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SCIENTIFIC INVESTIGATIONS

Adropin and Inflammation Biomarker Levels in Male Patients With Obstructive Sleep Apnea: A Link With Glucose Metabolism and Sleep Parameters

Josko Bozic, MD, PhD[1,2]; Josip A. Borovac, MD, BSc[1]; Tea Galic, DMD, PhD[2,3]; Tina Ticinovic Kurir, MD, PhD[1]; Daniela Supe-Domic, PhD[4]; Zoran Dogas, MD, PhD[2,5,6]

[1]Department of Pathophysiology, University of Split School of Medicine, Split, Croatia; [2]Department of Neuroscience, University of Split School of Medicine, Split, Croatia; [3]Study of Dental Medicine, University of Split School of Medicine, Split, Croatia; [4]Department of Medical Laboratory Diagnostics, University Hospital of Split, Split, Croatia; [5]Sleep Medicine Center, University of Split School of Medicine, Split, Croatia; [6]University Hospital of Split, Split, Croatia

Study Objectives: The main objectives of the study were to determine plasma adropin, systemic inflammation biomarker levels, and glucose metabolism parameters in patients with moderate and severe obstructive sleep apnea (OSA) compared to healthy controls.

Methods: In this study, we included 50 male patients with OSA (25 moderate and 25 severe) and 25 age- and sex-matched control subjects. All subjects underwent fasting sampling of peripheral blood for laboratory analyses.

Results: Adropin plasma levels were significantly lower in the severe OSA group in comparison with the moderate and control groups (4.50 ± 1.45 versus 6.55 ± 1.68 versus 8.15 ± 1.79 ng/mL, $P < .001$). Plasma biomarkers of systemic inflammation were significantly increased in patients with moderate OSA (interleukin [IL]-6 and tumor necrosis factor alpha [TNF-α]) and severe OSA (IL-6, TNF-α, high-sensitivity C-reactive protein) when compared with controls ($P < .001$). Adropin levels showed a significant negative correlation with IL-6 ($r = −.419$, $P < .001$), TNF-α ($r = −.540$, $P < .001$), fasting glucose ($r = −.331$, $P = .004$), hemoglobin A1c ($r = −.438$, $P < .001$), homeostatic model assessment insulin resistance index ($r = −.213$, $P = .046$), and polysomnographic parameters including apnea-hypopnea index ($r = −.615$, $P < .001$) and oxygen desaturation index ($r = −.573$, $P < .001$). A multivariate regression analysis showed that plasma adropin remained as a significant negative predictor of severe OSA status, when adjusted for age and body mass index and computed along with other inflammatory biomarkers in the regression model (odds ratio 0.069, 95% confidence interval 0.009–0.517, $P = .009$).

Conclusions: Plasma adropin concentrations significantly correlate with indices of disease severity in patients with OSA, suggesting that adropin potentially plays an important role in the complex pathophysiology of the disease.

Keywords: adropin, inflammation mediators, metabolism, obstructive sleep apnea, polysomnography

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BRIEF SUMMARY

Current Knowledge/Study Rationale: Obstructive sleep apnea (OSA) is a complex sleep disorder that is marked by cardiometabolic, autonomic, and inflammatory dysregulation. Adropin is a novel biopeptide that is implicated in energy homeostasis regulation and protective cardiometabolic activity. The rationale of this study was, therefore, to investigate plasma adropin levels among patients with OSA in comparison with healthy controls.

Study Impact: This study demonstrated that adropin levels in plasma were decreased in patients with OSA in comparison with controls and reflected disease severity. Moreover, adropin showed a good discriminatory power for OSA status and a significant negative correlation with certain indices of systemic inflammation, glucose metabolism, and polysomnographic parameters of sleep, suggesting its pathophysiological link with multiple dimensions of this complex disorder.

INTRODUCTION

Obstructive sleep apnea (OSA) is a common breathing disorder that occurs during sleep and is caused by partial or complete obstruction of the upper airway.[1] OSA is characterized by chronic hemodynamic and metabolic supply/demand mismatch and each episode of airway occlusion is responsible for induction of adverse hemodynamic, biochemical and autonomic events.[2] In a multitude of studies, associations between OSA and cardiometabolic and vascular dysregulation have been established.[3–5] OSA has been independently associated with hypertension, increased arterial stiffness, hypothalamic-pituitary-adrenal (HPA) dysregulation, inflammation, endothelial dysfunction, alterations in glucose metabolism, and cognitive and psychomotor impairment.[6–11]

A relationship between glucose metabolism and adropin, a novel peptide hormone, is of particular interest in this study. Adropin is highly conserved among different mammalian species and is encoded by the Energy Homeostasis Associated gene (*Enho*) that is expressed in many organs and tissues such as pancreas, liver, hypothalamus, kidney, endocardium, myocardium, and epicardium.[12,13] Adropin is also present in breast milk and maternal and cord blood during pregnancy.[14,15] Moreover, this recently described hormone is implicated in energy

homeostasis, chiefly through interaction with glucose and lipid metabolism.[12] Adropin deficiency has been associated with increased adiposity and insulin resistance.[16] A study performed on rats with hyperlipidemia showed that low-dose administration of adropin ameliorated lipid metabolism, reduced insulin resistance, and inhibited hepatocyte inflammation.[17] In terms of cardiovascular effects, recent studies reported significant associations of adropin with endothelial dysfunction and heart failure, whereas its ability to regulate blood pressure has been widely hypothesized.[18–21] The protective role of adropin on vascular function was achieved through increased endothelial nitric oxide (NO) bioavailability by upregulating the endothelial NO synthase (eNOS) expression through the vascular endothelial growth factor receptor-2 (VEGFR2) signaling pathways.[22] In a clinical setting, adropin plasma levels were significantly decreased among hypertensive individuals in comparison with controls, suggesting that adropin plasma level is an independent predictor of hypertension.[23]

Another intriguing relationship worth examining is the association between plasma adropin levels and inflammation biomarkers such as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and high-sensitivity C-reactive protein (hsCRP). The results obtained from the study that examined women with polycystic ovary syndrome demonstrated an inverse relationship between serum adropin levels and TNF-α, whereas the analysis showed that the homeostatic model assessment insulin resistance index (HOMA-IR) and TNF-α concentration both independently predicted adropin levels in plasma.[24] Similarly, a study conducted among obese children with OSA showed that plasma adropin levels significantly increased while systemic inflammatory and metabolic biomarkers significantly decreased after adenotonsillectomy was performed.[25] Some authors suggest that adropin could decrease messenger RNA expression of proinflammatory cytokines such as TNF-α and IL-6 by regulating the expression of inducible nitric oxide synthase (iNOS).[17] Keeping in mind that parameters of systemic inflammation are significantly increased in OSA and hypertensive subjects when compared to control groups, the role of adropin might be implicated in biological mediation of this pathophysiological relationship.[26,27]

Therefore, the main goals of this study were to determine plasma adropin levels in an adult male population of patients with moderate and severe OSA in comparison with controls. Additionally, we wanted to assess the relationship between adropin and inflammatory biomarkers (IL-6, TNF-α, hsCRP) in plasma along with parameters of sleep and glucose metabolism in OSA patients and a respective control group.

METHODS

The Ethics Committee of the University of Split School of Medicine approved the study. All procedures performed in this study were in accordance with the ethical standards of the institutional Ethics Committee and with the 1964 Declaration of Helsinki and its revision from 2008. Informed written consent was obtained from all participants included in the study.

Subjects

This study included male subjects with newly diagnosed OSA who were enrolled consecutively at the Sleep Medicine Center (Split, Croatia), between June 2013 and December 2014. The diagnosis of OSA was established based on the relevant clinical practice recommendations for the diagnosis of OSA in adults.[28] The apnea-hypopnea index (AHI) is an established index that reflects the severity of sleep apnea and is derived from the number of apnea and hypopnea events that occur per 1 hour of sleep.[29] Therefore, according to the severity of OSA, our participants were stratified into two groups: moderate OSA group (AHI 15–30 events/h, 25 patients) and severe OSA group (AHI > 30 events/h, 25 patients). Exclusion criteria were: (1) diagnosed diabetes mellitus, severe cardiovascular, neurological, psychiatric, respiratory, or renal disease, systemic autoimmune or inflammatory disease, acute or chronic immunocompromised state and active malignant disease; (2) regular use of drugs that could interfere with glucose metabolism or HPA axis, sedatives or narcotics, alcohol and drug abuse; (3) medical history of any OSA treatment prior to the study enrollment; (4) female patients.

We screened 34 healthy volunteers of whom 25 were prospectively included in the study as controls after applying exclusion criteria and obtaining results from sleep-related questionnaires. The control group consisting of 25 healthy male volunteers was matched with the group of patients with OSA for age and body mass index (BMI). The Snoring, Tiredness, Observed apnea and high blood Pressure (STOP) questionnaire was used as a screening tool for identification of subjects with a high risk of OSA development; therefore, subjects who scored ≥ 2 points on this questionnaire were excluded from the study.[30] Additionally, the Epworth Sleepiness Scale (ESS) for the evaluation of daytime sleepiness in common daily situations was used, excluding subjects who had an ESS score > 9. Polysomnography (PSG) assessment was not performed for the control subjects. The control group had the same exclusion criteria and assessment protocol as moderate and severe OSA patient groups.

After a detailed medical interview and physical examination, anthropometric measurements were performed for all the subjects included in the study. The calibrated scale was used for body weight and height measurement (Seca, Birmingham, United Kingdom). BMI was calculated as body weight (kg) divided by height squared (m^2). The midpoint between the inferior tip of the ribcage and the superior aspect of the iliac crest was used for waist circumference measuring, while subjects were standing upright. At the midpoint of the neck, between the mid-cervical spine and midanterior neck, was the place for measuring neck circumference, while subjects were standing upright. Arterial blood pressure was measured via sphygmomanometer with patients in a sitting position, at least twice between 8:00 and 9:00 AM, after 10 minutes of rest.

Sleep Assessment

All patients with OSA underwent full-night attended PSG at the Sleep Medicine Center during which the following measurements were continuously recorded: electrooculography, electroencephalography, mental and tibial electromyography,

electrocardiography, nasal airflow, pulse oximetry, thoracic and abdominal movements, and snoring intensity (Alice 5LE, Philips Respironics, Eindhoven, Netherlands). All data were stored on a personal computer and manually analyzed in accordance with the published American Academy of Sleep Medicine and European Sleep Research Society guidelines.[31–33] The validated,[30] Croatian language version of the ESS was used to measure excessive daytime sleepiness.

According to the guidelines, apnea was defined as a complete cessation of airflow for at least 10 seconds, whereas in hypopnea airflow is decreased by more than 50% for at least 10 seconds, in combination with a reduction in hemoglobin oxygen saturation of at least 3%.[31–33] Full-night PSG measurements that lasted fewer than 6 hours were not accepted, and in such cases, another sleep study was undertaken.

Blood Sampling and Laboratory Analysis

Patients underwent laboratory analysis 7 to 14 days after the sleep studies were performed. No treatment for OSA was administered to any patient in the period between PSG and blood testing. Venous blood samples were taken at 8:00 AM through a polyethylene catheter inserted into a forearm vein after fasting for 12 hours. All blood samples were analyzed in the same biochemical laboratory and by the same specialist in medical biochemistry, following standard laboratory procedures. Furthermore, the biochemist was highly experienced and was blinded to the subject's assignment in the study groups. Fasting plasma insulin levels were determined by electrochemiluminescence immunoassay (ECLIA) method (Roche Diagnostics GmbH, Mannheim, Germany). Fasting plasma glucose was analyzed using photometry with hexokinase method (Abbott, Chicago, Illinois, United States) and hemoglobin A1c (HbA1c) levels were measured by turbidimetric inhibition immunoassay (Roche Diagnostics GmbH, Mannheim, Germany).

Adropin (Phoenix Pharmaceuticals, Phoenix, Arizona, United States) and IL-6 (Roche Diagnostics GmbH, Mannheim, Germany) plasma levels were determined using an enzyme-linked immunosorbent assay test. TNF-α (Nal Von Minden Diagnostics GmbH, Regensburg, Germany) plasma levels were determined by ECLIA whereas the latex turbidimetric method was used for hsCRP (Abbott Laboratories, Chicago, Illinois, United States) level determination in plasma. Other laboratory measurements were performed using routine laboratory methods. Furthermore, insulin resistance was measured by the homeostatic model assessment index of insulin resistance (HOMA-IR). HOMA-IR is calculated as the product of the fasting serum insulin concentration (mU/L) and fasting plasma glucose concentration (mmol/L), divided by 22.5.[34]

Statistical Analysis

All data analyses were performed using IBM SPSS Statistics for Windows, version 23.0 (IBM Corp, Armonk, New York, United States). Continuous data were shown as means ± standard deviation (SD) whereas categorical variables were presented as whole numbers and percentages. Normality of data distribution was measured with the Kolmogorov-Smirnov test. The *t* test was used for measuring differences in PSG findings between moderate and severe OSA groups. The differences in parameters of glucose metabolism, adropin, mediators of systemic inflammation (TNF-α, IL-6, hsCRP), and other biochemical parameters between groups were analyzed using one-way analysis of variance (ANOVA) with *post hoc* Tukey Honestly Significant Difference test. A multivariate regression analysis with forward selection algorithm was used to determine a relationship between selected independent variables (plasma levels of adropin and mediators of systemic inflammation) with the probability of having a moderate and severe OSA status. A reported regression model was adjusted for age and BMI and inspected for goodness of fit by the Hosmer-Lemeshow test and χ^2 overall model fit. Adjusted odds ratios (OR), statistical significance (*P*), and 95% confidence interval (95% CI) were reported for regression analysis. Furthermore, Pearson correlation coefficients were used for evaluation of correlations of adropin levels in plasma with polysomnographic and glucose metabolism parameters. Finally, receiver operating characteristic (ROC) analysis was used to determine sensitivity and specificity of plasma adropin in the detection of OSA status. Area under the curve (AUC), *P*, standard error, and 95% CI were reported for respective ROC analysis. The statistical significance reported at all instances in provided data was 2-tailed, set at $P < .05$ level.

RESULTS

Patients' Baseline Characteristics

There were no significant differences among patients with OSA and control subjects in baseline anthropometric characteristics, except for waist circumference (109.16 ± 11.82 versus 101.72 ± 7.57 versus 103.48 ± 6.83 cm, $P = .013$) and neck circumference (45.58 ± 2.97 versus 42.80 ± 3.05 versus 39.68 ± 1.72 cm, $P < .001$) (**Table 1**).

The analyses of PSG data are presented in **Table 2**. Patients with moderate OSA had significantly lower AHI (21.69 ± 3.92 versus 48.26 ± 18.10 events/h, $P < .001$) and oxygen desaturation index (ODI) (19.66 ± 4.54 versus 46.68 ± 17.22 events/h, $P < .001$), whereas mean and minimum oxygen saturation were significantly higher in the moderate OSA group. In addition, there were no significant differences in excessive daytime somnolence measured by the ESS between groups.

There were significant differences in triglycerides plasma levels between control subjects and patients with moderate OSA in comparison with the patients with severe OSA (2.10 ± 1.32 versus 1.41 ± 0.74 versus 1.40 ± 0.63 mmol/L, $P = .017$) (**Table 1**).

Glucose Metabolism Parameters and Inflammatory Biomarkers

Glucose metabolism parameters of patients with OSA and control subjects are presented in **Table 1**. Fasting plasma glucose levels in patients with severe OSA were significantly higher than in patients with moderate OSA and the control group (5.51 ± 0.67 versus 5.08 ± 0.54 versus 4.83 ± 0.58 mmol/L, $P = .001$), as well as fasting plasma insulin levels (116.76 ± 87.56 versus 79.06 ± 58.80 versus 72.65 ± 43.13 pmol/L, $P = .043$), respectively, whereas patients with moderate OSA did not differ significantly from the control subjects. Additionally,

Table 1—Baseline characteristics of patients with OSA and control subjects.

Parameter	Control Group (n = 25)	Moderate OSA (n = 25)	Severe OSA (n = 25)	P*
Age (years)	52.52 ± 10.18	53.92 ± 10.75	52.04 ± 13.11	.833
Body weight (kg)	94.64 ± 7.18	92.88 ± 8.59	97.52 ± 10.89	.193
Body height (cm)	184.68 ± 4.80	180.92 ± 6.89	182.40 ± 7.24	.120
BMI (kg/m^2)	27.78 ± 2.23	28.42 ± 2.57	29.30 ± 2.74	.110
Systolic blood pressure (mmHg)	132 ± 9.24	133 ± 14.14	134 ± 12.66	.845
Diastolic blood pressure (mmHg)	85.6 ± 6.17	85 ± 6.77	85.6 ± 6.97	.934
Waist circumference (cm)	103.48 ± 6.83	101.72 ± 7.57 [b]	109.16 ± 11.82 [a]	.013
Neck circumference (cm)	39.68 ± 1.72	42.80 ± 3.05 [a,b]	45.58 ± 2.97 [a]	< .001
Urea (mmol/L)	6.13 ± 1.14	6.02 ± 0.99	6.11 ± 1.31	.943
Creatinine (μmol/L)	86.44 ± 12.15	89.44 ± 14.04	88.12 ± 18.13	.778
Uric acid (μmol/L)	361.96 ± 64.18	347.84 ± 67.88	374.76 ± 80.75	.414
Triglycerides (mmol/L)	1.40 ± 0.63	1.41 ± 0.74	2.10 ± 1.32 [a,c]	.017
Total cholesterol (mmol/L)	5.76 ± 0.66	5.79 ± 1.25	5.98 ± 1.21	.736
HDL cholesterol (mmol/L)	1.48 ± 0.32	1.37 ± 0.29 [b]	1.22 ± 0.24 [a]	.010
LDL cholesterol (mmol/L)	3.56 ± 0.75	3.70 ± 1.11	3.93 ± 1.03	.407
HbA1c (%)	5.29 ± 0.15	5.44 ± 0.25 [b]	5.79 ± 0.37 [a]	< .001
Fasting plasma glucose (mmol/L)	4.83 ± 0.58	5.08 ± 0.54 [b]	5.51 ± 0.67 [a]	.001
Fasting plasma insulin (pmol/L)	72.65 ± 43.13	79.06 ± 58.80 [b]	116.76 ± 87.56 [a]	.043
HOMA-IR	2.21 ± 1.34	2.63 ± 2.05 [b]	4.33 ± 3.63 [a]	.010

Data are presented as mean ± standard deviation. * = one-way ANOVA with *post hoc* Tukey Honestly Significant Difference test. Superscript letters indicate: a = comparison with control group ($P < .05$), b = comparison with severe OSA group ($P < .05$), c = comparison with moderate OSA group ($P < .05$). BMI = body mass index, HbA1c = glycated hemoglobin, HDL = high-density lipoprotein, HOMA-IR = homeostasis model assessment of insulin resistance, LDL = low-density lipoprotein, OSA = obstructive sleep apnea.

Table 2—PSG data of patients with moderate and severe OSA.

Parameter	Moderate OSA	Severe OSA	P*
AHI (events/h)	21.69 ± 3.92	48.26 ± 18.10	< .001
ODI (events/h)	19.66 ± 4.54	46.68 ± 17.22	< .001
Mean SpO_2 (%)	94.60 ± 1.97	92.52 ± 3.51	.025
Lowest SpO_2 (%)	83.76 ± 5.23	71.96 ± 13.04	< .001
TST (hours)	5.75 ± 1.67	6.8 ± 1.22	.014
Central apnea †	6.40 ± 9.51	23.32 ± 13.71	.001
Obstructive apnea †	56.28 ± 42.03	146.92 ± 105.35	.001
Mixed apnea †	6.40 ± 8.63	58.68 ± 35.38	< .001
Hypopnea †	79.40 ± 41.43	103.24 ± 73.83	.005

Data are presented as mean ± standard deviation. * = Student *t* test for independent samples. † = number of events per total sleep time. AHI = apnea-hypopnea index, SpO_2 = arterial oxygen saturation, ODI = oxygen desaturation index, OSA = obstructive sleep apnea, PSG = polysomnography, TST = total sleep time.

HOMA-IR was significantly higher in patients with severe OSA in comparison to patients with moderate OSA and control subjects (4.33 ± 3.63 versus 2.63 ± 2.05 versus 2.21 ± 1.34, $P = .010$) (**Table 1**).

Inflammatory biomarkers were significantly higher in patients with severe OSA in comparison with patients with moderate OSA and the control group: IL-6 (3.58 ± 1.55 versus 2.62 ± 0.71 versus 1.26 ± 0.69 pg/mL, $P < .001$) and TNF-α (8.67 ± 2.41 versus 5.79 ± 1.44 versus 2.35 ± 1.25 pg/mL, $P < .001$). Furthermore, plasma levels of hsCRP were also significantly higher in patients with severe OSA in comparison with controls, but without significant differences between other groups (**Figure 1**).

Adropin Plasma Levels in Patients With OSA and Control Subjects

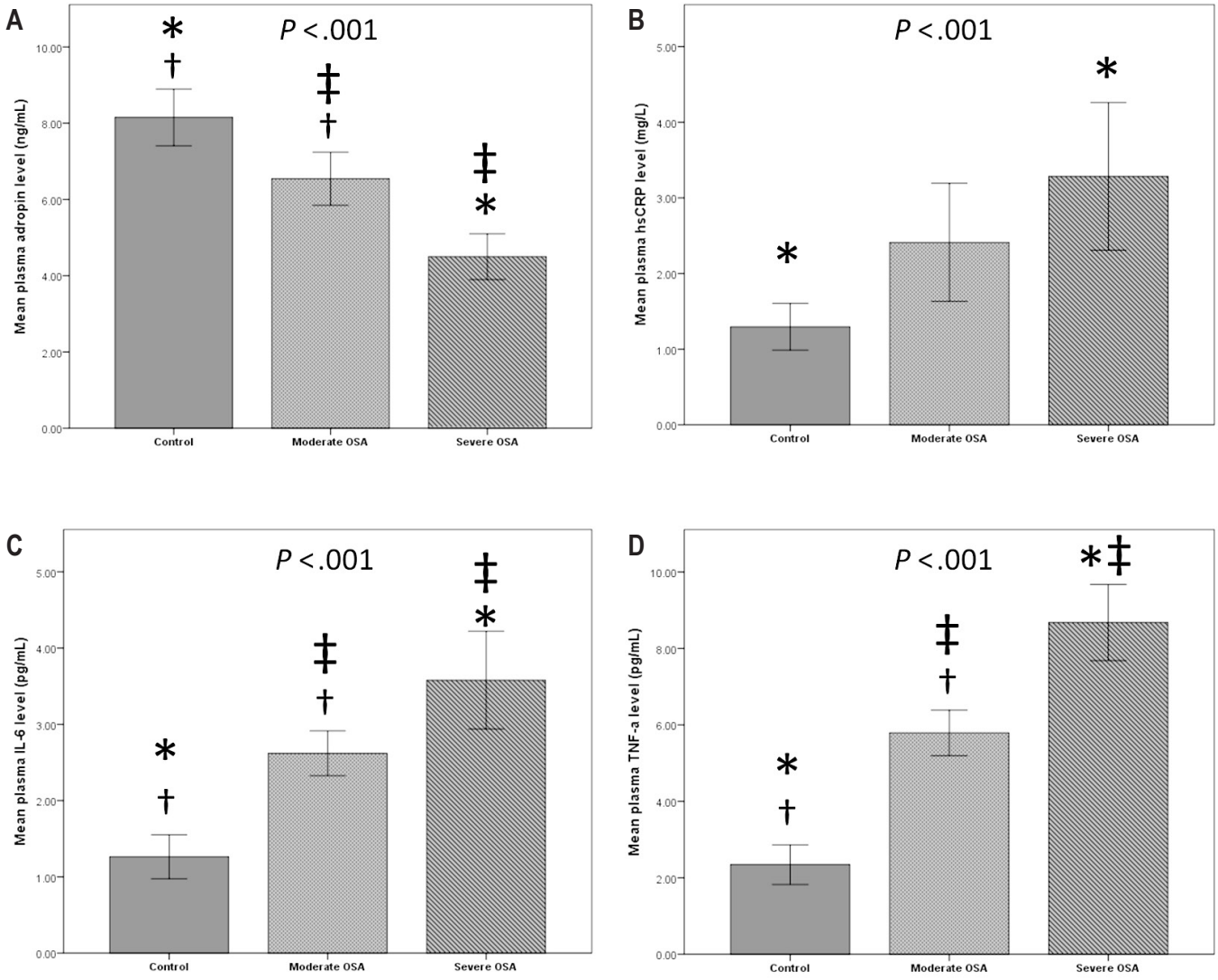
Adropin plasma levels were significantly lower in patients with severe OSA in comparison with the moderate OSA and control groups (4.50 ± 1.45 versus 6.55 ± 1.68 versus 8.15 ± 1.79 ng/mL, $P < .001$) (**Figure 1**).

Linear regression models were constructed in the 50 patients with OSA to examine the independent association of OSA severity and plasma adropin levels. After adjusting for age and a measure of adiposity (either BMI, waist circumference, or neck circumference), increasing AHI was independently associated with lower plasma adropin levels ($P < .001$). Similar results were obtained when AHI was replaced by ODI.

Among all included patients with OSA (both moderate and severe), plasma adropin levels were in significant negative correlation with plasma levels of IL-6 ($r = -.419$, $P < .001$) and TNF-α ($r = -.540$, $P < .001$). Contrary to these findings, the significant correlation between plasma adropin and hsCRP levels was not established ($r = -.182$, $P = .119$) (**Table 3**).

In addition, plasma adropin levels were in significant negative correlation with fasting glucose plasma levels ($r = -.331$, $P = .004$) and HbA1c ($r = -.438$, $P < .001$). Moreover, a significant negative correlation was established between adropin plasma levels and HOMA-IR ($r = -.213$, $P = .046$). There was no significant correlation established between plasma

Figure 1—Averaged plasma levels between OSA groups and controls.



(A) Adropin, **(B)** high-sensitivity C-reactive protein (hsCRP), **(C)** interleukin 6 (IL-6), and **(D)** tumor necrosis factor alpha (TNF-α). Data are presented as mean ± standard deviation with respective significance value (*P*). Tested with one-way analysis of variance (ANOVA) with *post hoc* Tukey Honestly Significant Difference test. *P* value at each graph represents the global one-way ANOVA significance whereas respective symbols represent *post hoc* statistical significance obtained when examining differences between particular groups. * = $P < .05$ between patients with severe obstructive sleep apnea (OSA) and controls. † = $P < .05$ between patients with moderate OSA and controls. ‡ = $P < .05$ between patients with moderate and severe OSA.

adropin levels and fasting plasma insulin ($r = -.173$, $P = .137$) (**Table 3**).

Regarding the PSG parameters, plasma adropin levels were in significant negative correlation with AHI ($r = -.615$, $P < .001$) (**Figure 2A**) and ODI ($r = -.573$, $P < .001$) (**Figure 2B**).

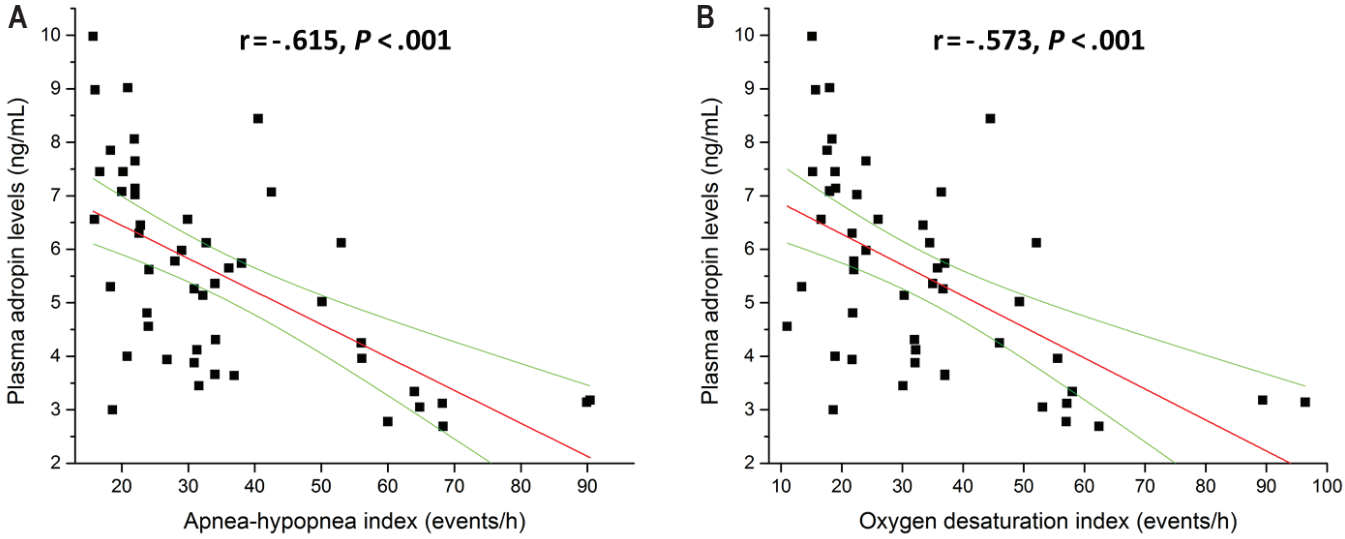
The ROC analysis (**Figure 3**) showed that AUC for plasma adropin levels in excluding OSA status was 0.831 ($P < .001$, standard error 0.048, 95% CI 0.737–0.925). Plasma adropin cutoff value less than 6.56 ng/mL provided 72% sensitivity and 80% specificity for the detection of positive OSA status.

A multivariate regression analysis showed that plasma adropin remained as a significant negative predictor of severe OSA status, when adjusted for age and BMI and computed along with other inflammatory biomarkers in the regression model (OR 0.069, 95% CI 0.009–0.517, $P = .009$) (**Table 4**). Furthermore, TNF-α was a significant positive predictor of severe OSA status (OR 24.864, 95% CI 1.338–46.194, $P = .031$). Other inflammatory biomarkers (IL-6 and hsCRP) were not found to be significant predictors in both regression models for moderate and severe OSA (**Table 4**).

DISCUSSION

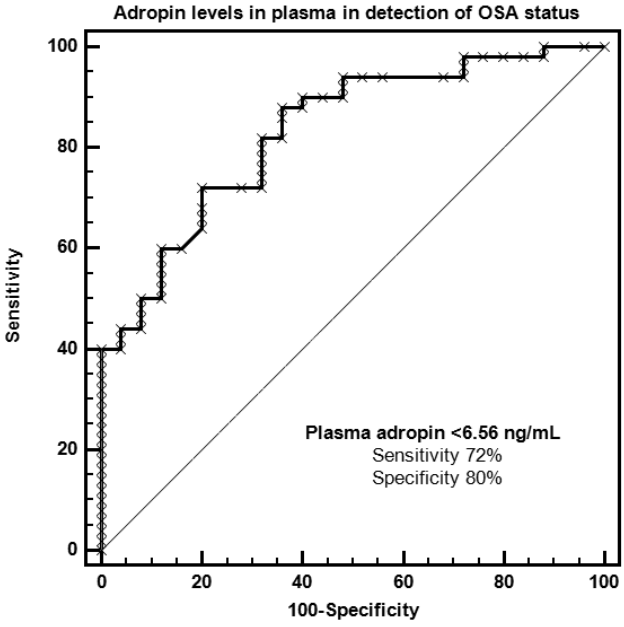
The results of this study showed that mean plasma adropin levels were lowest in patients with severe OSA, followed by the patients with moderate OSA, and the highest adropin levels in plasma were measured in healthy volunteers. This is complementary to results of a study performed among a pediatric OSA population where plasma adropin concentrations were

Figure 2—Correlations in composite of moderate and severe OSA patient groups (n = 50).



Correlations between plasma adropin levels and **(A)** apnea-hypopnea index, **(B)** oxygen desaturation index in composite of moderate and severe OSA patient groups. Red lines represent Pearson correlation coefficient and green lines represent respective 95% confidence intervals. OSA = obstructive sleep apnea.

Figure 3—Receiver operating characteristic analysis of adropin cutoff value in detection of OSA status.



OSA = obstructive sleep apnea.

Table 3—Correlations between plasma adropin levels, inflammation biomarkers, and glucose metabolism parameters in patients with moderate and severe OSA.

	Plasma Adropin Level (ng/mL)	
Parameter	***r***	***P****
Inflammation biomarkers		
TNF-α (pg/mL)	−.540	< .001
IL-6 (pg/mL)	−.419	< .001
hsCRP (mg/L)	−.182	.119
Glucose metabolism		
HOMA-IR	−.213	.046
Fasting plasma glucose (mmol/L)	−.331	.004
Fasting plasma insulin (pmol/L)	−.173	.137
HbA1c (%)	−.438	< .001

* = all *P* values are 2-tailed, significance level is set at $P < .05$. AHI = apnea-hypopnea index, HbA1c = glycated hemoglobin in plasma, HOMA-IR = homeostatic model assessment of insulin resistance, hsCRP = high-sensitivity C-reactive protein, IL-6 = interleukin 6, ODI = oxygen desaturation index, *r* = Pearson correlation coefficient, TNF-α = tumor necrosis factor alpha.

reduced in pediatric patients with OSA, especially when associated with endothelial dysfunction, compared with matched controls.[6] Moreover, adenotonsillectomy in children with OSA and endothelial dysfunction increased mean adropin levels and not in children with OSA without endothelial dysfunction. Similarly, a subsequent study by Kheirandish-Gozal et al. demonstrated that among obese children with OSA who underwent adenotonsillectomy, a significant decrease in proinflammatory cytokines such as IL-6 and IL-18 and adverse metabolic parameters in plasma were achieved while exhibiting a concomitant significant increase in plasma adropin levels.[25] It can be inferred that endothelial dysfunction is an important constituent in the adropin homeostasis. Our results showed that plasma levels of inflammatory biomarkers such as hsCRP, IL-6, and TNF-α were significantly increased in patients with moderate and severe OSA in comparison with the control group, thus reinforcing the concept that sleep disorders and obesity impose a significant inflammation burden on the affected individuals.

An important meta-analysis by Nadeem and colleagues showed that the levels of systemic inflammatory markers were higher in patients with OSA compared to healthy subjects.[27] Interestingly, a recent study showed that blood biomarkers such

as HbA1c, CRP, IL-6, uric acid, and erythropoietin were superior to ESS questionnaire in the initial screening for OSA.[35] Similarly, a study by Araújo et al. demonstrated that hsCRP and glucose levels were significantly increased in patients with OSA in comparison with the control group.[36] However, as far as we know, the role of adropin in plasma, especially in relation to established inflammatory biomarkers and parameters of sleep and glucose metabolism, has not been previously described for adult patients with OSA.

It has been well established that intermittent hypoxia, a hallmark feature of OSA, is responsible for upregulation of inflammatory pathways that lead to cardiovascular and metabolic disturbances in individuals with OSA. In line with this, a study by Thünstrom et al. showed that OSA with ODI ≥ 5 events/h was independently associated with increased inflammatory activity, especially with elevated hsCRP and IL-6 levels, when adjusted for confounders.[37] Similarly, these authors suggest that intermittent hypoxia, rather than the number of apneas and hypopneas, is the main contributor to enhanced inflammation status. However, literature data concerning inflammatory biomarkers in OSA are inconsistent and often confounded with other risk factors.[38] Also, sleep deprivation in the form of sleep restriction did not affect plasma adropin levels among middle-aged men and women in a study by St-Onge et al.[39]; however, sleep deprivation consistently increased plasma concentrations of inflammatory mediators.[40] A study by Salord et al. suggested that obesity as an independent risk factor could overwhelm the effects of OSA and metabolic syndrome on plasma levels of adipokines, proinflammatory cytokines, endothelial dysfunction, and atherosclerosis markers, reinforcing the notion that control comorbidities might significantly skew interpretation of plasma biomarker data in these patients.[41]

In our multivariate regression analysis, plasma adropin was a significant negative predictor of severe OSA and this effect was of borderline significance for moderate OSA. Importantly, our model was adjusted for age and BMI, because some authors emphasized that obesity might be an important confounder and could lead to misinterpretation of the results.[41] Regression analysis also showed that biomarkers of systemic inflammation in plasma (TNF-α, IL-6, hsCRP) generally portended an increased probability of having a moderate and severe OSA; however, these interactions were not significant when adjusted for age and BMI with the exception of TNF-α that was associated with an almost 25-fold increase in probability of having severe OSA. This is in agreement with findings reported in the recent meta-analysis of Li and Zheng showing that circulating TNF-α levels were significantly higher in patients with OSA and this difference increased as the severity of disease progressed confirming the promising role of TNF-α as a circulating biomarker for OSA development.[42] It is possible that small sample sizes of groups in our study may have limited our regression results, with respect to other inflammatory biomarkers to reach significant levels.

Another important finding in our study is a significant negative correlation of plasma adropin levels with mediators of systemic inflammation in patients with OSA. Namely, this relationship was confirmed for TNF-α and IL-6 in plasma whereas the correlation with hsCRP was found to be insignificant. In a population of patients with type 2 diabetes and those without diabetes, Wu et al. reported a modest but significant global correlation of adropin with hsCRP levels in plasma; however, this might have been influenced by a high burden of coronary artery disease (CAD) in this population as assessed by relevant angiographic indices.[43] Atherosclerosis and CAD are currently recognized as chronic inflammatory conditions and this paradigm was reinforced even more with the results of a recent CANTOS trial.[44] A negative association of adropin plasma levels with indices of systemic inflammation could be mechanistically mediated through upregulated eNOS activity by adropin via VEGFR2 2-phosphatidylinositol 3-kinase-Akt and VEGFR2-extracellular signal-regulated kinase pathways, consequently leading to increased proliferation, migration, and capillary-like tube formation as well as decreased permeability and TNF-α induced apoptosis of endothelial cells as demonstrated in preclinical study of Lovren et al., suggesting a protective role of adropin in endothelial homeostasis.[22] Similarly, adropin reduced messenger RNA expression levels of TNF-α and IL-6 in a preclinical model of hyperlipidemia, further establishing suppressive effects of adropin on systemic inflammation.[17] Adropin upregulates eNOS activity at the post-transcriptional level and NO activation negatively regulates mediators of inflammation.[45] Physiology of NO metabolism and recruitment is complex; however, current evidence suggests that NO derived from eNOS confers anti-inflammatory actions by abolishing leukocyte extravasation and leukocyte movement—processes that are tightly regulated by TNF-α.[46–49] Finally, increase in NO recruitment contributes to dilatation of vasculature, thus contributing to antihypertensive effects of adropin. Furthermore, our correlation analysis showed that plasma adropin levels have a significant inverse relationship with PSG parameters of sleep—AHI and ODI—indices that correlate with disease severity in OSA. These indices generally

Table 4—Multivariate regression analysis of adropin and inflammatory biomarkers in plasma as independent predictors for moderate and severe OSA.

Moderate OSA			
Plasma biomarker	**OR***	**95% CI**	***P*†**
Adropin	0.245	0.041–1.484	.126
TNF-α	11.288	0.651–19.562	.096
IL-6	17.558	0.105–29.307	.272
hsCRP	1.434	0.474–4.337	.523
Severe OSA			
Plasma biomarker	**OR***	**95% CI**	***P*†**
Adropin	0.069	0.009–0.517	.009
TNF-α	24.864	1.338–46.194	.031
IL-6	26.920	0.139–52.168	.220
hsCRP	2.064	0.604–7.048	.247

* = all OR in the regression model are adjusted for age and body mass index. † = all *P* are 2-tailed, significance level is set at $P < .05$. CI = confidence interval, hsCRP = high sensitivity C-reactive protein, IL-6 = interleukin 6, OR = odds ratio, OSA = obstructive sleep apnea, TNF-α = tumor necrosis factor alpha.

reflect the degree of hypoxic burden in OSA and hypoxia elicits detrimental effects on eNOS and NOS isoforms, thus sustaining and further aggravating endothelial dysfunction.[50] In the only study comparable to ours, performed in a pediatric OSA population, adropin levels did not significantly correlate with AHI or nadir SpO_2.[6]

Less pronounced but significant correlations between adropin levels in plasma and parameters of glucose metabolism were established as well. Namely, negative correlation with HbA1c levels followed by fasting plasma glucose and HOMA-IR score were observed. Clinical data on the relationship between adropin plasma levels and glucose parameters are lacking. However, a preclinical study performed in rats with hyperlipidemia showed how intraperitoneal administration of adropin significantly decreased HOMA-IR score, glycated hemoglobin, glucose and insulin levels in plasma.[17] Similarly, Yildirim et al. reported that adropin levels negatively correlated with fasting serum insulin levels, HOMA-IR score, and serum lipid markers including cholesterol, very low density lipoprotein, and triglycerides in the population of women with polycystic ovarian syndrome.[51] Mechanistically, this could possibly be mediated through interactions of adropin with eNOS and consequently increased systemic bioavailability of NO. Of note, NO activation has been implicated in mediating whole-body insulin sensitivity through the phosphatidylinositol 3-kinase pathway.[52] It could be reasonably hypothesized that decreased adropin levels are consequently contributing to decreased insulin sensitivity and increased systemic inflammation burden as demonstrated by significant association of adropin with HOMA-IR, TNF-α, and IL-6 in our study.

Finally, data obtained from ROC analysis demonstrated that adropin as a plasma biomarker showed a negative discriminatory value (AUC = 0.169, $P < .001$) in terms of OSA status detection. This result along with the findings obtained from regression analysis suggests that normal or higher-than-normal plasma adropin levels are unlikely to be measured in patients with OSA, particularly in those with severe disease, and these levels show a decreasing trend with the progression of OSA severity. It is important to highlight that normative adropin plasma values were not defined and were established based on the obtained values in our control group, with mean value being 8.15 ± 1.79 ng/mL (95% CI 7.40–8.89 ng/mL).

Our study has some limitations because it was a single-center, prospective analysis that included a relatively limited number of patients. Furthermore, our analyzed sample did not include female patients; therefore, our results might not be generalized to a whole population. Finally, a full-night PSG was not performed among healthy volunteers who obtained a low score (< 2) on the STOP questionnaire and for this reason it cannot be fully excluded that some of the healthy volunteers might have had OSA that was not detected. However, a low score obtained on the STOP questionnaire has a high negative predictive value for OSA status and is a highly sensitive instrument, especially for patients with moderate and severe OSA, as demonstrated in multiple validation cohorts and relevant systematic analyses.[53,54]

Moreover, we performed additional analysis using the full STOP-BANG questionnaire instead of the STOP questionnaire in order to increase sensitivity for moderate to severe and severe OSA. Our analysis showed that none of the 25 subjects had a high risk for OSA, and only 3 subjects were at intermediate risk category (scored 3 points), whereas most (22 of 25) had a low risk for OSA. Additionally, when the 3 patients in the intermediate risk category for OSA were excluded, no significant changes in any of the measured parameters were observed. Therefore, we can reasonably conclude that our volunteer subjects (control group) were highly unlikely to have clinically significant OSA, despite not being tested with full-night PSG.

In conclusion, this is the first study performed in an adult population of male patients with OSA that showed a significant association of adropin plasma levels with PSG sleep parameters, circulating markers of inflammation, and parameters of glucose metabolism. However, future clinical studies with larger patient enrollment are necessary to fully elucidate the role of adropin in terms of mechanisms of the disease.

ABBREVIATIONS

AHI, apnea-hypopnea index
BMI, body mass index
CAD, coronary artery disease
ECLIA, electrochemiluminescence immunoassay
eNOS, endothelial nitric oxide synthase
ESS, Epworth Sleeping Scale
HbA1c, glycated hemoglobin A1c
HOMA-IR, homeostatic model assessment insulin resistance index
HPA, hypothalamic-pituitary-adrenal axis
hsCRP, high-sensitivity C-reactive protein
IL-6, interleukin 6
iNOS, inducible nitric oxide synthase
NO, nitric oxide
ODI, oxygen desaturation index
OSA, obstructive sleep apnea
PSG, polysomnography
ROC, receiver operating curves
SpO_2, arterial oxygen saturation
STOP, snoring, tiredness, observed apnea, pressure
TNF-α, tumor necrosis factor alpha
VEGFR2, vascular endothelial growth factor receptor-2

REFERENCES

1. Caples SM, Gami AS, Somers VK. Obstructive sleep apnea. *Ann Intern Med.* 2005;142(3):187–197.
2. Floras JS. Hypertension and sleep apnea. *Can J Cardiol.* 2015;31(7):889–897.
3. Pedrosa RP, Barros IM, Drager LF, et al. OSA is common and independently associated with hypertension and increased arterial stiffness in consecutive perimenopausal women. *Chest.* 2014;146(1):66–72.
4. Nizankowska-Jedrzejczyk A, Almeida FR, Lowe AA, et al. Modulation of inflammatory and hemostatic markers in obstructive sleep apnea patients treated with mandibular advancement splints: a parallel, controlled trial. *J Clin Sleep Med.* 2014;10(3):255–262.

5. Baguet JP, Nadra M, Barone-Rochette G, Ormezzano O, Pierre H, Pépin JL. Early cardiovascular abnormalities in newly diagnosed obstructive sleep apnea. *Vasc Health Risk Manag*. 2009;5:1063–1073.
6. Gozal D, Kheirandish-Gozal L, Bhattacharjee R, Molero-Ramirez H, Tan HL, Bandla HP. Circulating adropin concentrations in pediatric obstructive sleep apnea: potential relevance to endothelial function. *J Pediatr*. 2013;163(4):1122–1126.
7. Drager LF, Diegues-Silva L, Diniz PM, et al. Obstructive sleep apnea, masked hypertension, and arterial stiffness in men. *Am J Hypertens*. 2010;23(3):249–254.
8. Vgontzas AN, Bixler EO, Lin HM, Prolo P, Trakada G, Chrousos GP. IL-6 and its circadian secretion in humans. *Neuroimmunomodulation*. 2005;12(3):131–140.
9. Tasali E, Ip MS. Obstructive sleep apnea and metabolic syndrome: alterations in glucose metabolism and inflammation. *Proc Am Thorac Soc*. 2008;5(2):207–217.
10. Bozic J, Galic T, Supe-Domic D, et al. Morning cortisol levels and glucose metabolism parameters in moderate and severe obstructive sleep apnea patients. *Endocrine*. 2016;53(3):730–739.
11. Galic T, Bozic J, Pecotic R, Ivkovic N, Valic M, Dogas Z. Improvement of cognitive and psychomotor performance in patients with mild to moderate obstructive sleep apnea treated with mandibular advancement device: a prospective 1-year study. *J Clin Sleep Med*. 2016;12(2):177–186.
12. Kumar KG, Trevaskis JL, Lam DD, et al. Identification of adropin as a secreted factor linking dietary macronutrient intake with energy homeostasis and lipid metabolism. *Cell Metab*. 2008;8(6):468–481.
13. Li L, Xie W, Zheng XL, Yin WD, Tang CK. A novel peptide adropin in cardiovascular diseases. *Clin Chim Acta*. 2016;453:107–113.
14. Aydin HI, Eser A, Kaygusuz I, et al. Adipokine, adropin and endothelin-1 levels in intrauterine growth restricted neonates and their mothers. *J Perinat Med*. 2016;44(6):669–676.
15. Aydin S, Kuloglu T, Aydin S. Copeptin, adropin and irisin concentrations in breast milk and plasma of healthy women and those with gestational diabetes mellitus. *Peptides*. 2013;47:66–70.
16. Ganesh Kumar K, Zhang J, Gao S, et al. Adropin deficiency is associated with increased adiposity and insulin resistance. *Obesity*. 2012;20(7):1394–1402.
17. Akcilar R, Emel Kocak F, Simsek H, et al. The effect of adropin on lipid and glucose metabolism in rats with hyperlipidemia. *Iran J Basic Med Sci*. 2016;19(3):245–251.
18. Chen M, Ouyang F, Zhou S. Adropin as a novel energy factor likely has the ability to regulate blood pressure. *Med Hypotheses*. 2015;85(2):234.
19. Goetze JP, Albrethsen J. Adropin: a new regulatory peptide in cardiovascular endocrinology. *Regul Pept*. 2014;190–191:41–42.
20. Topuz M, Celik A, Aslantas T, Demir AK, Aydin S, Aydin S. Plasma adropin levels predict endothelial dysfunction like flow-mediated dilatation in patients with type 2 diabetes mellitus. *J Invest Med*.2013;61(8):1161–1164.
21. Lian W, Gu X, Qin Y, Zheng X. Elevated plasma levels of adropin in heart failure patients. *Intern Med*. 2011;50(15):1523–1527.
22. Lovren F, Pan Y, Quan A, et al. Adropin is a novel regulator of endothelial function. *Circulation*. 2010;122(11 Suppl):S185–S192.
23. Gu X, Li H, Zhu X, et al. Inverse correlation between plasma adropin and et-1 levels in essential hypertension: a cross-sectional study. *Medicine*. 2015;94(40):e1712.
24. Kume T, Calan M, Yilmaz O, et al. A possible connection between tumor necrosis factor alpha and adropin levels in polycystic ovary syndrome. *J Endocrinol Invest*. 2016;39(7):747–754.
25. Kheirandish-Gozal L, Gileles-Hillel A, Alonso-Alvarez ML, et al. Effects of adenotonsillectomy on plasma inflammatory biomarkers in obese children with obstructive sleep apnea: a community-based study. *Int J Obesity*. 2015;39(7):1094–1100.
26. Abdel-Fadeil MR, Abedelhaffez AS, Makhlouf HA, Al Qirshi GA. Obstructive sleep apnea: Influence of hypertension on adiponectin, inflammatory markers and dyslipidemia. *Pathophysiology*. 2017; 24(4):305–315.
27. Nadeem R, Molnar J, Madbouly EM, et al. Serum inflammatory markers in obstructive sleep apnea: a meta-analysis. *J Clin Sleep Med*. 2013;9(10):1003–1012.
28. Kapur VK, Auckley DH, Chowdhuri S, et al. Clinical practice guideline for diagnostic testing for adult obstructive sleep apnea: an American Academy of Sleep Medicine clinical practice guideline. *J Clin Sleep Med*. 2017;13(3):479–504.
29. Ruehland WR, Rochford PD, O'Donoghue FJ, Pierce RJ, Singh P, Thornton AT. The new AASM criteria for scoring hypopneas: impact on the apnea hypopnea index. *Sleep*. 2009;32(2):150–157.
30. Pecotic R, Dodig IP, Valic M, Ivkovic N, Dogas Z. The evaluation of the Croatian version of the Epworth sleepiness scale and STOP questionnaire as screening tools for obstructive sleep apnea syndrome. *Sleep Breath*. 2012;16(3):793–802.
31. McNicholas W. Sleep-related breathing disorders: nosological classification, definitions, epidemiology. In: Bassetti C, Dogas Z, Peigneux P, ed. *Sleep Medicine Textbook*. 1st ed. Regensburg, DE: European Sleep Research Society; 2014:215–220.
32. Health Quality Ontario. Polysomnography in patients with obstructive sleep apnea: an evidence-based analysis. *Ont Health Technol Assess Ser*. 2006;6(13):1–38.
33. Epstein L, Kristo D, Strollo P Jr, et al. Clinical guideline for the evaluation, management and long-term care of obstructive sleep apnea in adults. *J Clin Sleep Med*. 2009;5(3):263–276.
34. Matthews D, Hosker J, Rudenski A, Naylor B, Treacher D, Turner R. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985;28(7):412–419.
35. Fleming WE, Ferouz-Colborn A, Samoszuk MK, et al. Blood biomarkers of endocrine, immune, inflammatory, and metabolic systems in obstructive sleep apnea. *Clin Biochem*. 2016;49(12):854–861.
36. Araújo LS, Fernandes JF, Klein MR, Sanjuliani AF. Obstructive sleep apnea is independently associated with inflammation and insulin resistance, but not with blood pressure, plasma catecholamines, and endothelial function in obese subjects. *Nutrition*. 2015;31(11–12):1351–1357.
37. Thunström E, Glantz H, Fu M, et al. Increased inflammatory activity in nonobese patients with coronary artery disease and obstructive sleep apnea. *Sleep*. 2015;38(3):463–471.
38. Unnikrishnan D, Jun J, Polotsky V. Inflammation in sleep apnea: an update. *Rev Endocr Metab Disord*. 2015;16(1):25–34.
39. St-Onge MP, Shechter A, Shlisky J, et al. Fasting plasma adropin concentrations correlate with fat consumption in human females. *Obesity*. 2014;22(4):1056–1063.
40. Faraut B, Boudjeltia KZ, Vanhamme L, Kerkhofs M. Immune, inflammatory and cardiovascular consequences of sleep restriction and recovery. *Sleep Med Rev*. 2012;16(2):137–149.
41. Salord N, Gasa M, Mayos M, et al. Impact of OSA on biological markers in morbid obesity and metabolic syndrome. *J Clin Sleep Med*. 2014;10(3):263–270.
42. Li Q, Zheng X. Tumor necrosis factor alpha is a promising circulating biomarker for the development of obstructive sleep apnea syndrome: a meta-analysis. *Oncotarget*. 2017;8(16):27616–27626.
43. Wu L, Fang J, Chen L, et al. Low serum adropin is associated with coronary atherosclerosis in type 2 diabetic and non-diabetic patients. *Clin Chem Lab Med*. 2014;52(5):751–758.
44. Ridker PM, Everett BM, Thuren T, et al. Antiinflammatory therapy with canakinumab for atherosclerotic disease. *N Engl J Med*. 2017;377(12):1119–1131.
45. Herman AG, Moncada S. Therapeutic potential of nitric oxide donors in the prevention and treatment of atherosclerosis. *Eur Heart J*. 2005;26(19):1945–1955.
46. Lefer DJ, Jones SP, Girod WG, et al. Leukocyte-endothelial cell interactions in nitric oxide synthase-deficient mice. *Am J Physiol*. 1999;276(6 Pt 2):H1943–H1950.
47. Cockrell A, Laroux FS, Jourd'heuil D, et al. Role of inducible nitric oxide synthase in leukocyte extravasation in vivo. *Biochem Biophys Res Commun*. 1999;257(3):684–686.
48. Sedgwick JD, Riminton DS, Cryster JG, Körner H. Tumor necrosis factor: a master-regulator of leukocyte movement. *Immunol Today*. 2000;21(3):110–113.

49. Cirino G, Fiorucci S, Sessa WC. Endothelial nitric oxide synthase: the Cinderella of inflammation? *Trends Pharmacol Sci*. 2003;24(2):91–95.
50. Ho JJ, Man HS, Marsden PA. Nitric oxide signaling in hypoxia. *J Mol Med*. 2012;90(3):217–231.
51. Yildirim B, Celik O, Aydin S. Adropin: a key component and potential gatekeeper of metabolic disturbances in polycystic ovarian syndrome. *Clin Exp Obstet Gynecol*. 2014;41(3):310–312.
52. Potenza Ma, Marasciulo FL, Chieppa DM, et al. Insulin resistance in spontaneously hypertensive rats is associated with endothelial dysfunction characterized by imbalance between NO and ET-1 production. *Am J Physiol Heart Circ Physiol*. 2005;289(2):H813–H822.
53. Chung F, Yegneswaran B, Liao P, et al. STOP questionnaire: a tool to screen patients for obstructive sleep apnea. *Anesthesiology*. 2008;108(5):812–821.
54. Abrishami A, Khajehdehi A, Chung F. A systematic review of screening questionnaires for obstructive sleep apnea. *Can J Anaesth*. 2010;57(5):423–438.

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Address correspondence to: Professor Zoran Dogas, MD, PhD, Sleep Medicine Center and Department of Neuroscience, University of Split School of Medicine, Soltanska 2, 21000 Split, Croatia; Tel: +385 21 557 905; Fax: +385 21 557 955; Email: zdogas@mefst.hr

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