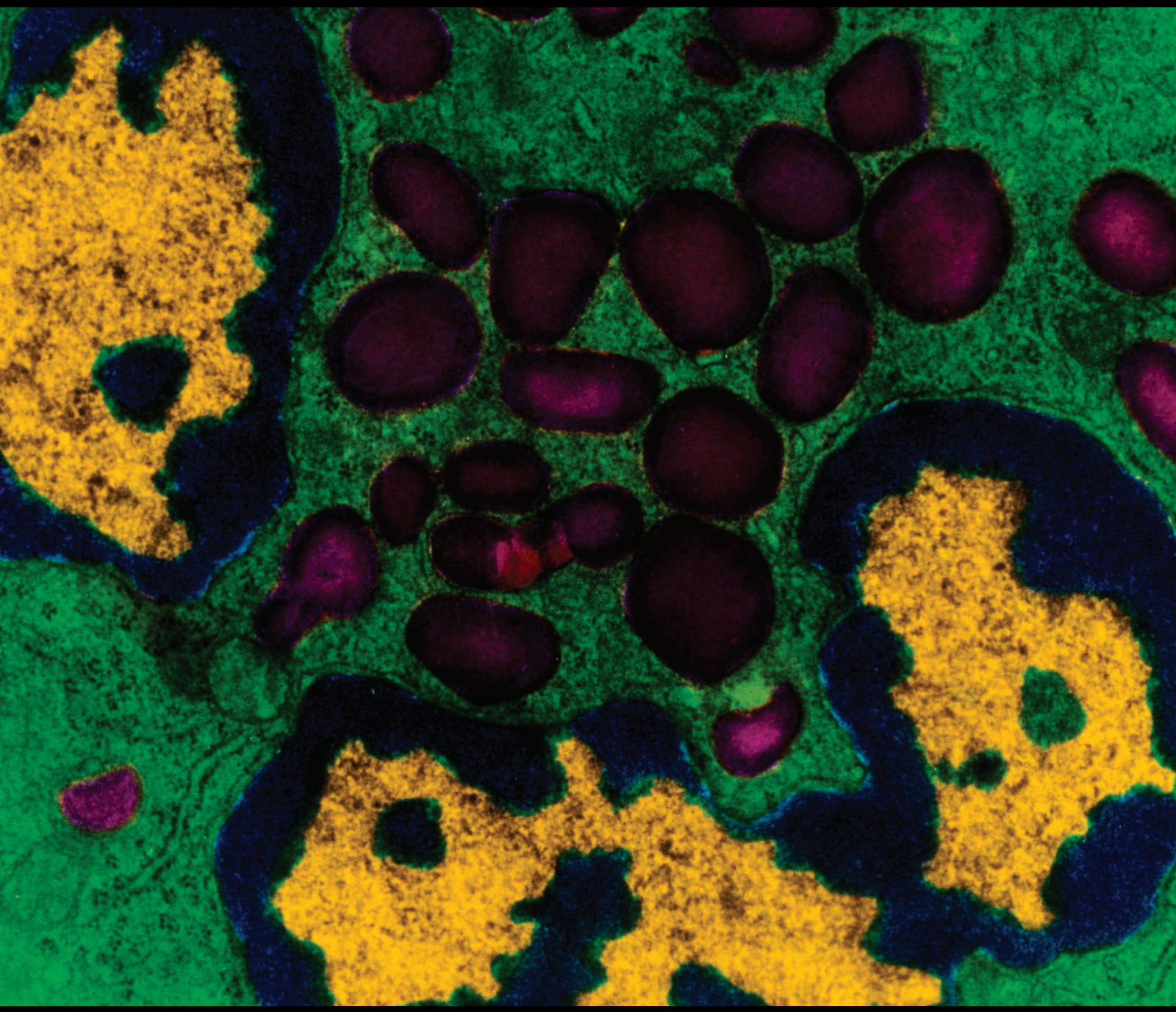
Mediators of Inflammation

Inflammation in Sleep Debt and Sleep Disorders

Guest Editors: Leila Kheirandish-Gozal, David Gozal, and Jean-Louis Pépin



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Editorial Board

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Editorial

Inflammation in Sleep Debt and Sleep Disorders

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Not only sleep is an essential physiological function, but also plays important roles in promoting growth, maturation, and overall health. There is increasing interest regarding the impact of sleep and its disorders on the regulation of inflammatory processes and end-organ morbidities, particularly in the context of neurocognitive, metabolic, and cardiovascular diseases and their complications [1–6]. Furthermore, jetlag and other perturbations of the circadian clock have also been linked to the regulation of fundamental regulatory properties underlying inflammatory processes and metabolic homeostasis [7, 8].

Sleep disorders such as obstructive sleep apnea syndrome (OSAS), a highly prevalent health problem across the age spectrum, are epidemiologically and mechanistically linked to metabolic deregulation. In the last decade, the emergence of increasing obesity rates has further led to remarkable increases in the prevalence of OSAS, along with more prominent neurocognitive, behavioral, cardiovascular, and metabolic morbidities [2, 9].

Although the underlying mechanisms leading to OSAS-induced morbidities are likely multifactorial and remain to be fully elucidated, activation of inflammatory pathways by OSAS has emerged as an important pathophysiological component of the end-organ injury associated with this disorder. To this effect, it would appear that OSAS could be viewed as a chronic, low-grade inflammatory disorder. Furthermore, the concurrent presence of obesity and OSAS poses a theoretically increased risk of OSAS-related complications.

In this special issue, studies covering aspects of inflammatory processes as they relate to sleep curtailment, sleep perturbation, or sleep disorders, such as OSAS, are presented and further reinforce the conceptual framework that sleep is a homeostatic regulator of inflammatory pathways and that perturbations in either sleep or inflammation will reciprocally affect each other.

M. S. Thimgan et al. report on the presence of elevated inflammatory gene transcripts such as prostaglandin-endoperoxide synthase 2 in the saliva of patients with OSAS and excessive daytime sleepiness (EDS), as well as in those with EDS, but in the absence of OSAS. M. L. Fung further elaborates on the potential contributions of the carotid body and other peripheral chemoreceptors to the recruitment of inflammatory pathways in the context of perturbed sleep and OSAS. The paper by L. Poulain et al. describes how an integral component of OSAS, namely, intermittent hypoxia, recruits TLR4 mechanisms that propagate inflammatory processes in both visceral adipose tissues and large blood vessels, ultimately promoting the emergence of insulin resistance. As a corollary of such processes, A. Gileles-Hillel et al. show that obese children with OSAS display evidence of enhanced levels of a selected array of inflammatory biomarkers in the circulation. Y. Nachalon et al. further demonstrate that treatment of OSAS by surgical removal of tonsils and adenoids leads to a reduction in the plasma levels of C-reactive protein (CRP), along with improved somatic growth. Furthermore, I. Bouloukaki et al. describe the association between the presence of erectile dysfunction in patients with severe OSAS and the concomitant elevation of inflammatory markers such as CRP, as well as tumor necrosis factor-α, interleukin-6, and interleukin-8. Finally, E. Paschetta et al.

draw the attention to the potential inflammatory pathways that underlie the causal association between OSAS and liver injury, particularly nonalcoholic steatohepatitis.

We hope that the readers of this special issue will find the studies presented here not only interesting, but also further stimulating discussion and promoting the incorporation of the conceptual frameworks developed herein into the clinical, research, and educational realms.

Leila Kheirandish-Gozal
David Gozal
Jean-Louis Pépin

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Research Article

Excessive Daytime Sleepiness Is Associated with Changes in Salivary Inflammatory Genes Transcripts

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Excessive daytime sleepiness (EDS) is a ubiquitous problem that affects public health and safety. A test that can reliably identify individuals that suffer from EDS is needed. In contrast to other methods, salivary biomarkers are an objective, inexpensive, and noninvasive method to identify individuals with inadequate sleep. Although we have previously shown that inflammatory genes are elevated in saliva samples taken from sleep deprived individuals, it is unclear if inflammatory genes will be elevated in clinical populations with EDS. In this study, salivary samples from individuals with sleep apnea were evaluated using the Taqman low density inflammation array. Transcript levels for 3 genes, including *prostaglandin-endoperoxide synthase 2 (PTGS2)*, were elevated in patients with sleep apnea. Interestingly, *PTGS2* was also elevated in patients with EDS but who did not have sleep apnea. These data demonstrate the feasibility of using salivary transcript levels to identify individuals that self-report excessive daytime sleepiness.

1. Introduction

Inadequate sleep is a pervasive problem in today's society. Insufficient sleep leads to decreased cognitive performance [1], increased sleepiness [2–4], reduced productivity [5], and increased traffic accidents [6]. Moreover, inadequate sleep increases the susceptibility of individuals to adverse health outcomes, including cardiovascular deficits [7], increased immune challenges [8, 9], longer recovery times after injury [10], increases in sympathetic tone [11, 12], and reduced lifespan [7]. Given the number and severity of consequences that accompany inadequate sleep, it would be helpful to have a simple and reliable test to identify vulnerable individuals before they experience adverse consequences.

One approach to accomplish this objective has been to test candidate biomarkers to determine whether they are consistently altered in subjects with inadequate or insufficient sleep. Biomarkers are objective, often endogenous factors that report changes in body chemistry and correlate with either disease state or the severity of the disease. Several biomarkers, including eyelid closures [13] and balance [14], as well as biochemical markers from blood [15, 16], cerebral spinal fluid [17–19], and breath analytes [20] have been evaluated as candidate biomarkers of disrupted sleep. Although each of these approaches has had limited success, assessment tools that can be used in real-world settings are not yet widely available. With that in mind, we have hypothesized that saliva, as a rich source of analytes, can be mined to identify biomarkers of sleepiness or inadequate sleep. Saliva is particularly well suited for monitoring sleepiness since it is a readily accessible biological fluid that can be easily collected using noninvasive procedures. Indeed, we have shown that transcripts for *α-amylase*, *Filamin-A*, *maleic enzyme*, *integrin*, *αM*, and *integrin*, *α5,* are all elevated in saliva samples following sleep deprivation [21–24]. Increased levels of salivary *α*-amylase activity have also been shown to correlate with increased sleepiness and decreased cognitive performance in an independent study [25]. However, since

endogenous factors are frequently modulated by a variety of physiological conditions (stress, circadian time, etc.), test of sleepiness should be comprised of a panel of independent analytes. Several studies have found that serum markers of inflammation are elevated in populations of individuals with sleep disorders [9]. For example, serum levels of interleukin 6 (IL-6) [26], interleukin 8 (IL-8) [27, 28], tumor necrosis factor-α (TNF-α) [29], C-reactive protein (CRP) [30], intracellular adhesion molecule (ICAM) [31], selectins [32], and vascular cell adhesion molecule (VCAM) [31] have all been shown to increase in multiple populations of patients with sleep apnea. Therefore, we wanted to determine if levels of salivary inflammation transcripts could be used to identify sleepiness in a clinical population, individuals diagnosed with sleep apnea.

2. Materials and Methods

2.1. Subjects and Samples. 8 controls, 14 patients confirmed to have sleep apnea, and 18 patients that were suspected to have sleep apnea during their initial screen but did not exhibit sleep apnea during their overnight assessment (sleepy) were evaluated for salivary transcript levels. Both the sleep apnea group and the "sleepy" group were referred to the Washington University Sleep Laboratory due to excessive sleepiness. Samples were taken from patients at ~9 pm after they had arrived at the sleep lab and prior to beginning polysomnography. The apnea/hypopnea index (AHI) was determined using polysomnography (PSG) as previously described [33]. Subjects were free from psychiatric disorders and prescription medicines [24]. Each subject was administered the Epworth Sleepiness Scale (ESS) and their body mass index (BMI) was calculated. BMI was unavailable for one subject in the "sleepy" group. Consent was received from all participants and all protocols were approved by the Washington University School of Medicine Institutional Review Board.

2.2. Low Density Arrays. Saliva was taken and cDNA was generated according to the protocol previously described [24]. Briefly, saliva was obtained when subjects chewed on a salivette (Sarstedt, Newton, NC). One mL of RNAlater (Life Technologies, Grand Island, NY) was added to the salivette and immediately frozen on dry ice and stored at −80°C. At the time of transcript analysis, salivettes were thawed and the saliva-RNAlater extracts were extracted by centrifugation. RNA was purified from cell-free lysates and reverse-transcribed using Superscript III (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Equal amounts of cDNA were loaded into an inflammation Taqman low density array (LDA) (Life Technologies, Grand Island, NY) and Taqman based qPCR was carried out using the 7600 real-time PCR system (Applied Biosystems, Foster City, CA). The LDAs were normalized to 18S RNA. Fold changes were determined using the method described in [34], known as the comparative C_T method. Transcript levels for the gene of interest were first normalized within subject to 18S RNA.

Table 1: Demographics for controls and sleep apnea.

	Controls (n = 8)	OSA (n = 14)
Gender	5M, 3F	11M, 3F
Age	40.8 ± 8.9	49.0 ± 12.2
AHI	—	48.5 ± 23.6
BMI*	25.7 ± 4.4	34.6 ± 7.1
ESS*	3.9 ± 2.7	10.8 ± 4.3

Ave. ± Std. Dev.; *$P < 0.05$.

2.3. Statistics. All demographic data is presented as average ± standard deviation. For transcript analysis, if a transcript was not detected in more than 77% of the group, the transcript was deemed undetectable and not analyzed. For all detectable genes, transcript levels for each subject were determined as a fold change from the average levels of the control group. Fold changes were converted to a $\log_2$ scale. From the log data, average and standard error were calculated. Groups of interest were compared to control levels using a two-tailed, unpaired Student's t-test and the significance level was set at 0.05. P values were corrected for multiple comparisons using the false discovery method (FDR) with a FDR level of 5% [35]. Transcript levels are presented as a geometric mean and standard error [36]. For ease of presentation, group data, including average, upper, and lower bounds, were then converted back to standard units by raising 2 to the power of the calculated value to obtain fold change values.

3. Results

3.1. Demographic Data for Patients with Sleep Apnea. We evaluated 14 patients with sleep apnea (11 male and 3 female) and 8 control subjects (5 male and 3 female) for salivary transcriptional changes associated with sleep apnea. Control subjects did not have any prior existing sleep or mental disorders, were not on prescription medications, had not consumed caffeine, and had not eaten 1 hour prior to providing a saliva sample. Subject age was not different between the two groups (Table 1). The subjects with sleep apnea had an average AHI of 48.5 ± 23.6 (mean ± S.D.). The subjects with sleep apnea had a significantly higher body mass index (BMI) than the controls. Consistent with previous results, subjects with sleep apnea also reported a higher score on the Epworth Sleepiness Scale.

3.2. Evaluation of Salivary Biomarkers Associated with Inflammation in Patients with Sleep Apnea. We evaluated salivary transcript levels from subjects with sleep apnea and control subjects using low density arrays. The LDA platform simultaneously evaluates the levels of 96 RNA species. Of the 96 genes tested, 21 transcripts were detected in 11 or more sleep apnea patients. Fold changes from control and P value for each gene are presented in Table 2. The P value listed was determined using a two-tailed Student's t-test comparing the controls to sleep apnea for all analyzed genes present in saliva. We performed a correction for multiple comparisons using a FDR set at 5%, which yielded a new P value equivalent to

Table 2: Salivary transcript levels in patients with sleep apnea compared to controls.

Gene	Abbreviation	Sleep apnea fold Δ from control[a]	*P* value
β-actin	ACTB	1.43 ± 0.38	0.191
Arachidonate 5-lipoxygenase	ALOX5	1.39 ± 0.36	0.238
Arachidonate 12-lipoxygenase	ALOX12	1.56 ± 0.69	0.404
Annexin A1	ANXA1	4.62 ± 1.15	0.003[b]
Annexin A3	ANXA3	1.26 ± 0.38	0.444
Annexin A5	ANXA5	2.05 ± 0.92	0.168
β-2-microglobulin	B2M	2.65 ± 0.70	0.007[b]
Caspase 1, apoptosis-related cysteine peptidase	CASP1	2.57 ± 0.81	0.025
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	2.66 ± 0.81	0.019
Intercellular adhesion molecule 1	ICAM1	0.44 ± 0.13	0.123
Interleukin 1 receptor, type II	IL1R2	1.62 ± 0.41	0.143
Interleukin 2 receptor, gamma	IL2RG	2.36 ± 0.72	0.054
Integrin, alpha M	ITGAM	1.50 ± 0.61	0.376
Integrin, beta 2	ITGB2	1.47 ± 0.31	0.167
Mitogen-activated protein kinase 1	MAPK1	1.72 ± 0.66	0.401
Mitogen-activated protein kinase 3	MAPK3	1.30 ± 0.41	0.585
Mitogen-activated protein kinase 14	MAPK14	1.85 ± 0.53	0.089
Phosphodiesterase 4B, cAMP-specific	PDE4B	1.49 ± 0.21	0.168
Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	PTGS2	3.73 ± 0.98	0.002[b]
Tumor necrosis factor receptor superfamily, member 1A	TNFRSF1A	1.32 ± 0.20	0.206
Tumor necrosis factor receptor superfamily, member 1B	TNFRSF1B	1.07 ± 0.44	0.900

[a]Mean ± SEM.
[b]Significant difference using 5% FDR post hoc test.

$\alpha = 0.0071$ [35]. Compared to controls, 3 transcripts exhibited significant changes in patients with sleep apnea (Table 2), *ANXA1*, *β2M*, and *PTGS2* (see Table 2 for abbreviations). These results indicate that several inflammatory transcripts are increased in patients with sleep apnea.

3.3. Demographic Data for Patients with an Elevated ESS but without Sleep Apnea. To assess whether the increased inflammatory markers were due primarily to hypoxia or whether they might be more closely associated with increased sleepiness, we evaluated transcriptional changes in an independent group of people that entered the sleep lab suspected of having sleep apnea due to initial screening but ultimately had a normal AHI as determined by PSG (sleepy). The "sleepy" group was composed of 6 males and 12 females. By definition, all individuals in this group had an AHI less than 5. However, all subjects reported ESS scores significantly higher than controls and similar to the subjects with sleep apnea (Tables 1 and 3). "Sleepy" subjects had a similar age to controls but an increased BMI.

3.4. Salivary Transcript Levels in "Sleepy" People. Twenty-one transcripts were detected in the saliva of 14 or more "sleepy" patients. As above, to facilitate comparisons with the apnea patients reported in Table 2, Table 4 includes

Table 3: Demographics for "sleepy" subjects and controls.

	Controls ($n = 8$)	Sleepy ($n = 18$)
Gender	5M, 3F	6M, 12F
Age	40.8 ± 8.9	44.1 ± 14.4
AHI	—	4.7 ± 5.2
BMI*	25.7 ± 4.4	34.2 ± 10.8
ESS*	3.9 ± 2.7	11.0 ± 5.0

Ave ± Std. Dev.; *$P < 0.05$.

transcripts for fold changes for each of the 21 transcripts. After and FDR correction for multiple comparisons ($\alpha = 0.005$), 2 transcripts were significantly changed in the "sleepy" population, *CASP1* and *PTGS2*. *PTGS2* can be considered an independent replicate of the apnea results and may deserve further consideration. Thus, inflammatory transcripts are elevated under conditions of EDS.

3.5. Transcriptional Changes Are Not due to Elevated BMI. One possible explanation for elevated inflammatory transcripts is that both the sleep apnea and "sleepy" patients have a significantly higher BMI compared to controls. If the observed relationship is due solely to BMI, then patients with high BMI should have increased levels of inflammatory transcripts compared to patients with lower BMI. Similarly, we

Table 4: Salivary transcript levels in "sleepy" patients compared to controls.

Gene	Abbreviation	Sleepy people fold Δ from control[a]	*P* value
β-actin	ACTB	1.86 ± 0.47	0.033
Arachidonate 5-lipoxygenase	ALOX5	1.30 ± 0.46	0.472
Arachidonate 12-lipoxygenase	ALOX12	0.53 ± 0.33	0.408
Annexin A1	ANXA1	2.86 ± 1.37	0.099
Annexin A3	ANXA3	2.64 ± 0.85	0.010
Annexin A5	ANXA5	2.29 ± 0.76	0.054
β-2-microglobulin	B2M	3.33 ± 1.34	0.013
Caspase 1, apoptosis-related cysteine peptidase	CASP1	5.36 ± 2.07	0.001[b]
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	2.35 ± 0.88	0.068
Intercellular adhesion molecule 1	ICAM1	0.61 ± 0.33	0.441
Interleukin 1 receptor, type II	IL1R2	2.04 ± 0.75	0.092
Interleukin 2 receptor, gamma	IL2RG	3.53 ± 2.25	0.118
Integrin, alpha M	ITGAM	1.64 ± 0.65	0.292
Integrin, beta 2	ITGB2	1.84 ± 0.52	0.102
Mitogen-activated protein kinase 1	MAPK1	1.55 ± 0.65	0.510
Mitogen-activated protein kinase 3	MAPK3	1.06 ± 0.42	0.921
Mitogen-activated protein kinase 14	MAPK14	2.33 ± 0.77	0.057
Phosphodiesterase 4B, cAMP-specific	PDE4B	1.69 ± 0.69	0.259
Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	PTGS2	3.80 ± 1.34	0.005[b]
Tumor necrosis factor receptor superfamily, member 1A	TNFRSF1A	1.87 ± 0.62	0.096
Tumor necrosis factor receptor superfamily, member 1B	TNFRSF1B	1.80 ± 0.88	0.322

[a]Mean ± SEM.
[b]Significant difference using 5% FDR post hoc test.

should detect a significant positive correlation between BMI and the level of salivary transcripts. To test this hypothesis we discretized the data by placing the subjects with a low BMI (≤30; $n = 24$) into a single group and comparing them to subjects with a high BMI (>38; $n = 16$). As seen in Table 5, transcripts were not significantly different between subjects in the lower BMI group compared to their counterparts with a higher BMI. In addition, we evaluated the relationship between BMI and transcript levels using a Pearson correlation and found no significant correlations. Examples of transcript expression levels for the genes with the lowest *P* values are plotted in Figure 1 as a function of BMI. These data indicate that, in this dataset, BMI does not account for increases in transcript levels in these patients.

4. Discussion

In this paper, we report that salivary transcript levels of inflammatory genes are increased both in patients with sleep apnea and in an independent cohort of patients who self-identified as sleepy and were referred to the sleep lab suspected of having sleep apnea. We have previously used these same human low density arrays as a discovery tool to identify genes that are modified following acute sleep deprivation in healthy adults [24]. In that study, we reported that *integrin, αM* (*ITGAM*), and *annexin A3* (*ANXA3*) were significantly increased following 24 h and 30 h of waking. Interestingly, neither *ITGAM* nor *ANXA3* were altered in either sleep apnea or "sleepy" subjects suggesting that they may be better suited for detecting acute sleep loss than for identifying patients with chronic sleep disruption. In contrast, salivary *PTGS2* was significantly increased in both sleep apnea patients and in the "sleepy" cohort but was not affected by acute sleep deprivation [24]. Thus, these data support our previous proposal that a panel of biomarkers will be required to accurately identify sleep deprived individuals and further suggest that different sets of biomarkers may be required to distinguish between acute and chronic sleep disruptions.

Sleep apnea has been particularly difficult to diagnose and treat and the incidence of sleep apnea has been difficult to determine. Estimates of sleep apnea have ranged from as low as 2–4% to as high as 24% of middle aged men [37], although other studies have estimated the likelihood of sleep apnea to be much higher still [38, 39]. Moreover, it has been suggested that sleep apnea is likely to be substantially underdiagnosed [38, 39]. Indeed, years may transpire between the onset of sleep apnea and the eventual diagnosis [40]. As the population ages and becomes more obese, the prevalence of sleep apnea is expected to increase even further [7, 41, 42]. During these intervening years, the severity and consequences of the sleep apnea may also increase [41, 42]. The associated

Table 5: Comparison of transcriptional changes in high and low BMI subjects.

Gene	Abbreviation	BMI < 30 fold $\Delta^{a,b}$	BMI > 30 fold $\Delta^{a,c}$	t-test
β-actin	ACTB	1.39 ± 0.29	1.65 ± 0.40	0.438
Arachidonate 5-lipoxygenase	ALOX5	1.46 ± 0.33	0.97 ± 0.28	0.117
Arachidonate 12-lipoxygenase	ALOX12	1.25 ± 0.47	0.48 ± 0.32	0.058
Annexin A1	ANXA1	3.06 ± 1.25	2.33 ± 1.03	0.529
Annexin A3	ANXA3	1.68 ± 0.40	1.63 ± 0.61	0.913
Annexin A5	ANXA5	1.74 ± 0.58	2.17 ± 0.75	0.519
β-2-microglobulin	B2M	1.95 ± 0.66	3.12 ± 0.91	0.156
Caspase 1, apoptosis-related cysteine peptidase	CASP1	2.68 ± 0.90	3.05 ± 1.42	0.741
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	2.14 ± 0.72	1.94 ± 0.57	0.771
Intercellular adhesion molecule 1	ICAM1	0.61 ± 0.22	0.62 ± 0.36	0.982
Interleukin 1 receptor, type II	IL1R2	1.88 ± 0.53	1.24 ± 0.34	0.153
Interleukin 2 receptor, gamma	IL2RG	2.65 ± 1.37	2.20 ± 0.93	0.713
Integrin, alpha M	ITGAM	1.56 ± 0.48	1.25 ± 0.51	0.537
Integrin, beta 2	ITGB2	1.52 ± 0.35	1.54 ± 0.38	0.949
Mitogen-activated protein kinase 1	MAPK1	1.77 ± 0.83	1.20 ± 0.37	0.355
Mitogen-activated protein kinase 3	MAPK3	1.41 ± 0.36	0.90 ± 0.34	0.156
Mitogen-activated protein kinase 14	MAPK14	2.18 ± 0.60	1.51 ± 0.45	0.211
Phosphodiesterase 4B, cAMP-specific	PDE4B	1.44 ± 0.36	1.44 ± 0.55	0.999
Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	PTGS2	2.43 ± 0.84	3.42 ± 1.13	0.338
Tumor necrosis factor receptor superfamily, member 1A	TNFRSF1A	1.47 ± 0.36	1.40 ± 0.33	0.841
Tumor necrosis factor receptor superfamily, member 1B	TNFRSF1B	1.72 ± 0.66	0.86 ± 0.36	0.099

[a]Mean ± SEM.
[b]$n = 15$–24.
[c]$n = 11$–15.

consequences of sleep apnea include increased daytime sleepiness [3] and an increased likelihood of automobile accidents [6]. Moreover, sleep apnea has been associated with a host of cardiovascular complications [43, 44] and increased rates of all-cause mortality [7]. Sleep apnea has also been associated with learning impairments [45]. The current gold standard of treatment, continuous positive airway pressure (CPAP), may be able to reverse some of the consequences of sleep apnea, such as sleepiness [2] and restoration of typical sleep stages [2], and may lower blood pressure [46] but the literature is less conclusive whether cognitive function is improved [4, 47]. Thus, a clinical goal is to have patients begin treatment for sleep apnea as soon as possible.

A simple cost-effective biomarker that can be easily used at the point of care may be a useful tool to convince people to go to the sleep lab for a more precise diagnosis and treatment. Since biomarkers are both objective and quantifiable, they are well suited for persuading an individual that they may have an underlying affliction that needs greater attention. In that regard, it is important to note that the well-known relationship between inflammation and sleep deprivation is an advantage since many physicians are likely to be familiar with this relationship and thus more willing to incorporate such a biomarker into their treatment practices. That is, numerous inflammatory markers, including IL-6 [20, 26, 48], IL-8 [27, 28, 48], TNF-α [26], CRP [49, 50], monocyte chemoattractant protein-1 (MCP-1) [27], ICAM [27, 51], selectins [51], VCAM [51], nitric oxide [52], and isoprostane [20], are elevated in body fluids of patients with sleep apnea. Moreover, proteomics have identified protein biomarkers in urine [53]. Interestingly, inflammatory markers have also been associated with sleep deprivation in serum [54, 55] and saliva in healthy adults [24]. In this study, transcript levels for *PTGS2* were elevated in people with inadequate sleep. Our results parallel findings in mice in which transcript levels for PTGS2 are elevated after acute sleep deprivation [56]. *PTGS2*, along with *PTGS1*, is a critical enzyme in prostaglandin synthesis. Prostaglandins initiate a set of molecular cascades that result in an inflammatory response. *PTGS2* is induced by several stimuli including proinflammatory signals and converts arachidonic acid to prostaglandin H2 [57]. Thus, *PTGS2* plays a role in the inflammatory pathway in addition to being a candidate biomarker for EDS.

It is interesting to note that many studies compare sleep apnea patients to healthy controls. Although this approach has been quite successful, our data suggest that other patients with chronic sleep disruption may also experience some of the same outcomes as patients with sleep apnea. If this turns out to be the case, more generally, assessing chronically sleepy individuals along with patients that have sleep apnea

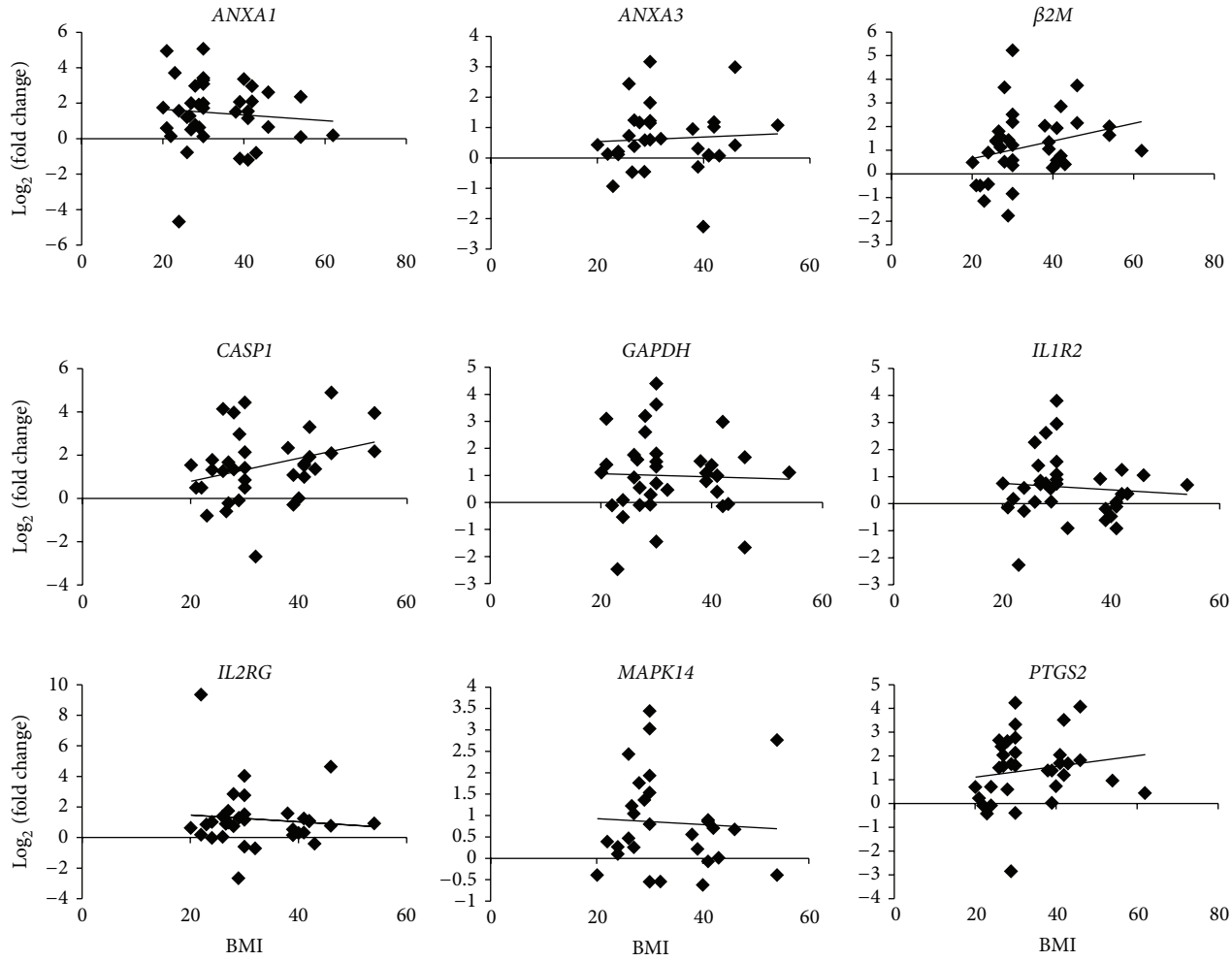

Figure 1: Correlation of transcript levels with BMI. The transcript levels were plotted against the log values of the transcript levels for each of the transcripts that were significant in the sleep apnea or "sleepy" groups. In addition the transcripts with the lowest *P* value were plotted to determine if there was a significant correlation with increased weight. For each transcript, there was no significant correlation with increased BMI.

may facilitate the identification of precise deficits that are due specifically to sleep loss rather than other features of sleep apnea, such as hypoxia [48]. Nonetheless, our results emphasize that *PTGS2* cannot distinguish between sleepy patients with and without sleep apnea. While this may seem disappointing, we believe that any diagnosis of a sleep disorder should be made by a qualified sleep physician after fully examining the patient. Indeed, it may not be possible to identify a biomarker that is specific to a single sleep disorder. One example of a promising biomarker is cerebrospinal fluid (CSF) levels of hypocretin-1/orexin (HCRT), which has emerged from research in the narcolepsy field [18, 58]. CSF levels of hypocretin-1/orexin are lower in narcoleptic patients. But for patients without cataplexy, decreased CSF levels of HCRT are not as predictive, even in combination with the genetic marker DBQ. Moreover, decreases in CSF levels of HCRT are also associated now, being associated with other diseases, including dementia with Lewy Body [59] and Alzheimer's disease [60] and Kleine-Levin syndrome [58, 61]. This example illustrates the difficulty of a single biomarker with a given sleep disorder. Nonetheless, a biomarker that can help primary physicians to identify sleepy individuals may be extremely useful for ensuring that they get the appropriate care in a timely manner.

Our protocol has two aspects that may be useful for the real-world application of biomarkers to identify patients with sleep disorders. First, acquiring saliva is a noninvasive procedure and does not, for example, require a private location for sample collection (e.g., urine) or specialized skills to acquire samples (e.g., blood). As a consequence, salivary biomarkers can be readily used both on the roadside and in a doctor's office with equal effectiveness. A second advantage of our protocol is our focus on salivary RNA coupled with the LDA platform for the rapid detection of 96 transcripts simultaneously. LDAs may be an effective way to screen multiple targeted transcripts at once without the burden that NextGen sequencing frequently demands. One advantage of using transcript levels as the biomarker is that nucleotide probes can be generated quickly and are very specific for the target intended as opposed to having to design an antibody

against a protein target. Given human variability, the different roles that genes can play throughout the body (pleiotropy), and the likelihood that a given analyte will be induced by a variety of environmental situations, it is likely that no single biomarker will be ideal for reliable diagnosis of sleep apnea. Nonetheless, our data suggest that *PTGS2* may be a particularly good candidate for inclusion in a panel of biomarkers.

There are two potential weaknesses of our study. The first is the sample size of our groups. Low sample sizes may identify significant differences within the subgroup that does not generalize to the rest of the population. In this study, we have tried to balance the difficulty of completing discovery experiments with the expense of a human study. Although our sample size was low for sleep apnea subjects, we were able to confirm the changes in *PTGS2* in a separate cohort of "sleepy" patients. This confirmation adds to our confidence that *PTGS2* is a good candidate for followup studies. A second weakness of this study is that we were not able to control for the levels of BMI between our control subjects and patients with sleep apnea or the "sleepy" cohort. To address this issue, we discretized the subjects into groups with low or high BMI. Means testing between the two groups indicated the subjects with low and high BMI were not different from one another. Moreover, Pearson correlation between BMI and transcript levels did not reveal any significant associations. Finally, when examined as a whole, we could find no general trend of inflammatory genes being elevated in subjects with higher BMI. Thus, while BMI is a potential confounder, it did not appear to unduly influence the results of this study.

Biomarkers have the capacity to serve an important need in maintaining human health. They provide an objective insight into the goings-on of the body. In particular, there is a need to identify individuals with sleep problems that result in EDS. Given the difficulty in identifying individuals, the consequences of sleep apnea and other sleep disorders, and the potential that therapy possesses, objective biomarkers can be a way to convince people to go to the sleep lab for diagnosis. We have demonstrated the feasibility of using saliva to identify changes in inflammatory transcripts that correlate with the presence of EDS. Saliva is an ideal source of biofluid for a potential source of biomarkers. Obtaining saliva is a noninvasive and relatively inexpensive process. Further experiments in larger and independent populations will determine the precision and validity of these biomarkers, but these experiments demonstrate that saliva has the potential to be objective biomarkers to identify subjects with EDS.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Toll-Like Receptor-4 Mediated Inflammation Is Involved in the Cardiometabolic Alterations Induced by Intermittent Hypoxia

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Objective. Intermittent hypoxia (IH) is a major component of sleep apnea syndrome as its cardiometabolic complications have been mainly attributed to IH. The pathophysiology is still poorly understood but there are some similarities with the obesity-associated cardiometabolic complications. As the latter results from inflammation involving toll-like receptor-4 (TLR4) signaling, we assessed this pathway in the cardiometabolic consequences of IH. *Methods*. Lean adult male TLR4-deficient ($TLR4^{-/-}$) mice and their controls (C57BL/6 mice) were exposed to either IH (FiO_2 21-5%, 1 min cycle, 8 h/day) or air (normoxic mice) for 4 weeks. Animals were assessed at 1-week exposure for insulin tolerance test and after 4-week exposure for morphological and inflammatory changes of the epididymal fat and thoracic aorta. *Results*. IH induced insulin resistance, morphological and inflammatory changes of the epididymal fat (smaller pads and adipocytes, higher release of TNF-α and IL-6) and aorta (larger intima-media thickness and higher NFκB-p50 activity). All these alterations were prevented by TLR4 deletion. *Conclusion*. IH induces metabolic and vascular alterations that involve TLR4 mediated inflammation. These results confirm the important role of inflammation in the cardiometabolic consequences of IH and suggest that targeting TLR4/NFκB pathway could represent a further therapeutic option for sleep apnea patients.

1. Introduction

Obstructive sleep apnea (OSA) is a public-health problem as it affects at least 10% of the middle aged men and represents a main cause of cardiovascular morbidity and mortality [1]. An independent association between OSA, insulin resistance, and type 2 diabetes has been consistently demonstrated by a number of cross-sectional, observational, and large population-based studies [2, 3]. Moreover, OSA patients have increased carotid intima-media thickness (IMT), an early sign of atherosclerosis, which correlates with nocturnal oxygen desaturation, independently of other cardiovascular risk factors [4, 5]. OSA severity may also predict occult coronary atherosclerosis in healthy overweight or obese male subjects [6]. Repetitive upper airway collapses during sleep result in intermittent hypoxia (IH) which is thought to be responsible for OSA-associated cardiometabolic complications, including atherosclerosis and insulin resistance [7]. Data obtained from animals exposed to IH, a validated experimental model of sleep apnea, showed that activation of the sympathetic

nervous system and systemic inflammation underlie IH-induced metabolic and vascular consequences [7–9]. We also recently demonstrated that IH-induced inflammatory changes of epididymal white adipose tissue (EWAT) contributed to these outcomes [10].

In obesity, it is increasingly recognized that chronic activation of inflammatory signaling pathways is causally linked to insulin resistance [11] and vascular alterations [12]. Recent studies suggest that these deleterious effects could be mediated, at least in part, through the activation of toll-like receptors (TLR), and in particular the TLR4. TLRs are a family of pattern-recognition receptors that play a critical role in the innate immune system by activating proinflammatory signaling pathways in response to microbial pathogens [13]. Lipopolysaccharide (LPS) binds to TLR4, triggering a downstream cascade, which leads to the activation of the proinflammatory nuclear factor kappa-B (NFκB) pathway and finally to the expression of numerous proinflammatory molecules, such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α [14]. Furthermore, studies on different strains of mice showed that expression and activation of TLR4 are involved in aortic inflammation [15] and atherogenesis [16]. Together these experimental data confirm the major role of TLR4/NFκB pathway in the crosstalk between inflammation, atherosclerosis, and metabolism dysfunctions.

In C57BL/6 mice, we previously found that IH-induced cardiovascular inflammation was characterized by an increased activity of NFκB in aortic [17] and cardiac tissues (unpublished data). We also reported that, in lean animals, EWAT exposed to IH became pathological, behaving like excess fat in obesity, as it exhibited increases in macrophage recruitment and secretion of IL-6 and TNF-α [10]. Collectively, these data suggest that cardiometabolic complications due to IH and obesity may share some pathophysiological mechanisms. Therefore, we assessed whether metabolic and vascular consequences induced by IH involved the proinflammatory TLR4/NFκB pathway activation.

2. Methods

2.1. Animals. Male TLR4-deficient mice (TLR4$^{-/-}$, C57BL/6 background) were developed initially by Dr. Shizuo Akira (Research Institute for Microbial Diseases, Osaka, Japan) and were obtained from the EMMA (European Mouse Mutant Archive) network in Orleans, France. Seventeen-week-old male TLR4$^{-/-}$ mice and their control groups (C57BL/6 mice) fed on a standard-chow diet were used. They were weighed throughout the experiments. The study was conducted in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council of Europe, European Treaties ETS 123, Strasbourg, 18 March 1986) and with the *Guide for Care and Use of Laboratory Animals* (NIH Publication no. 85-23, revised 1996).

2.2. Intermittent Hypoxia. TLR4$^{-/-}$ and their control C57BL/6 mice were divided into 2 subgroups, exposed to either intermittent hypoxia (IH) or normoxia (N). IH was performed as previously described [10]. The four groups of animals were exposed to the IH stimulus during daytime (n = 10 per cage, 8 h/day, cyclic 21-5% FiO_2, 60 s cycle (60 events/h), lowest blood oxygen saturation up to 60%) for 4 weeks. FiO_2 was measured with a gas analyzer (ML206, ADInstruments) throughout the experiment. Control animals (normoxic mice, N) were exposed to air in similar cages to reproduce similar noise and turbulences to those of the IH stimulus. Ambient temperature was maintained at 20–22°C.

During the first week of IH exposure, intraperitoneal insulin tolerance test (IpITT) was performed to assess global insulin sensitivity and, on the day following the last exposure period, fasted animals were sacrificed under anesthesia with intraperitoneal injection of ketamine (100 mg·kg^{-1}) and xylazine (10 mg·kg^{-1}) for further analysis.

2.3. Intraperitoneal Insulin Tolerance Test (IpITT). Mice were fasted for 5 hours and then weighted before blood was collected from the tail tip for baseline glucose determination (t = 0). Blood glucose was measured using the OneTouch Ultra glucometer. Insulin (0.5 IU·kg^{-1} body weight, Novo Nordisk A/S, Bagsvaerd, Denmark) was injected intraperitoneally, followed by further blood glucose measurements at 15, 30, 60, and 90 minutes after the injection. The lowest blood glucose level (nadir) following insulin administration was calculated for each experimental group.

2.4. Blood Cholesterol Measurements. At the time of sacrifice, blood was collected by cardiac puncture on EDTA tubes. The plasma fraction was collected after blood centrifugation during 10 minutes at 11000 rpm (4°C). Total cholesterol was measured in plasma by a colorimetric enzymatic reaction using the Infinity kit (ThermoElectron Corporation, Massachusetts, USA) according to the manufacturer's guidelines.

2.5. Epididymal White Adipose Tissue (EWAT) Alterations. Bilateral epididymal fat pads were collected, weighted, and either fixed in 90% ethanol for adipocyte morphology study or incubated for cytokine determinations.

2.5.1. Adipocyte Morphology. Ethanol-fixed, paraffin-embedded EWAT were sectioned (3.5 μm), deparaffinized in toluene and rehydrated in descending ethanol series, and then stained with hematoxylin-eosin to assess tissue morphology. Adipocyte size was measured from photographs (10 × 40 magnification) using the NIS-Elements microscope imaging software (Nikon).

2.5.2. Cytokine Secretion. Each EWAT pad was divided into two equal pieces and incubated at 37°C with mild shaking in RPMI medium. After 120 minutes of incubation, IL-6 and TNF-α were measured in the supernatants using an ELISA test according to manufacturer's instructions (R&D System Europe, Lille, France). Cytokine concentrations were expressed as ng/mL for 1 g of adipose tissue.

2.6. Assessment of Vascular Inflammation and Remodeling

2.6.1. Aortic Intima-Media Thickness (IMT). IH and N aortas were embedded in optimum cutting temperature (OCT) compounds (Tissue-Tek, Sakura Finetek Europe BV, Alphen aan den Rijn, The Netherlands), sectioned (10 μm), and stained. Hematoxylin-eosin staining was used to assess the intima-media thickness (IMT). Morphometric analysis (up to 15 measurements on 10 noncontiguous midthoracic descending aorta sections per animal) was performed with a light microscope (Nikon Eclipse 80i, Nikon) and the NIS-Elements microscope imaging software (Nikon Instruments Europe BV).

2.6.2. NFκB Activity. We investigated whether IH could activate NFκB by assessing the expression of its activated subunit NFκB-p50 which has translocated into the nucleus. Nuclear NFκB-p50 was determined in thoracic aorta of mice exposed to N or IH. Tissue homogenization and proteins extraction were performed according to manufacturer's instructions using a nuclear extract kit (Active Motif Europe, Rixensart, Belgium). Proteins concentration was evaluated using the BCA assay (ThermoScientific, Massachusetts, USA). Nuclear proteins were assayed for the presence of the activated p50 by ELISA using the TransAM NFκB-p65/p50/p52 kit (Active Motif Europe). NFκB activity was expressed in arbitrary units.

2.7. Statistical Analysis. Results were expressed as mean ± standard errors of the means (SEM) and analyzed using 2-way ANOVA and subsequent Bonferroni's multiple post hoc comparisons or Mann-Whitney U test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. TLR4 Deficiency Prevents IH-Induced Fat Tissue Inflammation and Remodeling. C57BL/6 mice exposed to IH had morphological and functional alterations of EWAT. They had smaller fat pads with smaller adipocytes (Figures 1(a), 1(c), and 1(d)), and EWAT released more TNF-α and IL-6 compared to normoxic controls (Figures 1(e) and 1(f)). All these alterations were absent in hypoxic TLR4$^{-/-}$ mice. Regarding body weight alterations, normoxic and hypoxic TLR4$^{-/-}$ mice were not different from their respective control animals (Figure 1(b)).

3.2. TLR4 Deficiency Prevents IH-Induced Insulin Resistance. After one-week exposure, the 4 experimental groups were assessed for insulin tolerance test (Figure 2(a)). C57BL/6 mice exposed to IH exhibited a decreased response to insulin as shown by a lower glucose decrement (Figure 2(b)) and a trend for a smaller glucose nadir compared to their normoxic controls (Figure 2(d)). The insulin response was not affected in hypoxic TLR4$^{-/-}$ mice: the response curve was almost superposable with those of the normoxic TLR4$^{-/-}$ animals (Figures 2(c) and 2(d)) and not significantly different from the curve of normoxic C57BL6 animals (Figure 2(a)).

3.3. TLR4 Deficiency Prevents IH-Induced Inflammatory Vascular Remodeling. Hypoxic C57BL/6 mice had morphological and functional alterations of their aorta, as they exhibited larger intima-media thickness (Figures 3(a) and 3(b)) and higher NFκB-p50 activity (Figure 3(c)). These alterations were not observed in hypoxic TLR4$^{-/-}$ mice (Figures 3(a), 3(b), and 3(c)). Plasma levels of total cholesterol were not different between the 4 experimental groups (Figure 3(d)).

4. Discussion

The pathophysiology of OSA-induced cardiometabolic consequences is still poorly understood. There are some similarities with cardiometabolic complications due to obesity, the latter resulting from inflammation involving TLR4 signaling. Here, we showed in nonobese C57BL/6 mice that IH induced morphological and inflammatory remodeling of vascular and white adipose tissues as well as insulin resistance. These alterations were prevented in hypoxic TLR4-deficient mice suggesting that IH-induced cardiometabolic consequences involve TLR4 signaling-mediated inflammation.

4.1. Methodological Considerations. As previously published by our group and others, we used a deep intermittent hypoxia which mimics severe sleep apnea whereas patients mainly suffer from mild to moderate sleep apnea [7, 17, 18]. Indeed, in the absence of additional factors such as obesity, high fat diet, and genetic vulnerability, IH needs to be severe enough to induce measurable and reproducible vascular alterations, especially in C57BL/6 mice which are atheroresistant animals [7].

4.2. IH-Induced EWAT Remodeling Involves TLR4 Mediated Inflammation. In lean C57BL/6 mice, we showed that IH induced EWAT alterations characterized by fat pad wasting and shrunken adipocytes. These results are in agreement with previous reports in lean C57BL/6 [19] and ApoE$^{-/-}$ [10] mice after 4 and 6 weeks of IH, respectively. Shrunken adipocytes are suggestive of lipolysis, as elevated circulating free fatty acids (FFAs) have been reported by us and others in IH-exposed animals [10, 20, 21] and in patients suffering from sleep apnea [22]. In the latter, plasma FFA levels were positively correlated with apnea-hypopnea index [22]. Activation of beta-adrenergic receptors is a well-known mechanism of lipolysis [23], and both IH and OSA are commonly associated with sympathoadrenergic activation [10, 24]. Adipose inflammation may have also contributed to lipolysis [25] as we found an increased release of the inflammatory cytokines TNF-α and IL6 from fat pads of hypoxic C57BL/6 mice. This is again consistent with previous reports in lean ApoE$^{-/-}$ mice after 6 weeks of IH [10], and more recently in 3T3-L1 adipocytes exposed to fluctuating oxygen concentration [26]. These inflammatory changes may be due to local hypoxia [27], which is known to be a leading cause of EWAT dysregulation [28], as well as to systemic effects such as elevated circulating FFAs [29] and activation of the sympathoadrenergic system [10].

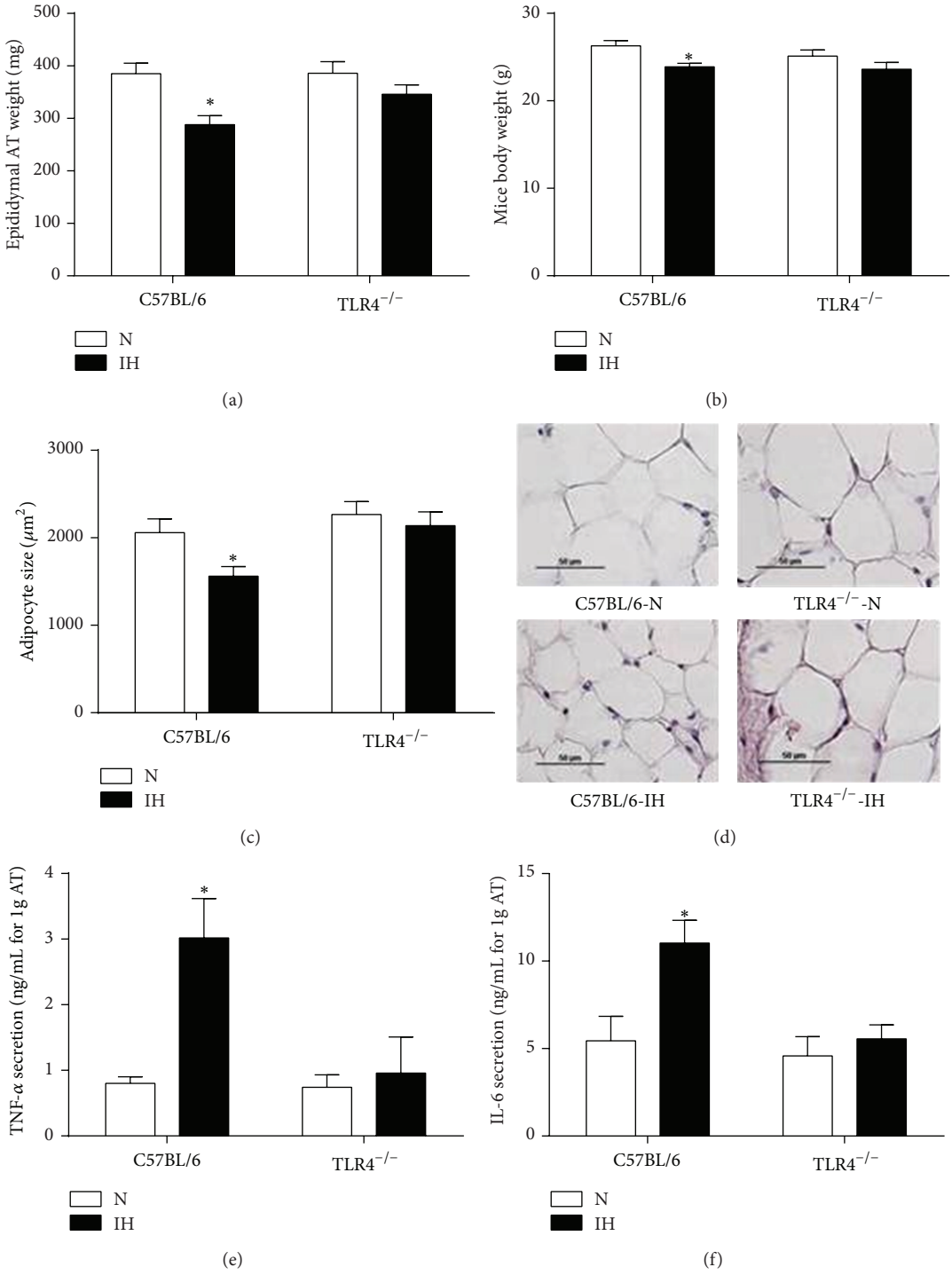

Figure 1: TLR4 signaling is involved in IH-induced epididymal fat alterations. Morphological and inflammatory changes of epididymal fat pads were studied in C57BL/6 and TLR4$^{-/-}$ mice exposed to intermittent hypoxia (IH) or normoxia (N) for 4 weeks. (a) Measurements of weight of bilateral epididymal fat pads (n = 13–15 per group), (b) mice body weights (n = 13–15 per group), (c) adipocyte size (n = 4–6 per group), and (d) representative photographs of adipose tissue remodeling. Inflammation was studied through the release of TNF-α (e) and IL-6 (f) (n = 6–8 per group). $^{*}P < 0.05$ IH/C57BL/6 versus N/C57BL/6.

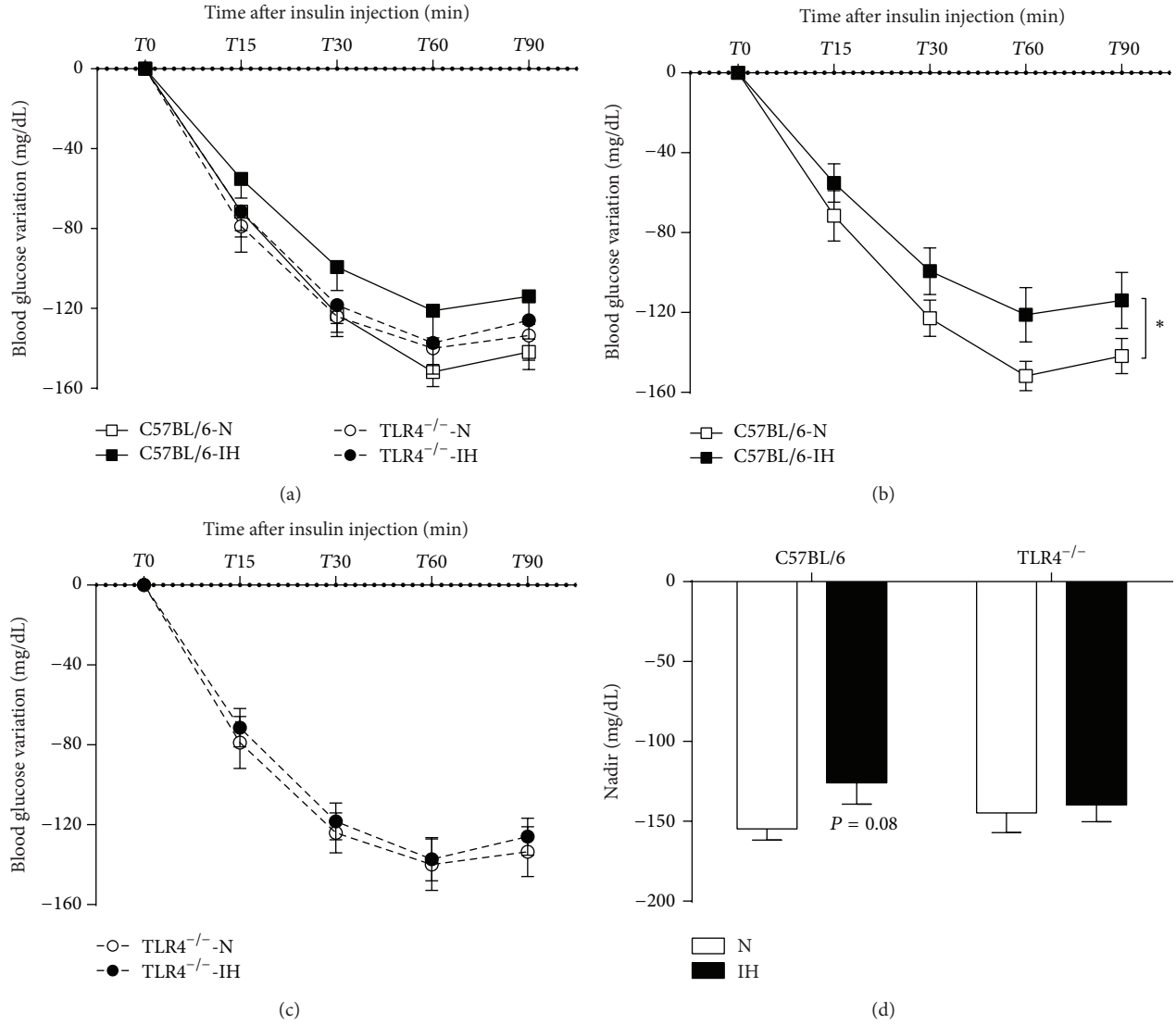

Figure 2: TLR4 signaling is involved in IH-induced insulin resistance. Glucose variation during the 90 minutes of the intraperitoneal insulin tolerance test (IpITT) in C57BL/6 or TLR4$^{-/-}$ mice exposed to 1 week of intermittent hypoxia (IH) or normoxia (N) (a). IpITT presented separately for C57BL/6 (b) and TLR4$^{-/-}$ mice (c). For each group, lowest blood glucose level (nadir) during the 90 minutes of the IpITT (d). $^{*}P < 0.05$ IH/C57BL/6 versus N/C57BL/6, $n = 13$–15 per group.

We found that both IH-induced morphological and inflammatory alterations of EWAT were prevented in TLR4-deficient mice. There is growing evidence from studies using murine models of obesity that activation of the proinflammatory TLR4/NFκB pathway constitutes one mechanism that links inflammation and metabolic disorders [30–32]. Although TLR4 activation is known to enhance lipolysis [33, 34], we were surprised to find that EWAT wasting and adipocyte hypotrophy were completely prevented in TLR4-deficient mice suggesting that TLR4 signaling could be the main mechanism of these consequences. As observed in mice models of diet-induced obesity [30, 32], TLR4 deficiency prevented the enhanced release of TNF-α and IL6 in our hypoxic mice. This strengthens the role of TLR4 in IH-induced inflammation, as well as the pathophysiological similarities with obesity, that is, a normal amount of fat under hypoxia behaving like excess fat in obesity [10].

4.3. IH-Induced Insulin Resistance Involves TLR4 Signaling. We found that IH induced insulin resistance in C57BL/6 mice. This is in agreement with previous findings obtained in various mouse strains (genetically obese, lean C57BL/6, and ApoE$^{-/-}$ mice) and duration of IH exposure (acute or chronic IH), using different methods to assess insulin sensitivity (HOMA-IR, ITT, hyperinsulinemic euglycemic clamp) [10, 19, 35, 36]. This confirms the role of IH in the alterations of glucose homeostasis observed in sleep apnea, the insulin resistance worsening with OSA severity, independently of obesity [37–39]. Such relationship has also been confirmed

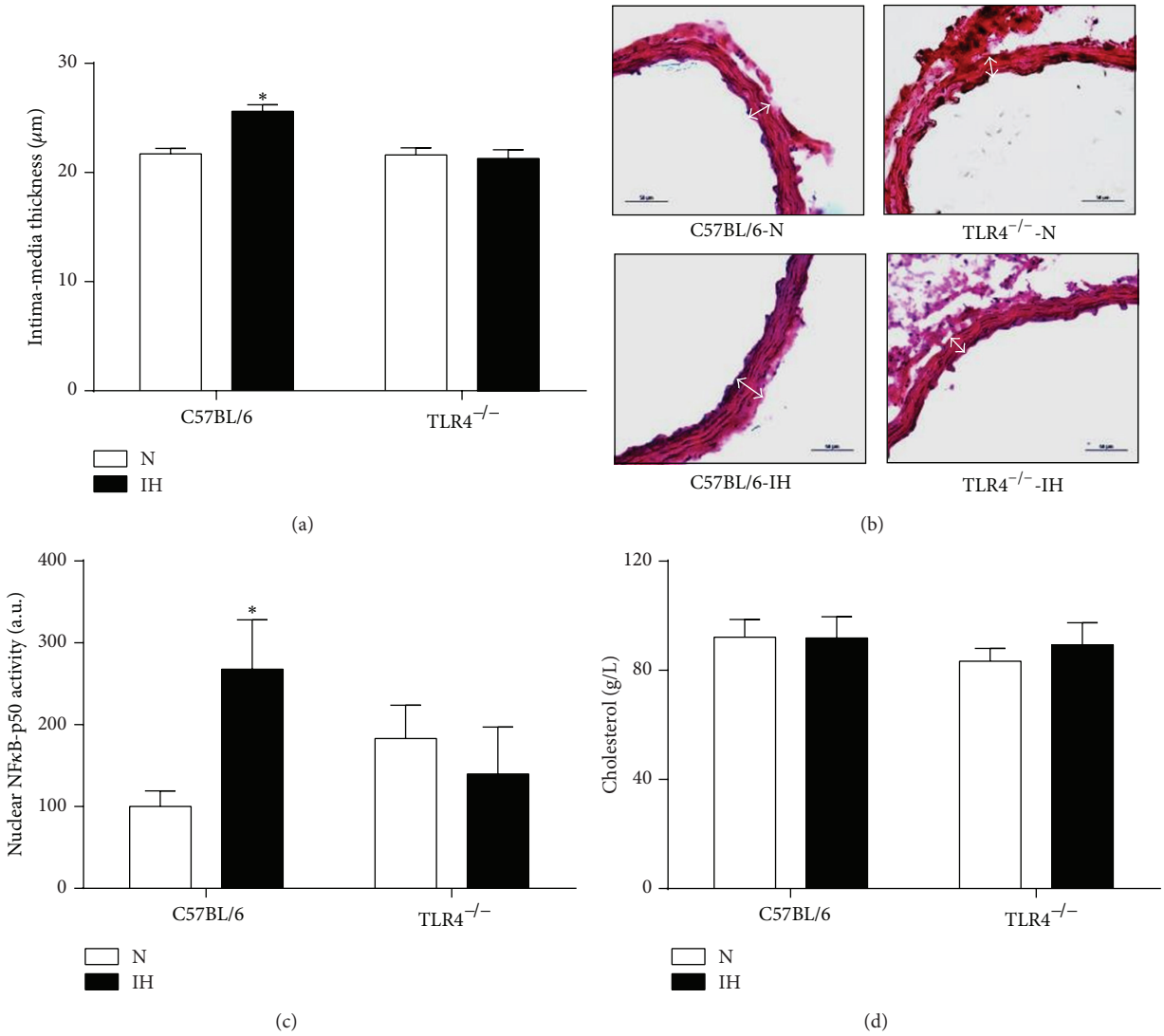

Figure 3: TLR4 signaling is involved in IH-induced vascular remodeling. Morphometric and inflammatory remodeling of aorta was assessed in C57BL/6 and TLR4$^{-/-}$ mice exposed to 4 weeks of intermittent hypoxia (IH) or normoxia (N). (a) Aortic intima-media thickness quantification, (b) representative photographs of aorta remodeling (wall thickness represented by white double-headed arrows), (c) quantification of activated NFκB (NFκB-p50 activity) in aorta (n = 8–11 per group). (d) Plasma levels of total cholesterol in C57BL/6 and TLR4$^{-/-}$ mice exposed to 4 weeks of IH or N, n = 12–16 per group. $^{*}P < 0.05$ IH/C57BL/6 versus N/C57BL/6.

experimentally in healthy humans exposed to IH [40]. Both chronic inflammation [11] and elevated FFA levels [41, 42] are established factors causing insulin resistance in obesity. Indeed proinflammatory cytokines secreted from adipocytes are considered as a key step in obesity-induced insulin resistance, as the sole TNF-α neutralization in obese rats is sufficient to improve insulin sensitivity [43]. Inversely, upstream activation of the inflammatory cytokine cascade using agonists of toll-like receptors leads to insulin resistance [33, 34]. In the present study, we observed increased release of TNF-α and IL6 from EWAT and decreased insulin sensitivity in hypoxic C57BL6 mice. Both parameters were not impaired in TLR4-deficient mice suggesting that TLR4 mediated inflammation was involved in this metabolic consequence. Body weight alterations did not contribute to these improvements, as hypoxic C57BL/6 and TLR4-deficient mice had similar body weight.

4.4. IH-Induced Vascular Remodeling Involves TLR4 Signaling. We found that IH induced vascular remodeling, including morphological (larger intima-media thickness) and inflammatory changes (higher NFκB-p50 activity). This confirms our previous results and others regarding the detrimental remodeling effects of IH on preatherosclerotic [17, 18] and atherosclerotic [21, 44, 45] processes. We confirmed in the present study that IH is indeed a powerful vascular stress, as only 28 days of exposure, which is very short compared to the duration of the human disease, induce early vascular alterations in the atheroresistant C57BL/6 mouse strain.

Besides the well-known role of the sympathoadrenergic system and the related hemodynamic alterations [18], accumulating evidence suggests that inflammation is involved early in the pathophysiology of IH-related atherosclerosis [45]. Regarding the aggravation of atherosclerosis by IH in ApoE-deficient mice, we recently demonstrated that this deleterious effect involved inflammatory alterations of EWAT, as EWAT lipectomy prevented the proatherogenic effect of IH [10]. Interestingly, this beneficial effect occurred while insulin resistance (a well-known risk factor for atherosclerosis) was not improved by EWAT lipectomy, suggesting that EWAT inflammation could be the main determinant of IH-induced atherogenicity. Here, we showed that EWAT from hypoxic C57BL/6 mice released higher amounts of inflammatory cytokines and that this effect, as well as the morphological and inflammatory changes of aorta, was prevented in TLR4-deficient animals. Given the role of TLR4 in adipose tissue inflammation and insulin resistance [46], and the relationship between adipose tissue inflammation, insulin resistance, and vascular dysfunction [12, 47], the beneficial effect of TLR4 deficiency on arterial remodeling could be explained by the prevention of adipose inflammation and insulin resistance. Here, the role of EWAT inflammation seems to be predominant compared to the light improvement of insulin response and the absence of cholesterol alterations in these animals. IH-induced dyslipidemic alterations are indeed inconstant in mice [45, 48–50], suggesting that dyslipidemia contributes only in part to the first steps of vascular remodeling in this model. Reduced IH-driven hemodynamic alterations could be a further explanation, as TLR4-deficient mice are less susceptible to hypertension [51, 52]. Finally, a direct effect on the vascular wall is also possible, as TLR4 has been evidenced in human [53] and murine [54] atherosclerotic plaques, and inhibition of TLR4 signaling pathway attenuated diet-induced atherosclerosis in $ApoE^{-/-}$ mice [16, 55]. A recent study also identified TLR4 signaling pathway as a direct key mediator of vascular inflammation and impairment of endothelial insulin signaling in the setting of obesity [15]. The direct effect on vasculature could even be predominant, as TLR4 deficiency prevented atherosclerosis in $LDLR^{-/-}$ mice, with no effect on adipose tissue inflammation and whole-body insulin sensitivity [56].

5. Conclusion

We showed in nonobese C57BL/6 mice that IH induced morphological and inflammatory remodeling of aorta and epididymal white adipose tissue, as well as insulin resistance. These alterations were prevented in TLR4-deficient mice suggesting that IH-induced cardiometabolic consequences involved inflammation mediated by TLR4 signaling. The precise mechanisms and the specific role of one type of tissue or cell (e.g., adipose tissue) remain to be determined as TLR4 knockout used in the study was not cell specific. TLR4 is indeed expressed on many cell types, predominantly those of the immune system, but also on nonhematopoietic cell types (e.g., endothelial, epithelial cells, etc.). Despite these limitations, as a practical point of view, the whole body is exposed to hypoxia during sleep apnea, and TLR4 in various cells is likely to be involved in the numerous complications of sleep apnea. Moreover the available treatment that blocks TLR4 activation (eritoran) is not cell specific. Whether our results can be extrapolated to the human disease remains to be determined. However, one clinical study has recently investigated the activation of TLR4 signaling pathway in OSA. The authors found increases in TLR4 expression, NFκB nuclear binding, and release of IFNγ, TNF-α, and IL-6 in circulating monocytes [57]. There are therefore similarities between these clinical findings and our experimental results suggesting that targeting TLR4/NFκB pathway could provide further therapeutic options for sleep apnea patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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Review Article

OSAS-Related Inflammatory Mechanisms of Liver Injury in Nonalcoholic Fatty Liver Disease

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Obstructive sleep apnoea syndrome (OSAS) is a common sleep disorder, affecting over 4% of the general population, and is associated with metabolic syndrome and cardiovascular disease, independent of obesity and traditional risk factors. OSAS has been recently connected to nonalcoholic fatty liver disease (NAFLD), the most common chronic liver disease in the world, which can be found in 30% of the general adult population. Several studies suggest that the chronic intermittent hypoxia (CIH) of OSAS patients may per se trigger liver injury, inflammation, and fibrogenesis, promoting NAFLD development and the progression from steatosis to steatohepatitis, cirrhosis, and hepatocellular carcinoma. In NAFLD patients, liver disease may be caused by hypoxia both indirectly by promoting inflammation and insulin resistance and directly by enhancing proinflammatory cytokine production and metabolic dysregulation in liver cells. In this review, we focus on molecular mechanisms linking OSAS to NAFLD, including hypoxia inducible factor (HIF), nuclear factor kappa B (NF-κB), YKL-40, unfolded protein response, and hypoxic adipose tissue inflammation, which all could provide novel potential therapeutic approaches for the management of NAFLD patients with OSAS.

1. Introduction

Obstructive sleep apnoea syndrome (OSAS) is a common sleep disorder in which complete or partial airway obstruction, caused by pharyngeal collapse during sleep, determines loud snoring or choking, frequent awakenings, disrupted sleep, and excessive daytime sleepiness. OSAS affects over 4% of the general population and 35–45% of obese individuals [1, 2]. The growing clinical relevance of OSAS is due to its emerging association with diabetes mellitus, metabolic syndrome, and cardiovascular disease (CVD), independent of other traditional cardiometabolic risk factors and obesity [3–6]. A lot of evidence suggests that the pathophysiological alteration in gas exchange (repetitive hypoxemic and hypercapnic events), called chronic intermittent hypoxia (CIH), can lead to increased proinflammatory cytokine production, endothelial dysfunction, oxidative stress, metabolic dysregulation, and insulin resistance [7–9]. In the latest years, OSAS has been associated with nonalcoholic fatty liver disease (NAFLD). Experimental evidence suggests that CIH may per whole trigger liver injury, inflammation, and fibrogenesis [10], and, interestingly, OSAS is also believed to be one of the elements promoting the evolution of NAFLD from steatosis to nonalcoholic steatohepatitis (NASH) [11]. NAFLD is the most common chronic liver disease in the world: about 30% of the general adult population and up to 60–70% of diabetic and obese patients suffer from this clinical condition [12]. NAFLD includes a group of liver damages, ranging from simple steatosis to NASH. NAFLD confers an increased risk of liver-related complications (hepatocellular carcinoma and end-stage liver disease), cardiovascular disease, and chronic kidney disease and is projected to be the leading

cause of liver transplantation by 2020 [13, 14]. The metabolic alterations connecting to NASH encompass insulin resistance and obesity, but mechanism(s) whereby repeated hypoxic events of OSAS can enhance liver disease progression is not completely clear.

This review focuses on molecular mechanisms linking OSAS to liver injury in NAFLD.

2. Chronic Intermittent Hypoxia and Fatty Liver Disease

CIH is a key feature in the pathophysiology of OSAS. The mechanism is probably similar to ischemia-reperfusion injury. In OSAS patients, some oxidative stress markers are augmented and they could increase inflammation, endothelial dysfunction, and development of atherosclerosis [15].

Recently, some studies have focused on the effects of hypoxia on metabolic pathways and on mechanisms of cell injury in NAFLD. Savransky et al. showed that CIH induces hyperglycemia and hepatic lipid peroxidation and enhances activity of nuclear factor kappa B (NF-κB), a master regulator of inflammatory response. Liver histology is characterized by swelling and significant increase in accumulation of glycogen in hepatocytes. Therefore, CIH may independently drive to mild liver injury also in the absence of factors that induce obesity [16]. Murine models have demonstrated that CIH leads to a significant rising in hepatic lipid peroxidation, α1-collagen mRNA and amount of myeloperoxidase, and proinflammatory cytokines (such as IL-1β, IL-6, the chemokine macrophage inflammatory protein-2, and TNF-α) [17]. The authors suggest that, in the chronic hypoxic conditions associated with OSAS, a high-fat diet could promote NAFLD. It has been demonstrated that hypoxia reduces insulin sensitivity in mice and could enhance expression of the lipogenic transcription factors sterol-regulatory-element binding protein-1c (SREBP-1c), peroxisome proliferator-activated receptor-γ (PPAR-γ), acetyl-CoA carboxylase 1 (ACC1), and acetyl-CoA carboxylase 2 (ACC2).

Enhanced SREBP-1c activity involves two mechanisms: activation of SREBP-1c transcription and stimulation of proteolytic cleavage of the SREBP-1c precursor form embedded in the membranes of the endoplasmic reticulum (ER) [18]. Within the ER membranes, the inactive SREBP proteins are associated with two proteins with a pivotal role in the control of the cleavage process: SREBP cleavage-activating protein (SCAP) and insulin-induced gene (*INSIG*). SCAP interacts with both newly synthesized SREBP precursor and Insig, which keep the SCAP/SREBP complex into the ER [19]. In the presence of specific signals such as insulin, SCAP dissociates from Insig and escorts SREBPs in coated protein II (COPII) vesicles from the ER to the Golgi apparatus, where SREBPs are proteolytically processed to yield the transcriptionally active form. The mature SREBP forms are released and translocate into the nucleus and the SREBP-1c mature isoforms stimulate the expression of lipogenic genes. Mounting animal and human evidence suggests that abnormalities of SREBP-1c function play an important pathogenetic role in contributing to the NAFLD phenotype [20, 21]. Insulin-resistant ob/ob mice have increased concentrations of SREBP-1c and also develop spontaneous fatty liver [22]: SREBP-1c activates, among other genes, ACC that produces malonyl-CoA, an intermediate in fatty acid synthesis, which inhibits CPT-1, the enzyme transferring fatty acyl-CoAs into the mitochondria for β-oxidation. PPAR-γ is necessary for regulation of insulin sensitivity and lipid metabolism. The overexpression of PPAR-γ in liver tissue causes lipid accumulation; it could represent a mechanism for hypoxia-induced fatty liver [23]. Moreover, hypoxia also reduces the expression of genes regulating mitochondrial β-oxidation (e.g., PPAR-α and carnitine palmitoyltransferase-1 (CPT-1)), which might decrease fat oxidation and promote lipid accumulation [11]. PPAR-α is highlyexpressed in the liver and mice lacking PPAR-α develop steatosis [24]. Moreover, PPAR-α has anti-inflammatoryproperties. PPAR-α suppresses the expression of proinflammatory genes, allowing the control and inhibition of inflammation [25]. Therefore, hypoxia per se can upregulate the expression of lipogenic genes and downregulate genes involved in lipid metabolism: it promotes hepatic triglyceride accumulation, necroinflammation, and fibrosis that promote the progression of NAFLD [26]. Consistent with experimental data, Nobili et al. found in paediatric NAFLD thatthe presence of OSAS was associated with the presence of NASH and of significant fibrosis, and the severity of sleep apnoea and nocturnal hypoxemia correlated with NAS score and fibrosis stage, independently of overall/abdominal obesity, metabolic syndrome, and insulin resistance [27]. In a population of obese children and adolescents with liver biopsy-proven NAFLD, Sundaram et al. have demonstrated that histological fibrosis was more severe in the subjects with NAFLD and OSA/hypoxemia compared with those without OSA/hypoxemia. Moreover, in this study, the severity and the duration of nocturnal hypoxemia were associated with both histological measures of NAFLD disease severity and elevated AST and ALT levels [28].

The duration of nocturnal haemoglobin desaturation independently predicted the number of liver-infiltrating leukocytes and activated Kupffer cells/macrophages, which are believed to play a key role in the pathogenesis of liver injury in NAFLD [29]. Furthermore, CIH directly activates hypoxia-inducible factor- (HIF-) 1a and HIF-2a, two key transcription factors regulating the expression of genes involved in hepatocyte de novo lipogenesis and free fatty acid oxidation and in Kupffer and hepatic stellate cell activation, eventually promoting hepatic steatosis, necroinflammation, and fibrogenesis [30, 31] (Figure 1).

3. Nuclear Factor Kappa B (NF-κB): A Link between OSAS and Hypoxic Liver Injury

The NF-κB family of transcription factors is constitutively expressed in all cell types, which has a central role as a transcriptional regulator in response to cellular stress. The two known pathways for NF-κB activation are the canonical (classical) Toll-like receptor signaling and the noncanonical (alternative) pathway which is particularly important in B cells. NF-κB exists as homodimer/heterodimer composed of members of the Rel protein family which includes RelA

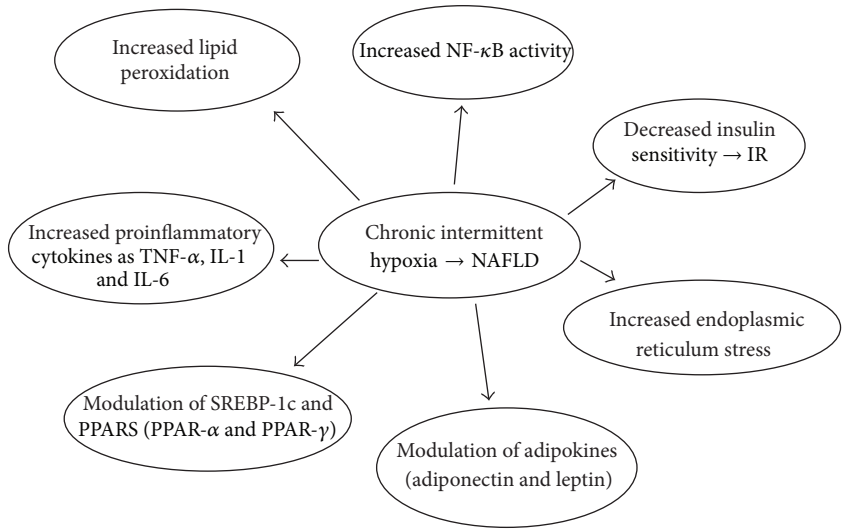

Figure 1: The relationship between nonalcoholic fatty liver disease (NAFLD) and chronic intermittent hypoxia (CIH). TNF: tumor necrosis factor. IL: interleukine. SREBP-1c: sterol-regulatory-element-binding protein-1c. PPAR: peroxisome proliferator-activated receptor. ER: endoplasmic reticulum.

(p65), p50 and its precursor p105 (NF-κB1), and RelB, cRel, and p52 and its precursor p100 (NF-κB2) in the alternative pathway. Inactive NF-κB dimers are stored in the cytoplasm, under inhibition control of IkB (inhibitor of NF-κB) family which bind to NF-κB and masks its nuclear localization signal [32]. IkB proteosome degradation occurs by phosphorylation via the activity of IkB kinases, IKKα, and IKKβ. In the classical pathway, proinflammatory cytokines like TNFα or oncogenes promote a kinase signalling cascade, leading to the phosphorylation of IkB and ubiquitination-mediated proteasomal degradation; finally, NF-κB is released and translocates into the nucleus. In the alternative pathway, activation of IKKα phosphorylates NF-κB precursors (p100/RelB); the proteasome then processes these precursors into the active p52/RelB heterodimer. Then, the activated NF-κB dimer affects the expression of genes involved in immune responses, proliferation, apoptosis, and expression of certain viral genes by binding to target DNA sequences [33]. NF-κB participates in the initiation and the progression of inflammation. Particularly, the cardiovascular and adipose tissue inflammation in OSAS seems to be related to an enhanced NF-κB activity in endothelial cells, adipose tissue macrophages, and adipocytes [34]; furthermore, NF-κB activation in hepatocytes and in stellate cells is associated with hepatic insulin resistance, hepatocyte apoptosis, and development of NASH and hepatocellular carcinoma in animals and humans [35, 36]. To date, systemic activation of NF-κB has been related to OSAS pathophysiology independently of HIF by novel mechanism activated in hypoxic conditions [37]. In hypoxic conditions, calcium is released from the endoplasmic reticulum (ER) and activates calcium-dependent IKK kinase transforming growth factor β activated kinase- (TAK-) 1; TAK-1 phosphorylates IκBα, thereby promoting RelA subunit release and NF-κB activation and translocation into the nucleus. Hypoxia also inhibits specific sumo proteases (Senps), resulting in an increased Sumo-2/3 (S2) chains on IkBα, which is sufficient to release RelA from IkBα and activate NF-κB [38]. In addition, NF-κB pathway is linked to oxidative stress and reactive oxygen species (ROS) by the production of inducible NO synthase (iNOS), cyclooxygenase- (COX-) 2, and metalloproteinase 9. ROS released from hypoxic hepatocyte have been shown to directly activate hepatic stellate cell through IkB-α phosphorylation and NF-κB signalling activation in cell culture [39]. The induction of NF-κB by CIH may lead to upregulation of multiple inflammatory cytokines and chemokines, including IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, I-10, IL-13, IL-15, IL-18, TNF-α, TNF-β, IFN-α, IFN-β, and macrophage inhibitory protein 1b [40].

The unfolded protein response (UPR) represents another mechanism linking hypoxia and liver injury. A pathogenic role of UPR in steatosis, inflammation and insulin resistance is supported by studies on obese and nutritional models of fatty liver [41] and on NAFLD patients [42].

The ER is a specialized cellular organelle synthesizing, folding, and assembling membrane and secretory proteins. Any condition perturbing ER homeostasis, as excessive protein synthesis or alterations in cellular redox balance or in calcium concentration, triggers a physiologic response, the UPR, which involves an increment of ER-folding capacities by increasing the transcription of ER-resident chaperones and protein foldases, to a downregulation of the protein load in the ER lumen by reducing protein synthesis and to an ER-associated degradation (ERAD) of irretrievably misfolded proteins [43].

The UPR is mediated by 3 transducer proteins that are integral membrane proteins of the ER: inositol-requiring kinase-1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase RNA-like ER kinase (PERK), which are normally maintained inactive by the linking of intraluminal ER chaperones, including the binding immunoglobulin protein (BiP)/glucose-regulated protein 78 (GRP78). During UPR, BiP is sequestered by the unfolded proteins and it

dissociates from the three ER-transmembrane transducers, leading to their activation. IRE1α is an inositol-requiring enzyme that regulates the expression of the transcription factor X box-binding protein 1 (XBP1) and regulates the activity of kinase c-Jun N-terminal kinase (JNK). ATF6 is a transcription factor that, like SREBP-1c and SREBP-2, translocates to the nucleus, upregulating chaperones/foldases such as GRP78, homocysteine-induced ER protein (HERP), calreticulin, and calnexin, which enhance the folding ability of the ER. PERK phosphorylates eukaryotic initiation factor 2α (eIF2α) causing a global mRNA translation attenuation and, at the same time, selectively enhances the translation of several mRNAs, including the activating transcription factor 4 (ATF4), which upregulates chaperones and antioxidant response genes and increases damaged ER repairing [44].

When the mechanisms of adaptation are saturated, the ER-folding capacity cannot be restored and the UPR over-activation results in pathologic conditions, such as the trigger of apoptosis through the activation of transcription factor C/EBP homologous protein (CHOP), of JNK, and of caspases [45].

Recent evidence has shown that UPR is a component of the cellular response to hypoxia and PERK has been identified as the kinase responsible for elf-2α phosphorylation in hypoxic conditions [46].

Several recent studies have linked the UPR to lipogenesis regulation and hepatic steatosis.

The degree of UPR contribution to hepatic steatosis may depend on the relative activation of the 3 transducer proteins IRE1a, PERK, and ATF6. IRE1a-dependent activation of JNK can lead to liver damage and hepatocyte apoptosis, a characteristic feature of NAFLD [47].

PERK-dependent factor Nrf2 transcription is part of an antioxidant pathway. In murine model, Nrf2 deletion results in rapid onset and progression of NASH. These data suggest that PERK plays a critical role in the defence against oxidative stress linked to NASH [48].

Different studies have shown that ATF6 can inhibit the transcriptional activity of SREBP2, regulating lipid storage in the liver [49].

To date, different experimental mice models have demonstrated that CIH leads to increased phosphorylation of PERK, pointing to an upregulation of the UPR in liver and adipose tissue, collectively suggesting that ER stress may be a key mediator of hypoxia-induced liver injury and NAFLD.

Extensive evidence supports the notion that metabolically active adipose tissue plays a role in the development of NASH through altered secretion of lipotoxic free fatty acids and of adipocytokines including adiponectin, leptin, TNFα, or IL-6 [50].

In obese patients, adipocytes dysfunction, apoptosis, and consequent macrophage accumulation are associated with local hypoxemia [51] supporting the idea that adipose tissue hypoxia is one of the causes of adipose tissue inflammation. Evidences demonstrated that, in hypoxic condition, adipocytes express more proinflammatory cytokines (i.e., leptin, TNFα, and IL-6) and less adiponectin than in normoxia [52]. In adipocytes, hypoxia reduces adiponectin secretion, directly or indirectly through TNFα stimulation [53], and stimulates lipolysis and inhibits uptake of FFA through a direct inhibitory effect on the fatty acid transporters (FATP1 and CD36) and on the transcription factor (PPAR-γ) [54]. From these data, one might assume that OSAS can associate with obesity making the adipocyte prone to dysfunction and death, aggravating liver and metabolic disease.

The concentration of TNF-α correlates with the severity of OSAS. TNF-α has a role in modulation of physiological sleep. Independently of obesity, in OSAS subjects, TNF-α is higher than in healthy population and its levels are lower after the introduction of CPAP therapy [55]. Furthermore, in OSAS patients, the secretion rhythm of TNF-α is changed: the nocturnal peak of secretion is substituted by an abnormal daytime peak [56].

During nocturnal hypoxia, adipocytes and circulating monocytes secrete IL-6 through the NF-κB pathway. IL-6 represents an important stimulus of CRP production in the liver [57] and has a role in inflammatory processes in sleep disorders. An increase of IL-6 has been shown in OSAS patients as compared to healthy controls [58], but IL-6 circadian rhythm is not altered in OSAS differently from TNF-α [59].

The increase in TNF-α and IL-1 is shown not only in OSAS. It is associated with an increase in insulin resistance [68], metabolic syndrome, and obesity [37]. The relationship between OSAS and CRP in obesity remains somewhat controversial, mainly, because of the major confounding effect of BMI. Nevertheless, several authors demonstrated that, in OSAS patients, CRP levels are independently associated with the severity of the sleep disturbance and of nocturnal hypoxemia independently of adiposity [69–71].

Adiponectin is another cytokine produced by adipocytes. The presence and severity of NAFLD are correlated to decreased adiponectin [72] and recently several authors have demonstrated that, in adipose tissue, the expression of adiponectin is decreased by hypoxia [73]; the consequence could be the increased expression of inflammatory cytokines. Moreover, TNF-α has been shown to inhibit adiponectin in adipose tissue [74]. Collectively, these data suggest that hypoxia might directly or indirectly inhibit adiponectin expression.

Leptin is a cytokine produced by adipocytes that have a role in the regulation of food intake, lipid and glucose metabolism, and the energy balance. In a rabbit model of OSAS, leptin activated inflammation and mediates cellular injury in association with IH [75]. It is known that the increased leptin production is associated with visceral obesity; also hypoxia can stimulate leptin production [76]. In several studies leptin is linked with OSAS independently of obesity [77]. Kapsimalis et al. confirmed that nocturnal hypoxemia is associated with leptin independently of obesity [71].

4. Hypoxia-Inducible Factors: A Key Factor of Hypoxia-Mediated Steatosis and Inflammation in the Liver

Hypoxia modulates target genes expression through a number of transcription factors, including hypoxia-inducible

factors (HIFs). HIFs are heterodimers consisting of an α and a β subunit. There are three α subunits: the hypoxia-inducible factor 1α (HIF1α), the hypoxia-inducible factor 2α (HIF2α), and the hypoxia-inducible factor 3α (HIF3α); HIFα subunits bind to a common β subunit called or aryl- hydrocarbon receptor nuclear translocator (ARNT) [78].

HIF-α subunits are constitutively produced; in normoxic cells, HIF-α subunits are immediately degraded through hydroxylation by three prolyl-hydroxylases (PHD1, PHD2, or PHD3). The proline residues hydroxylated are assembled on a multimeric protein complex that includes the Von Hippel-Lindau (VHL) protein. This leads to rapid ubiquitination and proteasomal degradation of HIF-α [79]. Under hypoxic conditions, the mechanism of degradation is inhibited and HIF-α subunits heterodimerize with HIF-1β; active HIF translocates to the nucleus and binds to the hypoxia responsive elements (HRE) of hundreds of hypoxia responsive genes, regulating gene transcription [80]. In addition, HIFs can modulate target gene expression causing chromatin conformational changes [81].

In OSAS patients, the chronic intermittent cycles of hypoxia and reoxygenation (CHI) can increase active HIFs levels. In animal model, CHI leads to hypercholesterolemia and hepatic lipid peroxidation, in the absence of obesity [61].

HIFs could play a role in the pathogenesis of NAFLD, affecting many metabolic pathways in hepatic cells (see Table 1). The increase of HIF1 and HIF2 is associated with the expression in the hepatocytes of genes important for lipogenesis and gluconeogenesis regulation, triglycerides storage, and fatty acid synthesis, uptake, and β-oxidation. HIF1 is a mediator of alcohol-induced lipid accumulation as part of the monocyte-chemoattractant protein-1 (MCP-1) pathway; a constitutive activation of HIF2 leads to an increase of hepatic fatty acid uptake and lipid storage, along with a reduction in fatty acid β-oxidation and in lipoprotein lipase activity [30, 60, 62].

In hepatocytes, HIFs also regulate the transcription of genes involved in inflammation and HIF2 overexpression stimulates proinflammatory cytokines synthesis (IL-1β and IL-6) in hepatocytes and in macrophages [30].

Interestingly, HIFs can modulate fibrogenesis and angiogenesis in hepatic Kupffer and stellate cells [30, 31, 65]. Hypoxic HIF1 level in stellate cells is both associated with upregulation of proangiogenic mediator, including vascular endothelial growth factor (VEGF) and placental growth factor (PGF), and macrophage migration inhibitory factor (MIF) release, which is implied in fibrosis development. In Kupffer cells, HIF1 activation leads to production of platelet-derived growth factor- (PDGF-) B, VEGF, and Angiopoietin-1, promoting fibrosis and neoangiogenesis. Furthermore, HIF-2 coordinates the reprogramming of cell metabolic pathways from aerobic to anaerobic and generates ATP in an oxygen-independent manner [82]. These observations could link the increases of HIF levels not only to NAFLD development and progression but also to the pathogenesis of hepatocellular carcinoma (HCC).

In animal models, the overexpression of HIF1α and HIF2α has been found to be related to hepatic steatosis, hepatitis, and fibrosis. Hepatocyte-specific deletion of HIFs protected from steatohepatitis, whereas disruption of VHL resulted in a robust accumulation of lipids in the liver and an increase in liver inflammation and fibrosis [30, 31, 60–62].

Collectively, these data suggest that activation of HIFs in chronic hypoxemic conditions could cause hepatic steatosis, inflammation, and fibrosis, leading to NAFLD and HCC development; HIFs pathway may represent a novel potential therapeutic target.

5. YKL-40: A Novel Biomarker of Inflammation

YKL-40, a member of the mammalian chitinase-like proteins, is a glycoprotein consisting of 383 amino acids with a molecular mass of 40 kDa [83]. YKL-40 is secreted by activated macrophages, neutrophils, and vascular smooth muscle cells during inflammation. YKL-40 represents a biomarker of systemic inflammation and high YKL-40 levels were found in numerous pathological conditions, such as atherosclerosis, diabetes, obstructive lung disease, asthma, liver fibrosis, inflammatory bowel disease, rheumatoid arthritis, and cancer [84].

In human liver, YKL-40 may be involved in extracellular matrix turnover and its expression is regulated by TNFα, through the modulation of upstream transcriptional complexes that interact with the YKL-40 promoter. Sarma et al. have identified the putative binding sites for NF-κB subunit P65 and CCAAT/enhancer-binding protein alpha (CEBPα) in the YKL40 promoter, demonstrating that the TNF mediated YKL-40 expression involves the NOTCH/NFKB signaling pathway [85].

YKL-40 has been found to be upregulated in alcoholic hepatitis and HCV patients [86, 87], and serum YKL-40 has been shown to be associated with degree of fibrosis progression and extracellular matrix synthesis in many chronic liver diseases [88]. However, in 95 patients with nonalcoholic fatty liver disease, Malik et al. observed that YKL-40 performed relatively poorly as marker of liver inflammation or fibrosis [89]; moreover, in 52 children with biopsy-verified NAFLD, Lebensztejn and coworkers did not find a correlation between YKL-40 and fibrosis stage [90].

In OSAS patients, Xang et al. observed a significant association between increments in serum YKL-40 concentrations and severity of OSAS; the YKL-40 levels were found independently correlated with AHI scores. In these patients, YKL-40 is probably secreted from activated inflammatory cells located in the upper airway or synthesized and released from differentiated VSMCs in response to tissue remodelling. Macrophages exposed to YKL-40 increase the release of proinflammatory and profibrogenic mediators, such as IL-8, IL-18, macrophage inflammatory protein- (MIP-) 1, matrix metallopeptidase- (MMP-) 9, and monocyte chemotactic protein- (MCP-) 1. This mechanism may contribute to OSAS development and progression [84].

In conclusion, YKL-40 expression is enhanced in numerous local inflammatory processes, including OSAS and

Table 1: Molecular targets and biological effects of hypoxia inducible factors (HIFs) in the liver.

Cell	Molecular target	Biological effect
	HIF1α	
Hepatocyte	↑ Lipid droplet binding protein adipose differentiation-related protein (ADFP)	↑ Triglyceride storage in lipid droplets [60]
	↑ Stearoyl-coenzyme A desaturase 1 (SCD-1)	↑ De novo lipogenesis [61]
	↑ Enolase 1 ↑ Lactate dehydrogenase 1	↑ Glycolysis ↑ Pyruvate metabolism [62]
	↑ Membrane glucose transporter- (GLUT-) 1 and GLUT-3	↑ Glucose uptake [63]
Hepatic stellate cells	↑ Plasminogen activator inhibitor- (PAI-) 1 ↑ Platelet-derived growth factor- (PDGF-) A and PDGF-B	↑ Hepatic stellate cell activation and fibrogenesis [64]
	↑ PDGF-B ↑ Transforming growth factor- (TGF-) β1 ↑ Vascular endothelial growth factor (VEGF) ↑ Chemokine receptor- (CCR-) 1 and 5	↑ Fibrogenesis ↑ Angiogenesis ↑ Carcinogenesis [31]
Kupffer cells	↑ Plasminogen activator inhibitor- (PAI-) 1 ↑ Platelet-derived growth factor- (PDGF-) A and PDGF-B	↑ Hepatic stellate cell activation and fibrogenesis [65]
	HIF2α	
Hepatocyte	↑ Lipid droplet binding protein adipose differentiation-related protein (ADFP)	↑ Triglyceride storage in lipid droplets [62]
	↓ Acyl-coenzyme A synthase long-chain family member 1 ↓ Carnitine-palmitoyltransferase I	↓ Mitochondrial fatty acid β-oxidation [62]
	↓ Acyl-CoA oxidase (Aco) ↓ Carnitine O-octanyltransferase	↓ Peroxisomal fatty acid β-oxidation [62]
	↑ Membrane fatty acid transporter cluster differentiation 36 (CD36)	↑ Fatty acid uptake from plasma [30]
	Sterol-regulatory-element binding protein-1c (Srebp-1c) Fatty acid synthase (Fas) Acetyl-CoA carboxylase (Acc) (↑ early after HIF2α activation and then ↓ after long-term HIF2α activation)	De novo lipogenesis (↑ early after HIF2α activation and then ↓ after long-term HIF2α activation) [30, 62]
	↑ Angiopoietin-like 3 (Angptl3)	↓ Tissue lipoprotein lipase activity ↓ ↑ Plasma triglycerides [62, 66]
	↓ Phosphoenolpyruvate-carboxykinase (Pepck) ↓ Glucose-6-phosphatase	↓ Gluconeogenesis [62, 67]
	↓ Peroxisome proliferator-activated receptor γ coactivator 1α (Pgc-1α) ↓ Hepatocyte nuclear factor 4 (Hnf4)	↓ Gluconeogenesis [62, 67] ↓ Mitochondrial fatty acid β-oxidation [62]
Macrophage	↑ Interleukin-1β ↑ Interleukin-6	↑ Inflammation [30]
	↑ Interleukin-1β ↑ Interleukin-6	↑ Inflammation [30]
Hepatic stellate cells	↑ Lysyl oxidase-like- (LOXL-) 1 and LOXL-2 ↑ Prolyl 4-hydroxylase α- (P4HA-) 1 and P4HA-2 ↑ Procollagen lysine ↑ 2-Oxoglutarate 5-dioxygenase 2 (PLOD2) ↑ Transglutaminase 2 (TGM2)	↑ Collagen synthesis and deposition ↑ Fibrogenesis [30]

chronic liver diseases, but the role of this protein in NAFLD development and progression needs to be clarified; moreover, further studies are needed to elucidate if the OSAS-dependent YKL-40 increase could trigger liver injury or affect the progression of a concomitant liver disease.

6. Insulin Resistance (IR) and Liver Injury in OSAS

Hypoxia can cause insulin resistance and inflammation causing development of NAFLD. In humans, OSAS has been

known as a risk factor for insulin resistance, independently of obesity [91]. In OSAS, glucose intolerance correlates positively with severity of the disease. Different studies have shown that CIH and sympathetic nerve discharge are a possible cause of alteration in insulin sensitivity. In healthy men undergoing an euglycaemic hyperinsulinemic clamp test, a short (30-minute duration) period of hypoxia was able to induce glucose intolerance and to increase plasma epinephrine levels [92].

Hypoxia can inhibit insulin receptor activation and trigger the formation of inflammatory cytokines which promote peripheral insulin resistance, while sympathetic activation causes insulin resistance by inducing glycogenolysis and gluconeogenesis [93].

It is certain that hypoxia inhibits respiratory function and biogenesis of the mitochondria and has also been found to decrease the number of mitochondria, alterations that have been linked to both insulin resistance and NAFLD [94, 95]. Hypoxia is identified to induce ER stress and inhibition of ER stress protects mice against insulin resistance and NAFLD [96].

In vitro exposure of human and murine adipocytes to prolonged hypoxia decreased phosphorylation of IRS-1 and IRS-2 and induced IR [97]. Furthermore, exposure to continuous hypoxia causes multiple changes in cell metabolism, including a switch to anaerobic glycolysis. In adipocytes, continuous hypoxia increased the expression of the glucose transporter- (GLUT-) 1 [98], glucose uptake, and release of lactate but decreased the expression of the insulin-dependent glucose transporter- (GLUT-) 4 [99].

The loss of the repressive effect of insulin on the expression of CYP2E1 could be a possible link between OSAS, insulin resistance, and NASH [100, 101]. CYP2E1 is a major microsomal source of oxidative stress and it could play a role in the pathogenesis of NASH [102]. Increased hepatocyte CYP2E1 expression may also result in the downregulation of insulin signalling, potentially contributing to the insulin resistance associated with NAFLD [103]. In the liver of mice exposed to hypoxia CYP2E1, mRNA and protein levels were increased. However, in the future, other studies are needed to elucidate if increased CYP2E1 is a cause or a consequence of insulin resistance.

7. Conclusion

Several studies correlate OSAS with liver inflammation and they suggest that CIH may play a role in the pathogenesis of NAFLD and in the progression from steatosis to steatohepatitis, cirrhosis, and liver cancer.

OSAS and obesity often coexist and share common molecular mechanisms that lead to metabolic disease, which could represent potentially therapeutic targets; in the future, it will be important to clarify the relative importance of these factors in the pathogenesis of liver disease.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Pathogenic Roles of the Carotid Body Inflammation in Sleep Apnea

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Breathing difficulties in sleep are a hallmark of sleep-disordered breathing commonly observed in patients with sleep disorders. The pathophysiology of sleep apnea is in part due to an augmented activity of the carotid body chemoreflex. Arterial chemoreceptors in the carotid body are sensitive to inflammatory cytokines and immunogenic molecules in the circulation, because cytokine receptors are expressed in the carotid body in experimental animals and human. Intriguingly, proinflammatory cytokines are also locally produced and released in the carotid body. Also, there are significant increases in the expression of proinflammatory cytokines, cytokine receptors, and inflammatory mediators in the carotid body under hypoxic conditions, suggesting an inflammatory response of the carotid body. These upregulated cytokine signaling pathways could enhance the carotid chemoreceptor activity, leading to an overactivity of the chemoreflex adversely effecting breathing instability and autonomic imbalance. This review aims to summarize findings of the literature relevant to inflammation in the carotid body, with highlights on the pathophysiological impact in sleep apnea. It is concluded that local inflammation in the carotid body plays a pathogenic role in sleep apnea, which could potentially be a therapeutic target for the treatment of the pathophysiological consequence of sleep apnea.

1. Introduction

Sleep apnea is the most prevalent form of sleep-disordered breathing, which is characterized by recurrent breathing pauses lasting over 10 seconds due to a repeated narrowing or closure of the upper airway and/or a periodic halt of the central drive for respiratory activities during sleep. Sleep apnea syndrome is closely associated with episodes of desaturated arterial blood oxygen, sleep fragmentation, and arousals in the patient. As a consequence of sleep apnea, it causes excessive daytime sleepiness and increases the risk of neurocognitive dysfunction and cardiovascular morbidities in young and adult patients [1, 2]. The pathophysiology and consequences of sleep apnea have been extensively studied over decades. A growing amount of evidence suggests that the carotid chemoreflex elicited by arterial chemoreceptors in the carotid body in response to episodes of hypoxia (intermittent hypoxia) in sleep apnea plays a role in the pathophysiological cascade [3]. Specifically, there are significant increases in the chemosensitivity and the activity of the carotid chemoreceptor induced by hypoxia in experimental animals and in patients with sleep apnea [4, 5]. Thus, mechanisms underlying the augmented activity of the carotid chemoreceptor are currently a focus of investigation.

Arterial chemoreceptors in the carotid body play an important physiological role in the chemotransduction of chemical changes in the arterial blood, which is essential to elicit the chemoreflex for rapid adjustment of respiratory and cardiovascular activities to maintain blood gases and pH homeostasis. Thus a lowered arterial oxygen or pH level causes an increase in the chemoreceptor activity of the carotid body [6]. The increased afferent activity via the carotid sinus nerve projects to the nucleus tractus solitarius in the medulla, through which the primary relaying neurons activate the central pathway of the chemoreflex. This in turn increases the central drive for ventilation and also alters cardiac and autonomic activities for the compensatory changes adjusting to metabolic needs [7].

The carotid body is bilaterally located at the bifurcation of the carotid artery, which is structurally analogous to vagal paraganglia. The major cell type in the carotid body is type-I glomus cells, which are chemosensitive and responsive to

chemical changes in the arterial blood. These cells form lobular clusters and are apposed to afferent nerve endings, which are essential for the sensory chemotransduction [6, 8]. These glomic clusters are encapsulated by type-II sustentacular cells, which are glial like and are recently known to play a role in the paracrine signaling in the chemotransduction and other housekeeping functions [9]. In responding to hypoxia, chemosensitive type-I glomus cells are depolarized with an inhibition of potassium currents causing an elevated level of intracellular calcium, which triggers the vesicular release of neurotransmitters acetylcholine and ATP as well as neuromodulators including catecholamines and neuropeptides from the chemosensitive cells [10]. This elevates the excitability of the nerve endings which increases the activity of the carotid sinus nerve of the carotid body for eliciting the chemoreflex.

The carotid body is highly vascularized with blood perfusion from the carotid artery far more than the local metabolism [6]. Thus, the chemosensory component of the carotid body is in a close diffusion distance responding to any changes in arterial oxygen tension or pH, circulating hormones or signaling molecules, and also substances locally produced by vascular cells and tissues acting as autocrines or paracrines. For example, signaling molecules in the arterial blood and locally produced in the carotid body including vasoactive peptides endothelin-1, angiotensin II, and also proinflammatory cytokines have been shown to modulate the excitability of the carotid chemoreceptor [11, 12]. Also, the cytokine receptors are functionally expressed in the carotid body [13–15]. These findings provide initial evidence for a role of cytokine signaling in the carotid body, which raises a number of questions on the following: (i) the physiological significance of the expression of cytokines and cytokine receptors in the carotid body, (ii) the regulation of the cytokine signaling pathway in the carotid body under physiological or pathophysiological conditions, and (iii) alterations of the carotid chemoreceptor activity by cytokines and the pathophysiological significance of carotid body inflammation in disease conditions.

2. The Carotid Body Is a Peripheral Sensor of Inflammatory Cytokines

Both the central and peripheral nervous systems play roles in the communication between the brain and the immune system, which is important for the regulation of immune and brain responses to inflammation under physiological or disease conditions. The circumventricular organs are important structures where central neurons respond to cytokines in the blood, whereas the visceral activity of the vagus nerve is crucial to the afferent projection of immunogenic signals to the central [16]. In addition, recent studies suggest a role of the peripheral chemoreceptor in the carotid body in the immunosensing of inflammatory cytokines, by which the elicitation of the activity of the chemoreflex pathway could be an important part of the ventilatory response and altered cardiovascular and autonomic activities adjusting to the inflammatory status.

It is well known that the biosynthesis and release of inflammatory cytokines, including interleukin- (IL-) 1β and IL-6 and tumor necrosis factor- (TNF-) α in the immune cells play important roles in the peripheral mediation of the physiological and pathophysiological changes under inflammatory and/or infective conditions. In experimental animals, it has been reported that IL-1 type-1 receptors are expressed in glomus cells of vagal paraganglia [17]. Also, the IL-1 receptors are expressed in the type-I glomus cells of the carotid body, suggesting an immunosensory function of the carotid body [13, 15, 18]. Functionally, extracellular application of IL-1β significantly decreases the outward potassium current in cultured glomus cells of the carotid body [19]. In addition, topical application of IL-1β to the carotid body increases the activity of the carotid sinus nerve in rats [19]. Also, exogenous IL-1β induces an elevated level of intracellular calcium response to hypoxia in the glomus cell of the carotid body [15]. Besides the expression of IL-1 receptors, components of the IL-6 receptor, namely, IL-6 receptor alpha chain and gp130, are expressed in the type-I glomus cell and possibly in type-II and vascular cells of the rat carotid body (Table 1). These receptors are functional because exogenous application of IL-6 to the glomus cells increases the intracellular calcium response to hypoxia [15]. These observations support a role of the carotid body in sensing proinflammatory cytokines via the cytokine receptors.

In addition to proinflammatory cytokines IL-1β and IL-6, Fernández et al. [20] reported that infusion of lipopolysaccharide (LPS) increases respiratory rate in the cat, which is abolished by sectioning the carotid and aortic nerves. The carotid chemoreceptor activity is also increased by the intravenous LPS administration [20]. The carotid chemoreceptor response to LPS could be mediated by toll-like receptor 4, which is the LPS canonical receptor, expressed in the carotid body. Also, LPS induces the release of TNF-α from immune cells [21, 22]. It raises the possibility that the effect of LPS may be mediated by TNF-α. Indeed, the TNF receptors, namely, TNF-r1 and TNF-r2, are expressed, respectively, in the type-I glomus cell and endothelial cell of the cat carotid body [20]. In the rat, the TNF receptors are also expressed in the type-I glomus cell and possibly type-II and vascular cells of the carotid body (Table 1). Exogenous application of TNF-α to the rat glomus cell increases the intracellular calcium response to hypoxia [15]. However, TNF-α attenuates the carotid chemosensory response to hypoxia in the cat [20]. Nevertheless, these findings support a role of the glomus cells in sensing and transmitting immune signals by responding to the proinflammatory cytokines via their corresponding receptors.

In human, inflammation of the carotid body has been reported to be related to aging or autoimmunity disease. The histological feature of carotid glomitis is associated with follicles of lymphocytes and also infiltration of immune cells with morphological changes in the type-I and type-II cells in the carotid body [23]. More recently, cytokine receptors for IL-1β, IL-6, IL-10, and TNF-α as well as toll-like receptors are also found to be expressed in the human carotid body [24, 25]. These findings are consistent with the findings in

Table 1: A summary of main findings in literatures reporting the expression of cytokines and cytokine receptors in the carotid body of rodents, cats, and humans; also changes in the expression level under hypoxic conditions, namely, sustained hypoxia (SH) or intermittent hypoxia (IH) are shown.

	Rat	Mouse	Cat	Human	SH	IH
IL-1β	IHC, PCR [15, 18, 29, 30, 52, 62]			IA [24]	+ [15, 29]	+ [18, 30, 52, 62]
IL-1r1	IHC, PCR, WB [13, 15, 18]			MA, IHC [24, 25]	+ [15]	+ [18]
IL-4				IA [24]		
IL-6	IHC, PCR [15, 18, 29, 30]			MA, IA [24, 25]	+ [15, 29]	+ [18]
IL-6Rα	WB [14]	MA [25]		MA, IHC [24, 25]		
gp130	IHC, WB [14, 15, 18]			MA [25]		
IL-8				IA [24]		
IL-10				IA [24]		
IL-10R		MA [25]				
TNF-α	IHC, PCR, WB [15, 18, 28–30, 52, 62]		IHC, PCR [20]	MA, IHC [24, 25]	+ [15, 29]	+ [18, 30, 52, 62]
TNF-r1	IHC, PCR, WB [15, 18, 28]	MA [25]	IHC, PCR [20]	MA [25]	+ [15]	+ [18]
TNF-r2	IHC, PCR, WB [28]	MA [25]	IHC, PCR [20]	MA [25]		

Techniques involved in the study—IA, immunoassay; IHC, immunohistochemistry; MA, microarray; PCR, polymerase chain reaction; WB, western blot; +, increased expression.

experimental animals, suggesting an immunosensing function of the carotid body in its response to proinflammatory cytokines and also other immunogenic molecules via the corresponding receptors expressed in chemosensitive type-I glomus cells and also other cell types in the organ.

3. Regulation of the Expression of Proinflammatory Cytokines and Cytokine Receptors in the Carotid Body

The carotid body and its chemoafferent activity could play a role in the communication between the immune system and the brain, in particular, in the inflammatory and infective status [26]. It raises the possibility that the cytokine signaling pathway in the carotid body may also be regulated by the inflammatory cytokines and immunogenic molecules in the circulation. Indeed, it has been shown that intraperitoneal injection of IL-1β increases the expression of IL-1 type-I receptors in the carotid body of the rat [27]. In addition, infusion of LPS increases the expression of TNF-r2 receptor in the rat carotid body [28]. These observations suggest that the cytokine receptors in the carotid body are regulated by inflammatory cytokines, which could modulate the chemosensory activity and its response to proinflammatory cytokines or hypoxia under inflammatory or disease conditions.

Besides the circulating source of cytokines, a growing amount of evidence supports that there is a local expression of proinflammatory cytokines in the carotid body under physiological or disease conditions. Fernández et al. [20] reported the expression of TNF-α is particularly significant following intravenous injection of LPS to induce endotoxemia in the cat and the expression is colocalized to the type-I and endothelial cells in the carotid body. This study provides evidence suggesting that the TNF signaling pathway in the carotid body, via activation of the chemoreflex, could be functionally important in the ventilatory and cardiovascular response to sepsis [26]. In the rat, the expression of TNFα has also been shown in the carotid body under physiological and hypoxic conditions (Table 1). In addition, proinflammatory cytokines IL-1β and IL-6 are expressed in type-I glomus cells (Table 1) and also in type-II cells of the carotid body [29, 30]. Furthermore, cytokines IL-1β, IL-4, IL-6, IL-8, and IL-10 are reported to be released from carotid bodies in patients with head and neck neoplasms [24]. Importantly, the release of the cytokines is increased following a prolonged period of hypoxia for an hour [24]. These findings suggest

that cytokines locally produced in the carotid body are an active component of the cytokine signaling pathway which modulates the activity of chemoreceptors under physiological or hypoxic conditions.

4. Sustained Hypoxia Induces Local Inflammation in the Carotid Body

Inflammation plays an important role in physiological processes, for instance, wound healing, and also a common clinical condition manifested in a chronic manner in diseases. In addition to infection and inflammation, evidence suggests that the cytokine signaling pathway in the carotid body is regulated by hypoxic conditions. In the circulation, plasma levels of cytokines are elevated in altitude natives and subjects sojourning at high altitude under a sustained hypoxic condition [31–33]. Also, chronic inflammation is one of the important clinical manifestations in patients with diseases associated with chronic hypoxemia, including chronic obstructive pulmonary disease [34, 35]. In fact, hypoxia and inflammation are interrelated and the inflammatory response to hypoxia can be adaptive or pathogenic in nature [36]. It is well known that inflammatory cytokines induced the expression of nuclear factor kappa B- (NFκB-) dependent genes encoding inflammatory mediators including cyclooxygenases and inducible nitric oxide synthase (iNOS), which could in turn modulate the expression of cytokines and chemokines. In this regard, the expression of cytokines and cytokine receptors in the carotid body may play roles in the physiological acclimatization to altitude, particularly in the modulation of carotid chemoreceptor activity, and also in the pathogenic cascade in disease conditions associated with chronic hypoxemia and inflammation.

We and others have shown that hypoxia induces increased expressions of proinflammatory cytokines IL-1β, IL-6, and TNF-α and the corresponding IL-1β receptor (IL-1r1), IL-6 receptor (gp130), and TNF receptor (TNF-r1) in the carotid body of rats exposed to sustained hypoxia for days up to 4 weeks [15, 29]. The upregulated expression of proinflammatory cytokines and cytokine receptors is localized to the type-I glomus cell and is also expressed in the type-II cell and immune cells in the carotid body in sustained hypoxia [15, 29]. Importantly studies show that exogenous cytokines induce an enhanced intracellular calcium response to hypoxia in the glomus cell of rats exposed to sustained hypoxia, suggesting that the upregulated expression of proinflammatory cytokines and cytokine receptors plays a functional role in the chemosensory function [15]. Also, the enhanced carotid chemoreceptor activity induced by sustained hypoxia is significantly blocked by a concurrent treatment of the animal with an anti-inflammatory drug ibuprofen or dexamethasone [29]. Furthermore, ibuprofen prevents the increase in the ventilatory response to hypoxia in rats exposed to sustained hypoxia [37]. These findings strongly supported the notion that the activity of chemosensitive glomus cells is enhanced by the locally produced proinflammatory cytokines, mediated by the cytokine receptors in a paracrine-autocrine signaling manner. In effect, the cytokine signaling pathway plays a more prominent role in the enhanced chemosensory activity under hypoxic conditions with an upregulation of the local expression of proinflammatory cytokines and cytokine receptors.

Evidence supports that activation of the cytokine pathway could lead to inflammation of the carotid body associated with an increased expression of inflammatory mediators and an enhanced carotid chemoreceptor activity under sustained hypoxic conditions. Indeed, the expression level of the inflammatory mediator iNOS is significantly increased in the carotid body of rats exposed to sustained hypoxia [15], which could be functionally significant in being involved in the endogenous production of NO in the carotid body under hypoxic conditions [38]. Activation of NF-κB pathway may be involved in the mechanistic aspects of the cytokine-induced iNOS expression and the hypoxia-induced inflammatory response of the carotid body [15, 29]. Furthermore, sustained hypoxia induces the expression of chemokines (monocyte chemoattractant protein- (MCP-) 1, chemokine receptor- (CCR-) 2, macrophage inflammatory protein- (MIP-) 1α, and MIP-1β) and adhesive molecule (intercellular adhesion molecule- (ICAM-) 1) as well as infiltration of immune cells in the carotid body of the rat [15, 29]. Anti-inflammatory drug ibuprofen or dexamethasone attenuates the infiltration of macrophages and the expression of cytokines and chemokines in the carotid body [29]. These findings give support to the notion that the upregulated expression of the cytokines and cytokine receptors significantly contributes to the local inflammation of the carotid body in sustained hypoxia.

Besides the upregulation of cytokines and cytokine receptors, the inflammatory response of the carotid body to sustained hypoxia could be mediated by endothelin-1. Liu et al. [39] reported that pharmacological blockade of the endothelin receptors with bosentan neutralizes the elevated expression of proinflammatory cytokines, chemokines, and macrophage infiltration in the carotid body. The expression of endothelin-1 and endothelin receptors in the carotid body is significantly upregulated in the sustained hypoxia [40, 41]. Thus, as aforementioned, inflammation of the carotid body is likely to be mediated by locally regulated paracrine-autocrine signals during sustained hypoxia. In addition, it is known that plasma levels of cytokines are elevated under hypoxic conditions. The circulating level of cytokines could also be an important stimulus to induce the upregulation of the cytokine signaling pathway in the carotid body, leading to the local inflammation.

Moreover, inflammation of the carotid body is associated with an increased expression of acid-sensitive iron channels (ASIC) in the chemoafferent neurons under sustained hypoxia, hinting an altered chemoreceptor function under a hypoxia-induced inflammatory condition resembling a condition of hyperalgesia/hyperexcitability induced by chronic pain/inflammation [42, 43]. Liu and colleagues [42] reported that inflammatory cytokines significantly increase ASIC expression in cultured petrosal ganglionic neurons, which are the chemoafferent neurons of the carotid body. Also the elevated ASIC expression in the animal exposed to sustained hypoxia is blocked by concurrent treatment of a nonsteroidal

anti-inflammatory drug ibuprofen. Importantly, the hypoxia-induced enhanced carotid chemoreceptor activity recorded from the carotid sinus nerve is significantly attenuated by an ASIC antagonist A-317567 and also by ibuprofen. These findings underscore an important role of ASIC in the carotid body inflammation, which leads to an enhanced carotid chemoreceptor activity under sustained hypoxic conditions. Thus the inflammatory response of the carotid body to sustained hypoxia may be an adaptive response of the carotid chemoreceptor to facilitate the ventilatory acclimatization to hypoxia at high altitude and also to adjust the ventilatory and autonomic activities in response to hypoxia under disease conditions.

In summary, evidence suggests that the increased expressions of cytokines and cytokine receptors play a role in the local inflammation of the carotid body induced by sustained hypoxia. In effect, activation of the cytokine signaling pathway increases the expression of inflammatory mediators and chemokines, which mediates the local inflammatory response of the carotid body. Thus, this upregulation could increase the locally produced cytokines in addition to the rise of circulating cytokines under hypoxic conditions, leading to activation of inflammatory signaling pathways. The chemosensitive type-I glomus cell plays an important role in this local inflammation because of the expression of the cytokines and the cytokine receptors. The elevation of cytokine levels could alter the response of the glomus cells to hypoxia, which may be one of the cellular mechanisms underlying the altered functions of the carotid body relevant to the cardiopulmonary control under sustained hypoxic conditions during physiological acclimatization to altitude and also in diseases associated with chronic hypoxemia.

5. Pathogenic Role of the Chemoreflex in Sleep Apnea

Recurrent apnea in patients suffering from sleep apnea leads to intermittent hypoxia, which adversely impact the neurocognitive and cardiovascular functions and increased risks for stroke and cardiovascular disease [1, 2]. The physiological compensatory response to arterial oxygen desaturation in sleep apnea is significantly mediated by the chemoreflex which triggers hyperventilation, parasympathoexcitation contributing to bradycardia, and increased sympathetic activities for the redistribution of blood flow in tissues and organ [7]. However, repeated episodes of hypoxia induce augmented activities of the carotid chemosensory activity and ventilatory hypoxic responses [44, 45]. Mounting evidence supports that the augmented activity of the carotid body induced by intermittent hypoxia plays an important role in the pathogenesis of sleep apnea [46, 47]. It has been proposed that augmented activities of the chemoreflex significantly contribute to a lowered activity of the central respiratory drive leading to breathing instability in sleep apnea and also in the pathophysiological impact of sleep apnea mediated by increased sympathetic activities leading to hypertension [3]. Indeed, denervation of the carotid body could normalize the elevated blood pressure induced by intermittent hypoxia in animals [48, 49]. Altered chemosensitivity of the chemoreflex has also been reported in patients with sleep apnea, which significantly contributes to the overactivity of the sympathetic activity mediated by the chemoreflex [50]. Recently, Marcus and colleagues [51] reported that denervation of the carotid body significantly attenuates disordered breathing patterns and renal sympathetic activities in rabbits with cardiac pacing simulating congestive heart failure. The mechanistic cause of the augmented carotid chemoafferent activity is believed to be multifactorial and it could be attributed to the oxidative stress and inflammation induced by intermittent hypoxia [47, 52, 53]. In addition, maladaptive changes in the paracrine-autocrine signaling in the carotid body have been proposed to be responsible for the pathogenic response to intermittent hypoxia [12].

Intermittent hypoxia induces elevated levels of reactive oxygen species (ROS) and nitrogen reactive species leading to oxidative stress, which causes cellular and tissue injuries in organs. The oxidative stress induced by intermittent hypoxia has been proposed as a significant factor contributing to the pathogenesis of sleep apnea [54, 55]. Indeed, superoxide dismutase which is mimetic attenuates the augmented carotid chemoreceptor activity induced by intermittent hypoxia, suggesting an involvement of ROS [44]. It has been proposed that proinflammatory cytokines play a role in the increase in cardiovascular morbidities as a pathophysiological consequence of sleep apnea in patients [56]. Supporting this idea, studies have demonstrated elevated levels of circulating proinflammatory cytokines including IL-6, TNF-α, and chemokines including MCP-1 in patients with obstructive sleep apnea [57–59]. In addition, it has been shown that proinflammatory cytokines and hypoxia increase the ROS production, involving the mitochondria and NADPH oxidase [60, 61]. As aforementioned, the elevated proinflammatory cytokines in the arterial blood increase the local expression of proinflammatory cytokines and cytokine receptors in the carotid body [15, 29]. Also, proinflammatory cytokines significantly enhance the intracellular calcium response to hypoxia in the chemosensitive glomus cells [15] and the carotid chemoafferent activity [19, 29]. It has been proposed that expressions of proinflammatory cytokines and cytokine receptors in the carotid body play pathogenic roles in the local inflammation and augmented activities of the carotid body under chronic intermittent hypoxic condition which is a hallmark feature of sleep apnea.

6. Intermittent Hypoxia Augments Proinflammatory Cytokine Signaling and Local Inflammation in the Carotid Body

Intermittent hypoxia induces a significant increase in the mRNA and protein expression of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) and cytokine receptors (IL-1r1, gp130, and TNF-r1) in the rat carotid body [18, 30, 52, 62]. The increased expression of proinflammatory cytokines and receptors is localized to lobules of chemosensitive type-I glomus cells and the proportional amount of the cells expressing cytokines and cytokine receptors is significantly

increased by a couple of days of intermittent hypoxia and it remains at an elevated level in hypoxia up to a week [18, 30, 52, 62]. Functional studies show that the effect of exogenous IL-1β, IL-6, and TNFα at 0.01–1 nM concentration dependently increased the intracellular calcium response to hypoxia by 10–40% in the glomus cell. The calcium response is significantly augmented in cells obtained from rats exposed to intermittent hypoxia [18]. These findings suggest that the increase in local expression of proinflammatory cytokines could increase the local release of cytokines under hypoxic conditions. Also the upregulated expression of the cytokine receptors augments the sensitivity of glomus cells to cytokines under hypoxic conditions. Thus these local changes in the cytokine signaling pathway could play a pathogenic role in the local inflammation and augmented activities of the carotid body induced by intermittent hypoxia.

7. Oxidative Stress and Inflammation in the Carotid Body in Intermittent Hypoxia

Reports have shown that intermittent hypoxia induces oxidative stress and inflammation in the carotid body. Hence, there are significant elevated levels of markers of oxidative stress including 8-isoprostane, malondialdehyde, and nitrotyrosine in the serum and the carotid body of rats exposed to intermittent hypoxia equivalent to a severe condition of sleep apnea for days to weeks [52, 63]. In addition, there are significant increases in the macrophage infiltration and the expression of chemokines (MCP-1, CCR2, MIP-1α, MIP-1β, and ICAM-1) in the carotid body [18, 52]. As mentioned, oxidative stress could lead to cellular injuries and inflammation, which could in turn induce the inflammatory response that could be an important contributing factor in the altered function of the carotid body in intermittent hypoxia. Indeed, the transcriptional upregulation of the chemokines could increase the invasion of immune cells in the carotid body participating in the inflammatory process. Thus, the augmented expression of chemokines induced by intermittent hypoxia plays a role in the inflammation of carotid body, which could also be mediated by oxidative stress induced by intermittent hypoxia.

Mechanistically, NADPH oxidase-dependent ROS generation has been shown to induce oxidative stress in local tissues and increase expressions of proinflammatory cytokines and chemokines via activation of NFκB pathway [64, 65]. Indeed, gene transcripts of NADPH oxidase subunits ($gp91^{phox}$ and $p22^{phox}$) are significantly increased in the carotid body in intermittent hypoxia [18, 63]. Importantly, treatment of anti-inflammatory drugs dexamethasone or ibuprofen significantly attenuates levels of oxidative stress and $gp91^{phox}$ as well as macrophage infiltration in the carotid body of the rat in intermittent hypoxia [18]. Thus, the upregulated proinflammatory cytokine pathway could be initially activated by tissue hypoxia [18, 29]. In turn, the inflammatory response recruits infiltrated macrophages which also contribute to the inflammatory cascade in the carotid body. More importantly, increased levels in the ROS and oxidative stress induced by intermittent hypoxia regulate the gene expression of inflammatory cytokines, chemokines, and adhesion molecules [66]. As a result, these significantly contribute to the upregulated cytokine pathways and local inflammation induced by intermittent hypoxia in the carotid body. Moreover, proinflammatory cytokines, including TNFα and IL-1β, and HIF-1α also cause the activation of NFκb [67, 68]. The interplay of these molecules and signaling pathways could lead to a positive regulatory loop that may further enhance and exaggerate the local inflammatory response in the carotid body. In fact, administration of anti-inflammatory drug dexamethasone or ibuprofen could attenuate the levels of macrophage infiltration and oxidative stress in the carotid body of rats exposed to intermittent hypoxia [18]. Also, ibuprofen prevents the elevated ventilatory response to hypoxia and arterial blood pressure in intermittent hypoxia, although it fails to normalize the augmented carotid chemosensory response to hypoxia [52, 62]. Thus, the activation of proinflammatory cytokine pathway could play mechanistic roles in mediating the local inflammation and in contributing to the altered function of the carotid body in intermittent hypoxia, leading to the pathophysiology and pathophysiological consequences of sleep apnea [12].

Besides, it has recently been shown that blockade of angiotensin II AT_1 receptors with losartan significantly attenuates the macrophage infiltration, oxidative stress, and inflammation in the carotid body in intermittent hypoxia [69]. There is an upregulation of the expression of a local renin angiotensin system in the carotid body in intermittent hypoxia, which significantly contributes to inflammation and oxidative stress [69]. Marcus et al. [63] also reported that losartan attenuates the expression of $gp91^{phox}$ and superoxide production in the rat carotid body in intermittent hypoxia. Also, activation of $gp91^{phox}$ mediates ROS production in the carotid body via AT_1 receptor [70, 71]. These findings support a pathogenic role of NADPH oxidase regulated by AT_1 receptors in the local inflammation of the carotid body. In effect, the upregulation of AT_1 receptors in the carotid body could increase the NADPH oxidase-mediated ROS production, which exaggerates the inflammation and oxidative stress induced by intermittent hypoxia.

8. Perspectives of the Inflammation of the Carotid Body-Clinical Implications in Sleep Apnea

In premature infants, altered functions of the carotid body have been known to be a key drive of the unstable breathing leading to apnea of prematurity. The clinical conditions are commonly associated with inflammation and infection. It has been proposed that the inflammation of carotid body could play a role in the changes in the chemosensitivity of the carotid body, which adversely affects the stability of breathing in the infant [72]. Thus, targeting the inflammation and the altered carotid body function may hint to a novel therapeutic strategy for treating the sleep apnea and its pathophysiological consequence. As shown in experimental studies, anti-inflammatory drug prevents the inflammatory response of the carotid body to intermittent hypoxia [18] and also the elevated ventilatory response to hypoxia and

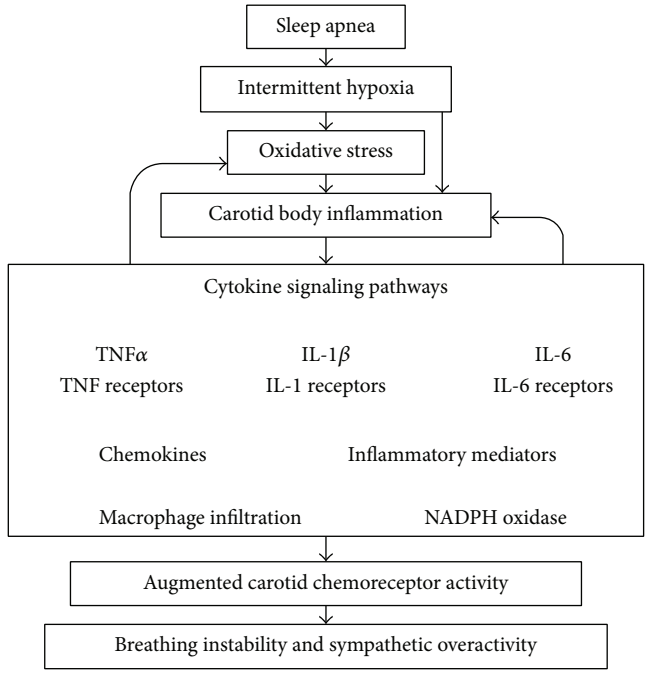

Figure 1: Schematic diagram shows the role of inflammation in the carotid body in the pathogenic cascade of sleep apnea. Arrows are the direction of the pathogenic effects of oxidative stress and inflammation in the carotid body leading to augmented activities of the carotid chemoreceptor and chemoreflex activity, significantly contributing to the pathophysiological consequence impacting on breathing instability and autonomic imbalance.

arterial blood pressure in intermittent hypoxia, despite the fact that the drug effect is less significant on the augmented chemoreceptor activity [52, 62]. Nevertheless, these findings suggest that local inflammation of the carotid body plays a role in the altered chemoreflex induced by intermittent hypoxia, although the inflammatory response may not be the sole mechanism responsible for the augmented activity of the chemoreceptor. Given that there are significant elevated levels of the expression of proinflammatory cytokines TNF-α and IL-6 and the expressions of endothelin-1 and angiotensin II in the carotid body [18, 30, 52, 62, 69, 73], the augmented carotid chemoreceptor response to hypoxia could be mediated by the paracrine-autocrine signaling molecules involving upregulated cytokine pathways, which contribute to the modulation of the chemoreceptor activity in intermittent hypoxia [12].

In patients, anti-inflammatory medications could be useful to alleviate the inflammation of the upper airway and also to reduce the adenoidal size in children with obstructive sleep apnea [74]. In clinical studies with small groups of adult patients, administration of etanercept, a TNF-α antagonist, significantly decreases the apnea-hypopnea index (AHI) and IL-6 levels in the patient [75]. Also sleep apnea is less common in patients with spondyloarthritis who received TNF-inhibitor therapy [76]. In contrast, the AHI is worsened after steroid treatment in the patients for 3 months [77]. Also oral indomethacin administration increases AHI by about 2-fold in healthy patients with mild obstructive sleep apnea, which could be related to reductions in cerebral blood flow and its response to CO_2 [78]. Thus, clinical studies are limited on the efficacy and effectiveness of the anti-inflammatory drugs in the patient with sleep apnea. The effect of the medication is highly dependent on the complex nature of the etiology and pathophysiological cause of sleep apnea in patients. As compared to the findings in experimental animals, the inflammatory response is more related to the impact of intermittent hypoxia equivalent to a severe level of AHI. Future clinical studies in large patient group with stratification of the inflammatory status and chemosensory function may be useful to address the therapeutic potential of targeting inflammation in the carotid body in sleep apnea.

9. Summary and Conclusion

The expression of proinflammatory cytokines and inflammatory mediators in the carotid body plays a pathogenic role in sleep apnea. As shown in Figure 1, the cytokine signaling pathway is part of the mechanistic cascade involved in the augmented carotid chemoreceptor activity because of the local upregulation of the proinflammatory cytokines, cytokine receptors, and inflammatory mediators in the carotid body. This in effect could significantly contribute to the increased chemosensory activity and the afferent activity of the chemoreflex, causing breathing instability in sleep apnea. In addition, the increased chemoafferent activity could lead to an increase in the sympathetic activity via the chemoreflex. This in turn could significantly contribute to the pathophysiological consequence of the sleep apnea, namely, endothelial dysfunction leading to arterial hypertension and increased risks for cerebrovascular and cardiovascular disease. In experimental animals, studies have shown

that anti-inflammatory drugs could significantly attenuate the inflammation of the carotid body and the augmented carotid chemoreceptor activity induced by chronic intermittent hypoxia. In parallel to the conventional treatment of patients with continuous positive airway pressure for sleep apnea, pharmacological intervention has been proposed to alleviate the pathophysiological impact of sleep apnea aiming to reduce the cardiovascular morbidities and mortalities in the patients with sleep apnea. To this end, in addition to the antihypertensive drugs, anti-inflammatory drugs may also be implicative according to the recent advance in the experimental study highlighting a pathogenic role of the local inflammation of the carotid body. Also it is well known that oxidative stress induced by intermittent hypoxia exerts a systemic impact on multiple physiological systems and organs, leading to significant levels of systemic and chronic inflammation in patients with sleep apnea. Thus, it is proposed that the anti-inflammatory drugs could have significant effect not only on targeting the locally upregulated cytokine pathways in the carotid body, but also on the systemic inflammation in other systems and organs as well. Future studies in this direction warrant further investigations and clinical trials have yet to be conducted to confirm the effectiveness of the treatment with anti-inflammatory drugs in patients with sleep apnea.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Research Article

Response to Statin Therapy in Obstructive Sleep Apnea Syndrome: A Multicenter Randomized Controlled Trial

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Rationale. Accumulated evidence implicates sympathetic activation as inducing oxidative stress and systemic inflammation, which in turn lead to hypertension, endothelial dysfunction, and atherosclerosis in obstructive sleep apnea (OSA). Statins through their pleiotropic properties may modify inflammation, lipid profile, and cardiovascular outcomes in OSA. *Methods.* This multicenter, randomized, double-blind study compared the effects of atorvastatin 40 mg/day versus placebo over 12 weeks on endothelial function (the primary endpoint) measured by peripheral arterial tone (PAT). Secondary endpoints included office blood pressure (BP), early carotid atherosclerosis, arterial stiffness measured by pulse wave velocity (PWV), and metabolic parameters. *Results.* 51 severe OSA patients were randomized. Key demographics for the study population were age 54 ± 11 years, 21.6% female, and BMI 28.5 ± 4.5 kg/m^2. In intention to treat analysis, mean PAT difference between atorvastatin and placebo groups was 0.008 (−0.29; 0.28), $P = 0.979$. Total and LDL cholesterol significantly improved with atorvastatin. Systolic BP significantly decreased with atorvastatin (mean difference: −6.34 mmHg (−12.68; −0.01), $P = 0.050$) whereas carotid atherosclerosis and PWV were unchanged compared to the placebo group. *Conclusion.* In OSA patients, 3 months of atorvastatin neither improved endothelial function nor reduced early signs of atherosclerosis although it lowered blood pressure and improved lipid profile. This trial is registered with NCT00669695.

1. Introduction

Obstructive sleep apnea (OSA) syndrome represents a serious health hazard and is recognized as an independent risk factor for adverse cardiovascular outcomes such as hypertension, arrhythmias, stroke, and coronary heart disease [1]. Sympathetic activation, oxidative stress, and systemic inflammation have been shown to be the main intermediary mechanisms linking intermittent hypoxia (IH), the marker of OSA, with deleterious cardiovascular and metabolic consequences leading to enhanced cardiovascular morbidity and mortality [2, 3]. Prior to the occurrence of cardiovascular

events, sleep apnea is associated with several subclinical cardiovascular alterations including nocturnal hypertension and early atherosclerosis that are related to both vasculature remodeling (such as increased intima-media thickness [4], arterial plaque formation, and arterial stiffness [5–8]) and endothelial dysfunction [9]. OSA patients exhibit altered endothelial function with desensitization of the alpha and beta 2-adrenergic receptors, altered NO-dependent vasodilatation, and hypersensitivity to vasoconstriction (induced by angiotensin II (Ang II)) [10–12]. Alterations in endothelial function precede the development of morphological atherosclerotic changes and subsequent clinical complications [13]. Digital pulse amplitude augmentation in response to hyperemia (EndoPAT) is one of the validated methods for measuring endothelial function. EndoPAT has the advantage of being easy to perform compared to other endothelial function assessment techniques [14]. Measurements are automated with low interobserver and intraobserver variability. A main advantage of the system is that the contralateral arm serves as an internal control that can be used to correct for any systemic drift in vascular tone during the test. There is demonstration that peripheral arterial tone (PAT) values allow quantifying cardiovascular risk [15] and predicting late adverse cardiovascular events [16].

In OSA patients, reversing early disorders in the cardiovascular system before the occurrence of major clinical events, such as myocardial infarction or stroke, may be a means of reducing cardiovascular risk.

Continuous positive airway pressure (CPAP) the first line therapy for OSA has been suggested in small size randomized controlled trials (RCTs) as being able to reverse some of these subclinical alterations [17] as well as endothelial dysfunction [18]. However, CPAP acceptance is poor in some subgroups of OSA patients [19] and recent large RCTs demonstrate that CPAP alone is not enough to reduce cardiometabolic risk in OSA patients [20]. Thus a crucial issue is to develop alternative or combined treatments to address early, or delay, deleterious OSA-related cardiovascular consequences.

Statins were initially introduced for the prevention of cardiovascular risk because of their lowering lipid effects. During the last decade, numerous in vivo and in vitro studies have described pleiotropic effects of statins, independent of their lipid-lowering properties. Some of the reported pleiotropic effects of statins may impact intermediate mechanisms underlying cardiovascular risk in OSA patients. Simvastatin treatment is able to reduce sympathetic tone and normalize autonomic function in chronic heart failure (CHF) rabbits by inhibiting central Ang II mechanisms and therefore the superoxide pathway [21]. Statins are also able to reduce IH-induced hypertension to improve carotid compliance and to reduce cardiac infarction hypersensitivity on IH exposed rats [22]. These beneficial vascular effects have also been reported in normolipidemic patients with isolated systolic hypertension where statins reduce large artery stiffness and blood pressure [23]. Statins are also known to stabilize atherosclerosis plaques, induce inhibition of vascular smooth muscle cell proliferation as well as platelet aggregation, and reduce vascular inflammation [24–26].

Statins through their pleiotropic properties that impact intermediary mechanisms might modify cardiovascular outcomes in OSA. The aim of this study was thus to determine the effect of 3 months of atorvastatin treatment on endothelial function, blood pressure, and early signs of atherosclerosis in OSA patients, through a randomized double-blind placebo-controlled trial.

2. Material and Methods

The study was conducted in accordance with applicable good clinical practice requirements in Europe, French law, ICH E6 recommendations, and ethical principles of the Helsinki Declaration (South Africa 1996 and Edinburgh 2000). The study was approved by an independent ethics committee (Comité de Protection des Personnes, Grenoble, France, IRB0005578) and registered on the ClinicalTrials.gov site (NCT00669695). Written informed consent was obtained from all included patients. An external data quality control was performed systematically for some criteria (such as informed consent, complications, and adverse events) and by a random selection of 10% of the case report forms for other criteria.

2.1. Patients. This multicenter, randomized, double-blind, parallel group study compared atorvastatin 40 mg/day versus placebo over 12 weeks. The primary endpoint was change in endothelial function from baseline to 12 weeks, measured by PAT. Other endpoints included office blood pressure (BP), early carotid atherosclerosis (intima-media thickness (IMT) and carotid diameters), arterial stiffness measured by pulse wave velocity (PWV), and metabolic and inflammatory parameters.

Patients were recruited from sleep laboratories of 3 university hospitals (Grenoble and Angers, France and Geneva, Switzerland). Only subjects diagnosed with OSA (apnea-hypopnea index (AHI) > 30/h) aged over 18 years and who gave written informed consent were eligible. The study was conducted following the CONSORT recommendations [27].

Patients presenting any of the following criteria were not included: history of stroke, coronary heart disease, chronic respiratory failure, hypothyroidism, already on statin treatment, multiple antihypertensive medications, pregnant or lactating women, alcohol consumption > 3 units/day, treatment by itraconazole, ketoconazole, protease inhibitor, fibrates, antivitamin K, diltiazem, verapamil, erythromycin, clarithromycin, or cyclosporine.

The determination of the sample size was based on published data on the beneficial effect of an oral appliance using the same endpoint [28], showing that PAT improved from 1.77 ± 0.4 at baseline to 2.0 ± 0.4 after treatment. We hypothesized that the beneficial effect of statin treatment would be similar. A change of approximately 0.23 in this variable was anticipated after 3 months of atorvastatin treatment. No study to date has demonstrated the range of improvement in PAT values after an intervention that is predictive of reduced morbidity or mortality. The sample size calculated to obtain significant differences with 80% statistical power and an alpha error of 0.05 showed that 57 patients per group were

necessary. Thus, 60 patients per group were initially planned. An interim analysis initially planned was scheduled after the inclusion of 25 patients per group.

2.2. Study Design and Treatment

2.2.1. Baseline Visit. Patients underwent an overnight polysomnography. After waking up and while still in a fasting state, a peripheral blood sample was drawn. Then, endothelial function was assessed by PAT and arterial stiffness measured by PWV. A carotid ultrasonography was performed to assess IMT and carotid diameters. The Epworth sleepiness scale was completed and arterial blood gases analysis was performed in order to exclude obesity hypoventilation syndrome. Patients were then randomized to receive statin or placebo treatment. The randomization was made by an independent statistician. Investigators, patients, and the study team were blinded to treatment allocation. Patients randomly allocated to the statin group received 40 mg/day atorvastatin (Tahor, Pfizer Laboratories, France) during 12 weeks. Patients randomly allocated to the control group received placebo (lactose, LC2 Laboratories, France) similarly administered. In order to maintain the double blind status, atorvastatin tablets were encapsulated in capsules identical to the lactose placebo capsules (by LC2 Laboratories, France).

2.2.2. Three-Month Visit. Twelve weeks after the baseline visit, the same parameters were measured to compare the effect of the statin treatment with placebo.

2.3. Study Outcomes. The primary endpoint was the effect of atorvastatin treatment on endothelial function between baseline and 12 weeks, as measured by PAT. The secondary objectives of the study were to determine the effect of atorvastatin treatment on BP, IMT and carotid diameters, arterial stiffness (PWV evaluation), and metabolic parameters. The primary analysis was in intention to treat.

2.4. Study Procedures

2.4.1. Polysomnography (PSG). Overnight sleep studies were scored manually according to standard criteria [29] and an AHI was calculated from the number of apneas and hypopneas per hour according to international guidelines [30].

2.4.2. Blood Pressure Measurements. Clinical BP was measured using a mercury sphygmomanometer on three occasions, in line with European Society of Hypertension-European Society of Cardiology guidelines [31]. Systolic BP (SBP) and diastolic BP (DBP) were measured. Mean arterial BP (MABP) was calculated as DBP + 1/3(SBP-DBP).

2.4.3. Endothelial Function. After BP measurements, endothelial function was assessed by reactive hyperaemia using the finger plethysmographic methodology (PAT) with the EndoPAT device (Itamar Medical Ltd, Caesarea, Israel) as previously described [32]. PAT index was calculated as the natural logarithm of the average amplitude of PAT signal 90 to 120 seconds after deflation divided by average amplitude of the PAT signal during the 210 seconds prior to cuff inflation [15].

Carotid-to-femoral pulse wave velocity (PWV) was used for the arterial stiffness evaluation. To determine the carotid-to-femoral PWV, two pulse transducers were fixed on the skin over the right common carotid and femoral arteries. The time delay was measured with a Complior device, between the troughs of simultaneously recorded pulse waves and averaged over 10 consecutive cycles. The carotid-femoral PWV was calculated as the distance between the arterial sites divided by the time delay. Increased PWV was defined as PWV > 12 m/s [33].

2.4.4. Carotid Ultrasonography. B-mode ultrasonography was performed using an HP Sonos 2500 (Hewlett Packard) machine with a sectorial 7.5 MHz probe. The method used to determine the mean common carotid IMT and luminal diameter has been previously described [4]. Both common carotid arteries were studied consecutively in the long axis with a probe incidence allowing good quality images. The IMT was defined as the distance separating the most internal parts of these lines and the luminal diameter by the distance between the blood-intima interfaces on the anterior and posterior walls. The images were recorded in end-diastole and then analyzed by specific validated software (TIMC Laboratory, CHU Grenoble, France). IMT and diameter measurements were carried out on areas free of plaques and then averaged. The IMT and luminal diameter values were the mean values for the two common carotid arteries. Carotid ultrasonography was performed by two operators who were blinded to the other study data. The analysis of carotid parameters using the specific software was performed by the same operator throughout the entire study.

2.4.5. Metabolic Parameters. After peripheral blood sampling, plasma glucose and serum triglyceride levels were measured automatically (Modular 700, Roche, Meylan, France). Serum insulin was measured using a radio-immunometric sandwich assay (CIS bio international, Gif-Sur-Yvette, France).

2.4.6. Inflammatory Markers. The high-sensitivity C-reactive protein (hs-CRP) level was measured using automated immunonephelometry (Behring Nephelometer II Analyzer, Dade Behring, Germany). Urinary leukotriene E4 (LTE4, a validated marker of proinflammatory cysteinyl leukotriene production) and 11-dehydro-thromboxane B2 (11-DHTXB2) were quantified using liquid chromatography-tandem mass spectrometry [34].

2.5. Statistical Analysis. Statistical analysis was performed by using NCSS 97 software (Kaysville, Utah, USA). The data were analyzed in intention to treat, which includes all patients who signed the informed consent form. Missing baseline data were replaced by the median of each group and missing data at 12 weeks were replaced by the median of the opposite group (maximum bias method). Baseline data were compared by a Student or a Mann-Whitney test for

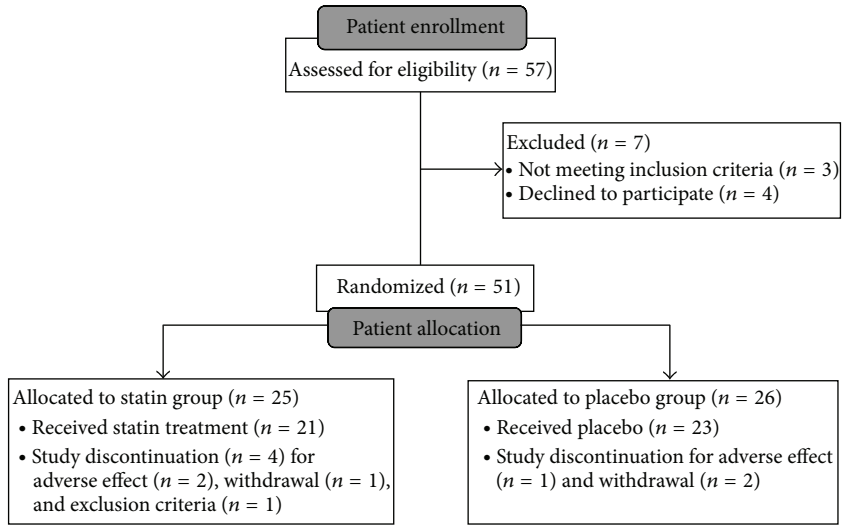

Figure 1

continuous data (depending on the validity of the normality of distributions) and by a Chi-Square test for categorical data. For the analysis of data evolution between baseline and 12 weeks, a repeated measure two-way analysis of variance (ANOVA) was performed, followed by a Bonferroni post hoc test when necessary. When normality was not respected, a transformation of variables was used. All P values were two-tailed and a P value <0.05 was considered significant. Study design and data are reported here in accordance with the CONSORT criteria [27].

3. Results

3.1. Patient Characteristics. Fifty-one patients were included and randomized ($n = 25$ in the statin group, $n = 26$ in the placebo group) between 16 May 2008 and 1 June 2012 (36 patients in Grenoble between 16 May 2008 and 20 January 2012, 11 patients in Angers between 13 May 2011 and 1 June 2012, and 4 patients in Geneva between 16 February 2009 and 23 September 2010). Three patients (2 from the statin group and 1 from the placebo group) presented adverse effects such as myalgia or digestive disorders. Three patients withdrew: 1 from the statin group and 2 from the placebo group (see flowchart presented in Figure 1).

Key demographics for the study population included age 54 ± 11 years, 21.6% female, BMI 28.5 ± 4.5 kg/m^2. There were no significant difference regarding baseline demographic data between the groups and no differences in baseline BP and sleep apnea characteristics. None of the patients was hypercapnic. Baseline HDL cholesterol levels were significantly higher in the statin group (Table 1).

After 12 weeks of treatment, adherence was not significantly different between the two groups (94.7 ± 0.07% in the statin group versus 96.6 ± 0.05% in the placebo group).

3.2. Primary Outcome Analysis: Endothelial Dysfunction. After 12 weeks, there was no improvement in endothelial function when the statin intervention group was compared with the placebo group. The mean difference in PAT measurements between the groups was 0.008 (−0.29; 0.28), $P = 0.979$ (Table 2). This intermediary analysis, initially planned in the study protocol, revealed no positive effect of treatment on the primary endpoint. Moreover, as a simulated calculation with 3 times the number of included patients also showed no positive effect of treatment, the study was then stopped based on futility for primary outcome PAT measured endothelial function.

3.3. Secondary Outcome Analyses

3.3.1. Change in Clinical BP. SBP significantly decreased after 12 weeks of atorvastatin treatment with a mean difference between groups of −6.34 mmHg (−12.68; −0.01), $P = 0.050$ (Table 3 and Figure 2).

3.3.2. Effect on Arterial Stiffness. After 12 weeks, there was no effect of statin treatment in reducing arterial stiffness compared with the placebo group. The mean difference in PWV measurements between the groups was 0.54 m/s (−0.45; 1.52), $P = 0.189$ (Table 2).

3.3.3. Effect on Carotid IMT and Diameters. After 12 weeks of treatment both carotid IMT and left and right luminal carotid diameters remained unchanged in both groups (Table 2).

3.3.4. Changes in Biological Parameters. Total and LDL cholesterol levels significantly improved after 12 weeks of atorvastatin treatment ($P < 0.0001$), whereas HDL cholesterol was unchanged compared to the placebo group. Moreover, in both groups, glycemia, insulinemia, the HOMA index reflecting insulin resistance, and the glycated hemoglobin A1c level did not change (Table 4).

Changes in Inflammatory Markers. In both groups hs-CRP, LTE4, and 11-DHTXB2 were unchanged after 12 weeks of treatment (Table 5).

Table 1: Baseline characteristics of placebo and statin patients.

	Placebo	Statin	*P* value
n	26	25	
Age (years)	56 ± 9	51 ± 12	NS
Male gender (%)	73	84	NS
BMI (kg/m^2)	28.70 ± 3.94	28.28 ± 5.12	NS
History			
Smoking (%)	50.0	60.0	NS
Alcohol (%)	38.5	56.0	NS
Diabetes (%)	0.0	4.0	NS
Dyslipidemia (%)	3.9	12.0	NS
Respiratory characteristics			
AHI (n/h)	45.47 ± 13.10	43.26 ± 19.19	NS
Mean SaO_2 (%)	92.57 ± 1.44	92.70 ± 2.40	NS
SaO_2 < 90% (%)	10.33 ± 13.20	11.66 ± 18.11	NS
Clinical BP			
SBP (mmHg)	127.67 ± 15.27	127.78 ± 15.07	NS
DBP (mmHg)	79.02 ± 11.61	79.16 ± 11.60	NS
MAP (mmHg)	95.22 ± 11.93	95.37 ± 12.21	NS
HR (bpm)	63 ± 8	64 ± 10	NS
Biological parameters			
Total cholesterol (g/L)	2.25 ± 0.64	2.46 ± 0.94	NS
LDL cholesterol (g/L)	1.41 ± 0.45	1.60 ± 0.67	NS
HDL cholesterol (g/L)	0.54 ± 0.23	0.74 ± 0.52*	**0.011**
Triglycerides (g/L)	1.55 ± 1.10	1.16 ± 0.59	NS

Data are mean ± SD or percentage. BMI, body mass index; SaO_2, oxygen saturation; SaO_2 < 90%, percentage of recording time spent at a SaO_2 < 90%; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HR, heart rate. * P < 0.05 by Student or Mann-Whitney test. For qualitative data, a Chi-2 test or a Fisher test was performed. NS, not significant.

4. Discussion

This multicenter, randomized, double-blind, and parallel group study in OSA patients was the first to investigate the effect of statin treatment on OSA-related cardiovascular outcomes.

OSA impairs macro- and microvascular endothelial function compared to healthy controls. PAT reflects changes in digital microvessel dilatation which is only partly dependent on nitric oxide [14]. It has been demonstrated that PAT and flow-mediated dilatation (FMD) measurements have significant but differing relations with cardiovascular and metabolic risk factors. However, there is only a weak link between the two assessments [35]. In fact, FMD and PAT measure different aspects of vascular biology and provide distinct information regarding vascular function in conduit versus smaller digital vessels [35]. Using PAT, we showed that 3 months of atorvastatin neither improved endothelial function nor reduced early signs of atherosclerosis or arterial stiffness. In this relatively healthy population of OSA patients, we did not confirm the beneficial effect on vascular compliance that we have previously reported with statins in rats exposed to intermittent hypoxia [22]. This result is in accordance with another human study showing that, despite improvement in the lipid profile, 6 weeks of atorvastatin treatment (40 mg/day) failed to improve endothelial dysfunction in the first-degree relatives of patients with premature coronary artery disease [36]. The atorvastatin dosage used here (40 mg/day) may have potentially been too low to improve endothelial function and atherosclerosis markers in OSA patients. Indeed, a higher atorvastatin dosage (80 mg/day) was shown to improve endothelial function assessed by flow-mediated dilation [37] and to reduce large artery stiffness [23] in normolipidemic hypertensive patients. An alternative explanation might be that we did not include patients with comorbidities such that a majority of our OSA patients did not exhibit sufficient endothelial dysfunction or severe stiffening at baseline to detect an effect.

However and importantly, we showed that this statin dosage is able to lower systolic office blood pressure in OSA patients. This observation is in accordance with previous results from our group showing that in rodents statin treatment reduces IH-induced blood pressure elevation [22]. However, outside the area of sleep apnea, such a beneficial effect has also been reported in normolipidemic patients with isolated systolic hypertension [23, 37].

In vascular smooth muscle cells statins are known to inhibit hypoxia-induced endothelin-1 via accelerated degradation of HIF-1α [38]. Statins have also been shown, in the CHF rabbit, to reduce sympathetic tone, inhibiting central Ang II mechanisms and therefore the superoxide pathway

Table 2: Cardiovascular parameters at baseline (J0) and after 12 weeks (M3) of placebo or statin treatment.

	J0	M3	Change M3 − J0	Difference in change (95% CI)
PAT				
Placebo ($n = 26$)	2.09 ± 0.56	2.18 ± 0.45	0.09 ± 0.47	0.008 (−0.29; 0.28)
Statin ($n = 25$)	2.16 ± 0.62	2.24 ± 0.45	0.09 ± 0.54	
Carotid-to-femoral PWV (m/s)				
Placebo ($n = 26$)	9.41 ± 1.91	9.49 ± 1.88	0.08 ± 2.07	0.54 (−0.45; 1.52)
Statin ($n = 25$)	8.38 ± 1.33	8.99 ± 1.39	0.61 ± 1.37	
Right carotid IMT (μm)				
Placebo ($n = 26$)	666.35 ± 165.33	667.96 ± 155.37	1.61 ± 155.91	0.02 (−70.38; 70.35)
Statin ($n = 25$)	615.00 ± 98.65	616.60 ± 92.99	1.60 ± 82.78	
Left carotid IMT (μm)				
Placebo ($n = 26$)	726.54 ± 174.15	691.44 ± 130.30	−35.10 ± 123.69	17.42 (−45.98; 80.81)
Statin ($n = 25$)	676.68 ± 148.02	659.00 ± 112.14	−17.68 ± 99.81	
Mean carotid IMT (μm)				
Placebo ($n = 26$)	696.73 ± 161.55	682.81 ± 130.51	−13.92 ± 119.44	5.34 (−51.34; 62.02)
Statin ($n = 25$)	647.16 ± 108.56	638.58 ± 93.26	−8.58 ± 77.68	
Right carotid luminal diameter (μm)				
Placebo ($n = 26$)	6782 ± 1052	6539 ± 708	−243 ± 888	83 (−305; 472)
Statin ($n = 25$)	6515 ± 536	6355 ± 525$^{\$}$	−160 ± 396	
Left carotid luminal diameter (μm)				
Placebo ($n = 26$)	6491 ± 816	6576 ± 770	85 ± 637	−73 (−360; 215)
Statin ($n = 25$)	6357 ± 527	6369 ± 602	12 ± 338	

Data are mean ± SD or percentage. PAT, peripheral arterial tone; PWV, pulse wave velocity; IMT, intima-media thickness. Analysis of data by repeated measure two-way ANOVA, followed by a Bonferroni post hoc test when necessary.
$^{\$}P < 0.05$ for value visit.

Table 3: Blood pressure at baseline (J0) and after 12 weeks (M3) of placebo or statin treatment.

	J0	M3	Change M3 − J0	Difference in change (95% CI)
SBP (mmHg)				
Placebo ($n = 26$)	127.66 ± 15.26	127.46 ± 12.77	−0.21 ± 9.74	−6.34 (−12.68; −0.01)
Statin ($n = 25$)	127.78 ± 15.07	121.23 ± 9.68*	−6.55 ± 12.64	
DBP (mmHg)				
Placebo ($n = 26$)	79.02 ± 11.61	80.64 ± 12.33	1.62 ± 10.57	−3.98 (−9.98; 2.03)
Statin ($n = 25$)	79.16 ± 11.60	76.80 ± 8.55	−2.36 ± 10.78	
MAP (mmHg)				
Placebo ($n = 26$)	95.22 ± 11.93	96.31 ± 11.52	1.09 ± 9.17	−4.74 (−10.15; 0.67)
Statin ($n = 25$)	95.37 ± 12.21	91.72 ± 8.26	−3.65 ± 10.03	

Data are mean ± SD. SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure. $^{*}P < 0.05$ versus baseline data by repeated measure two-way ANOVA, followed by a Bonferroni post hoc test when necessary.

[21]. There is growing evidence that NAD(P)H oxidase-derived reactive oxygen species induced by Ang II play an important role in the central regulation of autonomic activity and cardiovascular function in various pathological states. As previously shown in IH exposed rats [39], the hypertensive effect and cardiac infarction hypersensitivity induced by IH were abolished by antioxidant treatments (such as tempol and melatonin) which were able to normalize DHE level and NADPH expression. All these mechanisms might potentially be involved in the blood pressure lowering effect of statins observed here in OSA patients.

In this study, the impact of statin in reducing blood pressure (around 6 mmHg mean difference) is clinically relevant. This is particularly true in view of the limited impact of CPAP treatment in reducing BP [40, 41]. Indeed, among patients treated for hypertension, even 1 to 2 mmHg mean differences in office blood pressure are already associated with reduced odds of stroke and major cardiovascular events [42–44]. Law et al. have underlined the importance of lowering blood pressure in everyone over a certain age, rather than measuring it in everyone and treating it in some [44]. Larger reductions in blood pressure are known to produce

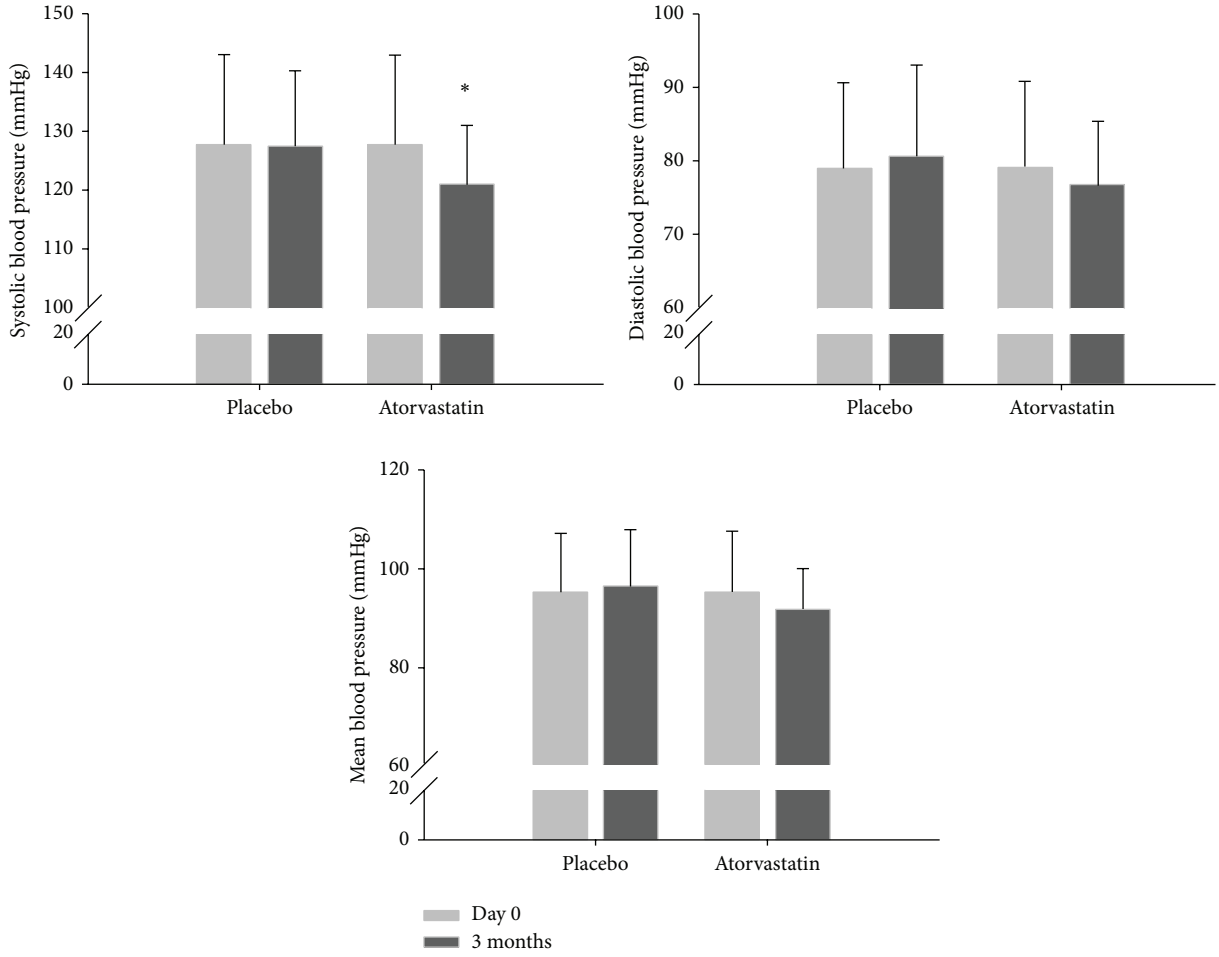

Figure 2

larger reductions in the risk of all major cardiovascular events [43].

Finally, we also showed that statin treatment improved the lipid profile in normolipidemic OSA patients, in accordance with previous studies on normolipidemic hypertensive patients [23, 37]. We observed here that 3 months of statin treatment induced a decrease of 2 mmol/L in total cholesterol and of 1.68 mmol/L in LDL cholesterol that could significantly reduce cardiovascular risk. Indeed, lowering LDL cholesterol concentration by an average of 1.8 mmol/L under statins is able to reduce the risk of ischaemic heart disease events by about 60% and stroke by 17% [45].

Recent large RCTs demonstrate that CPAP alone is not sufficient to address cardiometabolic risk in OSA patients [20]. Thus, a combination of statin treatment with CPAP therapy could be useful to better control blood pressure in OSA patients and to reduce associated cardiovascular morbidity and mortality. Indeed, in view of the high risk of cardiovascular disease (CVD) in patients with OSA, the use of statins in this group of patients, irrespective of their baseline cholesterol levels, should be encouraged [46]. While absolute risk assessment is essential when considering primary prevention for CVD, an uncritical application of the Framingham risk equation may result in the underuse of statins in patients with OSA. Thus, when the Framingham risk tool is used to manage statin treatment for primary CVD prevention in OSA patients, a lower CVD risk threshold than that recommended by current guidelines may need to be set [46]. Evidence from large registries and long-term prospective trials is now required to determine the potential synergic beneficial effects and the rate of new cardiovascular events in patients with OSA receiving combined statin and CPAP therapy.

Finally, combined statin and CPAP therapy should be put in a realistic perspective compared to the undisputed effects of weight loss and/or exercise not only on blood pressure but also on the cardiometabolic consequences of sleep apnea [20, 47, 48].

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Table 4: Metabolic parameters at baseline (J0) and after 12 weeks (M3) of placebo or statin treatment.

	J0	M3	Change M3 − J0	Difference in change (95% CI)
Total cholesterol (g/L)				
Placebo ($n = 26$)	2.25 ± 0.64	2.36 ± 1.24	0.11 ± 1.18	−0.89 (−1.50; −0.27)
Statin ($n = 25$)	2.45 ± 0.94[$]	1.68 ± 0.42[$]	−0.78 ± 0.99[£]	
LDL cholesterol (g/L)				
Placebo ($n = 26$)	1.41 ± 0.45	1.34 ± 0.46	−0.07 ± 0.38	−0.65 (−0.96; −0.34)
Statin ($n = 25$)	1.60 ± 0.66	0.88 ± 0.30	−0.72 ± 0.67	
HDL cholesterol (g/L)				
Placebo ($n = 26$)	0.54 ± 0.23	0.74 ± 0.91	0.21 ± 0.93	−0.31 (−0.78; 0.17)
Statin ($n = 25$)	0.74 ± 0.52	0.64 ± 0.49	−0.10 ± 0.74	
Triglycerides (g/L)				
Placebo ($n = 26$)	1.55 ± 1.10	1.27 ± 0.69	−0.28 ± 0.83	0.09 (−0.27; 0.46)
Statin ($n = 25$)	1.16 ± 0.59	0.97 ± 0.43	−0.18 ± 0.36	
Glycemia (mmol/L)				
Placebo ($n = 26$)	5.18 ± 0.51	5.03 ± 0.56	−0.15 ± 0.57	0.06 (−0.34; 0.46)
Statin ($n = 25$)	5.18 ± 0.76	5.01 ± 0.57	−0.09 ± 0.83	
Insulinemia (mUI/L)				
Placebo ($n = 26$)	9.84 ± 8.02	8.20 ± 6.15	−1.63 ± 5.12	−2.27 (−8.96; 4.42)
Statin ($n = 25$)	11.07 ± 16.38	7.16 ± 3.19	−3.90 ± 15.56	
HOMA				
Placebo ($n = 26$)	2.39 ± 2.05	1.83 ± 1.47	−0.56 ± 1.42	−0.66 (−2.83; 1.51)
Statin ($n = 25$)	2.83 ± 5.19	1.61 ± 0.77	−1.22 ± 5.11	
Hemoglobin A1c (%)				
Placebo ($n = 26$)	5.60 ± 0.42	5.64 ± 0.40	0.04 ± 0.25	0.04 (−0.12; 0.19)
Statin ($n = 25$)	5.64 ± 0.41	5.71 ± 0.43	0.08 ± 0.30	

Data are mean ± SD. LDL, low-density lipoprotein; HDL, high-density lipoprotein; HOMA, homeostasis model assessment of insulin resistance.
[$]$P < 0.05$ for value group, [§]$P < 0.0001$ value visit, [£]$P < 0.0001$ value interaction, by repeated measure two-way ANOVA, followed by a Bonferroni post hoc test when necessary.

Table 5: Inflammatory parameters at baseline (J0) and after 12 weeks (M3) of placebo or statin treatment.

	J0	M3	Change M3 − J0	Difference in change (95% CI)
hs-CRP (mg/L)				
Placebo ($n = 26$)	3.9 ± 4.0	2.4 ± 1.8	−1.5 ± 4.4	2.5 (−3.8; 8.8)
Statin ($n = 25$)	3.8 ± 6.1	4.8 ± 13.7	1.0 ± 15.4	
Urinary LTE4 (pg/mg creatinine)				
Placebo ($n = 26$)	83.8 ± 72.9	88.4 ± 73.7	4.6 ± 42.8	14.1 (−10.4; 38.6)
Statin ($n = 25$)	68.8 ± 31.1	87.5 ± 50.9	18.7 ± 44.4	
Urinary 11-DHTXB2 (pg/mg creatinine)				
Placebo ($n = 26$)	784.5 ± 463.5	848.0 ± 635.9	63.6 ± 355.6	41.0 (−156.9; 238.9)
Statin ($n = 25$)	704.6 ± 279.9	809.1 ± 374.6	104.6 ± 347.2	

Data are mean ± SD. hs-CRP, high-sensitivity C-reactive protein; LTE4, leukotriene E4; 11-DHTXB2, 11-dehydro- thromboxane B2; TXB2, thromboxane B2.

Authors' Contribution

Marie Joyeux-Faure and Renaud Tamisier contributed equally to this work and should be considered as co-first authors. Frédéric Gagnadoux and Jean-Louis Pepin (principal investigator) equally managed this work and should be considered as co-last authors.

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Clinical Study

Inflammation and Growth in Young Children with Obstructive Sleep Apnea Syndrome before and after Adenotonsillectomy

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Background. Obstructive sleep apnea (OSA) is associated with growth impairment that usually improves following effective treatment. In this study we investigated the mechanisms underlying the growth processes in young children diagnosed with OSA, before and after adenotonsillectomy (T&A). *Methods*. Young children (6–36 months old) were enrolled and evaluated before and several months after T&A surgery for height, weight, circulating high sensitive C-reactive protein (CRP), and insulin-like growth factor 1 (IGF-1) levels. Caloric intake was assessed by a validated Short Food Frequency Questionnaire (SFFQ). *Results*. Following T&A, children added 4.81 cm and 1.88 kg to their height and weight, respectively ($P < 0.001$ for both) and had a significant increase in BMI Z score ($P = 0.002$). Increased caloric intake of 377 kcal/day was noted ($P < 0.001$), with increased protein and decreased fat intake. The decrease in CRP levels correlated with the increase in body weight in boys ($P < 0.05$, adjusted for caloric intake). *Conclusions*. Adenotonsillectomy results in enhanced somatic growth in young children that correlates with a decrease in systemic inflammation and caloric intake increment. Our findings imply that systemic inflammation may have an important role in this OSA-related morbidity.

1. Background

Obstructive sleep apnea syndrome (OSA) is a common problem among children and especially boys, with estimated prevalence of 2-3% [1], with most children being diagnosed at the age of 2–6 years. At night OSA is characterized by interrupted sleep, snoring, and disrupted breathing. At daytime, children present hyperactive behavior, cognitive deficits, and attention problems. Adenotonsillar hypertrophy is considered the leading cause of pediatric OSA and tonsillectomy and adenoidectomy (T&A) is still considered and clinically used as a first line treatment for children with OSA [2].

There is growing evidence on OSA's associated neurobehavioral [3], cognitive [4], cardiovascular [5], and metabolic morbidities [6]. Furthermore, new studies found an improvement in cognitive [7] and cardiovascular [8] functions after T&A.

In the last 30 years there were several studies, as well as case studies, suggesting OSA as a major risk factor for growth failure [9, 10]. These studies were conducted mostly among kindergarten and school children and showed an improvement of somatic growth accompanied with an increased level of IGF-1 and the ratio growth hormone (GH)/IGF-1 after T&A [11, 12]. The very few studies that followed somatic growth in toddlers have also shown an improvement of somatic growth, but none of them have ever tried to estimate the role of growth hormone in the process [11, 12]. There are several possible mechanisms for growth retardation among children with OSA, including increased

energy expenditure during sleep due to labored breathing, decreased appetite, and enlarged tonsils that serve as a mechanical barrier limiting the amount of food swallowed. Another possible explanation is interruption in the axis of growth hormone (GH) secretion. GH is secreted from the pituitary gland mainly during slow-wave sleep in a pulsatile fashion. GH secretion positively correlates with IGF-1 and IGFBP-3 levels which triggers bone growth. Notwithstanding the direct effects of GH on growth plate chondrocytes, it is now accepted that the indirect local and systemic effects of GH function in a highly coordinated manner regulate growth plate activities and linear bone growth [13].

In children with OSA there is a decrease in the slow-wave sleep component, and an increase in its percentage of the total sleep time is noted following T&A [14]. This phenomenon was revealed by others and serves as a reasonable explanation to growth retardation before T&A and the improvement in growth after surgery [15].

Another possibility that may explain growth impairment is systemic inflammation.

Linear bone growth is adversely affected in children with chronic kidney disease (CKD) and other chronic inflammatory disorders. CKD patients have been reported to have elevated circulating levels of IL-6 and TNFα, similar to patients with OSA [16, 17]. The (GH)/(IGF-1) pathway is anabolic to the skeleton and inflammatory cytokines compromise bone growth through several mechanisms, which include interference with the systemic as well as the tissue-level GH/IGF-1 axis [18]. Recent studies have shown that, in children, OSA is accompanied by systemic inflammation, reflected by increased levels of markers like circulating C-reactive protein (CRP), that decrease after T&A [19]. Interestingly, the role of systemic inflammation, in regard to growth, in such children was not studied so far. Therefore, we assessed growth in OSA young children before and after T&A and evaluated potential mechanisms that may influence growth in these children.

2. Methods

The study was approved by Soroka University Medical Center Human IRB Committee, and informed consent was obtained from the legal caretaker of each participant.

2.1. Patients. Young children with PSG proven OSA were enrolled and followed prospectively before T&A surgery and 4–6 months afterwards.

Inclusion criteria were children older than 6 and younger than 36 months of age who were diagnosed with an obstructive apnea-hypopnea index (AHI) > 5 events/hour of sleep in an overnight polysomnographic evaluation and informed consent from the legal caregiver who agreed was obtained. Exclusion criteria were craniofacial, neuromuscular, syndromatic, or defined genetic abnormalities, any known previous allergies, no upper respiratory infection use of any corticosteroids or antibiotics within 4 weeks preceding the initial sleep study, and any children that had had T&A or adenoidectomy in the past.

Overnight Polysomnography. All participating children underwent polysomnography in our sleep center. No sleep deprivation or sedation was implemented. Children were studied in a dedicated quiet, darkened room with an ambient temperature of 24°C in the company of one of their parents.

Polysomnography was performed with a computerized, commercially available, sleep monitoring system (SensorMedics Inc., Yorba Linda, CA, USA). Data was streamed to an optical disk for later analysis. Polysomnography was performed as previously described [8]. All of the studies were initially scored by a certified technician. The scores were then blindly reviewed by 2 physicians experienced in pediatric polysomnography. Analysis of the polysomnograms was performed with standard techniques [20]. In brief, sleep staging was performed with the standard criteria published by the AASM in 2007 [21] and not by the latest revision of 2012 since we recruited our last child in February 2012. The AHI was defined as the airflow with continued chest wall and abdominal movement over at least two breaths [21]. Hypopneas were defined as a ≥50% decrease in nasal flow with a corresponding ≥3% decrease in SpO_2 and/or arousal or awakening [21]. We determined the mean and nadir SpO_2 values. Arousals were defined as recommended by the revised AASM rules [21].

2.2. Evaluation of Somatic Growth. In each visit the child was evaluated for height and weight, using the same equipment on both encounters. Anthropometric data was analyzed by the endocrinologist (N.L.) for Z score using the software "Growth Analyzer 3.5" of The Dutch Growth Foundation (http://growth-analyser.sharewarejunction.com). Z score represents standard deviations gap from the mean to child's age.

2.3. Dietary Assessment. A Short Food Frequency Questionnaire (SFFQ) for energy assessment was completed for each child on both encounters. This SFFQ is based on a list of items reported by 160 mothers of infants at the age of 6 to 24 months describing the previous day's food items offered or consumed. This SFFQ was validated in previous studies [22, 23]. All SFFQs were assessed for caloric intake and for the differential percent of protein, fat, and carbohydrates in diet.

It was filled by the primary caregiver at the two encounters.

2.4. Inflammatory and Endocrinologic Markers. Circulating hsCRP was measured by means of particle-enhanced immunonephelometry using the BN ProSpec system (Newark, DE). All samples were assessed in duplicate and assayed at two dilutions. Data are presented in mg%. Blood draws were performed at the morning of T&A and 4–6 months following surgery also at the morning hours at the pediatric sleep clinic. IGF-1 levels were measured using an immunoradiometric assay after extraction (DSL, Webster, TX, USA). Interassay coefficient of variation (CV) was <14%; data are presented in nmol/L. The markers were measured

Table 1: Pre- and postsurgery characteristics.

	Before surgery	After surgery	P value
Age (m)	23 ± 6	28 ± 6	<0.001
Time to follow-up (m)		5 ± 2	NA
Weight (kg)	11.11 ± 2.59	13 ± 2.48	<0.001
Z score weight (STD)	−1.29 ± 1.84	−0.55 ± 1.51	0.002
Height (cm)	83.28 ± 8.74	88.09 ± 6.34	<0.001
Z score height (STD)	−1.18 ± 1.9	−0.49 ± 1.44	0.223
BMI (kg/m^2)	15.85 ± 1.67	16.7 ± 1.69	0.037
Z score BMI (STD)	−0.51 ± 1.24	0.36 ± 1.18	0.007
Circulating CRP (mg%)	0.72 ± 0.9	0.21 ± 0.02	0.131
Circulating IGF-1 (nmol/L)	4.32 ± 2.71	6.69 ± 2.69	0.104
Daily caloric intake (kcal)	837 ± 233	1214 ± 412	<0.001
Apnea-hypopnea index (events/h)	16.8 ± 7.7	NA	
Oxygen minimal (nadir) saturation (%)	78.6 ± 8.4	NA	
Arousals index (events/h)	18.6 ± 11.3	NA	
Total sleep time (min)	429.6 ± 174.3	NA	

at the endocrinology and immunology laboratories at the Soroka University Medical Center.

2.5. Postsurgical Sleep Evaluation. All parents filled a validated questionnaire given on the day of surgery and at the postsurgical follow-up visit in our pediatric sleep clinic [24]. In brief, it includes eight items scored by the parent in a 0–4 scale (0 = never; 4 = most of the time). Questions included are being hard to awake, witnessed apnea, breathing difficulties, snoring, sweating, mouth breathing, awakening, and restless sleep.

2.6. Data Analysis. Results are presented as means ± SD, unless otherwise stated. All analyses were conducted using statistical software SPPS version 17.0. All numeric data were subjected to statistical analyses with either t tests or 2-way analysis of variance procedures for repeated measures, as described by Neuman-Keuls. Post hoc tests were performed as appropriate. A two-tailed $P < 0.05$ was considered statistically significant.

3. Results

20 children were enrolled. Four children were lost to followup and were not different than the remaining participants. All 16 children (10 boys and 6 girls) were evaluated before and after T&A. Their mean age at the first encounter was 23 ± 6 months (range 7–33 months), and the mean time of follow-up was 5±2 months (range 3.8–6.2 months).

The demographic, anthropometric, and polysomnographic findings are shown in Table 1.

Most children showed significant improvements in their height (4.81 cm) and weight (1.88 kg) following adenotonsillectomy (average: $P < 0.001$ for both). Thus BMI also increased by 0.85 ± 0.42 kg/m^2 ($P = 0.037$).

At diagnosis, mean Z scores for height, weight, and BMI were −1.18 ± 0.32, −1.29 ± 0.35, and −0.51 ± 0.15, respectively.

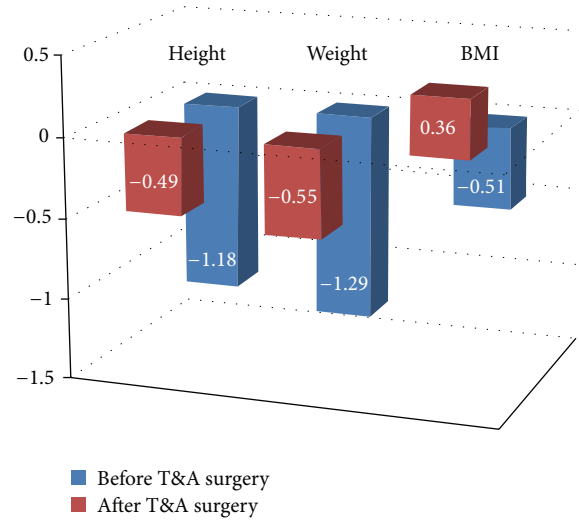

Figure 1: A graphic presentation of the improvement in Z score for weight, height, and BMI following T&A surgery. It shows the "catch-up" growth that is demonstrated by the gap of standard deviation to the average values of children of the same age (Z score = 0) for weight, height, and BMI.

After treatment, there were significant improvements in weight Z scores ($P = 0.002$) and in BMI Z scores ($P = 0.007$) as shown in Figure 1.

IGF-1 and hsCRP circulating levels were improved but did not reach statistical significance. The decrease in systemic inflammation was reflected by decrements of 0.51 ± 0.28 mg% in CRP levels after T&A ($P = 0.131$), while small increases in IGF-1 levels also emerged (2.37 ± 1.41 nmol/L; $P = 0.104$).

SFFQ were filled by the parents and compared the caloric intake (Table 1). On average, the children added 377.6 ± 292.4 calories to their daily diet after surgery ($P < 0.001$). There were a significant increase in protein intake (2.0 ± 1.4%; $P = 0.045$; Figure 2) and decrease in fat intake (4.49 ± 2.36%;

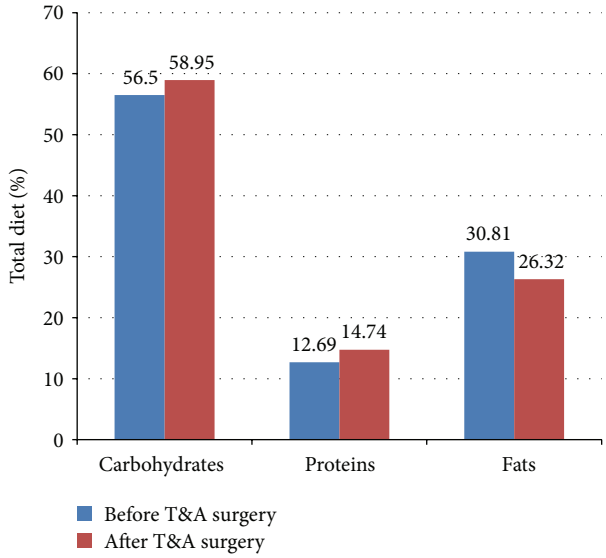

Figure 2: A graphic presentation of the change in dietary composition before and after T&A surgery. A rise in % carbohydrates (NS) and % proteins intake ($P = 0.045$) was noted, accompanied by a decrease in % fat ($P = 0.016$) in diet.

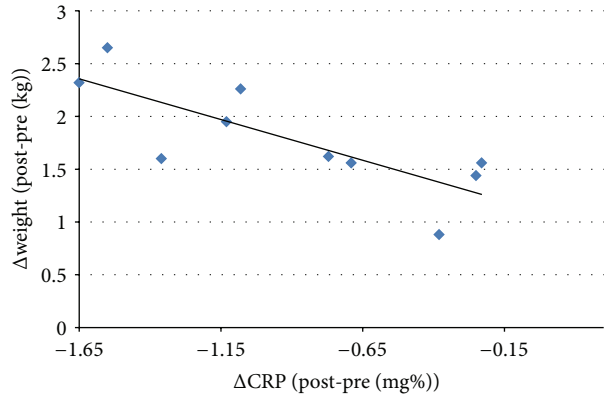

Figure 3: A correlation between the decrease in circulating CRP levels after surgery and the weight the child gained after surgery. The greater the fall in CRP levels the more the weight the boys gained after surgical intervention ($r = -0.775$, $P = 0.041$).

$P = 0.016$). There was also an increase in consumption of carbohydrates of 2% that failed to reach statistical significance ($P = 0.44$).

A multivariate analysis identified a significant negative correlation between systemic inflammation and weight in boys, such that, with more prominent decreases in circulating CRP levels, weight gain following surgery was magnified ($r = -0.775$; $P = 0.041$; Figure 3).

4. Discussion

This study found a major improvement in anthropometric parameters accompanied by a decrease in systemic inflammation and an increase in caloric intake and endocrinologic markers following T&A. Furthermore, for the first time it has been proved that even young children with OSA show improvement in somatic growth that is associated in boys with a reduction in systemic inflammation.

This study also identified, for the first time, a significant change in diet composition in children with OSA after surgery. Interestingly, the improvement in somatic growth correlated with an improvement in systemic inflammation but did not correlate with the changes in caloric intake.

We demonstrated that children aged 6–36 months who suffer from sleep disordered breathing are indeed smaller, for height and weight, than their peers before surgery. This gap was minimized following T&A, as shown in the rise in the Z scores.

This gap narrowing can be referred to as the "catch-up" growth.

It has been long known that children with OSA improve their somatic growth after surgery, but so far a comprehensive assessment of children 6–36 months was not done. Previous studies that were held in this age group evaluated children for anthropometric parameters before and after T&A and found an improvement of somatic growth [11, 12]. None of them tried to measure circulating levels of endocrine factors or the presence of systemic inflammation and their effect on growth. Furthermore, no dietary assessment has ever been performed in this age group and no one tried to estimate how surgical intervention affects the diet composition.

Several mechanisms have been proposed during the years to try and explain growth retardation in children with OSA. Among them are the presence of systemic inflammation that is well documented in children with OSA [8, 14, 25] and dysphagia from hypertrophied tonsils that serve as a mechanical barrier and obstruct food entry, thus reducing caloric intake [26]. One of the day symptoms of OSA is hyperactive behavior which results in increased motor activity that increases energy expenditure, thus contributing to growth failure [27]. Another possible mechanism is interruption to sleep architecture that interferes with growth hormone secretion [13].

This study tried to find the factor that imposes the biggest influence on "catch-up" growth in these children. Circulating CRP and IGF-1 levels were measured to estimate level of systemic inflammation and growth hormone-insulin-like growth factor 1 (GH-IGF-1) axis, respectively. In order to assess caloric intake, parents completed a Short Food Frequency Questionnaire (SFFQ) for energy assessment for each child on both encounters. This SFFQ is based on a list of items reported by 160 mothers of infants at the age of 6 to 24 months describing the previous day's food items offered or consumed and was validated in previous studies [22, 23].

Other studies that assessed the relationship between nutrition and T&A surgery did show a rise in carbohydrates consumption after T&A, but these studies used 24-hour dietary-recalls and were performed in preschool children [28]. This is the first study that performed dietary assessment in toddlers with OSA before and after T&A using a validated questionnaire. Furthermore, this SFFQ evaluates meal compound during the month prior to each encounter and is less prone to recall bias.

Analyzing the SFFQs showed that all children but one increased their caloric intake after surgical intervention. Moreover, looking at their dietary compound, a rise of 2% in protein and a decline of 4.49% in fat consumption were significantly shown. A nonsignificant rise of 2% percent in carbohydrates was also noticed. Although these changes were found to be significant, their actual effect on growth pattern is not clear, and there is certainly a need to repeat the SFFQ in a larger group in order to draw conclusions.

In parallel to the rise in caloric intake, the children narrowed the weight gap between them and their peers. These two findings can be linked because T&A surgery removes the hypertrophied tissue of tonsils and adenoids, thus removing a mechanical barrier that limits food swallow [19]. This barrier causes dysphagia and obstructs food entry hence causing a lower caloric intake. Removing this barrier enables the child to eat and swallow more easily and increases his caloric intake according to physiologic requirements and gaining weight accordingly [20].

In addition, the change in diet compound not only might indicate hormonal changes that influence appetite but also may represent a rise in anabolic process in the body that is manifested by weight gain. Though being statistically significant, these changes in dietary compound are not marked enough to explain the rise in somatic growth. This should be studied on a larger cohort.

Another possible explanation to the improvement in weight is the decline in systemic inflammation. Previous studies in children with OSA reported high circulating levels of inflammatory factors such as CRP. These inflammatory factors seemed to normalize and improved after surgical intervention. Those studies showed a decline of up to 50% in CRP levels after T&A [14, 25]. We hypothesized that an improvement in systemic inflammation will be followed by an improvement in somatic growth. There was an average decrease of 0.535 mg% in CRP level after surgery; however, the change was not significant probably due to small sample and this should be studied on a larger cohort.

Additional explanation is the improvement in GH-IGF-1 axis. GH mediates growth and is secreted in a pulsatile fashion. Its secretion is increased during sleep, especially slow-wave sleep. IGF-1 mediates the effects of growth hormone in tissues, especially the bone, and promotes its growth. Previous studies reported on shortening of the relative part of slow-wave sleep and improvement in its relative part after T&A [13]. A meta-analysis that tried to determine the role of T&A on GH-IGF-1 axis did find an increase in IGF-1 and IGFBP-3 serum levels, but all the studies were conducted on older children [29]. This study tried to assess whether toddlers will raise their IGF-1 levels after surgery. The children showed an increase in IGF-1 levels after T&A. This may explain the improvement in their weight but it was not significant probably due to a small sample.

In order to find the factor that imposes the biggest influence on "catch-up" growth in OSA children, a multivariate analysis was performed. There was a significant negative correlation between systemic inflammation and weight change in boys. The more prominent the decrease in circulating CRP levels was, the more weight was gained by the child. In contrast, there was no significant correlation between caloric intake and weight. This might support the assumption that the major factor influencing somatic growth in children with OSA is systemic inflammation and not the increase in caloric intake *per se*. The exact role in which systemic inflammation influences growth is not clear. One hypothesis is that local mediators effect bone growth, similar to children with chronic kidney disease [18]. The growth hormone (GH)/insulin-like growth factor 1 (IGF-1) pathway is anabolic to the skeleton and inflammatory cytokines compromise bone growth through a number of different mechanisms, which include interference with the systemic as well as the tissue-level GH/IGF-1 axis. It is known that suppressor of cytokine signaling 2 (SOCS2) expression is increased in inflammatory conditions including CKD and is a recognized inhibitor of GH signaling [18].

Therefore, SOCS2 signaling represents a critical pathway in growth plate chondrocytes through which OSA's activated proinflammatory cytokines alter both GH/IGF-1 signaling and cellular function.

Our report is the first to note the correlation between enhanced somatic growth and decreased systemic inflammation following surgery. It corroborates previous observations regarding other OSA's related morbidities (cardiovascular and neurocognitive) that improved following surgery and correlated with decreased systemic inflammation [8, 30].

Finally, although our findings may be of interest, there is a need to address the limitations of our study.

This report is based on a group of 16 children. Although they were well studied, there may be bias in the outcomes due to the small sample size. Second, we did not assess the resolution of OSA in a repeated PSG. The questionnaire we used shows that the overall symptom improvement was very good (all 8 questions with a scale of 1–5: preop 3.7 ± 1.3; postop 1.3 ± 0.4; $P = 0.002$), but questionnaires can be limited. The sleep disordered breathing scale predicts polysomnographic results to an extent useful for research but it is not always reliable enough for individual patients. However, the sleep disordered breathing scale may predict OSA-related neurobehavioral morbidity and its response to adenotonsillectomy as well as or better than polysomnography [31].

We also need to point out the fact that the children studied represent very accurately the average child in our lab in terms of BMI but not in other countries. Although we also see obese children in our sleep lab, most of the young children are normal or underweight for their age [8]. Therefore, the population recruited in our study may not be representative of current trends in the United States, for example, but still reflects what pediatric sleep specialists encounter in many other countries.

In summary, this study assessed the effect of potential mechanisms on growth in young children treated for OSA. Moreover, it tried to identify the major factor that influences this "catch-up" growth. We found that young children improve their anthropometric measures substantially following surgical removal of their lymphadenoid tissues. We have learned that the increase in caloric intake is accompanied by a change in the dietary composition, that is, an increase in protein and a decrease in fat consumption. The most

prominent result is the degree to which T&A changes in systemic inflammation as reported by hsCRP to correlate at least in boys with the degree of catch-up weight gain. This may point to the important role systemic inflammation plays in the growth processes of children with OSA.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Inflammatory Markers and Obstructive Sleep Apnea in Obese Children: The NANOS Study

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Introduction. Obesity and obstructive sleep apnea syndrome (OSA) are common coexisting conditions associated with a chronic low-grade inflammatory state underlying some of the cognitive, metabolic, and cardiovascular morbidities. *Aim.* To examine the levels of inflammatory markers in obese community-dwelling children with OSA, as compared to no-OSA, and their association with clinical and polysomnographic (PSG) variables. *Methods.* In this cross-sectional, prospective multicenter study, healthy obese Spanish children (ages 4–15 years) were randomly selected and underwent nocturnal PSG followed by a morning fasting blood draw. Plasma samples were assayed for multiple inflammatory markers. *Results.* 204 children were enrolled in the study; 75 had OSA, defined by an obstructive respiratory disturbance index (RDI) of 3 events/hour total sleep time (TST). BMI, gender, and age were similar in OSA and no-OSA children. Monocyte chemoattractant protein-1 (MCP-1) and plasminogen activator inhibitor-1 (PAI-1) levels were significantly higher in OSA children, with interleukin-6 concentrations being higher in moderate-severe OSA (i.e., AHI > 5/hrTST; $P < 0.01$), while MCP-1 levels were associated with more prolonged nocturnal hypercapnia ($P < 0.001$). *Conclusion.* IL-6, MCP-1, and PAI-1 are altered in the context of OSA among community-based obese children further reinforcing the proinflammatory effects of sleep disorders such as OSA. This trial is registered with ClinicalTrials.gov NCT01322763.

1. Introduction

Sleep-disordered-breathing (SDB) is a group of common disorders characterized by habitual snoring along with varying degrees of gas exchange alterations and sleep fragmentation [1]. Obstructive sleep apnea (OSA) is the most prevalent of those disorders affecting 1–4% of children with a peak incidence around 2–8 years [2]. In recent years, it has become apparent that the frequency of OSA is markedly increased by the concurrent presence of obesity [3] and the coexistence of these 2 conditions has been linked to a higher risk for development of end-organ morbidities, including neurocognitive and behavioral impairments and cardiovascular and metabolic dysfunction [4–8]. In addition to increased

oxidative stress, activation and propagation of inflammatory pathways in the context of immune dysregulation have been implicated in the deleterious consequences of OSA [9, 10], with the cumulative evidence strongly supporting the concept that pediatric OSA is a chronic, low grade inflammatory condition [11–16]. In this context, it is now recognized that OSA causes, albeit not always, systemic elevation in the levels of inflammatory mediators, such as CRP, TNFα, IL-6, and INF-γ [17–23], and the concomitant reduction of anti-inflammatory substances, such as IL-10, thereby tilting the balance toward a heightened proinflammatory state [24].

Similarly, obesity has long been recognized as an indolent and persistent inflammatory condition in which the sustained activity of such processes promotes the occurrence of insulin resistance and vascular dysfunction [25–29]. OSA and obesity frequently coexist in children and have been assumed to interact and promote each other [30–32].

However, the potential contributions of OSA to the proinflammatory profile of obese children have not been critically delineated, particularly considering the incongruent inflammatory phenotypes that have been previously reported in obese children [33]. Thus, we hypothesized that community-recruited obese children with OSA would display significant differences in their plasma levels of specific biomarkers, including inflammatory markers. The aim of the present study was to assess and better delineate the potential effects of perturbed sleep, such as occurs in OSA, on a panel of inflammatory cytokines and adipokines in a large cohort of obese children.

2. Subjects and Methods

204 obese children (ages 4–15 years) were recruited from the community in a multicenter prospective study in Spain—the NANOS study. A detailed account of the study design is described elsewhere [34]. Briefly, obese children were prospectively enrolled through primary care centers across Spain during 2007–2010 and were randomly selected to participate in the study. The study was approved by a human subject committee in each of the participating centers and is in accordance with the STROBE statement. The study was registered at ClinicalTrials.gov under NCT01322763. Informed consent was obtained from each subject or legal guardian, and assent was obtained from children above 12 years old. Data was coded so each investigator in the research network was blinded to subjects' personal information and thus ensuring confidentiality. Samples and data from subjects included in this study were provided by the Basque Biobank for research OEHUN (http://www.biobancovasco.org/) and were processed following standard operating procedures with appropriate approvals from the Ethical and Scientific Committees.

The general medical and sleep histories were obtained from all participating children and the parents filled a validated Spanish version of the Pediatric Sleep Questionnaire (PSQ) [35]. Every child then underwent a thorough medical examination followed by an overnight sleep study (PSG). The morning after the PSG, blood was drawn in fasting conditions.

2.1. Overnight Polysomnography. PSG tests were conducted in a sleep laboratory under standardized conditions. The studies were scored, after removal of movement and technical artifacts, according to the standard criteria defined by the American Academy of Sleep Medicine (AASM) [36]. Briefly, obstructive sleep apnea was defined as cessation of airflow with continued chest wall and abdominal movements for the duration of at least of two breaths. Hypopnea was defined as a decrease in nasal flow greater than 50%, corresponding to at least 4% decrease in the oxygen saturation (SpO_2) as measured by pulse oximetry and/or terminated by a 3-second EEG arousal. The obstructive apnea-hypopnea index (AHI) was defined as the number of apneas and hypopneas per hour of total sleep time (TST). The obstructive respiratory disturbance index (RDI) was calculated from the number of respiratory-effort-related arousals and the number of apneas and hypopneas per hour of TST. Children with OSA were defined as those having RDI $\geq$ 3/hr of TST in accordance with the clinical practice guidelines in Spain [37]. In addition, nadir and mean SpO_2, as well total sleep time during which SpO_2 is below 90% or end-tidal CO_2 > 50 mmHg occurred, were recorded. Oxygen desaturation index (ODI) was defined as the number of desaturation events $\geq$ 4% per hour of TST. Of note, in our subanalyses we defined moderate-to-severe OSA, as the presence of obstructive AHI > 5/hrTST.

2.2. Inflammatory Mediator Assays. Plasma was separated from the whole blood morning samples drawn from each child and stored in −80°C until assay. Commercially available ELISA kits specific for each cytokine were used to measure levels of IL-6, IL-18, MCP-1, adiponectin, MMP-9, apelin C, leptin (all individual kits from RayBiotech, Inc., Norcross, GA, USA), adropin (Peninsula laboratories LLC, San Carlos, CA, USA), osteocrin (MyBioSource, San Diego, CA, USA), and PAI-1 (Assaypro LLC, St. Charles, MO, USA). Assays were performed according to manufacturers' recommendations.

2.3. Statistical Analysis. Descriptive data for continuous variables are presented as means ± standard deviation (SD) and for categorical variables as percentages or ratios. Analyses for comparisons between clinical and laboratory values among OSA and no-OSA groups were performed using Student's t-tests for continuous and χ^2 tests for categorical variables followed by Fisher exact tests. Group comparisons were conducted using one-way ANOVA followed by Bonferroni correction for multiple comparisons. Pearson's correlation was used to compare between the marker levels and clinical parameters. Multivariate linear regression analysis was applied to assess relationships of significantly different markers between the two groups of children. Statistical significance was assumed at two-tailed $P < 0.05$. Statistical analyses were performed using SPSS software (version 21.0; SPSS Inc., Chicago, IL).

Table 1: Antropometric measures in OSA and no-OSA obese children.

	Total (n = 204)	No-OSA (n = 129)	OSA (n = 75)	P value
Age (years)	10.8 ± 2.6	11 ± 2.4	10.4 ± 2.8	0.1
Gender (male/female)	111/93	72/57	39/36	0.6
Height (m)	1.5 ± 0.16	1.5 ± 0.16	1.46 ± 0.17	0.1
Weight (Kg)	64.3 ± 21.1	65.2 ± 20.6	62.7 ± 22.1	0.4
BMI	27.9 ± 4.3	27.9 ± 4.1	28 ± 4.6	0.8
BMI%	96.8 ± 0.6	96.7 ± 0.6	96.8 ± 0.4	0.4
Neck circumference (cm)	34.1 ± 3.8	33.9 ± 3.8	34.3 ± 3.7	0.5
Waist circumference/hip circumference	0.9 ± 0.07	0.9 ± 0.07	0.9 ± 0.07	0.6

*Data presented as mean ± SD.

Table 2: Polysomnographic characteristics in OSA and no-OSA obese children.

	Total (n = 204)	No-OSA (n = 129)	OSA (n = 75)	P value
AHI (/hrTST)	3.6 ± 9.5	0.6 ± 0.6	9 ± 14.2	<0.001†
Time in Bed (min)	479.2 ± 45.8	482.8 ± 47	473.1 ± 43.4	0.1
Total sleep time (min)	379.6 ± 70.2	384.1 ± 70.7	372 ± 69.4	0.2
Sleep Efficiency%	78.9 + 12.8	78.9 ± 12.3	78.9 ± 13.9	0.9
Number of arousals	67.3 ± 62.5	48.2 ± 32.9	99.4 ± 84.1	<0.001†
Arousal index (/hrTST)	11.2 ± 11.2	7.9 ± 6.1	17 ± 15.1	<0.001†
Respiratory disturbance index (/hrTST)	6 ± 10.6	1.4 ± 1	14 ± 14.5	<0.001†
Obstructive RDI (/hrTST)	5.5 ± 10.3	1 ± 0.9	13.3 ± 13.9	<0.001†
Central RDI (/hrTST)	0.3 ± 1	0.2 ± 0.4	0.6 ± 1.7	0.01†
Baseline SpO_2 (%)	98.1 ± 1.4	98.3 ± 1.3	98 ± 1.7	0.2
Mean SpO_2 (%)	96.4 ± 1.5	96.7 ± 1.2	96.1 ± 1.9	0.008†
Nadir SpO_2 (%)	90.5 ± 5.2	91.4 ± 3.5	89.1 ± 7	0.003†
Time SpO_2 < 90%	1.1 ± 7.2	0.5 ± 3.3	2.3 ± 11.4	0.1
Oxygen desaturation index (/hrTST)	2.3 ± 9	0.7 ± 1.2	5.1 ± 14.2	0.001†
Peak end-tidal CO_2 (mmHg)	46.2 ± 6.9	46.1 ± 6.1	46.2 ± 8.3	0.9
Total Sleep time with end-tidal CO_2 > 50 mmHg (hours)	3.6 ± 11.8	1.6 ± 5.6	7.1 ± 17.7	0.003†

†Statistically significant difference.

3. Results

3.1. Demographic Data. 204 obese children from the community (ages 4–15 years) were recruited from the NANOS study, 111 boys and 93 girls, all fulfilling obesity criteria, that is, BMI above the 95% for age and gender [38]. The prevalence of OSA in this group of obese children was 36.7%. The 2 groups of children, those with (OSA) and without OSA (no-OSA), had similar demographic and anthropometric characteristics (Table 1).

3.2. Sleep Studies. PSG findings are summarized in Table 2 for the 2 groups. As would be anticipated from the OSA and no-OSA category allocation, most of the PSG variables differed, and most particularly for respiratory parameters and the number of arousals from sleep (Table 2). In contrast, there were no significant differences in either the total duration of sleep and total time in bed (Table 2). These findings support the idea that disruption of sleep architecture, that is, sleep fragmentation, rather than sleep deprivation, is the salient sleep perturbation among children with OSA [4].

3.3. Plasma Inflammatory Mediators in Obese Children: OSA versus No-OSA. Among the inflammatory markers included in the present study, 2 markers were significantly higher in the OSA group, namely, PAI-1 (Table 3; $P = 0.01$) and MCP-1 (Table 3; $P = 0.03$). In a subset of children with more severe OSA (i.e., AHI > 5/hrTST), significantly higher levels of IL-6 emerged ($P = 0.009$; Table 3). In addition, MCP-1 levels of ≥30 pg/mL and PAI-1 of ≥3.3 ng/mL conferred a modestly higher risk of OSA (OR = 2, $CI_{95\%}$ = 1.1–3.6, $P = 0.02$; OR = 1.8, $CI_{95\%}$ = 1–3.2, $P = 0.04$, resp.).

To further examine the global contribution of inflammatory markers to the overall inflammatory state of each child, we constructed a cumulative "inflammatory score" (IS), whereby each marker was standardized using z-score transformation. The IS was then calculated by summarizing all the individual z scores. Please note that the z scores for adiponectin and adropin were calculated and multiplied by −1, since their plasma levels have been reported to decrease in states of increased inflammation and obesity. The IS was significantly higher in the OSA as compared to no-OSA groups (Table 3; $P = 0.04$).

Table 3: Inflammatory markers in OSA and non-OSA obese children.

	Total ($n = 204$)	No-OSA ($n = 129$)	OSA ($n = 75$)	P value
IL-6 (pg/mL)	7.5 ± 3.8 [7–8.1]	7.3 ± 3.2 [6.7–7.8]	8 ± 4.8 [6.8–9.1]	0.2
IL-18 (pg/mL)	170.2 ± 96.8 [156.9–183.6]	163.2 ± 80.8 [149.1–177.2]	182.4 ± 119.2 [155.1–209.9]	0.17
PAI-1 (ng/mL)	3.3 ± 1.2 [3.1–3.5]	3.2 ± 1.2 [2.9–3.4]	3.6 ± 1.3 [3.3–3.9]	0.01†
MCP-1 (pg/mL)	35.1 ± 16.9 [32.8–37.5]	33.2 ± 15.2 [30.6–35.9]	38.4 ± 19.1 [34–42.8]	0.03†
Apelin C (ng/mL)	127.9 ± 118.9 [111.5–144.3]	125.9 ± 80.8 [111.9–140]	131.3 ± 165.8 [93.1–169.4]	0.7
Adropin (ng/mL)	0.8 ± 0.3 [0.79–0.87]	0.8 ± 0.3 [0.75–0.85]	0.87 ± 0.32 [0.79–0.94]	0.1
Adiponectin (μg/mL)	28.1 ± 13.3 [26.2–29.9]	26.8 ± 12.1 [24.6–28.9]	30.3 ± 14.9 [26.8–33.7]	0.07
MMP-9 (μg/mL)	0.9 ± 0.6 [0.85–1]	0.9 ± 0.5 [0.8–0.97]	1 ± 0.8 [0.85–1.2]	0.1
Osteocrin (ng/mL)	8.5 ± 12.6 [6.7–10.2]	7.8 ± 7.2 [6.5–9.1]	9.7 ± 18.5 [5.5–14]	0.3
Leptin (ng/mL)	19.1 ± 8.1 [17.9–20.2]	18.5 ± 8.2 [17.1–19.9]	20 ± 8 [18.1–21.8]	0.2
IS	0 ± 4.3 [−0.49–4.9]	−0.5 ± 3.4 [−1.1–0.13]	0.8 ± 5.4 [−0.43–2.1]	0.04†

*Data presented as mean ± SD [$CI_{95\%}$]. †Statistically significant difference; IS: inflammatory cumulative score.

No differences in inflammatory marker levels emerged between boys and girls in the full cohort, except for higher plasma levels of leptin among girls (17.1 versus 21.3 ng/mL, $P < 0.001$). Of note, girls had slightly lower baseline and mean SpO_2 levels during the PSG (mean difference ~0.5%, $P = 0.01$) and a trend toward lower BMI% (96.8 versus 96.7%, $P = 0.05$).

3.4. Correlation Analyses. First, we examined whether the various biomarkers were associated with both PSG-derived measures and anthropometric measurements in the full cohort ($n = 204$; Table 3). Higher MCP-1 levels correlated with ODI ($r = -0.171$; $P = 0.02$), with $TCO_2 > 50$ ($r = 0.352$; $P < 0.001$) and with peak CO_2 levels ($r = 0.168$; $P = 0.02$). These correlations remained statistically significant after adjusting for age, gender, and BMI. Leptin was positively associated with higher BMI, older age, female gender, and shorter sleep duration, and such associations remained significant even after adjusting for other confounders ($P \leq 0.006$). Higher leptin levels were also associated with lower sleep efficiency (after adjusting for age), but this effect disappeared when adjusted for BMI. Adiponectin was negatively correlated with age and BMI ($r = -0.3$; $P < 0.001$), while age-adjusted adiponectin levels were borderline associated with BMI ($P = 0.054$).

Additionally, IS had a strong positive correlation with BMI ($r = 0.241$, $P < 0.001$), neck circumference ($r = 0.226$, $P < 0.001$), age ($r = 0.154$, $P = 0.01$), and $TCO_2 > 50$ ($r = 0.294$, $P < 0.001$) and was inversely associated with TST ($r = -0.172$, $P = 0.007$) and sleep efficiency ($r = -0.142$, $P = 0.026$). In a linear regression model that included all of the above variables that had significant correlations with IS, BMI and $TCO_2 > 50$ independently predicted higher IS ($\beta = 0.296$, $P = 0.001$; $\beta = 0.360$, $P < 0.001$).

Next, we examined whether any of the specific markers was potentially useful in predicting clinically relevant components of sleep-disordered breathing among the 75 children with OSA, that is, sleep fragmentation, intermittent hypoxemia, and hypercapnia. Pearson correlation coefficients (PCC) are presented and only the results that remained statistically significant after age adjustment are presented below, given the considerable changes in marker levels as a function of age (Table 4). Significant associations were observed for MCP-1 levels and ODI ($r = -0.276$; $P = 0.01$), Nadir SpO_2 ($r = 0.232$; $P = 0.02$), and $TCO_2 > 50$ ($r = 0.412$; $P < 0.001$). MCP-1 association with ODI remained significant after adjusting for age, sex, and BMI. Leptin was associated with lower TST ($r = -0.413$, $P < 0.001$). Adropin was associated with lower total time in bed ($r = -0.363$; $P = 0.001$), baseline SpO_2 ($r = -0.471$; $P < 0.001$), peak CO_2 ($r = -0.389$; $P = 0.001$), and $TCO_2 > 50$ ($r = -0.335$; $P = 0.007$). MMP-9 was associated with lower total time in bed ($r = -0.310$; $P = 0.007$) and with higher $TCO_2 > 50$ (0.273; $P = 0.03$). Finally, apelin

Table 4: Univariate associations among inflammatory markers and PSG measures in children with OSA.

Marker	Clinical variable	PCC	P value
MCP-1	Oxygen desaturation index	−0.276	0.017
	Nadir SpO_2	0.232	0.02
	$TCO_2 > 50$	0.412	0.001
Leptin	Total sleep time	−0.413	<0.001
Adropin	Total time in bed	−0.363	0.001
	Baseline SpO_2	−0.471	<0.001
	$TCO_2 > 50$	−0.335	0.007
	Peak CO_2	−0.389	0.001
IL-18	Baseline SpO_2	−0.290	0.01
MMP-9	$TCO_2 > 50$	0.273	0.03
	Total time in bed	−0.310	0.007
Apelin C	$TCO_2 > 50$	0.511	<0.001

C exhibited a strong positive correlation with $TCO_2 > 50$ ($r = 0.511$; $P < 0.001$).

In a multivariate analysis that included all the marker levels in the OSA group aiming at correcting for inter-marker correlations, age-adjusted MCP-1 levels remained the only inflammatory mediator that independently predicted $TCO_2 > 50$ ($\beta = 0.322$, $P = 0.03$). Furthermore, age-adjusted leptin levels in the OSA group independently predicted lower TST ($\beta = -0.252$, $P = 0.04$). Inflammatory score (IS) was correlated in the OSA group with higher $TCO_2 > 50$ ($r = 0.359$, $P = 0.002$) and had borderline association with neck circumference ($r = 0.213$, $P = 0.049$). Only higher $TCO_2 > 50$ independently predicted higher IS ($\beta = 0.356$, $P = 0.003$) in the OSA group in a model that included age, BMI, and neck circumference.

4. Discussion

Current findings provide incremental evidence that the presence of OSA operates as an independent contributor to the increased systemic inflammation that occurs in obese children. Our data indicate that the levels of two blood markers, namely, PAI-1 and MCP-1, were increased among obese children with OSA, such that plasma concentrations of MCP-1 > 30 pg/mL and PAI-1 > 3.3 ng/mL provide reliable prediction on the presence of OSA. In addition, in a subset of obese children with moderate-to-severe OSA, IL-6 levels were also significantly higher. Furthermore, the overall inflammatory status, as inferred from the inflammatory score (IS), an arbitrary additive summation of the relative levels of all the current markers assayed in this study, was significantly increased in the OSA group, indicating heightened overall inflammatory load in OSA. Interestingly, IS also exhibited significant associations with BMI and total sleep time and efficiency as well as with the duration of hypercapnia.

Before discussing the potential implications of our findings, we will initially focus on those 3 inflammatory mediators that were markedly elevated in the OSA group, MCP-1, PAI-1, and IL-6. Monocyte chemoattractant protein 1 (MCP-1) is a central member of the C-C chemokine superfamily responsible for attracting mononuclear cells to inflammatory sites [39]. MCP-1 increases with obesity, plays a role in recruiting macrophages into adipose tissue in adult obese patients [40–42], and is associated with insulin resistance and with type 2 diabetes [43]. This cytokine, which is also highly expressed in the inflamed vasculature, is a potent attractor of lipid-activated monocytes involved in the inflammatory signaling cascade related to vascular dysfunction, atherosclerosis, and cardiac events [44, 45]. In children, there is also evidence that MCP-1 increases with obesity [46, 47]. In the context of OSA, MCP-1 elevations have been reported in adult patients, and treatment with CPAP reduced MCP-1 levels [48, 49]. The negative association reported herein between ODI and MCP-1 levels was unexpected considering that MCP-1 gene expression increases in response to hypoxia and appears to correlate with the degree of hypoxemia in adult patients with OSA [50]. PAI-1 is an inhibitor of tissue plasminogen activator and primarily functions as a suppressor of plasma fibrinolysis. PAI-1 increases in plasma are believed to play a role in the pathophysiology of endothelial dysfunction and atherothrombosis [51]. PAI-1 has been recently shown to have a strong correlation with known cardiometabolic risk factors in adults and is proposed as a biomarker for metabolic syndrome [52]. Similarly, higher PAI-1 levels have been associated with higher risk for microvascular complications in children, as well as with poorer diabetes control and hyperlipidemia in patients with type 1 diabetes [53]. In the context of OSA, higher levels of PAI-1 have been previously described in adults [54, 55]. Here, we show for the first time that obese children with OSA have higher plasma levels of PAI-1, supporting the notion that such alterations may reflect an underlying risk for vascular dysfunction, even if measures of endothelial function were not specifically acquired. Indeed, early development of endothelial dysfunction in pediatric OSA has been the subject to recent and intense research efforts which have led to the demonstration that the microvascular bed is a target of OSA [7, 8, 56–58]. Interleukin-6 is a ubiquitously expressed proinflammatory cytokine and well-established risk factor for adverse cardiovascular outcomes [59]. IL-6 signaling pathways are involved in the liver synthesis of C-reactive protein (CRP), and CRP is elevated in children with sleep-disordered breathing, whereby both IL-6 and CRP levels correlate with degree of hypoxemia and sleep disruption, independently of the degree of obesity [60]. Elevated IL-6 levels have been now repeatedly described in both adults and children with OSA [61, 62], and genetic variations in the IL-6 gene are associated with pediatric OSA and may account for the increased CRP levels seen in those children [23]. Thus, the increased IL-6 levels in the moderate-severe group of OSA children may provide a useful indicator for the presence of a more severe clinical phenotype. However, we cannot exclude the possibility that the different genomic background in this population may account for a decreased likelihood of finding elevated IL-6 plasma concentrations as recently reported in a comparison of US and Greek children [23].

Our study is the first to examine a large pediatric cohort of obese children from the community (i.e., not clinically

referred children) and evaluated these children in an unbiased fashion for the presence of sleep-disordered breathing. These were therefore a priori healthy children without any preexisting conditions except for the presence of obesity. All previous studies in which the proinflammatory effects and metabolic consequences of obesity were explored consisted of symptomatic, clinically-referred obese children being evaluated for management of their obesity and with a high prevalence of OSA, precluding systematic determination of the relative contribution of OSA to the inflammatory profile of obesity [3, 18, 19, 63, 64]. As reported above, the increase in individual inflammatory markers and in the overall IS among the OSA group was independent of the degree of obesity. Furthermore, all 3 markers altered by OSA are ascribed pathophysiological roles in cardiovascular dysfunction, thereby suggesting that OSA in obese children might predispose them to a more severe cardiovascular phenotype and to earlier development of cardiovascular morbidities. Based on our previous study showing that obese children with OSA have a significantly higher proportion of abnormal endothelial function [7], more aggressive diagnostic and intervention measures appear to be warranted by the concurrent presence of obesity and symptoms of OSA. Conversely, children with milder forms of sleep-disordered breathing, that is, RDI < 3/hrTST, had lower systemic inflammatory markers, potentially justifying the expectant approach strategy as recently recommended [65].

An interesting association emerged between increased BMI and leptin levels and decreased total sleep time during the overnight PSG. Such association concurs with epidemiological studies showing that sleep loss is associated with increased obesity, increased appetite, and elevated leptin levels in adults [66], and with similar recent findings in children [67]. Of note, reduced duration is not a primary feature of OSA, as confirmed by the similar total sleep time in OSA and no-OSA children in the present study.

The strong association between prolonged hypercapnia and increased inflammation deserves comment. Obesity-hypoventilation syndrome (OHS) is a relatively infrequent condition in children that is characterized by airway obstruction and CO_2 retention [68]. OHS is relatively underdiagnosed, and in adults it has been associated with impaired daily functioning and increased risk for diabetes and cardiovascular morbidity (including systemic and pulmonary hypertension, ischemic heart disease, and right-heart failure), as well as with higher risk of hospitalization and death [69–72]. The occurrence of alveolar hypoventilation during sleep is much more common in obese children with OSA when compared with children with OSA who are not obese [73, 74], and the present study illustrates for the first time the possibility that children with increased CO_2 retention may represent a high risk group.

In summary, systemic inflammation is more pronounced in obese children with OSA, further buttressing the contributions of perturbed sleep and gas exchange abnormalities to the inflammatory cascade. Further studies are needed to investigate the role of PAI-1 as a marker of endothelial dysfunction and the role of hypercapnia on increased inflammation and end-organ injury in obese and nonobese children with OSA.

Conflict of Interests

The authors have no conflict of interests to declare.

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Research Article

Abnormal Cytokine Profile in Patients with Obstructive Sleep Apnea-Hypopnea Syndrome and Erectile Dysfunction

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Patients with obstructive sleep apnea-hypopnea syndrome (OSAHS) show a high prevalence of erectile dysfunction (ED). Although the underlying pathogenesis is still unknown, endothelial dysfunction, induced by inflammatory cytokines, chemokines, and adhesion molecules, has been proposed as a possible mechanism. The aim of this study was to assess whether OSAHS is associated with activation of the inflammatory cytokine system in patients with ED compared to the matched OSAHS patients with normal sexual function. Thirty-one patients with severe OSAHS and ED were included. Fifteen patients with severe OSAHS and without ED served as controls. Serum concentrations of high-sensitivity C-reactive protein (hsCRP), tumor necrosis factor-α (TNF-a), interleukin-6 (IL-6), interleukin-8 (IL-8), and adiponectin were measured after the diagnostic polysomnography. We found that hsCRP levels were significantly elevated in OSAHS patients with ED compared to controls. Similarly, TNF-a levels, IL-6, and IL-8 were elevated in OSAHS patients with ED compared to controls. Serum adiponectin levels were lower in OSAHS-ED patients, but the difference did not reach statistical significance. The presence of ED in patients with severe OSAHS is associated with elevated levels of inflammatory markers, underlining a possible involvement of endothelial dysfunction in the pathogenesis of ED.

1. Introduction

Obstructive sleep apnea-hypopnea syndrome (OSAHS) is a common disease characterized by repetitive episodes of partial or complete upper-airway obstruction during sleep. Although estimates of disease prevalence are in the range of 3–7% for adult men and 2–5% for adult women in the general population [1], with prevalence rates reaching up to 33% in certain populations [2], OSAHS tends to be underdiagnosed in clinical practice [3]. OSAHS is associated with various neurobehavioral and cardiovascular sequelae, among which sexual dysfunction remains the least studied. However, erectile dysfunction (ED) is a highly prevalent condition in patients with OSAHS [4, 5], and its frequency and severity appear to correlate with the severity of OSAHS [6]. In addition, up to 91% of patients with erectile dysfunction may be diagnosed with OSAHS [7].

ED has been defined as the persistent inability to attain and maintain an erection sufficient to permit satisfactory sexual performance [8]. The prevalence of ED is also high, varying in different countries between 3% and 71% according to age [9]. In Greece, Doumas and colleagues reported an ED prevalence of 14.1% in normotensive patients compared to 35.2% in patients with essential hypertension [10]. There is a growing body of evidence suggesting that ED is predominantly a disease of vascular origin, with endothelial cell dysfunction as the unifying link [11]. Circulating markers of endothelial cell damage that have previously been reported include cytokines and chemokines, soluble adhesion molecules, and acute-phase reactants. Several inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-a (TNF-a), and high-sensitivity C-reactive protein (hsCRP), have been investigated and could be proposed as laboratory markers for potential use in ED

[12]. Moreover, in vitro studies demonstrate that the above inflammatory factors suppress the production of adiponectin, which is released from adipose cells and exerts a variety of anti-inflammatory effects [13].

The underlying mechanisms by which OSAHS and ED interact are still unknown. Several mechanisms have been proposed, including nerve involvement caused by hypoxemia, low levels of testosterone, and vascular endothelial dysfunction through diverse pathways such as hypoxemia, oxidative stress, and sympathetic activation [14]. There is convincing evidence for endothelial dysfunction in OSAHS; however, there are contradictory findings if administration of continuous positive airway pressure (CPAP) reverse endothelial dysfunction associated with OSAHS [15, 16]. Endothelial dysfunction, as already mentioned, is also a key finding in the patient with ED [11], suggesting a mechanistic paradigm whereby OSAHS may lead to ED. However, limited information exists regarding the pathophysiologic basis of ED in OSAHS, in particular, regarding how intermittent nocturnal hypoxemia is likely to contribute to impaired penile tumescence by promoting endothelial dysfunction, which might be mediated by elevated levels of inflammatory markers as well as decreased levels of anti-inflammatory markers. Therefore, the aim of this study was to assess whether OSAHS is associated with activation of the inflammatory cytokine system in patients with ED compared to patients with OSAHS and normal sexual function, evaluating a wide range of inflammatory (hsCRP, IL-6, IL-8, and TNF-a) and anti-inflammatory (adiponectin) markers.

2. Materials and Methods

2.1. Study Population. From June 2011 to June 2012, patients were recruited from a prospective study cohort comprising consecutive patients, between 18 and 65 years old, who visited the Sleep Disorders Center, Department of Thoracic Medicine, Medical School University of Crete, for evaluation of suspected sleep-disordered breathing. The inclusion criteria were the following: newly diagnosed OSAHS by polysomnography (PSG) according to standard criteria, severe OSAHS, and an above-elementary school education. The exclusion criteria were the following: refusal to participate, previous CPAP treatment, hypertension, coronary artery disease, congestive heart failure, history of life-threatening arrhythmias, cardiomyopathy, history of stroke, chronic renal disease, endocrine dysfunction, such as diabetes mellitus, Cushing's syndrome, and abnormal pituitary function, chronic obstructive pulmonary disease, morbid obesity (BMI $> 40\,kg/m^2$), dyslipidemias, undertreatment with cardiovascular medication, or pharmacologically treated depression, with severe cognitive impairment, with a family or personal history of mental illness, with drug or alcohol abuse, with concurrent oncologic diseases, metabolic or neurological disorders known to induce peripheral neuropathy or ED, deep vein thrombosis, peripheral vascular disease, connective tissue disorders, and history of narcolepsy. Moreover, if patients were diagnosed with ED before the enrolment of this study or had already undergone therapy with medications that affect erectile function, such as b-blockers and H_2 blockers, they were also eliminated from the study. All subjects provided written informed consent, and ethical approval was provided by the University Hospital Ethics Committee.

2.2. Data Collection: Study Design. The study subjects underwent a detailed evaluation that included age (years), body mass index (BMI) (kg/m^2), clinical history focused on sleep-related symptoms, associated conditions and comorbidities, medication use, smoking history, and alcohol intake. Furthermore, a urology specialist assessed sexual history and analyzed the use of medications that may interfere with erection function. Each patient was asked to complete the Greek version of the validated International Index of Erectile Function (IIEF) Questionnaire as a comprehensive tool for assessing erectile function [17]. In addition, the Epworth Sleepiness Scale (ESS) was used to assess the degree of diurnal somnolence [18].

After signing the informed consent form, the PSG was scheduled. The patients arrived at the Sleep Disorders Center at least 2 hours before their normal bedtime and went to sleep at their habitual bedtimes, and blood samples were collected the following morning for biochemical and haematological analyses. A sleep physician coordinated any necessary clinical followup and made the results of the exams available for each patient. During these appointments, they received one-on-one counselling by a sleep physician regarding the results of their PSG studies, basic information on OSAHS, its known effects on comorbid conditions, proper sleep hygiene, adjunctive/conservative methods to improve sleep, and the importance of treatment adherence.

2.3. Questionnaires

2.3.1. IIEF. The IIEF is divided into five domains (erectile function, intercourse satisfaction, orgasm, sex drive, and overall satisfaction). The erectile function domain consists of six questions (questions 1 to 5 and question 15) and has a maximum score of 30 and a minimum score of 6. Each question is scored on a 5-point Likert scale, with 5 representing the best score. A score of less than 26 is indicative of ED [17].

2.3.2. ESS. The ESS is currently the most used subjective test of daytime sleepiness in clinical practice. It is a simple, self-administered, and eight-item questionnaire that measures the risk of falling asleep in eight situations that are commonly met. Total score >10 is considered excessive daytime sleepiness. The higher the score (from 10 to 24), the greater the reported subjective daytime sleepiness [18].

2.4. Polysomnography. All patients underwent a single-night full diagnostic PSG (Alice 4, 5, Diagnostics System, Respironics, USA) according to standard techniques, with monitoring of the electroencephalogram (EEG) using frontal, central, and occipital leads, electrooculogram (EOG),

electromyogram (EMG), flow (by oronasal thermistor and nasal air pressure transducer), thoracic and abdominal respiratory effort (induction plethysmography), oximetry, and body position. Snoring was recorded by a microphone placed on the anterior neck. Polysomnographic recordings were manually interpreted over 30-second periods, in accordance with the 2007 guidelines of the American Academy of Sleep Medicine (AASM) [19]. The scorer was always the same person, blinded to the clinical condition of the patients and the previous results of the questionnaires. The determination of sleep stages and arousals was performed according to the AASM 2007 criteria using EEG montages, including frontal, central, and occipital leads [19].

Apnea was defined as a cessation of airflow (≥90%) for at least 10 seconds, and hypopnea was defined as a ≥30% reduction of airflow (from the nasal pressure transducer signal) lasting at least 10 seconds with ≥4% desaturation. The Apnea-Hypopnea Index (AHI) was calculated as the number of apnea and hypopnea events per hour of sleep.

2.5. Blood Collection and Analysis. Shortly after the conclusion of the overnight sleep recordings, venous blood was collected from all subjects between 8:00 a.m. and 9:00 a.m., following an overnight fast, for measurement of hsCRP, TNF-a, IL-6, IL-8, and adiponectin. All venous samples were centrifuged within 30 minutes at 3000 rpm for 15 min, and serum was separated into multiple aliquots and stored at −80°C until analysis. hsCRP levels, expressed in mg/dL, were measured by particle-enhanced immunonephelometry using BN Systems (Dade Behring Inc.; Newark, USA). Quantitative measurements of TNF-a, IL-6, and IL-8 in serum were made using an automated chemiluminescence analyzer (Immulite 1000, DPC) with reagents from the same manufacturer. For these three markers, the results were expressed as pg/mL. Serum levels of adiponectin were determined using a quantitative sandwich enzyme immunoassay technique (Quantikine Human Adiponectin Immunoassay, R&D Systems), which measures total human adiponectin (low, middle, and high molecular weight forms). The results were expressed as ng/mL.

2.6. Statistical Analysis. Values are expressed as mean (SD) or median value with interquartile range (IQR), depending on the data distribution. Comparisons between groups were performed using the nonparametric Mann-Whitney U test. The degree of OSAHS, assessed by the AHI, in the whole group and separately in patients with OSAHS-ED and controls was correlated with the levels of CRP and other cytokines using Pearson's correlation analysis. $P < 0.05$ was considered the threshold for statistical significance. All statistics were calculated using the Statistical Package for Social Sciences software, version 17.0.0 (SPSS, Chicago, Illinois, USA).

3. Results

During the study period, 1034 patients underwent diagnostic PSG for a suspected sleep disorder. Of them, 404 patients volunteered to participate in this study and completed the IIEF Questionnaire. Most of the participants were middle aged (42.6 ± 9.3 years old) and moderately obese (BMI: 33.3 ± 5.05 Kg/m^2). The overall ED prevalence, based on IIEF score, was 40.9% (165 patients) of the study population. The patients with severe OSAHS had higher incidence of ED compared with those moderate or mild OSAHS patients (53.5% versus 27.1%; $P = 0.005$). The mean IIEF score was significantly lower in patients with severe OSAHS (23.84 ± 6.38 versus 26.1 ± 4.9, $P = 0.001$). Of the 404 patients, most of them were not eligible for further evaluation because of the presence of at least one of the exclusion criteria. Finally, 31 patients with severe OSAHS who suffered from ED (IIEF < 26) and met the inclusion criteria agreed to participate in the study and were available for analysis. Fifteen patients with normal erectile function, who had similar age, BMI, and AHI distributions to the study group, served as controls. Table 1 shows the baseline demographic and PSG characteristics of the ED patients and controls. Apart from their IIEF scores, there were no statistically significant differences between the two groups.

Table 1: Baseline clinical and PSG characteristics of controls and OSAHS patients with ED (OSAHS-ED).

	Controls ($n = 15$)	OSAHS-ED patients ($n = 31$)
Age (years)	47.9 ± 7.9	48.5 ± 8.55
IIEF	28.1 ± 1.2	14.35 ± 6.6*
BMI (kg/m^2)	32.6 ± 3.21	32.4 ± 3.74
AHI (events/h of sleep)	47.82 ± 23.56	48.09 ± 26.39
ODI	30 ± 18	46 ± 22
TST (min)	313 ± 95	274 ± 75
SE (%)	71 ± 16	81 ± 55
AI	42 ± 8	44 ± 17
NREM (min)	291 ± 91	255 ± 66
SWS (min)	25 ± 16	23 ± 10
REM (min)	24 ± 16	23 ± 13
Mean SaO_2 (%)	92.3 ± 3.87	92.5 ± 3.6
Minimum SaO_2 (%)	80.14 ± 8.91	79.32 ± 9.68

Values are mean ± (SD).
PSG: polysomnography; OSAHS: obstructive sleep apnea-hypopnea syndrome; ED: erectile dysfunction; IIEF: International Index of Erectile Function Questionnaire; BMI: body mass index; AHI: Apnea-Hypopnea Index; ODI: oxygen desaturation index; TST: total sleep time; SE: sleep efficiency; AI: Arousal Index; SaO_2: oxygen saturation; SWS: slow wave sleep.
*$P < 0.001$.

Patients with ED had significantly higher median (interquartile range (IQR)) plasma levels of hsCRP [0.32 (0.38) versus 0.1 (0.17) mg/dL, $P < 0.001$] than controls (Figure 1). TNF-a was significantly elevated in ED patients compared to controls [13.8 (5.8) versus 11.55 (2.2) pg/mL, $P = 0.01$] (Figure 2). Furthermore, significantly higher levels of IL-6 [4.38 (2.74) versus 2 (0.39) pg/mL, $P < 0.001$] (Figure 3) and IL-8 [8.29 (5.2) versus 4.98 (7.0) pg/mL, $P = 0.034$] were observed in ED patients (Figure 4).

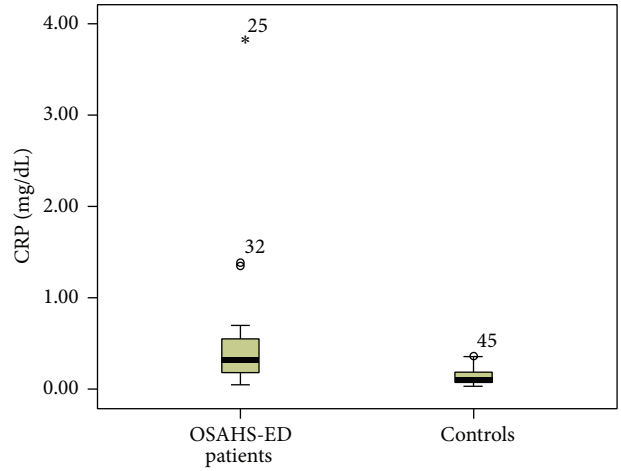

Figure 1: C-reactive protein (CRP) levels (mg/dL) are significantly higher in OSAHS patients with ED (OSAHS-ED) compared to controls ($P < 0.001$). CRP concentrations are shown as median (interquartile range) in the boxes.

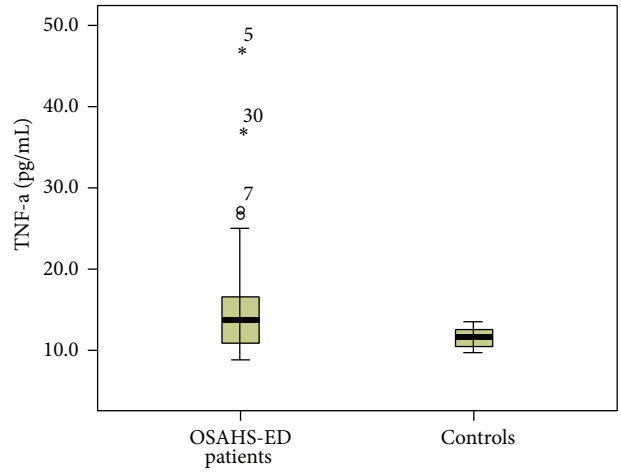

Figure 2: Tumor necrosis factor-α (TNF-a) levels (pg/mL) are significantly higher in OSAHS patients with ED (OSAHS-ED) compared to controls ($P = 0.01$). TNF-a concentrations are shown as median (interquartile range) in the boxes.

Adiponectin levels were lower in OSAHS-ED patients, but the difference did not reach statistical significance [4680.6 (3154) versus 4864.6 (8418) ng/mL, $P = 0.5$] (Figure 5).

The degree of OSAHS in the whole group assessed by the AHI showed a statistical significant correlation only with CRP values ($P = 0.013$ and $r = 0.52$) and not with the other measured cytokines. It is notable that the significant association between OSAHS severity and CRP was observed only in OSAHS patients with ED ($P < 0.001$, $r = 0.896$ and $P = 0.013$, $r = 0.52$, OSAHS patients with and without ED, resp.).

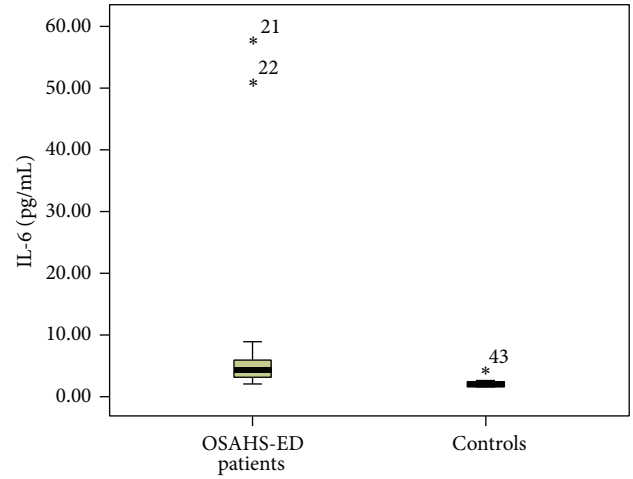

Figure 3: Interleukin-6 (IL-6) levels (pg/mL) are significantly higher in OSAHS patients with ED (OSAHS-ED) compared to controls ($P < 0.001$). IL-6 concentrations are shown as median (interquartile range) in the boxes.

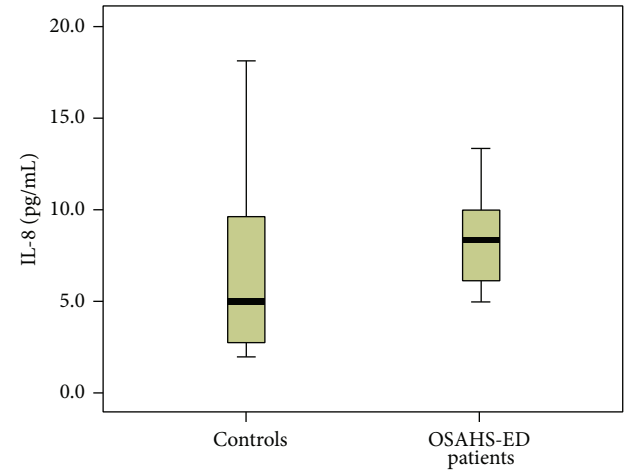

Figure 4: Interleukin-8 (IL-8) levels (pg/mL) are significantly higher in OSAHS patients with ED (OSAHS-ED) compared to controls ($P = 0.034$). IL-8 concentrations are shown as median (interquartile range) in the boxes.

4. Discussion

Sexual dysfunction represents a significant health problem and may have a strong negative impact on the quality of life. Therefore, the identification of potentially modifiable risk factors, such as OSAHS, may be important for disease prevention and treatment. Although ED is a frequent occurrence in male patients with OSAHS, the precise mechanisms mediating this morbidity are currently unknown.

The present study is the first to investigate low-grade inflammation and an altered endothelial state in patients with ED and severe OSAHS by evaluating a wide spectrum of circulating markers and mediators. With a view to explaining the association between ED and OSAHS, patient selection was strict enough to exclude the presence of other

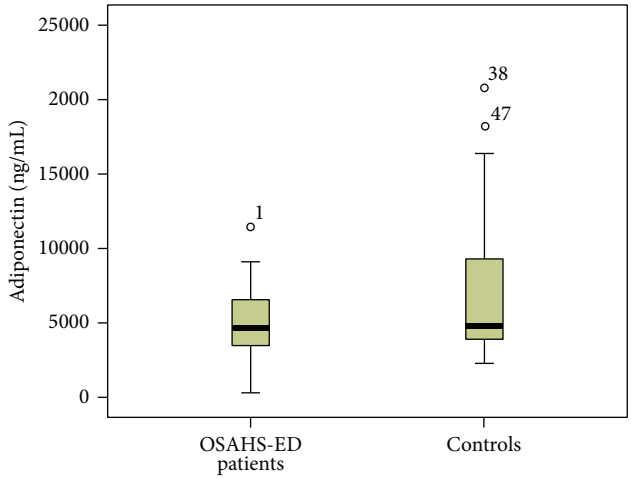

Figure 5: Adiponectin levels (ng/mL) were lower in OSAHS patients with ED (OSAHS-ED) compared to controls, but the difference did not reach statistical significance ($P = 0.5$). Adiponectin concentrations are shown as median (interquartile range) in the boxes.

comorbidities that are also known as risk factors for ED. Our results showed that the combination of ED and OSAHS was associated with higher levels of inflammatory markers compared to OSAHS alone.

Previous studies have shown that ED could be a disease of low-grade inflammation [20, 21]. Vlachopoulos et al. [21] demonstrated an increase in the levels of inflammatory markers such as hsCRP, IL-6, IL-1β, and TNF-a in patients with ED, suggesting that low-grade systemic inflammation is present in these subjects, similar to that seen in insulin resistance, obesity, type 2 diabetes mellitus, hypertension, hyperlipidaemia, and metabolic syndrome X. Moreover, other investigators reported that ED was associated with increased levels of TNF-a and CRP, which increased progressively with the severity of penile vascular disease, supporting the role of these markers in the pathophysiology of ED [22, 23]. Furthermore, recently, Matos et al. showed that there was an association between TNF-a levels and ED complaints in men independent of OSAHS [24]. It is worth noting that, in a recent study, reduced levels of adiponectin, an anti-inflammatory cytokine that attenuates endothelial cell adhesion molecules, and the levels of inflammatory cytokines, such as TNF-a, IL-8, and IL-6, were observed in patients with ED [25].

There are several explanations regarding why this link between ED and OSAHS exists, implicating hormonal, neural, and endothelial mechanisms. An important link connecting ED and OSAHS is endothelial dysfunction. Patients with OSAHS tend to have lower levels of nitric oxide (NO), which is responsible for vasodilatation and erection [26]. NO suppresses the production of TNF-a and IL-1. Similarly, oxidative stress, one of the promoters of endothelial dysfunction, is enhanced in OSAHS, promoting ED. The combination of repetitive hypoxemia and sleep deprivation in OSAHS patients may be associated with NO deficiency, resulting in increased levels of well-known inflammatory cytokines, such as IL-6, IL-8, adhesion molecules, and hsCRP [27, 28]. Increased expression of these inflammatory cytokines may contribute to endothelial dysfunction, which may cause ED [14]. Indeed, a number of studies have demonstrated a causal relationship between OSAHS and endothelial dysfunction, which was improved or not by CPAP treatment [16, 29, 30]. A chronic hypoxic condition also contributes to low levels of adiponectin; however, there are conflicting opinions regarding adiponectin levels in patients with OSAHS. Although a few studies reported that adiponectin was more strongly correlated with AHI in patients who had OSAHS, compared with various other factors such as age and obesity [31–34], other studies have reported that adiponectin levels were largely unaffected by the syndrome [35–37].

The elevated markers of endothelial damage and decreased anti-inflammatory adiponectin found in our study underline the involvement of endothelial dysfunction in the pathogenesis of ED, which also comprises a pathophysiological link between this entity and OSAHS. Considering OSAHS as a chronic disease state, it must play a role in the development of ED, leading to subsequent overall dissatisfaction with sexual function. However, as the two groups of patients were matched for severity of OSAHS, one could argue why one group would experience the elevated cytokine levels and ED while the other group was spared. Possibly, the disparity in responses among the two groups is based on the heterogeneity of the magnitude of end-organ morbidity in sleep apnea among patients and shows that not everyone will be affected to the same extent. Furthermore, the observation that treatment for sleep apnea restores erectile function argues for a direct role of OSAHS in the pathogenesis of ED [38, 39]. Therefore, OSAHS could be considered as a sensitive predictor for ED. These novel findings clearly warrant further research aimed at defining the roles of inflammatory markers and associated factors, such as testosterone levels and hormonal disorders, in subjects who are at high risk of ED in the early detection of low-grade systemic inflammation and in the prevention, prediction, and prognosis of ED. Subsequently, larger scale studies should be conducted and performed including subjects with comorbidities and various age groups. Screening and multidisciplinary approach must be adopted in patients at risk of ED, and there is a need not only for clinical, but also for public health interventions.

The present study had some limitations that deserve comment. Firstly, the analysis was conducted on a small population and not based on a power calculation, depriving the ability to conduct more robust statistical methods. This was due to the difficulty of including only middle-aged patients with newly diagnosed OSAHS who had no comorbidities and were not under treatment with cardiovascular medication. In addition, a high refusal rate limited the patients' participation and may have been related to embarrassment at reporting such symptoms. Secondly, ED was assessed based exclusively on the IIEF Questionnaire, a self-reported, subjective estimation of erectile function in men, and not on objective measures, such as nocturnal penile tumescence, testosterone measurements. However,

this questionnaire has been validated by multiple studies for evaluating erectile function in various patient populations and has been shown to be clinically appropriate for the evaluation of erectile function; therefore, it can serve as an indirect indicator of ED. Thirdly, we included only patients with severe OSAHS, so further studies are necessary in order to investigate possible relations between ED and cytokine profile in OSA patients with moderate or even mild disease. Finally, patients with other additional risk factors for ED were excluded, which does not represent well the situation found in clinical practice. However, as per Huang et al., subclinical endothelial dysfunction may underlie organic ED in young patients without well-known related risk factors [40].

5. Conclusions

In conclusion, our results showed that the presence of ED in patients with severe OSAHS is associated with higher levels of inflammatory markers and lower levels of an anti-inflammatory marker, adiponectin, compared to patients with OSAHS of the same severity but without ED. The increased markers of endothelial damage underline a possible involvement of endothelial dysfunction in the pathogenesis of ED.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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