalog.takara-bio.co.jp/en/PDFFiles/MK107_e.pdf

Table 1: Results of Laminin EIA

Specimen ID	**ABS**	**X-INT**	**Laminin (ng/ml)**
NG-008	0.241	52.98	423.81
NG-009	0.224	48.81	390.48
NG-013	0.240	52.62	420.95
NG-015	0.240	52.74	421.90
NG-016	0.267	59.17	473.33
NG-017	0.230	50.36	402.86
NG-020	0.202	43.57	348.57
NG-028	0.324	72.62	580.95
NG-029	0.262	57.98	463.81
NG-033	0.352	79.29	634.29
OG-080	0.263	58.21	465.71
OG-081	0.336	75.48	603.81
OG-082	0.241	52.86	422.86
OG-083	0.234	51.19	409.52
OG-084	0.283	62.98	503.81
OG-085	0.183	39.17	313.33

NG = non OSAS; OG = OSAS; ABS = absorbance at 450 nm; X-INT = X intercept, (sample ABS – intercept / slope)

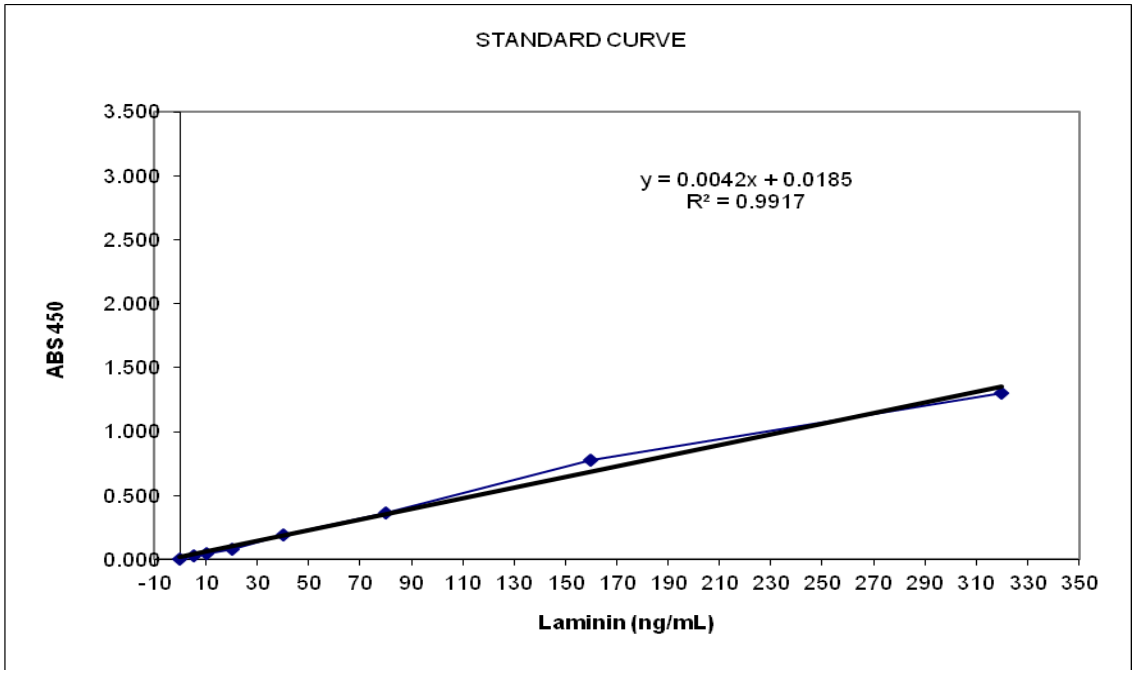


Figure 1: Standard Curve for Laminin Assay

APPENDIX K

CELL ADHESION ASSAY – DETAILED PROCEDURES AND RESULTS

Procedure

1. Coat tissue-culture wells by overnight incubation at 4 degrees C with various concentrations of 400uL purified matrix proteins: Collagen (1.5ug/mL); Vitronectin (0.5ug/mL); Fibronectin (5ug/mL); and Gelatin (0.5%){Currently we are using 10ug/mL for all matrix proteins}.
2. Aspirate protein solutions and saturate wells with 400uL of 1% BSA in PBS for 1 hour at 37 degrees C
3. Wash cells x 2 with PBS
4. Detach cells, at confluence on 10cm culture dish (~$4x10^6$ cells), by 5mM EDTA treatment in PBS (2mL) for 10mins at RT
5. Add 8mL of serum-free tissue-culture medium
6. Transfer cells to 50mL conical centrifuge tubes
7. Centrifuge for 4mins, 1000rpm at RT
8. Resuspend cells in 10mL serum-free tissue-culture medium
9. Centrifuge for 4mins, 1000rpm at RT
10. Resuspend cells in 10mL serum-free tissue-culture medium
11. Aliquot 50uL of cell solution and stain with equal volume of Trypan Blue
12. Count viable cells (non-viable cells stain blue) with hemocytometer (add 10uL of cell dilution under cover-slip, average counts in 4 grid quadrants and use the following formula: {[n x 10^4/1mL] x total volume}
13. Dilute to volume that will allow for 5 x 10^4 cells/well
14. Plate in triplicate at 5 x 10^4 cells/well and allow them to attach for various specified time points @ 37 degrees C
15. Rinse wells X2 with PBS to remove unbound cells, and then fix adherent cells with 3.7% PFA in PBS for 10min at RT
16. Wash wells X3 with PBS on shaker for 5mins each
17. Stain adherent cells (1 mL/well) with Coomassie blue for 1 hour on shaker at RT
18. Wash X3 with PBS on shaker for 5mins each
19. Evaluate cell adhesion/spreading with inverted optical microscope by counting stained cells in 6 separate 20x fields
20. Average cell counts and make relative to 103 cells/field (5 x 10^4 cells/484 fields at 20X = 103 cells/field maximum; 1 well/12 well plate = 22mm diameter; surface area = 379.94mm^2; 20X = 1mm diameter; surface area 0.785mm^2. Thus, 20X has 484 fields in 1 well of 12 well plate)

Table 1: Raw Cell Counts for Control Group

	No Coat		Laminin		BSA	
	Well One	Well Two	Well One	Well Two	Well One	Well Two
NG008						
Image 1	31	7	6	5	2	3
Image 2	25	4	5	5	4	6
Image 3	24	11	10	5	4	5
Image 4	29	8	5	4	3	5
Image 5	24	5	3	2	4	3
Image 6	30	4	6	3	8	4
NG009						
Image 1	7	2	3	7	5	3
Image 2	6	2	3	7	2	2
Image 3	8	1	6	4	6	8
Image 4	8	1	4	4	11	4
Image 5	8	8	6	11	10	10
Image 6	9	5	3	4	9	9
NG013						
Image 1	6	11	2	15	1	3
Image 2	7	14	4	14	4	5
Image 3	20	14	5	5	2	2
Image 4	18	16	5	6	2	7
Image 5	16	21	3	15	2	3
Image 6	15	18	2	7	2	6
NG015						
Image 1	8	8	8	5	5	3
Image 2	12	4	11	2	0	4
Image 3	8	8	2	5	1	3
Image 4	8	11	4	4	1	4
Image 5	7	5	7	2	5	2
Image 6	9	6	10	10	3	2
NG016						
Image 1	11	5	12	2	2	3
Image 2	12	20	12	1	5	3
Image 3	9	16	5	6	8	4
Image 4	11	38	5	5	7	8
Image 5	20	42	5	8	7	6
Image 6	10	30	6	5	5	4

Table 2: Raw Cell Counts for Control Group Continued

	No Coat		Laminin		BSA	
	Well One	Well Two	Well One	Well Two	Well One	Well Two
NG017						
Image 1	149	179	10	17	14	10
Image 2	126	57	16	4	7	13
Image 3	69	78	19	10	6	15
Image 4	33	105	14	19	11	22
Image 5	20	70	20	9	11	19
Image 6	26	136	21	13	16	18
NG020						
Image 1	84	16	15	13	12	17
Image 2	60	10	8	11	14	12
Image 3	64	52	8	20	13	22
Image 4	9	91	15	14	22	5
Image 5	29	47	7	16	16	11
Image 6	13	16	13	8	14	12
NG028						
Image 1	11	1	4	4	9	8
Image 2	8	3	4	5	8	2
Image 3	7	3	4	4	11	16
Image 4	6	15	4	4	8	15
Image 5	7	14	5	5	4	7
Image 6	7	13	5	13	8	4
NG029						
Image 1	4	9	5	6	4	8
Image 2	11	9	5	6	6	9
Image 3	15	3	7	8	9	5
Image 4	12	9	6	8	13	4
Image 5	8	8	3	17	6	4
Image 6	24	10	6	8	18	5
NG033						
Image 1	16	18	8	6	12	17
Image 2	6	20	8	6	15	7
Image 3	7	32	10	9	10	8
Image 4	36	48	10	2	25	12
Image 5	39	31	13	15	13	10
Image 6	8	38	12	14	16	25

Table 3: Raw Cell Counts for OSAS Group

	No Coat		Laminin		BSA	
	Well One	Well Two	Well One	Well Two	Well One	Well Two
OG080						
Image 1	12	16	3	3	4	1
Image 2	5	18	5	3	7	14
Image 3	18	8	7	4	8	3
Image 4	25	18	3	6	7	5
Image 5	12	8	5	5	5	24
Image 6	15	19	5	3	4	10
OG081						
Image 1	10	17	7	9	3	6
Image 2	8	21	5	9	5	4
Image 3	18	20	6	2	2	4
Image 4	26	18	10	10	6	2
Image 5	18	17	4	3	6	2
Image 6	31	12	6	11	6	3
OG082						
Image 1	17	9	5	14	6	15
Image 2	24	6	6	7	8	17
Image 3	8	10	5	7	8	5
Image 4	7	18	14	5	8	19
Image 5	9	14	21	12	19	16
Image 6	11	10	13	5	4	10
OG083						
Image 1	26	20	7	3	8	19
Image 2	14	15	8	4	8	14
Image 3	15	11	7	10	10	9
Image 4	23	24	8	7	15	8
Image 5	25	16	10	13	41	7
Image 6	20	11	4	7	19	24
OG084						
Image 1	12	8	7	9	2	7
Image 2	6	12	7	3	8	3
Image 3	9	11	2	5	8	28
Image 4	9	5	2	4	4	18
Image 5	7	10	6	3	10	18
Image 6	8	12	8	5	11	28
OG085						
Image 1	14	9	3	14	10	16
Image 2	8	6	21	5	16	20
Image 3	14	7	20	15	12	11
Image 4	10	62	19	12	19	10
Image 5	16	17	8	13	28	7
Image 6	13	17	8	10	19	23

Tables 1-3 represent the raw cellular counts for 6 random photos from each subject wells under the three conditions; coating with Laminin, coating with BSA and Laminin, and no coating. These were done in duplicate (well 1 and well 2). These were then averaged and made relative to 103 cells/field.

APPENDIX L

QUANTITATIVE PCR – DETAILED PROCEDURES AND RESULTS

TaqMan® Gene Expression Assays Protocol (PN 4333458)

1. **Prepare cDNA Sample**
2. Isolate total RNA utilizing the Qiagen RNeasy Mini Kits and following manufacturer's instructions
3. Perform reverse transcription utilizing the Ambion High Capacity RNA to cDNA kit and following manufacturer's instructions
4. Evaluate the cDNA
5. **Prepare the reaction mix and load the plate**
6. Thaw and mix reagents
7. Calculate the number of reactions
8. Prepare the PCR reaction mix
9. Load the plate
10. **Run the real-time PCR reaction**
11. Create the plate document
12. Run the plate
13. **Analyze the results**

Table 1: Calculations for qPCR

cDNA Reaction Calculations				q-rtPCR Reaction Calculations (amt/rxn)			Sample Master Dilutions for 10 positive cDNA samples		Sample Master Dilutions for 4 negative cDNA samples	
Sample ID	RNA concentration (ng/ul)	Vol added to cDNA reaction mix (ul)	Amt RNA in cDNA reaction (ug)	cDNA rxn Vol added to q-rtPCR rxn (ul/rxn)	cDNA rxn Amt in q-rtPCR reaction (ng/rxn)	Vol of Rnase free dH2O added to q-rtPCR rxn (ul/rxn)	Vol cDNA sample for (+). Make 10 (40ul total)	Vol H2O for (+). Make 10 (40ul total)	Vol neg cDNA sample for (-). Make 4 (16ul total)	Vol H2O for (-). Make 4 (16ul total)
N-8	77.31	9	0.7	0.9	**30**	3.1	8.6	31.4	**3.4**	12.6
N-9	16.87	9	0.15	1.6	**12**	2.4	15.5	24.5	**7.2 (14ng/rxn)**	8.8
N-13	251.97	8	2	0.3	**30**	3.7	3.0	37.0	**1.2**	14.8
N-15	34.2	9	0.3	1.6	**23**	2.4	15.5	24.5	**7.2 (27ng/rxn)**	8.8
N-16	263.12	7.6	2	0.3	**30**	3.7	3.0	37.0	**1.2**	14.8
N-17	242.47	8	2	0.3	**30**	3.7	3.0	37.0	**1.2**	14.8
N-20	185.89	9	1.7	0.4	**30**	3.6	3.5	36.5	**1.4**	14.6
N-28	188.01	9	1.7	0.4	**30**	3.6	3.5	36.5	**1.4**	14.6
N-29	808.05	2.5	2	0.3	**30**	3.7	3.0	37.0	**1.2**	14.8
N-33	23.07	9	0.21	1.5	**16**	2.5	15.0	25.0	**7.2 (19ng/rxn)**	8.8
O-80	167.07	9	1.5	0.4	**30**	3.6	4.0	36.0	**1.6**	14.4
O-81	505.07	4	2	0.3	**30**	3.7	3.0	37.0	**1.2**	14.8
O-82	266.92	7.5	2	0.3	**30**	3.7	3.0	37.0	**1.2**	14.8
O-83	301.01	6.6	2	0.3	**30**	3.7	3.0	37.0	**1.2**	14.8
O-84	77.28	9	0.7	0.9	**30**	3.1	8.6	31.4	**3.4**	12.6
O-85	167.44	9	1.5	0.4	**30**	3.6	4.0	36.0	**1.6**	14.4

PCR Quantification Detailed Report
PCR Base Line Subtracted Curve Fit Data (FAM)
Contains All Available Data

General Data

Data File Name	**LAM AND CDC42 PLATE 1 RESULTS.opd**
Data File Path	**C:\Program Files\Bio-Rad\iQ5\Users\Newsome**
Collected Data	**Collected Data**
Current Date	**12/8/2010 9:01:33 AM**
Run Date	**12/2/2010 12:06:04 PM**
User aborted the run	**No**
Active RMEs	**Original**
Active Well Factors	**Persistent**
Background Readings Valid	**Yes**
RME Valid	**Yes**
Well Factors Valid	**No, data is 986 day(s) old.**
Plate Setup File Name	**Plate 1 8-28.pts**
Plate Setup File Path	**C:\Program Files\Bio-Rad\iQ5\Users\admin**
Protocol File Name	**Lamini and CDC42.tmo**
Protocol File Path	**C:\Program Files\Bio-Rad\iQ5\Users\admin**
Computer name	**ISD26426**
Created by app	**iQ5.exe (v2.0.148.60623. (OS-Microsoft Windows NT 5.1.2600.0.Service Pack 2, CLR-1.1.4322.2463, Culture-en-US).)**
Created by user	**BioRad\admin**
Creation Date	**12/2/2010 12:06:04 PM**
Created in Security Edition	**No**
Last Creation GUID	**36311f57-d258-4019-9bdd-5ebe8bdc43ea**
Modified by user	**BioRad\admin**
Last modified date	**12/2/2010 12:06:04 PM**
OS Build and Service Pack	**2600 (Service Pack 2)**
Report differs from last save	**No**

Notes:

Protocol:

Cycle 1: (1X)		
Step 1:	95.0 °C	for 10:00.
Cycle 2: (40X)		
Step 1:	95.0 °C	for 00:15.
Step 2:	60.0 °C	for 01:00.

Data collection and real-time analysis enabled.

Modified Protocol:

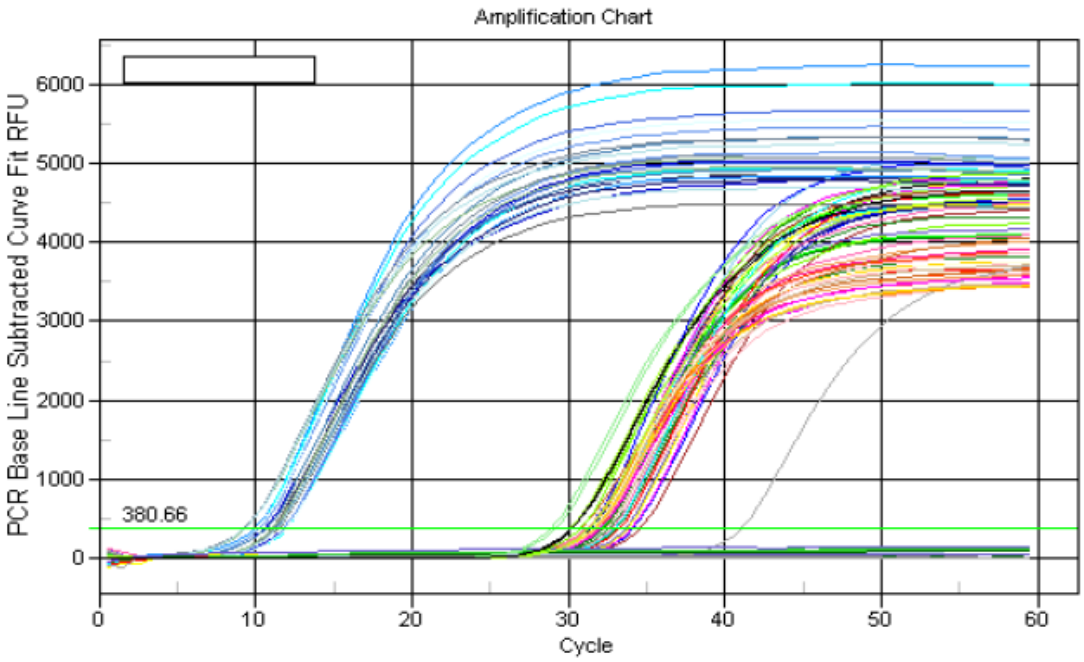
Cycle 1: (1X)		
Step 1:	95.0 °C	for 10:00.
Cycle 2: (60X)		

Step 1:	95.0 °C	for 00:15.
Step 2:	60.0 °C	for 01:00.

Data collection and real-time analysis enabled.

PCR Quantification Data

PCR Amp/Cycle Chart



Standard Curve Data

Standard Curve Chart

Not available

Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	100.0	0.000	0.000	0.000

Fluor	Units Changed?	Quantity Units	Original Units
FAM	No	copy number	copy number

Number of valid standard wells: **None**

Standard Curve Spreadsheet Data

Fluor	Well	Type	Ident.	Rep	Ct	Log SQ	SQ	SQ Mean	SQ SD	Ct Mean	Ct SD	Set Point
FAM	A01	Unkn	N-8 LAM	1	31.83	N/A	0.00E+00	0.00E+00	0.00E+00	31.71	0.198	N/A
FAM	A02	Unkn	N-8 LAM	1	31.82	N/A	0.00E+00	0.00E+00	0.00E+00	31.71	0.198	N/A
FAM	A03	Unkn	N-8 LAM	1	31.48	N/A	0.00E+00	0.00E+00	0.00E+00	31.71	0.198	N/A
FAM	A04	Unkn	N-9 LAM	2	34.10	N/A	0.00E+00	0.00E+00	0.00E+00	34.25	0.316	N/A
FAM	A05	Unkn	N-9 LAM	2	34.05	N/A	0.00E+00	0.00E+00	0.00E+00	34.25	0.316	N/A

FAM	A06	Unkn	N-9 LAM	2	34.62	N/A	0.00E+00	0.00E+00	0.00E+00	34.25	0.316	N/A	
FAM	A07	Unkn	N-13 LAM	3	33.68	N/A	0.00E+00	0.00E+00	0.00E+00	33.71	0.079	N/A	
FAM	A08	Unkn	N-13 LAM	3	33.65	N/A	0.00E+00	0.00E+00	0.00E+00	33.71	0.079	N/A	
FAM	A09	Unkn	N-13 LAM	3	33.80	N/A	0.00E+00	0.00E+00	0.00E+00	33.71	0.079	N/A	
FAM	A10	Unkn	N-15 LAM	4	32.59	N/A	0.00E+00	0.00E+00	0.00E+00	32.11	0.435	N/A	
FAM	A11	Unkn	N-15 LAM	4	31.74	N/A	0.00E+00	0.00E+00	0.00E+00	32.11	0.435	N/A	
FAM	A12	Unkn	N-15 LAM	4	32.00	N/A	0.00E+00	0.00E+00	0.00E+00	32.11	0.435	N/A	
FAM	B01	Unkn	N-8 CDC42	5	31.20	N/A	0.00E+00	0.00E+00	0.00E+00	31.29	0.100	N/A	
FAM	B02	Unkn	N-8 CDC42	5	31.27	N/A	0.00E+00	0.00E+00	0.00E+00	31.29	0.100	N/A	
FAM	B03	Unkn	N-8 CDC42	5	31.40	N/A	0.00E+00	0.00E+00	0.00E+00	31.29	0.100	N/A	
FAM	B04	Unkn	N-9 CDC42	6	32.73	N/A	0.00E+00	0.00E+00	0.00E+00	32.88	0.127	N/A	
FAM	B05	Unkn	N-9 CDC42	6	32.97	N/A	0.00E+00	0.00E+00	0.00E+00	32.88	0.127	N/A	
FAM	B06	Unkn	N-9 CDC42	6	32.92	N/A	0.00E+00	0.00E+00	0.00E+00	32.88	0.127	N/A	
FAM	B07	Unkn	N-13 CDC42	7	31.98	N/A	0.00E+00	0.00E+00	0.00E+00	31.88	0.117	N/A	
FAM	B08	Unkn	N-13 CDC42	7	31.91	N/A	0.00E+00	0.00E+00	0.00E+00	31.88	0.117	N/A	
FAM	B09	Unkn	N-13 CDC42	7	31.76	N/A	0.00E+00	0.00E+00	0.00E+00	31.88	0.117	N/A	
FAM	B10	Unkn	N-15 CDC42	8	32.28	N/A	0.00E+00	0.00E+00	0.00E+00	32.06	0.220	N/A	
FAM	B11	Unkn	N-15 CDC42	8	32.07	N/A	0.00E+00	0.00E+00	0.00E+00	32.06	0.220	N/A	
FAM	B12	Unkn	N-15 CDC42	8	31.84	N/A	0.00E+00	0.00E+00	0.00E+00	32.06	0.220	N/A	
FAM	C01	Unkn	N-8 18S	9	10.56	N/A	0.00E+00	0.00E+00	0.00E+00	10.32	0.214	N/A	
FAM	C02	Unkn	N-8 18S	9	10.19	N/A	0.00E+00	0.00E+00	0.00E+00	10.32	0.214	N/A	
FAM	C03	Unkn	N-8 18S	9	10.20	N/A	0.00E+00	0.00E+00	0.00E+00	10.32	0.214	N/A	
FAM	C04	Unkn	N-9 18S	10	9.92	N/A	0.00E+00	0.00E+00	0.00E+00	9.99	0.065	N/A	
FAM	C05	Unkn	N-9 18S	10	9.99	N/A	0.00E+00	0.00E+00	0.00E+00	9.99	0.065	N/A	
FAM	C06	Unkn	N-9 18S	10	10.05	N/A	0.00E+00	0.00E+00	0.00E+00	9.99	0.065	N/A	
FAM	C07	Unkn	N-13 18S	11	10.99	N/A	0.00E+00	0.00E+00	0.00E+00	10.89	0.200	N/A	
FAM	C08	Unkn	N-13 18S	11	11.02	N/A	0.00E+00	0.00E+00	0.00E+00	10.89	0.200	N/A	
FAM	C09	Unkn	N-13 18S	11	10.66	N/A	0.00E+00	0.00E+00	0.00E+00	10.89	0.200	N/A	
FAM	C10	Unkn	N-15 18S	12	9.25	N/A	0.00E+00	0.00E+00	0.00E+00	9.31	0.054	N/A	
FAM	C11	Unkn	N-15 18S	12	9.36	N/A	0.00E+00	0.00E+00	0.00E+00	9.31	0.054	N/A	
FAM	C12	Unkn	N-15 18S	12	9.32	N/A	0.00E+00	0.00E+00	0.00E+00	9.31	0.054	N/A	
FAM	D01	Unkn	N-8 (-) LAM	13	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A	
FAM	D02	Unkn	N-9 (-) LAM	14	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A	
FAM	D03	Unkn	N-13 (-) LAM	15	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A	
FAM	D04	Unkn	N-15 (-) LAM	16	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A	
FAM	D05	Unkn	N-8 (-) CDC42	17	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A	
FAM	D06	Unkn	N-9 (-) CDC42	18	41.00	N/A	0.00E+00	0.00E+00	0.00E+00	41.00	0.000	N/A	
FAM	D07	Unkn	N-13 (-) CDC42		19	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	D08	Unkn	N-15 (-) CDC42		20	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	D09	Unkn	N-8 (-) 18S	21	28.87	N/A	0.00E+00	0.00E+00	0.00E+00	28.87	0.000	N/A	
FAM	D10	Unkn	N-9 (-) 18S	22	30.22	N/A	0.00E+00	0.00E+00	0.00E+00	30.22	0.000	N/A	
FAM	D11	Unkn	N-13 (-) 18S	23	30.11	N/A	0.00E+00	0.00E+00	0.00E+00	30.11	0.000	N/A	
FAM	D12	Unkn	N-15 (-) 18S	24	30.23	N/A	0.00E+00	0.00E+00	0.00E+00	30.23	0.000	N/A	
FAM	E01	Unkn	N-16 LAM	25	32.13	N/A	0.00E+00	0.00E+00	0.00E+00	32.11	0.101	N/A	
FAM	E02	Unkn	N-16 LAM	25	32.20	N/A	0.00E+00	0.00E+00	0.00E+00	32.11	0.101	N/A	
FAM	E03	Unkn	N-16 LAM	25	32.00	N/A	0.00E+00	0.00E+00	0.00E+00	32.11	0.101	N/A	
FAM	E04	Unkn	N-17 LAM	26	31.21	N/A	0.00E+00	0.00E+00	0.00E+00	31.48	0.233	N/A	
FAM	E05	Unkn	N-17 LAM	26	31.57	N/A	0.00E+00	0.00E+00	0.00E+00	31.48	0.233	N/A	
FAM	E06	Unkn	N-17 LAM	26	31.65	N/A	0.00E+00	0.00E+00	0.00E+00	31.48	0.233	N/A	
FAM	E07	Unkn	N-20 LAM	27	31.41	N/A	0.00E+00	0.00E+00	0.00E+00	31.50	0.082	N/A	
FAM	E08	Unkn	N-20 LAM	27	31.57	N/A	0.00E+00	0.00E+00	0.00E+00	31.50	0.082	N/A	
FAM	E09	Unkn	N-20 LAM	27	31.53	N/A	0.00E+00	0.00E+00	0.00E+00	31.50	0.082	N/A	
FAM	E10	Unkn	N-28 LAM	28	32.98	N/A	0.00E+00	0.00E+00	0.00E+00	33.03	0.157	N/A	
FAM	E11	Unkn	N-28 LAM	28	32.90	N/A	0.00E+00	0.00E+00	0.00E+00	33.03	0.157	N/A	
FAM	E12	Unkn	N-28 LAM	28	33.20	N/A	0.00E+00	0.00E+00	0.00E+00	33.03	0.157	N/A	
FAM	F01	Unkn	N-16 CDC42	29	31.93	N/A	0.00E+00	0.00E+00	0.00E+00	31.97	0.040	N/A	
FAM	F02	Unkn	N-16 CDC42	29	31.95	N/A	0.00E+00	0.00E+00	0.00E+00	31.97	0.040	N/A	
FAM	F03	Unkn	N-16 CDC42	29	32.01	N/A	0.00E+00	0.00E+00	0.00E+00	31.97	0.040	N/A	
FAM	F04	Unkn	N-17 CDC42	30	31.16	N/A	0.00E+00	0.00E+00	0.00E+00	31.56	0.358	N/A	
FAM	F05	Unkn	N-17 CDC42	30	31.68	N/A	0.00E+00	0.00E+00	0.00E+00	31.56	0.358	N/A	
FAM	F06	Unkn	N-17 CDC42	30	31.84	N/A	0.00E+00	0.00E+00	0.00E+00	31.56	0.358	N/A	
FAM	F07	Unkn	N-20 CDC42	31	30.81	N/A	0.00E+00	0.00E+00	0.00E+00	31.08	0.253	N/A	
FAM	F08	Unkn	N-20 CDC42	31	31.30	N/A	0.00E+00	0.00E+00	0.00E+00	31.08	0.253	N/A	
FAM	F09	Unkn	N-20 CDC42	31	31.15	N/A	0.00E+00	0.00E+00	0.00E+00	31.08	0.253	N/A	
FAM	F10	Unkn	N-28 CDC42	32	31.55	N/A	0.00E+00	0.00E+00	0.00E+00	31.57	0.060	N/A	
FAM	F11	Unkn	N-28 CDC42	32	31.52	N/A	0.00E+00	0.00E+00	0.00E+00	31.57	0.060	N/A	
FAM	F12	Unkn	N-28 CDC42	32	31.64	N/A	0.00E+00	0.00E+00	0.00E+00	31.57	0.060	N/A	
FAM	G01	Unkn	N-16 18S	33	11.77	N/A	0.00E+00	0.00E+00	0.00E+00	11.80	0.028	N/A	
FAM	G02	Unkn	N-16 18S	33	11.82	N/A	0.00E+00	0.00E+00	0.00E+00	11.80	0.028	N/A	
FAM	G03	Unkn	N-16 18S	33	11.82	N/A	0.00E+00	0.00E+00	0.00E+00	11.80	0.028	N/A	
FAM	G04	Unkn	N-17 18S	34	11.97	N/A	0.00E+00	0.00E+00	0.00E+00	11.96	0.011	N/A	
FAM	G05	Unkn	N-17 18S	34	11.95	N/A	0.00E+00	0.00E+00	0.00E+00	11.96	0.011	N/A	
FAM	G06	Unkn	N-17 18S	34	11.96	N/A	0.00E+00	0.00E+00	0.00E+00	11.96	0.011	N/A	
FAM	G07	Unkn	N-20 18S	35	11.43	N/A	0.00E+00	0.00E+00	0.00E+00	11.34	0.083	N/A	
FAM	G08	Unkn	N-20 18S	35	11.33	N/A	0.00E+00	0.00E+00	0.00E+00	11.34	0.083	N/A	
FAM	G09	Unkn	N-20 18S	35	11.26	N/A	0.00E+00	0.00E+00	0.00E+00	11.34	0.083	N/A	
FAM	G10	Unkn	N-28 18S	36	11.15	N/A	0.00E+00	0.00E+00	0.00E+00	11.18	0.113	N/A	
FAM	G11	Unkn	N-28 18S	36	11.08	N/A	0.00E+00	0.00E+00	0.00E+00	11.18	0.113	N/A	
FAM	G12	Unkn	N-28 18S	36	11.30	N/A	0.00E+00	0.00E+00	0.00E+00	11.18	0.113	N/A	

FAM	H01	Unkn	N-16 (-) LAM	37	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	H02	Unkn	N-17 (-) LAM	38	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	H03	Unkn	N-20 (-) LAM	39	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	H04	Unkn	N-28 (-) LAM	40	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	H05	Unkn	N-16 (-) CDC42		41	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000
N/A												
FAM	H06	Unkn	N-17 (-) CDC42		42	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000
N/A												
FAM	H07	Unkn	N-20 (-) CDC42		43	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000
N/A												
FAM	H08	Unkn	N-28 (-) CDC42		44	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000
N/A												
FAM	H09	Unkn	N-16 (-) 18S	45	29.17	N/A	0.00E+00	0.00E+00	0.00E+00	29.17	0.000	N/A
FAM	H10	Unkn	N-17 (-) 18S	46	30.76	N/A	0.00E+00	0.00E+00	0.00E+00	30.76	0.000	N/A
FAM	H11	Unkn	N-20 (-) 18S	47	31.28	N/A	0.00E+00	0.00E+00	0.00E+00	31.28	0.000	N/A
FAM	H12	Unkn	N-28 (-) 18S	48	30.25	N/A	0.00E+00	0.00E+00	0.00E+00	30.25	0.000	N/A

Run Parameters

Hot Start?	**No**
Temperature Control Mode:	**Algorithmic**
Volume:	**25 ul**

Data Analysis Parameters

Display Controls

Fluor	Display Mode
FAM	SinglePoint

Data Selection

Fluor	Data Window Size	Center
FAM	99%	End

Digital Filtering

Fluor	Global Filter Enabled?	PCR Digital Filter Type	Smoothing Filter Desired Width
FAM	Off	Weighted Mean	5

PCR Data Analysis Method

Fluor	Data Analysis Method
FAM	PCR Base Line Subtracted Curve Fit

PCR Baseline Data Analysis Parameters

Baseline Calculation

Fluor	Baseline Method	Auto Baseline Cycle Calculation?	Global Baseline Cycles Start	Global Baseline Cycles End
FAM	Data Window	Yes	N/A	N/A

Overriden Baseline Cycles None

Threshold Calculation

Fluor	Use Auto Threshold?	Auto Calculated Threshold Value	User Defined Threshold Value
FAM	Yes	380.66	380.66

Excluded Wells

Excluded Well Count: 0

Modified Wells

Modified Well Count: 0

End

Figure 1: qPCR Data Output – Plate One

PCR Quantification Detailed Report
PCR Base Line Subtracted Curve Fit Data (FAM)
Contains All Available Data

General Data

Data File Name	**LAM AND CDC42 PLATE 2 RESULTS.opd**
Data File Path	**C:\Program Files\Bio-Rad\iQ5\Users\Newsome**
Collected Data	**Collected Data**
Current Date	**12/8/2010 9:05:00 AM**
Run Date	**12/2/2010 2:41:00 PM**
User aborted the run	**No**
Active RMEs	**Original**
Active Well Factors	**Persistent**
Background Readings Valid	**Yes**
RME Valid	**Yes**
Well Factors Valid	**No, data is 986 day(s) old.**
Plate Setup File Name	**Plate 2 29-85.pts**
Plate Setup File Path	**C:\Program Files\Bio-Rad\iQ5\Users\Newsome**
Protocol File Name	**Lamini and CDC42.tmo**
Protocol File Path	**C:\Program Files\Bio-Rad\iQ5\Users\Newsome**
Computer name	**ISD26426**
Created by app	**iQ5.exe (v2.0.148.60623. (OS-Microsoft Windows NT 5.1.2600.0.Service Pack 2, CLR-1.1.4322.2463, Culture-en-US).)**
Created by user	**BioRad\admin**
Creation Date	**12/2/2010 2:41:00 PM**
Created in Security Edition	**No**
Last Creation GUID	**8cf04a44-a481-4c65-ab72-39c6742b8056**
Modified by user	**BioRad\admin**
Last modified date	**12/2/2010 2:41:00 PM**
OS Build and Service Pack	**2600 (Service Pack 2)**
Report differs from last save	**No**

Notes:

Protocol:

Cycle 1: (1X)
Step 1: 95.0 °C for 10:00.
Cycle 2: (40X)
Step 1: 95.0 °C for 00:15.
Step 2: 60.0 °C for 01:00.
Data collection and real-time analysis enabled.

Modified Protocol:

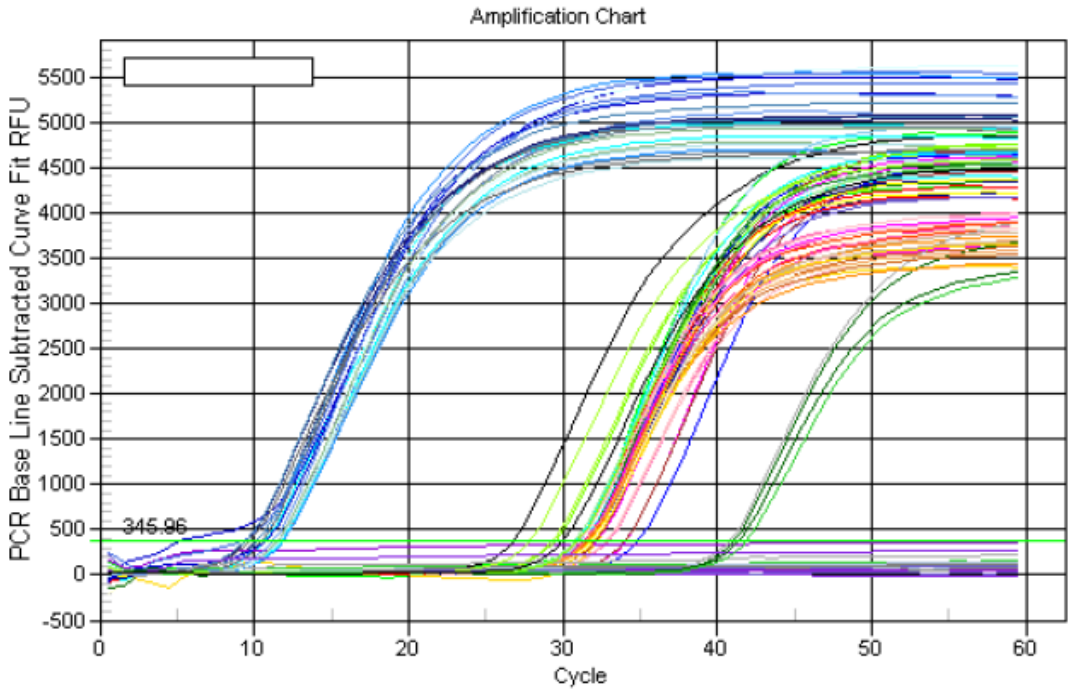
Cycle 1: (1X)
Step 1: 95.0 °C for 10:00.
Cycle 2: (60X)

Step 1:	95.0 °C	for 00:15.
Step 2:	60.0 °C	for 01:00.

Data collection and real-time analysis enabled.

PCR Quantification Data

PCR Amp/Cycle Chart



Standard Curve Data

Standard Curve Chart

Not available

Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	100.0	0.000	0.000	0.000

Fluor	Units Changed?	Quantity Units	Original Units
FAM	No	copy number	copy number

Number of valid standard wells: **None**

Standard Curve Spreadsheet Data

Fluor	Well	Type	Ident.	Rep	Ct	Log SQ	SQ	SQ Mean	SQ SD	Ct Mean	Ct SD	Set Point
FAM	A01	Unkn	N-29 LAM	1	31.25	N/A	0.00E+00	0.00E+00	0.00E+00	31.49	0.217	N/A
FAM	A02	Unkn	N-29 LAM	1	31.67	N/A	0.00E+00	0.00E+00	0.00E+00	31.49	0.217	N/A
FAM	A03	Unkn	N-29 LAM	1	31.55	N/A	0.00E+00	0.00E+00	0.00E+00	31.49	0.217	N/A
FAM	A04	Unkn	N-33 LAM	2	34.83	N/A	0.00E+00	0.00E+00	0.00E+00	34.24	0.509	N/A
FAM	A05	Unkn	N-33 LAM	2	33.97	N/A	0.00E+00	0.00E+00	0.00E+00	34.24	0.509	N/A

FAM	A06	Unkn	N-33 LAM	2	33.93	N/A	0.00E+00	0.00E+00	0.00E+00	34.24	0.509	N/A
FAM	A07	Unkn	O-80 LAM	3	31.49	N/A	0.00E+00	0.00E+00	0.00E+00	31.33	0.140	N/A
FAM	A08	Unkn	O-80 LAM	3	31.28	N/A	0.00E+00	0.00E+00	0.00E+00	31.33	0.140	N/A
FAM	A09	Unkn	O-80 LAM	3	31.22	N/A	0.00E+00	0.00E+00	0.00E+00	31.33	0.140	N/A
FAM	A10	Unkn	O-81 LAM	4	31.41	N/A	0.00E+00	0.00E+00	0.00E+00	31.40	0.145	N/A
FAM	A11	Unkn	O-81 LAM	4	31.26	N/A	0.00E+00	0.00E+00	0.00E+00	31.40	0.145	N/A
FAM	A12	Unkn	O-81 LAM	4	31.55	N/A	0.00E+00	0.00E+00	0.00E+00	31.40	0.145	N/A
FAM	B01	Unkn	N-29 CDC42	5	31.87	N/A	0.00E+00	0.00E+00	0.00E+00	31.78	0.075	N/A
FAM	B02	Unkn	N-29 CDC42	5	31.73	N/A	0.00E+00	0.00E+00	0.00E+00	31.78	0.075	N/A
FAM	B03	Unkn	N-29 CDC42	5	31.76	N/A	0.00E+00	0.00E+00	0.00E+00	31.78	0.075	N/A
FAM	B04	Unkn	N-33 CDC42	6	32.85	N/A	0.00E+00	0.00E+00	0.00E+00	32.77	0.080	N/A
FAM	B05	Unkn	N-33 CDC42	6	32.70	N/A	0.00E+00	0.00E+00	0.00E+00	32.77	0.080	N/A
FAM	B06	Unkn	N-33 CDC42	6	32.75	N/A	0.00E+00	0.00E+00	0.00E+00	32.77	0.080	N/A
FAM	B07	Unkn	O-80 CDC42	7	31.82	N/A	0.00E+00	0.00E+00	0.00E+00	31.72	0.084	N/A
FAM	B08	Unkn	O-80 CDC42	7	31.68	N/A	0.00E+00	0.00E+00	0.00E+00	31.72	0.084	N/A
FAM	B09	Unkn	O-80 CDC42	7	31.67	N/A	0.00E+00	0.00E+00	0.00E+00	31.72	0.084	N/A
FAM	B10	Unkn	O-81 CDC42	8	31.58	N/A	0.00E+00	0.00E+00	0.00E+00	31.83	0.293	N/A
FAM	B11	Unkn	O-81 CDC42	8	31.75	N/A	0.00E+00	0.00E+00	0.00E+00	31.83	0.293	N/A
FAM	B12	Unkn	O-81 CDC42	8	32.15	N/A	0.00E+00	0.00E+00	0.00E+00	31.83	0.293	N/A
FAM	C01	Unkn	N-29 18S	9	5.23	N/A	0.00E+00	0.00E+00	0.00E+00	9.57	3.764	N/A
FAM	C02	Unkn	N-29 18S	9	11.78	N/A	0.00E+00	0.00E+00	0.00E+00	9.57	3.764	N/A
FAM	C03	Unkn	N-29 18S	9	11.72	N/A	0.00E+00	0.00E+00	0.00E+00	9.57	3.764	N/A
FAM	C04	Unkn	N-33 18S	10	10.35	N/A	0.00E+00	0.00E+00	0.00E+00	10.87	0.694	N/A
FAM	C05	Unkn	N-33 18S	10	10.60	N/A	0.00E+00	0.00E+00	0.00E+00	10.87	0.694	N/A
FAM	C06	Unkn	N-33 18S	10	11.65	N/A	0.00E+00	0.00E+00	0.00E+00	10.87	0.694	N/A
FAM	C07	Unkn	O-80 18S	11	10.42	N/A	0.00E+00	0.00E+00	0.00E+00	10.17	0.218	N/A
FAM	C08	Unkn	O-80 18S	11	10.03	N/A	0.00E+00	0.00E+00	0.00E+00	10.17	0.218	N/A
FAM	C09	Unkn	O-80 18S	11	10.06	N/A	0.00E+00	0.00E+00	0.00E+00	10.17	0.218	N/A
FAM	C10	Unkn	O-81 18S	12	11.71	N/A	0.00E+00	0.00E+00	0.00E+00	11.71	0.100	N/A
FAM	C11	Unkn	O-81 18S	12	11.80	N/A	0.00E+00	0.00E+00	0.00E+00	11.71	0.100	N/A
FAM	C12	Unkn	O-81 18S	12	11.60	N/A	0.00E+00	0.00E+00	0.00E+00	11.71	0.100	N/A
FAM	D01	Unkn	N-29 (-) LAM	13	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	D02	Unkn	N-33 (-) LAM	14	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	D03	Unkn	O-80 (-) LAM	15	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	D04	Unkn	O-81 (-) LAM	16	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	D05	Unkn	N-29 (-) CDC42		17	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000
N/A												
FAM	D06	Unkn	N-33 (-) CDC42		18	40.68	N/A	0.00E+00	0.00E+00	0.00E+00	40.68	0.000
N/A												
FAM	D07	Unkn	O-80 (-) CDC42		19	40.97	N/A	0.00E+00	0.00E+00	0.00E+00	40.97	0.000
N/A												
FAM	D08	Unkn	O-81 (-) CDC42		20	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000
N/A												
FAM	D09	Unkn	N-29 (-) 18S	21	31.52	N/A	0.00E+00	0.00E+00	0.00E+00	31.52	0.000	N/A
FAM	D10	Unkn	N-33 (-) 18S	22	29.44	N/A	0.00E+00	0.00E+00	0.00E+00	29.44	0.000	N/A
FAM	D11	Unkn	O-80 (-) 18S	23	29.20	N/A	0.00E+00	0.00E+00	0.00E+00	29.20	0.000	N/A
FAM	D12	Unkn	O-81 (-) 18S	24	26.68	N/A	0.00E+00	0.00E+00	0.00E+00	26.68	0.000	N/A
FAM	E01	Unkn	O-82 LAM	25	30.85	N/A	0.00E+00	0.00E+00	0.00E+00	31.06	0.249	N/A
FAM	E02	Unkn	O-82 LAM	25	31.34	N/A	0.00E+00	0.00E+00	0.00E+00	31.06	0.249	N/A
FAM	E03	Unkn	O-82 LAM	25	30.99	N/A	0.00E+00	0.00E+00	0.00E+00	31.06	0.249	N/A
FAM	E04	Unkn	O-83 LAM	26	31.56	N/A	0.00E+00	0.00E+00	0.00E+00	31.88	0.279	N/A
FAM	E05	Unkn	O-83 LAM	26	32.08	N/A	0.00E+00	0.00E+00	0.00E+00	31.88	0.279	N/A
FAM	E06	Unkn	O-83 LAM	26	31.99	N/A	0.00E+00	0.00E+00	0.00E+00	31.88	0.279	N/A
FAM	E07	Unkn	O-84 LAM	27	31.84	N/A	0.00E+00	0.00E+00	0.00E+00	31.76	0.088	N/A
FAM	E08	Unkn	O-84 LAM	27	31.76	N/A	0.00E+00	0.00E+00	0.00E+00	31.76	0.088	N/A
FAM	E09	Unkn	O-84 LAM	27	31.67	N/A	0.00E+00	0.00E+00	0.00E+00	31.76	0.088	N/A
FAM	E10	Unkn	O-85 LAM	28	30.89	N/A	0.00E+00	0.00E+00	0.00E+00	30.88	0.046	N/A
FAM	E11	Unkn	O-85 LAM	28	30.83	N/A	0.00E+00	0.00E+00	0.00E+00	30.88	0.046	N/A
FAM	E12	Unkn	O-85 LAM	28	30.92	N/A	0.00E+00	0.00E+00	0.00E+00	30.88	0.046	N/A
FAM	F01	Unkn	O-82 CDC42	29	31.47	N/A	0.00E+00	0.00E+00	0.00E+00	31.37	0.097	N/A
FAM	F02	Unkn	O-82 CDC42	29	31.28	N/A	0.00E+00	0.00E+00	0.00E+00	31.37	0.097	N/A
FAM	F03	Unkn	O-82 CDC42	29	31.35	N/A	0.00E+00	0.00E+00	0.00E+00	31.37	0.097	N/A
FAM	F04	Unkn	O-83 CDC42	30	30.91	N/A	0.00E+00	0.00E+00	0.00E+00	30.94	0.026	N/A
FAM	F05	Unkn	O-83 CDC42	30	30.94	N/A	0.00E+00	0.00E+00	0.00E+00	30.94	0.026	N/A
FAM	F06	Unkn	O-83 CDC42	30	30.96	N/A	0.00E+00	0.00E+00	0.00E+00	30.94	0.026	N/A
FAM	F07	Unkn	O-84 CDC42	31	31.56	N/A	0.00E+00	0.00E+00	0.00E+00	31.50	0.110	N/A
FAM	F08	Unkn	O-84 CDC42	31	31.58	N/A	0.00E+00	0.00E+00	0.00E+00	31.50	0.110	N/A
FAM	F09	Unkn	O-84 CDC42	31	31.38	N/A	0.00E+00	0.00E+00	0.00E+00	31.50	0.110	N/A
FAM	F10	Unkn	O-85 CDC42	32	31.86	N/A	0.00E+00	0.00E+00	0.00E+00	31.86	0.032	N/A
FAM	F11	Unkn	O-85 CDC42	32	31.83	N/A	0.00E+00	0.00E+00	0.00E+00	31.86	0.032	N/A
FAM	F12	Unkn	O-85 CDC42	32	31.89	N/A	0.00E+00	0.00E+00	0.00E+00	31.86	0.032	N/A
FAM	G01	Unkn	O-82 18S	33	10.64	N/A	0.00E+00	0.00E+00	0.00E+00	10.24	1.132	N/A
FAM	G02	Unkn	O-82 18S	33	8.96	N/A	0.00E+00	0.00E+00	0.00E+00	10.24	1.132	N/A
FAM	G03	Unkn	O-82 18S	33	11.11	N/A	0.00E+00	0.00E+00	0.00E+00	10.24	1.132	N/A
FAM	G04	Unkn	O-83 18S	34	11.78	N/A	0.00E+00	0.00E+00	0.00E+00	11.69	0.089	N/A
FAM	G05	Unkn	O-83 18S	34	11.66	N/A	0.00E+00	0.00E+00	0.00E+00	11.69	0.089	N/A
FAM	G06	Unkn	O-83 18S	34	11.61	N/A	0.00E+00	0.00E+00	0.00E+00	11.69	0.089	N/A
FAM	G07	Unkn	O-84 18S	35	9.63	N/A	0.00E+00	0.00E+00	0.00E+00	9.48	0.126	N/A
FAM	G08	Unkn	O-84 18S	35	9.43	N/A	0.00E+00	0.00E+00	0.00E+00	9.48	0.126	N/A
FAM	G09	Unkn	O-84 18S	35	9.39	N/A	0.00E+00	0.00E+00	0.00E+00	9.48	0.126	N/A
FAM	G10	Unkn	O-85 18S	36	11.47	N/A	0.00E+00	0.00E+00	0.00E+00	11.47	0.089	N/A

FAM	G11	Unkn	O-85 18S	36	11.39	N/A	0.00E+00	0.00E+00	0.00E+00	11.47	0.089	N/A
FAM	G12	Unkn	O-85 18S	36	11.56	N/A	0.00E+00	0.00E+00	0.00E+00	11.47	0.089	N/A
FAM	H01	Unkn	O-82 (-) LAM	37	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	H02	Unkn	O-83 (-) LAM	38	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	H03	Unkn	O-84 (-) LAM	39	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	H04	Unkn	O-85 (-) LAM	40	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000	N/A
FAM	H05	Unkn	O-82 (-) CDC42		41	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000
N/A												
FAM	H06	Unkn	O-83 (-) CDC42		42	N/A	N/A	0.00E+00	0.00E+00	0.00E+00	.00	0.000
N/A												
FAM	H07	Unkn	O-84 (-) CDC42		43	41.24	N/A	0.00E+00	0.00E+00	0.00E+00	41.24	0.000
N/A												
FAM	H08	Unkn	O-85 (-) CDC42		44	41.77	N/A	0.00E+00	0.00E+00	0.00E+00	41.77	0.000
N/A												
FAM	H09	Unkn	O-82 (-) 18S	45	30.67	N/A	0.00E+00	0.00E+00	0.00E+00	30.67	0.000	N/A
FAM	H10	Unkn	O-83 (-) 18S	46	30.85	N/A	0.00E+00	0.00E+00	0.00E+00	30.85	0.000	N/A
FAM	H11	Unkn	O-84 (-) 18S	47	27.76	N/A	0.00E+00	0.00E+00	0.00E+00	27.76	0.000	N/A
FAM	H12	Unkn	O-85 (-) 18S	48	29.89	N/A	0.00E+00	0.00E+00	0.00E+00	29.89	0.000	N/A

Run Parameters

Hot Start? **No**
Temperature Control Mode: **Algorithmic**
Volume: **25 ul**

Data Analysis Parameters

Display Controls

Fluor	Display Mode
FAM	SinglePoint

Data Selection

Fluor	Data Window Size	Center
FAM	99%	End

Digital Filtering

Fluor	Global Filter Enabled?	PCR Digital Filter Type	Smoothing Filter Desired Width
FAM	Off	Weighted Mean	5

PCR Data Analysis Method

Fluor	Data Analysis Method
FAM	PCR Base Line Subtracted Curve Fit

PCR Baseline Data Analysis Parameters

Baseline Calculation

Fluor	Baseline Method	Auto Baseline Cycle Calculation?	Global Baseline Cycles Start	Global Baseline Cycles End
FAM	Data Window	Yes	N/A	N/A

Overriden Baseline Cycles None

Threshold Calculation

Fluor	Use Auto Threshold?	Auto Calculated Threshold Value	User Defined Threshold Value
FAM	Yes	345.96	345.96

Excluded Wells

Excluded Well Count: 0

Modified Wells

Modified Well Count: 0

End

Figure 2: qPCR Data Output – Plate Two

TABLE 2: Raw qPCR Data Values

Laminin C_t Values

Sample ID	N-8	N-13	N-15	N-16	N-17	N-20	N-28	N-29	O-80	O-81	O-83	O-84	O-85
replicate 1	32.12	33.95	32.88	32.48	31.56	31.72	33.30	31.25	31.49	31.41	31.56	31.84	30.89
replicate 2	32.12	33.92	32.03	32.55	31.84	31.84	33.20	31.67	31.28	31.26	32.08	31.76	30.83
replicate 3	31.77	34.08	32.34	32.32	31.92	31.81	33.56	31.55	31.22	31.55	31.99	31.67	30.92
AVG C_t	32.0	34.0	32.4	32.5	31.8	31.8	33.4	31.5	31.3	31.4	31.9	31.8	30.9
STDEV	0.2	0.1	0.4	0.1	0.2	0.1	0.2	0.2	0.1	0.1	0.2	0.1	0.0

CDC42 C_t Values

Sample ID	N-8	N-13	N-15	N-16	N-17	N-20	N-28	N-29	O-80	O-81	O-83	O-84	O-85
replicate 1	31.2	31.98	32.28	31.93	31.16	30.81	31.55	31.87	31.82	31.58	30.91	31.56	31.86
replicate 2	31.27	31.91	32.07	31.95	31.68	31.3	31.52	31.73	31.68	31.75	30.94	31.58	31.83
replicate 3	31.4	31.76	31.84	32.01	31.84	31.15	31.64	31.76	31.67	32.15	30.96	31.38	31.89
AVG C_t	31.3	31.9	32.1	32.0	31.6	31.1	31.6	31.8	31.7	31.8	30.9	31.5	31.9
STDEV	0.1	0.1	0.2	0.0	0.3	0.2	0.1	0.1	0.1	0.2	0.0	0.1	0.0

18S C_t Values

Sample ID	N-8	N-13	N-15	N-16	N-17	N-20	N-28	N-29	O-80	O-81	O-83	O-84	O-85
replicate 1	10.56	10.99	9.25	11.77	11.97	11.43	11.15	5.23	10.42	11.71	11.78	9.63	11.47
replicate 2	10.19	11.02	9.36	11.82	11.95	11.33	11.08	11.78	10.03	11.8	11.66	9.43	11.39
replicate 3	10.2	10.66	9.32	11.82	11.96	11.26	11.3	11.72	10.06	11.6	11.61	9.39	11.56
AVG C_t	10.3	10.9	9.3	11.8	12.0	11.3	11.2	9.6	10.2	11.7	11.7	9.5	11.5
STDEV	0.2	0.2	0.0	0.0	0.0	0.1	0.1	3.1	0.2	0.1	0.1	0.1	0.1

Samples in Yellow are OSA Controls

Samples in Green are OSA Positives

Table 3: qPCR Delta Ct Data

	Δ C_t (AVG Target Gene - AVG 18S Reference)												
Sample ID	N-8	N-13	N-15	N-16	N-17	N-20	N-28	N-29	O-80	O-81	O-83	O-84	O-85
Laminin	21.7	23.1	23.1	20.6	19.8	20.5	22.2	21.9	21.2	19.7	20.2	22.3	19.4
CDC42	21.0	21.0	22.8	20.2	19.6	19.7	20.4	22.2	21.6	20.1	19.3	22.0	20.4

Samples in Yellow are OSA Controls

Samples in Green are OSA Positives

Sample ID	AVG Δ Ct OSA Controls	STDEV	AVG Δ C_t OSA Positives	STDEV	OSA Positives Δ C_t-OSA Controls Δ Ct ($\Delta\Delta C_t$)	Fold Difference (2^-$\Delta\Delta C_t$)
Laminin	21.6	1.1	20.5	1.0	-1.1	2.1
CDC42	20.9	1.1	20.7	1.0	-0.2	1.1

APPENDIX M

RAW DATA

Table 1: Raw Subject Data

ID #	AGE (yrs)	Height (m)	Weight (kg)	BMI (kg/m2)	SBP (mmHg)	DBP (mmHg)	Neck (cm)	Waist (cm)	Hip (cm)	ESS	Berlin	ApneaLink Score	AHI
NG008	43	1.57	111.6	44.99	129	88	40.8	112.5	139	15	Low	5	
NG009	40	1.61	139.3	53.53	158	100	39.5	136.5	155	3	Low	6	
NG013	47	1.51	116.1	50.84	133	82	39.0	115	142	6	Low	8	
NG015	46	1.75	176.9	58.01	138	89	42.0	144	177	12	Low	4	
NG016	33	1.70	130.2	44.95	133	86	39.0	116	145	12	Low		0.2
NG017	24	1.75	140.2	45.63	140	80	40.5	132	145	12	Low		0.5
NG020	45	1.64	110.7	40.92	125	87	40.0	128	135	6	Low	4	
NG028	30	1.66	149.7	54.08	171	99	51.0	137	145	4	Low	4	
NG029	39	1.69	109.8	38.47	181	110	40.0	108	131	4	Low	2	
NG033	35	1.55	115.2	47.99	175	94	42.0	128.5	142.5	3	Low	2	
OG080	45	1.73	138.8	46.5	118	78	38.1	133.5	160	15	High		16.4
OG081	37	1.52	85.7	36.9	118	80	38.1	108	116	12	High		36.9
OG082	38	1.65	131.5	48.3	98	62	48.3	142	166	5	High		18.3
OG083	38	1.74	157.9	52.1	130	86	42.3	135	167	18	High		15
OG084	44	1.68	158.8	56.6	132	94	42.5	152	168	13	High		51
OG085	42	1.68	151.5	53.9	130	88	39.4	168	175	5	High		17.2