Molecular Signatures of Obstructive Sleep Apnea in Adults: A Review and Perspective

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The consequences of obstructive sleep apnea (OSA) are largely mediated by chronic intermittent hypoxia and sleep fragmentation. The primary molecular domains affected are sympathetic activity, oxidative stress and inflammation. Other affected domains include adipokines, adhesion molecules and molecules that respond to endoplasmic reticulum stress. Changes in molecular domains affected by OSA, assessed in blood and/or urine, can provide a molecular signature for OSA that could potentially be used diagnostically and to predict who is likely to develop different OSA-related comorbidities. High-throughput discovery strategies such as microarrays, assessing changes in gene expression in circulating blood cells, have the potential to find new candidates and pathways thereby expanding the molecular signatures for OSA. More research is needed to fully understand the pathophysiological significance of these molecular signatures and their relationship with OSA comorbidities.

Many OSA subjects are obese, and obesity is an independent risk factor for many comorbidities associated with OSA. Moreover, obesity affects the same molecular pathways as OSA. Thus, a challenge to establishing a molecular signature for OSA is to separate the effects of OSA from obesity. We propose that the optimal strategy is to evaluate the temporal changes in relevant molecular pathways during sleep and, in particular, the alterations from before to after sleep when assessed in blood and/or urine. Such changes will be at least partly a consequence of chronic intermittent hypoxia and sleep fragmentation that occurs during sleep.

Keywords: Obstructive sleep apnea, molecular mechanisms, chronic intermittent hypoxia, sleep fragmentation, pathophysiology

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applied. In this perspective we describe current data and indicate areas of opportunity. We propose that assessing changes in relevant biomarkers in blood overnight, for example, comparing levels before and after sleep (or an overnight urine test) assessing specific molecules as well as global gene and protein expression, is feasible and likely will be used in the future to assess OSA severity and inter-individual differences in the effects of the disorder. This approach has the potential to transform the practice of medicine in this area by providing diagnostic information as well as showing who will likely benefit from therapy. To fully understand the pathophysiological significance of these molecular signatures, and their relationship with OSA comorbidities, more research in this field related to both OSA and its consequences is needed.

OSA: PATHOGENETIC MECHANISMS AND CONSEQUENCES

OSA is a common condition with a number of adverse consequences. During sleep, individuals with OSA have repeated episodes of declines in breathing (apneas) or cessation of breathing (apneas) due to upper airway obstructions. These obstructions result in the following: interruption of sleep with frequent arousals (sleep fragmentation); loss of REM sleep and slow wave sleep (stage 3–4); repetitive decreases in oxygen saturation with rapid reoxygenation causing cyclical deoxygenation/reoxygenation; and repeated changes in intrathoracic pressure and episodic hypercapnia (Figure 2). A particular advantage in studying this common condition is that there is a safe, effective therapy—nasal continuous positive airway pressure (CPAP)—that can quickly reverse the occurrence of sleep disordered breathing events, and the majority of patients show reasonable compliance (device use > 4 hours per night).

One of the important questions in a quest to find molecular signatures of a disease such as OSA is which cells or tissue to choose for measurement. Blood, due to its interaction with all organ systems and tissues in the body, its diverse physiological roles as well as being an easily accessible tissue, is an attractive option. Blood is also accepted as a surrogate tissue for conditions where the target tissue is inaccessible (liver, lung), or other surrogate tissues that can potentially be used include urine, saliva, and breath condensate. In relevant molecular pathways in blood, urine, and breath condensate have been shown in OSA in various animal and human studies.

Chronic Intermittent Hypoxia

Much attention on the effects of OSA has focused on the role of chronic intermittent hypoxia (CIH). This can be reproduced in animal models, e.g., in rats and mice, in which a specific pattern of repetitive deoxygenation/reoxygenation can be produced so that causality can be established. CIH can also be produced in cell culture systems to investigate fundamental mechanisms.

CIH in mice/rats has been shown to lead to a large number of adverse effects: increased sympathetic activity and hypertension, increased catecholamine levels, liver dysfunction, learning deficits, with associated damage to cortical and hippocampal neurons; persistent hyperremolomucides with oxidative damage to wake-active neurons; insulin resistance; atherosclerosis when combined with a high-fat diet; and vascular remodeling. The activation of the proinflammatory transcription factor nuclear factor κB (NF-κB) pathways has been shown in response to CIH. There is also activation of the transcriptional factor hypoxia induction factor-1 (HIF-1α), a key factor in oxygen homeostasis, which causes direct activation of > 50 downstream expression, is feasible and likely will be used in the future to assess OSA severity and inter-individual differences in the effects of the disorder. This approach has the potential to transform the practice of medicine in this area by providing diagnostic information as well as showing who will likely benefit from therapy. To fully understand the pathophysiological significance of these molecular signatures, and their relationship with OSA comorbidities, more research in this field related to both OSA and its consequences is needed.

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Figure 1—Possible ways to assess molecular signatures in specific cells or tissues.

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**Figure 2**—A schematic illustrating the pathogenetic mechanisms for the consequences of obstructive sleep apnea (OSA) that indicate the areas for potential molecular signatures for the disorder.

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**Figure 2**—A schematic illustrating the pathogenetic mechanisms for the consequences of obstructive sleep apnea (OSA) that indicate the areas for potential molecular signatures for the disorder.
molecules such as erythropoietin (EPO)\textsuperscript{99} in response to CIH in animal and cell models.\textsuperscript{113,125-128} Activation of HIF-1α plays an important role in the sympathetic response, increased blood pressure and increased triglyceride levels in animal models.\textsuperscript{113,127,128}

In a recent paper it has been shown that NF-κB is a key transcriptional activator of HIF-1α, linking the proinflammatory and hypoxic response pathways together.\textsuperscript{129} Some cell model systems have shown preferential activation of NF-κB over HIF-1α activation\textsuperscript{121,122} and one study in rat carotid body showed increased expression of HIF-2α and HIF-3α in response to CIH.\textsuperscript{130}

In studies of chronic intermittent hypoxia, the oxygen levels that the animal models and cell cultures are usually exposed to are more severe than the degree of hypoxia typically found in most human subjects with OSA.\textsuperscript{97,98,112,124} Thus, the generalizability of the findings needs to be questioned. The level of oxygen saturation in tissue is usually not reported in rodent studies.\textsuperscript{100,102,106,112,115} Another difference between patients with OSA and studies in animal and cell models is duration of the insult. Human subjects with OSA may suffer over a long period of time from this condition before they seek medical assistance and are diagnosed.\textsuperscript{113} However, in animal or cell models CIH is usually administered for days or weeks.\textsuperscript{105,113,122,132} Some investigators have begun to address these issues by measuring the levels of oxygen saturation in tissue and produce less severe hypoxia within each CIH cycle to better simulate the hypoxia levels found in OSA patients in their models.\textsuperscript{111,118} A study with longer duration of insult in mice, i.e., 6 months, has also recently been reported.\textsuperscript{116}

The effect of intermittent hypoxia has also been assessed in humans. Studies looking at the effects of isocapnic (iso-CO\textsubscript{2}) hypoxia for 5 minutes (breathing in 10% O\textsubscript{2}) in awake healthy human subjects showed that isocapnic hypoxia causes increased sympathetic activation, increase in blood pressure and hyperventilation through peripheral chemoreceptor stimulation.\textsuperscript{133,134} This effect is strongly enhanced by a voluntary apnea at the end of the 5 minute period of hypoxia.\textsuperscript{135} Moreover, administration of 100% oxygen to OSA patients during obstructive events diminishes the sympathetic response to apneas.\textsuperscript{135} These studies therefore support the findings from animal models that short duration hypoxia leads to increased sympathetic activation.

Cyclical hypoxia with reoxygenation is thought to be like repeated ischemia-reperfusion damage with increased reactive oxygen species (ROS) production during the restoration of oxygen as occurs in an ischemic region when blood flow is restored (ischemia reperfusion).\textsuperscript{78,92,136} Thus, OSA is an oxidative stress disorder. CIH and ROS are the central focus of a model developed by Lavie about the pathogenesis of OSA comorbidities.\textsuperscript{92,137} According to this model, increased ROS production will lead to activation of NF-κB and hence increased expression of a number of downstream NF-κB target genes, e.g., proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), IL-6 as well as adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1).\textsuperscript{92,137} Increases in inflammatory cytokines and adhesion molecules are proposed to lead to activation of various cells such as monocytes, lymphocytes and endothelial cells leading to endothelial dysfunction and cardiovascular disease.\textsuperscript{92,137} The evidence supporting this model, both directly and indirectly, comes from studies in both animal models (as described above) and human studies, as will be described below.

\section*{Sleep Fragmentation}

CIH is not the only physiological challenge that occurs during apneic events; there is also the challenge of sleep fragmentation with repeated arousals. Each of these arousals is associated with a burst of sympathetic activity\textsuperscript{138-140} and cardiac changes including surges in blood pressure.\textsuperscript{141,142} Repetitive arousals lead to elevated cortisol and lipid levels,\textsuperscript{143} increased metabolism,\textsuperscript{144} and neurobehavioral deficits as a consequence of sleep fragmentation.\textsuperscript{144,145} The role of sleep fragmentation has, however, not received as much attention as chronic intermittent hypoxia. We do know that short sleep duration and chronic partial sleep deprivation over several days are associated with increased risk of hypertension, weight gain, insulin resistance and type 2 diabetes.\textsuperscript{146-150} Chronic partial sleep deprivation is also associated with neurobehavioral deficits\textsuperscript{151-154} and increased levels of inflammatory markers such as CRP,\textsuperscript{155} IL-6,\textsuperscript{156} and TNF-α.\textsuperscript{156} Even partial sleep deprivation for a single night has been shown to cause increased mRNA expression for IL-6 and TNF-α in circulating white cells.\textsuperscript{157}

\section*{Intrathoracic Pressure Changes}

The effect of recurrent changes in intrathoracic pressure has been little studied and is often neglected in models of the patho-genetic mechanisms for the consequences of OSA.

The recurring obstructive apneas, which occur with forceful inspiratory effort against an occluded airway, cause significant decreases in intrathoracic pressure.\textsuperscript{158} The molecular signature for intrathoracic pressure changes may be atrial natriuretic peptide (ANP), a volume-regulating hormone that functions to decrease the volume within the vascular system as a result of fluid overload.\textsuperscript{159} The intrathoracic pressure changes in OSA may cause increased venous dilation of the right atrium causing a “false” signal of fluid overload in the heart and hence increased atrial release of ANP.\textsuperscript{159} CIH may, however, also cause pulmonary vasconstriction, leading to right atrial dilation and increased ANP release.\textsuperscript{160}

In support of this hypothesis, ANP has been found to be increased in OSA subjects\textsuperscript{161,162} and decreased with CPAP treatment.\textsuperscript{161,163-165} ANP increases excretion of both urine and sodium-causing nocturia, which occurs commonly in OSA patients.\textsuperscript{159} The increase in ANP has been associated with either the intrathoracic pressure changes\textsuperscript{163} or intermittent hypoxia.\textsuperscript{163,166} Other studies have, however, questioned the role of OSA in ANP secretion and have found no significant elevation of ANP levels in patients with OSA.\textsuperscript{167,169} The short half-life of ANP (2-3 minutes) may be a factor in these negative results.\textsuperscript{170,171}

Subjects with what has been called the upper airway resistance syndrome (UARS) show increased upper airway resistance and changes in intrathoracic pressure during sleep, which is usually followed by arousals but occurs without any significant hypoxia.\textsuperscript{172} Studies on patients with UARS show that inspiratory efforts causing intrathoracic pressure changes are correlated with both systolic and diastolic blood pressure increases as well as an increased prevalence of hypertension.\textsuperscript{173} However, in a pig model, central apneas, which are characterized by no respiratory effort or intrathoracic pressure changes, caused more changes in mean arterial pressure, systemic vas-
cular resistance and cardiac output than obstructive apneas.174 Another study found similar systemic blood pressure response to obstructive and nonobstructive apneas in an anesthetized primate model.175

A sleep apnea model in rats has been recently described where upper airway collapse and reopening is induced by subjecting rats to recurrent positive and negative pressures by means of a nasal mask valve.176 Overexpression of inflammatory biomarkers, such as TNF-α, was found in the larynx and soft palate tissue in response to these recurrent pressure changes over a period of 5 hours.176 However, the blood oxygen levels were not reported, and the potential effect of intermittent hypoxia therefore not addressed. Interestingly, snoring-like vibrations applied short-term have also been found to produce similar inflammatory changes in upper airway tissue,177 supporting the hypothesis that mechanical stimuli may cause inflammatory changes in the upper airway.

These results collectively suggest that intrathoracic pressure changes are not a major contributor to the systemic changes in patients with OSA, but potentially a cause for upper airway inflammation. This pathogenetic mechanism requires further investigation.

CLINICAL EVIDENCE FOR THE MOLECULAR AND PATHOLOGICAL CONSEQUENCES OF OSA

Primary Molecular Domains Affected by OSA

Sleep fragmentation138-140 and CIH96-98,135 lead to increased sympathetic activity. Sleep fragmentation and CIH also cause an inflammatory response, and CIH causes oxidative stress. This suggests that the molecular signature for OSA will most likely be found using measures of the following: sympathetic activation, oxidative stress and inflammation. Described below is the evidence for each of these domains and potential biomarkers for each domain.

What Is the Evidence of Increased Sympathetic Activity in OSA?

OSA patients have increased sympathetic activity measured by microneurography (direct recording of muscle sympathetic nerve activity) compared to controls during the daytime,178,179 that decreases with CPAP treatment.180-182 Plasma and urine norepinephrine levels which reflect systemic sympathetic neuronal activation,183 are increased in OSA patients compared to controls,184,185 but this is not shown in all studies.179 Many studies have also shown that CPAP treatment reduces noradrenaline levels.182,186-191 The results for epinephrine levels, which reflect adrenomedullary hormonal activation,183 are less convincing, as levels were similar in OSA subjects compared to well-matched controls in 2 studies.184,185 Some studies have found a small decrease with CPAP,192,193 but not all.191 Withdrawal of CPAP for one night does not result in a significant increase in norepinephrine levels,194 but withdrawal for one week significantly increases urinary norepinephrine levels during the daytime but not the nighttime.195

Factors influencing the decrease in sympathetic activity with CPAP treatment include CPAP compliance,180 length of treatment,181 and whether subjects are initially normotensive or hypertensive.187 The increase in blood pressure found in many untreated OSA patients is considered, at least partly, a result of their high sympathetic activity.196,197

Increased sympathetic activation, such as occurs in OSA, leads to enhanced lipolysis with release of free fatty acids (FFAs), particularly from visceral fat cells.198,200 Elevated FFAs then activate inflammatory pathways,201,202 and act as mediators of insulin resistance.203,204 Interestingly, to date, little research has been done on FFAs in OSA. One recent study found that FFA levels, body mass index (BMI) and AHI were independently associated with insulin resistance in OSA patients but visceral and subcutaneous fat were not.205 As increased sympathetic activity, inflammation and insulin resistance are all associated with OSA, we propose that elevated FFAs likely have a role in the pathogenesis of OSA consequences. This role remains to be determined.

What is the Evidence That There Is Oxidative Stress in OSA?

The concepts described would posit that there should be evidence of oxidative stress in patients with OSA due to the CIH. A number of studies support this hypothesis.18,19,86,90,91,206-209 Oxidative stress is, however, difficult to assess.210-212 Many of the purported measures such as the commonly used thiobarbituric acid reactive substances (TBARS) are nonspecific and were not validated as reliable measures in a multi-institutional study in a rat model designed to assess reliability of methods to assess oxidative stress.213 Thus, current data need to be interpreted with caution, particularly since many studies have small sample sizes and lack appropriate controls.

Increases in the following purported measures of oxidative stress have been reported in OSA, usually compared to controls and in some studies consequent decreases with CPAP: plasma, exhaled breath condensate and nighttime urinary 8-isoprostane levels as measured with an enzyme immunoassay90,91,209, plasma levels of malondialdehyde (MDA)86; urinary o,o-dityrosine86; plasma levels of TBARS206,207; urine levels of 8-hydroxy-2’-deoxyguanosine (8-OhdG), a marker of DNA oxidation208; reactive oxygen species (ROS) production in specific subpopulations of monocytes, granulocytes, and neutrophils upon in vitro stimulation.18,19 However, studies showing no oxidative stress in OSA are also reported.214-216 Many of the studies performed have either used poorly matched control groups206-208 or used measures that are controversial regarding their ability to assess oxidative stress.217-219 One study which assessed the reliability of many different oxidative stress measurements in a small group of OSA subjects found that only plasma MDA and urine o,o-dityrosine were appropriate measurements of oxidative stress86 (This study did not measure 8-isoprostanes.) Given the non-reliability of many of the measures of oxidation, as revealed by recent studies,86,213 one needs to consider which measure is optimal to assess oxidative changes.

From the many candidates available, the best currently available biomarker for oxidative stress, and the one accepted in the field for in vivo studies,210-213 is 8-isoprostane (8-iso-PGF<sub>2</sub>α), a marker of lipid peroxidation, measured by gas chromatography/ negative ion chemical ionization mass spectrometry (GC/NICI-MS).210-213 Using immunoassays as has been done in earlier OSA research90,91,209,212 is more cost-effective but information regard-
What is the Evidence that OSA Increases the Inflammatory State?

Based on the overall oxidative stress model described above,92,137 increased ROS production should cause increased expression of inflammatory cytokines through activation of NF-κB in OSA patients. However, this is only one postulated model. As stated above, visceral fat and increased sympathetic activity can increase free fatty acid levels which cause an increase in inflammatory cytokines in the absence of ROS.198-202

Much of the focus on inflammation in OSA research has been on the markers TNF-α, IL-6, and CRP. Most studies show the following: higher levels of TNF-α and/or IL-6 and/or CRP in plasma of subjects with OSA compared to BMI-matched controls,11,74,85,90,221-227 and reductions in these inflammatory markers with CPAP therapy when assessed post-sleep.85,209,223,224 There are, however, also negative studies showing no difference in TNF-α, IL-6, and CRP levels in OSA patients versus matched controls and after CPAP therapy.73,75,85,228-232 Obesity is the primary factor affecting the levels of the inflammatory markers in many of these negative studies,23,228,229,231 illustrating the confounding effect of obesity on OSA research and the importance of having well-matched controls. (This issue of confounding by obesity is discussed more fully below.) These differences between studies support our concept of inter-individual differences and heterogeneity of responses in OSA patients that we also describe more fully below.

Other inflammatory effects such as systemic increases in interleukin-8 (IL-8), granulocyte chemotactic protein-2 (GCP-2), monocyte chemotactic protein-1 (MCP-1) levels, and inflammatory cell infiltration in the upper airway have been shown in OSA subjects.85,233-235 Whether the inflammatory changes in the upper airway occur because of hypoxia, snoring, the mechanical stress of recurrent pressure changes or systemic inflammation or all of the above remains to be answered.236 Moreover, whether at least some of the systemic increases in inflammatory biomarkers are due to a “spill-over” effect from the upper airway remains unknown.

Given available evidence on the effects of OSA, the inflammatory markers with the strongest rationale to study further are TNF-α and IL-6. Assessment of each of these has particular challenges. TNF-α may be useful to study dynamic changes. Rapid increase in TNF-α in plasma following apneic events has been described.88 TNF-α is, however, very sensitive to handling and the blood needs to be processed and frozen immediately for accurate measurements of its levels.237,238 This procedure should, of course, be applied to all measured biomarkers to avoid artificial changes in their levels. The challenge for IL-6 is that its levels can be artificially elevated over time when blood is obtained from an indwelling intravenous line due to local production by the endothelium.239-242 This does not apply to TNF-α.242

Systemic inflammation is, however, also produced by diseases other than obstructive sleep apnea. Thus, there are a number of confounding effects that need to be considered in assessing these biomarkers (Figure 3). This aspect is discussed more fully below.

Secondary Domains Affected by OSA

The three domains described above represent, in our opinion, the key variables that are affected by the pathogenetic processes in OSA. However, other domains are also affected, such as adipokines,243-259 adhesion molecules,260,261 and possibly stress in the endoplasmic reticulum (ER).211 We now describe evidence for each of these.

What is the Evidence for Changes in Adipokines?

White adipose tissue (WAT) is a metabolically active tissue that produces over 50 molecules termed adipokines with various functions.262,263 Oxidative stress,243-248,264-266 inflammation,243,249-257,264,266-271 and sympathetic activation259,272 can all affect the expression of adipokines. Therefore OSA potentially affects adipokine levels. A more detailed discussion of the function of the more investigated adipokines now follows.

Leptin:

Leptin is a pleiotropic cytokine with a circadian rhythm in expression, which is produced mainly by WAT and has a regulatory role in body adiposity with high levels acting as a satiety signal.273-277 Leptin also has an immunomodulatory role278-281 and is both activated by proinflammatory mediators249-251 and works as a stimulant of proinflammatory cytokine production such as IL-6 and TNF-α.282 Leptin levels are increased in obesity,271 but due to a central leptin resistance that occurs in obesity, it fails its regulatory role in reducing adiposity.277,283 However, despite the metabolic leptin resistance, high leptin levels still induce sympathetic activation of tissues such as the heart and the adrenal glands,283 which may contribute to the low-grade systemic inflammation and development of hypertension in obesity.283-285 Leptin acts as an independent risk factor for cardiovascular disease.286-288 Hyperleptinemia and hyperin-
Adiponectin: Adiponectin, produced by mature adipocytes, increases oxidation of fatty acids and has inhibitory effects on glucose synthesis by the liver. Unlike leptin, adiponectin shows no circadian rhythm but has some apparently random fluctuations in levels across the day and night. Despite being produced by adipocytes, adiponectin levels decrease with increased fat stores, and a decrease in adiponectin levels is associated with obesity, cardiovascular disease, insulin resistance and type 2 diabetes. It has an anti-inflammatory function, as it inhibits NF-kB activation and hence production of IL-6 and TNF-α. It also induces production of the anti-inflammatory interleukin-10 (IL-10). Oxidative stress, TNF-α, and IL-6 all inhibit adiponectin production, hence potentiating their effects.

Adiponectin levels have been shown to be lower both in the morning and evening in OSA patients compared to BMI-matched controls. However, other small studies have found increased adiponectin levels in the evening in OSA patients and no difference in the morning from controls. One study found a nocturnal decrease in adiponectin levels in severe OSA, which was not found in controls or milder OSA. Results from studies looking at the effect of CPAP therapy on adiponectin levels are also conflicting. Two studies found no immediate changes in adiponectin levels in the morning with CPAP, while one study found a decrease after 2 days on CPAP (time of day of measurement is unclear), and another found a reduction in a nocturnal decrease of adiponectin after one night of CPAP. Two small studies have also given conflicting results about long-term effects of therapy. One found an increase in adiponectin levels after long-term CPAP therapy, but the other no change from baseline levels. Finally, a larger study that measured visceral and subcutaneous abdominal fat (by computerized tomography) in untreated OSA patients found no relationship between morning adiponectin levels and OSA severity, but did find a relationship with visceral fat area and body weight.

Therefore the question whether adiponectin has a role in the pathogenetic consequences of OSA or whether its levels are affected by OSA remains unclear. Further studies assessing both abdominal fat volume and adiponectin levels across the day and night in OSA patients, when untreated and on CPAP, are needed to address this issue as OSA possibly affects the usually random secretion of adiponectin and causes it to decrease across the night.

Resistin:

Resistin is almost exclusively expressed in WAT in murine models but is expressed in high levels in other tissues such as bone marrow and macrophages in humans and associated with the immune system. Resistin has been implicated in insulin resistance based on research in rodent models, but the data from human studies are controversial. Resistin appears to have a role in inflammation and has been shown to be both activated by, and cause activation of, IL-6 and TNF-α through the NF-κB pathway in peripheral blood mononuclear cells. In adipocytes, however, IL-6, TNF-α and epinephrine either have no effect on or downregulate resistin production. Antioxidant treatment causes decreased resistin levels in serum, indicating that oxidative stress has some regulatory role in resistin production. Resistin has been shown to induce the production of adhesion molecules and be increased in patients with coronary artery disease, suggesting that it has a role in atherogenesis.
Two small studies to date have looked at the relationship between resistin and OSA in adults. One study looking at obese subjects with severe OSA found that resistin levels were related to obesity, inflammation and atherogenesis, not to OSA, and did not change with CPAP.338 The other study looked at less obese OSA subjects with different disease severity and controls. They found that resistin levels and inflammation levels were related but also found increased resistin levels with increased OSA severity and a reduction with CPAP.339 A study in children with OSA found no relationship between resistin levels and OSA.340 However, since inflammatory cytokines affect resistin levels and vice versa, and resistin has a potential role in atherogenesis, further studies are needed to address whether OSA alters resistin levels. This relationship between resistin and OSA is potentially dependent on OSA severity and obesity levels.

Other Newly Discovered Adipokines:

Other adipokines, recently discovered, include visfatin, apelin, vaspin, and hepciden.326 These adipokines have not yet been studied in the context of OSA but hepciden has been suggested as a marker of OSA.341 Studies looking at the relationship between OSA, obesity and these adipokines are of interest as they are regulated by many of the same molecular processes as are found in OSA pathophysiology (e.g., hypoxia and inflammation).243-245,252,264-272 This is a future direction for research.

What is the Evidence of Changes in the Unfolded Protein Response in OSA?

Stress in the endoplasmic reticulum (ER), caused by a disruption of protein folding and buildup of unfolded proteins in the ER,352 occurs in response to stressors such as hypoxia355,354 and cholesterol loading355 and in conditions such as obesity356,357 and type 2 diabetes.352,358 ER stress causes the unfolded protein response (UPR), an adaptive response which increases the up-regulation of ER chaperones and downregulates protein translation in order to reestablish normal function of the ER.352 The UPR has been shown to occur in the liver of obese mouse models356 and fat of obese subjects (not in lean).359 UPR has a role in initiating insulin resistance in obesity,356 as well as in promoting atherosclerosis355 and possibly inflammation.359

Increased expression of the prototypical molecular chaperone termed binding immunoglobulin protein (BiP, also known as GRP78), phosphorylation of PKR-like ER kinase (PERK) and the eukaryotic initiation factor 2-α (eIF2α) can be used as molecular signals that indicate activation of the UPR.352,358,360 A recent study provides support for the presence of ER stress in OSA as CIH in mice leads to increased phosphorylation of PERK and other changes signalling upregulation of the UPR in motoneurons.361 Total sleep deprivation has also been found to activate the UPR in the brain of different animal models.25,262-267 Research on the UPR in OSA is of interest, and it is conceivable that it will be activated across the sleep period in circulating cells.

Potential Confounding Effects on the Molecular Signatures of OSA

Confounding Effects of Obesity

Obesity, in particular central obesity, is the most important risk factor for OSA.368-374 OSA with complaints of excessive sleepiness affects 4% of middle-aged males and 2% of middle-aged females,39 but among obese subjects, these percentages are much higher.39,371,372,375-378 OSA prevalence in morbibly obese subjects requiring bariatric surgery has been found to be between 40% and 94%.372,375-378 Both obesity and OSA have been shown to be independent risk factors for insulin resistance, hypertension, and cardiovascular disease (for reviews on OSA, see 379-381). Insulin resistance is also an independent risk factor for hypertension and cardiovascular disease.382 Intervention studies based on treating subjects with OSA with CPAP have shown improvements in insulin resistance303,383,384 and hypertension.385-390 An observational study found improvement in glucose control in obese type 2 diabetics with OSA treated with CPAP,391 but a randomized trial of CPAP therapy in very obese type 2 diabetic OSA patients found no improvements in glucose control with CPAP.392 Meta-analysis of treatment trials suggests that the effects of treatment of OSA on blood pressure are modest.386

Because OSA and obesity commonly coexist and have been shown to have similar clinical consequences, it is important to consider their relative roles in causing adverse clinical consequences; it is also important to delineate the relative importance of shared common pathways and whether there are unique pathways related to OSA that mediate clinical consequences. The key pathogenic mechanisms resulting from OSA, i.e., oxidative stress, inflammation and sympathetic activation, also occur in obesity,393-395 and have a role in insulin resistance.399-406 Thus, at this mechanistic level both obesity and OSA affect the same processes, and their relative roles in oxidative burden, the inflammatory state (see Figure 3), and sympathetic activation need to be assessed. Unfortunately, the large literature on obesity simply ignores this issue. In a recent meta-analysis of studies demonstrating a link between obesity and cardiovascular disease published in Lancet,407 sleep disordered breathing was not assessed in a single study, which, from a scientific perspective, is an important omission. This is an important question to address, since there is a safe, effective therapy available for OSA.
CPAP treatment of OSA in obese individuals has the potential to alter the cardiovascular consequences of obesity. A critical issue is how to determine the relative role of OSA. One commonly used strategy is to assess differences between OSA patients and controls without OSA but matched for BMI. Matching for BMI is likely, however, not to be sufficient. Abdominal visceral fat has been shown to be a stronger risk factor than other fat tissue for adverse health consequences and is associated more strongly with hypertension, insulin resistance, diabetes, the metabolic syndrome than other fat deposits. Further evidence that visceral fat rather than other fat deposits is causal (not only associative) for insulin resistance is, however, needed. Visceral fat is also a risk factor for OSA and has been shown to be increased in OSA patients compared to BMI-matched controls. Visceral fat, therefore, plays an important part in understanding of OSA pathophysiology moving forward and needs to be directly assessed in studies of OSA.

An alternative and more powerful strategy to separate the effects of obesity and OSA is to use a within-subject design, i.e., assess differences before and after effective CPAP therapy. This strategy has been used in multiple studies. There are, however, problems with this strategy. First, there are data to indicate that successful CPAP therapy reduces the amount of visceral fat, albeit by a small amount (8% to 16% over a period of 3-6 months). Thus, changes with CPAP treatment could be due, at least in part, to reductions in visceral fat mass. Moreover, in patients with OSA who are effectively treated with CPAP, there could be irreversible effects of OSA. OSA is a chronic, slowly progressive disorder, and it can be present for years before it is diagnosed. Residual sleepiness in OSA has been described, i.e., persistent sleepiness even on effective CPAP therapy. This effect might be mediated, at least in part, by oxidative damage to wake-active neurons. There could also be vascular wall remodeling that occurs during the years with untreated OSA. Currently the magnitude of irreversible effects of untreated OSA is largely unknown. Studies are needed to estimate the reversible effects of OSA (differences pre-to post-CAP in effectively treated individuals) and irreversible effects of OSA, i.e., estimate the difference between patients with OSA after effective treatment when compared to controls with similar levels of visceral fat but not with OSA.

Since both free fatty acids and proinflammatory cytokines are produced by visceral fat, it is likely that for equivalent degrees of OSA, there will be an enhanced production of these biomarkers in obese subjects as a consequence of OSA in comparison to lean subjects with OSA. Since obesity in the absence of OSA can lead to production of these biomarkers, we would anticipate that obese individuals with OSA will have higher levels of biomarkers even after effective treatment of OSA than lean subjects. Comparison of biomarkers in individuals with different degrees of visceral fat, all of whom are effectively treated for OSA, will provide the much needed estimate of the effect of obesity per se. In such studies, waist circumference can be used, since waist circumference is increasingly recognized to be the best proxy for the degree of visceral adiposity. But direct measurement of both visceral and subcutaneous fat distribution would add more definitive information, as waist circumference cannot distinguish between subcutaneous and visceral fat. A study by Vgontzas et al emphasizes the importance of matching for visceral fat between groups in studies of OSA. In this study, plasma levels of IL-6, TNF-α, and leptin in the evening and morning were measured. Obese OSA patients were found to have the highest levels of the 3 biomarkers, obese controls without OSA intermediate levels, and lean controls the lowest. However, the OSA patients also had more visceral fat than the obese controls, confounding the results. Thus, directly assessing visceral fat is required to answer these questions.

Other Coexisting Conditions

Systemic inflammation, a key aspect of pathological mechanisms for the consequences of OSA, also occurs in cardiovascular disease, type 2 diabetes, asthma, and smoking. Oxidative stress is another key element in OSA but is also increased with type 2 diabetes, smoking, cardiovascular disease, the metabolic syndrome, age, and joint diseases. Sympathetic activation has also been shown to be increased in cardiovascular disease, the metabolic syndrome, and insulin resistance—all conditions commonly found in OSA patients. Thus, establishing the actual role of OSA is challenging, and will require study designs that deal with the confounding effect of obesity and the coexistence of other conditions that affect the same mechanisms as OSA does.

INTER-INDIVIDUAL DIFFERENCES IN OSA

Variability in the Nature of OSA

Differences between individuals with OSA may be directly related to the nature of their sleep disordered breathing events. Some individuals with OSA have marked sleep fragmentation with little, if any, intermittent hypoxia, while others may show marked intermittent hypoxia with lesser degrees of fragmentation. Within an individual the magnitude of hypoxia and arousals may vary across the sleep period. We propose the need for studies that capture this source of variability by recruiting subjects from both extremes of the spectrum (with regard to severity of intermittent hypoxia and number of arousals) and assess associations between changes in biomarkers and the nature of the sleep disordered breathing events both between and within individuals.

Heterogeneity in the Biological Response

The change in molecular pathways between different individuals for identical degrees of sleep disordered breathing is also unlikely to be the same. There will likely be inter-individual differences in response. Such inter-individual differences might be seen as a challenge but represent, in our view, another opportunity. We know that many individuals with OSA...
do not have excessive sleepiness, and only about 50% of OSA subjects develop hypertension. The question is why—why do some subjects with OSA develop hypertension and cardiovascular disease while others do not? One possibility is that individuals who develop hypertension and cardiovascular disease have increased pathogenetic responses or decreased adaptive response for equivalent degrees of OSA.

Such heterogeneity in response might be related to differences in protective mechanisms. Individuals vary in their antioxidant ability, which is complex, as it involves dietary factors and multiple intrinsic antioxidant systems. Candidates for measurement are numerous, including antioxidant enzymes (such as superoxide dismutases and glutathione peroxidases), non-enzymatic antioxidants (such as glutathione, bilirubin, and vitamins C and E), and melatonin, which has a role in the regulation of antioxidant enzyme activity and expression. Measurements of total antioxidant capacity that are meant to functionally assess the total antioxidants in plasma or serum are also used but the reliability of these assays and what they actually measure is questionable.

The question whether there is a change in antioxidant levels in OSA patients remains unanswered. The studies done so far have been relatively small, used various methods for assessing antioxidant ability, and have contradictory results. Levels of anti-inflammatory molecules such as IL-10 can also vary between individuals. IL-10 has a primary role in regulating inflammatory responses and has a role in protecting against atherosclerosis, insulin resistance, and type 2 diabetes. Thus, individuals with OSA and comorbidities may have lower IL-10 levels than those without comorbidities.

IL-10 levels have been reported to be lower in non-obese children with OSA than controls and increased after tonsillectomy and adenoidectomy in children with OSA. One study showed that IL-10 levels were decreased in the evening in OSA patients compared to ill-matched controls but another study with better controls found no difference.

Adaptive responses that affect sympathetic activation are also potentially heterogeneous between individuals with OSA. Likely candidates include differential downregulation of adrenergic receptors and differences in norepinephrine clearance rates, which act to diminish the stimulatory response and the increased sympathetic activity in OSA.

Differences between those with OSA with comorbidities and those without could be genetic in origin. There is now a large literature on genes conferring risk for hypertension, insulin resistance, and cardiovascular disease. Polymorphisms in genes affecting oxidative stress, inflammation, and sympathetic activity are also of high interest. There is a limited but growing number of candidate gene studies addressing the question of gene variants and their pathophysiological effect in the OSA population.

More studies in this field are clearly needed, preferentially using more high-throughput technology such as genome-wide association, custom genotyping arrays such as the new cardiovascular chip to look for single nucleotide polymorphisms associated with increased risk for comorbidities in individuals with OSA, copy number variations as well as gene-gene and gene-environment interactions.

THE CONCEPT OF A TEMPORAL MOLECULAR SIGNATURE FOR OSA

One approach to understanding the clinical variability in the consequences of OSA in different subjects is to evaluate the molecular signatures of the disorder. There are certain aspects that we believe make OSA unique: a) the pathogenetic events occur in a temporally controlled fashion, i.e., during sleep; and b) the nature of the pathogenetic events can be characterized, e.g., breathing cessations, sleep fragmentation, hypoxia, cyclical deoxygenation/reoxygenation, etc. These concepts would argue that if we are to develop a molecular signature of OSA we should focus on the temporal changes taking place across the sleep period and relate these to the postulated pathogenetic events. Such temporal changes during sleep might, of course, in part, be related to circadian factors and/or alterations in sleep stages. There is, for example, circadian control of molecules (such as melatonin) and functions (such as body temperature) and a sleep-stage specific regulation of others, such as plasma catecholamine concentration, heart rate variability, thermoregulation, and sweating. To initially evaluate this source of temporal variability it will be necessary to study OSA subjects before and while on an effective therapy with CPAP as well as matched controls with no OSA. The changes in sleep stages usually seen with successful CPAP treatment are harder to control for but can at least be addressed as covariates in statistical models of larger studies, as there are substantial inter-individual differences in the sleep stage architecture in untreated OSA patients and changes with treatment.

Thus, we believe that the optimal molecular signature for OSA is likely to be change in relevant biomarkers across the sleep period; that is to measure the change from pre-sleep to post-sleep. An advantage of assessing an overnight change, instead of a single time point, is that many of the molecular mechanisms activated by OSA are shared by other common conditions (as discussed earlier), particularly obesity, cardiovascular disease, and type 2 diabetes. Assessment of biomarkers at a single time point will be confounded by these conditions, whereas overnight changes due to the challenge of OSA will give, in our view, a clearer picture of the contribution of OSA.

Given the postulated relationship between breathing events during sleep and the molecular process we are assessing, there is a strong rationale for the overnight change to be an ideal molecular signature for OSA. Previous data to support this assertion are, however, limited since most studies have simply measured biomarkers in a single sample in the morning, i.e., post-sleep. Available data from studies looking at overnight change in OSA, which are scant, support this hypothesis that studying change across the sleep period will give more information than a single sample: a) progressive increase in the oxidative stress marker MDA across the night in patients with OSA that correlates with the percentage of time with SaO<sub>2</sub> < 85%; b) an almost 3-fold increase in plasma TNF-α levels after the first hypoxic apneic event of the night with oxygen saturation below 85% in OSA patients compared to pre-sleep levels; c) 24-hour measurements of erythropoietin that showed attenuation in oscillation and changed peak values from untreated to

SLEEP, Vol. 32, No. 4, 2009
CPAP treated subjects;\textsuperscript{4,8} and d) acute changes in sympathetic activity and blood pressure during apneas.\textsuperscript{196}

Our postulate that overnight measurements of change in biomarkers will provide superior diagnostic ability for OSA than post-sleep measurements only is that it addresses the problems of coexisting conditions such as obesity. It takes advantage of the temporal nature of OSA. This postulate needs to be tested further, as there are limited studies addressing this issue to date and more research is clearly needed. We summarize in Table 1 what we propose are the advantages of overnight measures (repeated samples) compared to assessments at a single time-point.

### COMBINING A TEMPORAL STRATEGY WITH NEWER APPROACHES

#### Gene Expression Profiling

Microarrays are increasingly being used to evaluate potential molecular signatures of disease.\textsuperscript{3,499,500} They offer the advantage of providing a broad unbiased approach to study changes in gene expression of thousands of genes simultaneously and the ability to discover new pathways and molecules affected by a disease such as OSA.\textsuperscript{1,2} The main disadvantage is the cost of this high-throughput technology.

Whether looking at the gene expression of specific genes or using a microarray approach, investigators need to be aware of the sensitivity of mRNA to storage and handling. mRNA expression continues to change after blood or other tissue is extracted, if the tissue is not immediately suspended in chemicals to kill the cells and the tissue is kept frozen.\textsuperscript{491-498} For example, after 4 hours of storage at room temperature of whole blood with EDTA, there is a 10- to 100-fold increase in expression of IL-8, c-myc, and c-fos.\textsuperscript{491} Care must also be taken with the statistical analysis of microarray data, as well as with other high-throughput methods, such as proteomics, metabolomics, and lipidomics, because of the multiple comparison issue. Complex statistical analyses have been designed for this purpose.\textsuperscript{499-506}

Table 1—A Comparison of Biomarker Assessment at a Single Time-point Versus Overnight Measurement

<table>
<thead>
<tr>
<th>Time of Measurement</th>
<th>Single Time-Point</th>
<th>Overnight Measurement (repeated)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Generally in the morning following OSA events</td>
<td>During OSA events and at different sleep stages</td>
</tr>
<tr>
<td>Circadian rhythm detection</td>
<td>No</td>
<td>Shows changes in amplitude and shifts in circadian rhythm</td>
</tr>
<tr>
<td>Assessment of dynamic changes during sleep</td>
<td>No</td>
<td>Detects rapid changes in biomarkers (e.g., TNF-α, 8-isoprostanes)</td>
</tr>
<tr>
<td>Assessment of confounders</td>
<td>Limited</td>
<td>Shows acute effects of OSA vs. combined chronic effects of OSA and comorbidities</td>
</tr>
<tr>
<td>Reliability of measurement</td>
<td>Single sample more affected by measurement errors and biological variations</td>
<td>Multiple samples less affected by measurement errors and biological variations</td>
</tr>
</tbody>
</table>

Proteomics

Proteomics is a discovery strategy, similar to gene expression profiling, that examines expression of proteins and post-translation-al modifications of thousands of proteins simultaneously.\textsuperscript{4,36,37,507,508} The benefits of proteomics is to obtain information regarding changes in protein quantities, post-translational modifications, and protein-protein interactions.\textsuperscript{509} 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.\textsuperscript{510}

Proteomics is more complicated than genomics and the state of the technology is not as developed as microarrays. 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\textsuperscript{10} as well as the myriad post-translational modifications that alter the protein signatures of samples (see 510). The most employed proteomics technologies today include 2-dimensional gel electrophoresis and/or HPLC coupled to different mass spectrometry approaches. A discussion on the advantages and disadvantages of the different technologies is beyond the scope of this paper but for an excellent review see Gulciçek.\textsuperscript{510}
An important issue with proteomics is that of protein abundance; abundant proteins tend to be overrepresented in studies, while less abundant proteins are often not detected. This is especially important/true in plasma proteomics, in which 99% of plasma is comprised of 22 highly abundant proteins, and most of the clinically interesting biomarkers are found at much lower levels. Immunoaffinity depletion of samples as well as the use of fluorescent dyes with greater dynamic ranges are being used to address this problem. Proteomics in blood can be done either on plasma or serum. The use of plasma is considered superior to serum for at least certain proteome measurements, as approximately 40% of signals found in serum are not found in plasma because of ex vivo generation during clotting. Urine proteomics is also considered a promising method for new biomarker discoveries in relation to various systemic diseases. Urine analysis has the potential to find changes in circulating proteins and peptides that are small enough to pass through the glomerular filter; it is therefore not isolated to diseases related to the kidneys but all diseases that have an effect on circulating molecules.

Only two studies have been published looking at proteomic patterns in relation to OSA. Both studies come from the same research group investigating OSA in children. One study assessed the serum proteomics patterns associated with OSA in children and found that 3 proteins differed in expression between children with OSA and those with snoring only. Assessment of the proteins could predict OSA diagnosis with a sensitivity of 93% and specificity of 90%. One of the 3 proteins was identified as osteocalcin, a precursor for a γ-carboxyglutamic acid-containing protein that has been used as a biomarker for growth retardation, an important consequence of OSA in children. The other study assessed the difference in first-morning urine samples between children with OSA and controls and found significant differences in the expression of 2 proteins: gelsolin (severs actin filaments when activated) and perlecan (a heparin sulfate proteoglycan), between the 2 groups. These interesting findings need to be replicated by other studies.

**Metabolic Profiling**

Metabolic profiling, the global analysis of metabolites found in a tissue or cell under specific conditions (also known as metabolomics) and the response of a tissue or cell to a disease or drug toxicity (also known as metabolomics), is still in its infancy and currently has its share of technical problems. Metabolic profiling, however, has the potential to shed further light on the molecular signature of diseases such as OSA in conjunction with other high-throughput techniques.

No studies using this approach have been done in the field of OSA but studies looking at diabetes and cardiovascular disease show initial promising results. This will likely be a topic of more studies in the future.

**Investigating a Cellular Window**

Measuring circulating biomarkers in plasma or assessment of molecules in urine can be used to estimate the changes in biomarkers due to OSA. Another approach is to assess changes in specific cell populations in blood. This provides a more cellular approach to the quest of finding biomarkers for OSA, and gives researchers the opportunity to look at intracellular mechanisms without employing invasive measures to look at affected organs. Such cellular approaches have already been applied successfully to study the effects of obesity and cardiovascular disease, hypoxia and seizures. While earlier studies using this approach often used all white blood cells to study changes, there is, not surprisingly, a heterogeneity in response of different cell types. 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 has been implicated in insulin resistance/type 2 diabetes and atherosclerosis, respectively, both of which are considered comorbidities of OSA. Monocytes have also been shown to be affected specifically by OSA, e.g., by NF-κB activation, increased production of TNF-α, and IL-6. Other subpopulations of leukocytes such as lymphocytes have also been examined in patients with OSA. Different subpopulations of T lymphocytes are activated and more cytotoxic in OSA patients. Lymphocytes also have less repair capacity for DNA damage in OSA patients. Therefore, studying molecular changes in different subpopulations of circulating cells provides additional opportunities for establishing molecular signatures for OSA.

**CONCLUSION**

Throughout this review and perspective, we have provided information to support our assertion that there are major opportunities to establish molecular signatures for OSA. These opportunities include both hypothesis-driven studies on specific pathways and use of broad discovery strategies such as expression profiling, proteomics, and metabolomics. Investigating changes in gene or protein expression in specific circulating cells will likely provide the clearest signatures.

Part of the challenge for establishing a molecular signature for OSA is that obesity, which is commonly associated with OSA, leads to activation of the same pathways as does OSA. This suggests that we need approaches to separate the effects of OSA from that of obesity per se. Examining the overnight change in relevant biomarkers across the sleep period is, we propose, the optimal strategy to do so.

It is conceivable that since changes in molecules in key pathways across the sleep period will be the consequence of the aberrant breathing events, considerable information about these events can be obtained from simple blood and/or urine tests, without the need for overnight recording of many physiological variables. It is likely that the changes across the sleep period will reflect the degree of free radical production, as a consequence of cyclical intermittent hypoxia, and the degree of hypoxia potentially assessed by looking at HIF-1α expression in circulating cells as well as levels of proteins whose level is affected by HIF-1α.

It is conceivable that establishing molecular signatures for OSA will provide not only diagnostic information, but also prognostic information, i.e., which person with OSA is likely to develop specific consequences. We need to focus on inter-individual differences within the OSA population and the heterogeneity in response to the disease between individuals. It is important to evaluate the sources of this inter-individual variability. Developing and validating molecular signatures for OSA will...
be a major part of this effort. It will complement genetic studies to make the goal of personalized sleep medicine a reality.

The biomarkers studied in the OSA field to date, are neither sensitive nor specific enough to be a molecular signature for OSA since changes in levels of the biomarkers studied are also found in many of the comorbidities of OSA. One of the major thrusts of this review is to urge investigators to collect data in a manner that allows us to evaluate what happens temporally to biomarkers across the sleep period, by sampling at times that are more specific to elucidating differences due to OSA. These studies are not at present at the stage of clinical application but the experimental approaches outlined here are likely to lead in the future to new approaches to diagnose as well as providing prognostic information. The future clinical utility in routine practice will of course be determined by the results of such studies.

Thus, much investigation remains to be done. We now have the tools to study candidate pathways and also take broader unbiased approaches. When combined with assessment of temporal change across the sleep period, we can fully address whether, as we propose, measuring molecular signatures of OSA will provide critical new and relevant information for diagnosis and management of patients with obstructive sleep apnea.

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