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Evaluation the Possible Role of Interleukin-6 and Tumor Necrosis Factor- Alpha in Pathogenesis of Obstructive Sleep Apnea in Obese Patients: A Case-Control Study

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Abstract

Background: According to a mounting body of evidence, recent observations have highlighted considerable association between obstructive sleep apnea (OSA) syndrome and patients' obesity and inflammation, however the exact underlying mechanisms remain to be fully understood. In this study, the relationship between OSA and Interleukin-6 and Tumor necrosis factor- alpha was assessed in obese patients and their serum concentrations were compared to non-OSA obese subjects. **Materials and Methods:** This case-control study was conducted on forty-six obese OSA patients (body mass indices, BMI>30) and 42 obese but otherwise healthy individuals who were admitted to the pulmonary or obesity clinics of the Hazrat-e Rasool General Hospital (Tehran, Iran) between November 2019 and May 2020 were included. The participants completed the NOSAS, EPWORTH and STOPBANG questionnaires. Tumor Necrosis Factor-Alpha (TNF-α) and Interleukin-6 (IL-6) serum concentrations were determined using the enzyme-linked immunosorbent assay (ELISA) method. **Results:** Compared to the non-OSA group, OSA patients had higher systolic and diastolic blood pressure, pCO_2, bicarbonate (HCO_3) and hemoglobin and lower high-density lipoprotein (HDL) values. IL-6 and TNF-α serum levels were not significantly different between both groups. Univariate and multivariate linear regression models showed that BMI, systolic blood pressure, pCO_2 and HCO_3 can positively affect the serum TNF-α and systolic blood pressure and HCO_3 can also positively affect the serum IL-6 values in patients with the OSA. **Conclusion:** This investigation suggests that among the OSA patients, the heightened inflammatory profile may be influenced by the high BMI. Furthermore, the exclusive relationship between different disease biomarkers and inflammatory agents in OSA patients is intriguing and needs further research.

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Keywords: Obesity; Obstructive Sleep Apnea; Interleukin-6; Tumor Necrosis Factor-Alpha; Inflammation

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Introduction

Obstructive sleep apnea (OSA) is caused by the collapse of the pharynx resulting in the upper respiratory tract blockage and subsequent manifestation of nocturnal symptoms such as observed apneas, sweating, choking sensation, restless sleep and also daytime symptoms including sleepiness, memory loss, headache and concentration difficulties [1]. Based on previous studies, metabolic syndrome, advanced age, snoring, high body mass index (BMI), elevated blood pressure and male gender have all been associated with the OSA [2, 3].

Oxidative stress and systemic inflammation may play an important role in the pathogenesis of OSA and associated comorbidities. Intermittent nocturnal hypoxemia present in OSA may act in a similar fashion to cardiac ischemia-reperfusion injury, leading to increased oxygen-derived free radical production and subsequent local and systemic inflammation [4, 5].

Interleukin-6 (IL-6) and Tumor necrosis factor- alpha (TNF-α) are prominent well-studied inflammatory cytokines. TNF-α exerts its effects generally via the NF-κB signaling pathway, a process inducing the activity of proteins such as nitric oxide synthase, cyclooxygenase 2, and adenosine A1 receptors, all of which participating in sleep regulation [6]. Studies exploring the effects of continuous positive airway pressure (CPAP) therapy on inflammation or even TNF-α targeted approaches have all reported promising disease attenuation [7, 8]. In addition to TNF-α, studies have revealed that intermittent hypoxia and sleep irregularities can result in increased IL-6 production [9-11] and similar to TNF-α, CPAP therapy in OSA patients has attenuated inflammatory status such as reduced IL-6 levels [12, 13]. According to existing data, the inflammation and high levels of inflammatory cytokines, such as the TNF-α and IL-6 are inevitably associated with the OSA [6].

Given the established association between obesity and inflammation [14, 15] and the connection between inflammatory cytokines and obesity with OSA, inflammatory cytokine profile of obese OSA patients is rarely studied. In the current study the serum levels of TNF-α and IL-6 between obese OSA and non-OSA subjects were compared to see if the presence of obesity can interfere with the detection of the heightened inflammatory profile of OSA patients. Furthermore, the relationship between these cytokines and characteristic disease markers was assessed.

Materials and Methods

Study population

In the current Case-Control study, participants were selected from the obese (BMI>30) patients of pulmonary or obesity clinics of the Hazrat-e Rasool General Hospital (Tehran, Iran) between November 2019 and May 2020. All available cases were included using the census method.

All participants completed the NOSAS (Neck circumference, Obesity (based on BMI), Snoring, Age and Sex) [16, 17], EPWORTH (Epworth sleepiness scale questionnaire consisting of 8 questions measuring the general daytime drowsiness) [18] and STOPBANG (Consisting of four subjective and four objective questions including snoring, tiredness, observed apnea, high blood pressure, BMI, age, neck circumference and gender) [19] screening questionnaires. Accordingly, polysomnography tests were conducted for patients who were deemed as moderate and high potential of risk of OSA. The patients were assigned to the OSA, and non-OSA group (based on polysomnography results) were assigned to the non-OSA group as 1:1 ratio and both groups were matched base on gender.

Exclusion criteria for the study consisted of BMI of <30, uncontrolled diabetes, uncontrolled hypothyroidism, active rheumatoid disease, autoimmune disease and cancer.

Prior medical history and treatment history were gathered from the patients via appropriate questionnaires and a complete blood laboratory analyses. The latter tests included fasting blood sugar (FBS), hemoglobin (Hb), blood urea nitrogen (BUN), Cholesterol, low-density and high-density lipoproteins (LDL

and HDL), triglyceride (TG), Creatinine, aspartate aminotransferase (AST), platelet count (PLT), alanine aminotransferase (ALT), erythrocyte sedimentation rate (ESR), thyroid stimulating hormone (TSH). For this purpose, 10 mL blood sample was collected from each participant via venipuncture of forearm veins.

IL-6 and TNF-α measurements

Quantitative double antibody sandwiched ELISA was implemented to determine concentrations of IL-6 and TNF-α in sera samples using ELISA kits (Demeditec Diagnostics GmbH, Kiel, Germany). These tests were conducted following the manual instructions provided by the manufacturer. Briefly, for IL-6 measurement, sera samples, calibrators and standards were incubated using incubation buffer for 1 hour at room temperature. Following the washing step (three times), enzyme-labeled reagent containing horse radish peroxidase (HRP)-conjugated secondary antibody and specimen diluent solution (containing human plasma, bovine serum albumin, benzamidin and thymol) were added to the wells followed by 1 hour incubation period at room temperature. This step completed the double antibody sandwiched complex (coated anti body - IL-6 - conjugated anti body). After another washing step, choromogenic solution (containing tetramethylbenzidine) was added to the wells and incubated at room temperature and dark environment for 15 min. This reaction was quenched using the stop solution (containing hydrochloric acid). In the last step, the optical density (OD) was measured at 450 nm via spectrophotometry using a microplate reader (ELx800, BioTek Instruments Inc., Winooski, VT, USA). IL-6 concentration in samples was determined using the plotted standard curve derived from standard samples.

For TNF-α, samples, calibrators and standards were incubated using incubation buffer for 2 hours at room temperature. After washing step, zero calibrator (containing human plasma, benzamidin and thymol) and conjugated antibody were added to the wells, completing the sandwich complex. After two hours in room temperature and washing step, choromogenic solution was added to the wells followed by 15-minute incubation period at room temperature and dark environment. In the last step, after the addition of stop solution, optical density (OD) was measured at 450 nm and TNF-α concentrations were calculated in a similar fashion to IL-6.

Ethics approval and consent to participate

This study was reviewed and approved by scientific advisory and ethical committees of Iran University of medical sciences (Registration number: IR.IUMS.FMD.REC.1398.317) and before signing written informed consent forms, all patients were given complete explanation about the study procedures and protocol.

Statistical Analysis

In the current study, the minimum sample size was calculated 48 subjects for each group by an expected R of 0.3, odds ratio (OR)=1.5, significance level α=0.05, and power of 70%. The data distribution and equality of variances were assessed using the Kolmogrov-Smirnov and Levene's tests respectively. Based on the results of these tests, the differences between the two study groups were analyzed using the student T-test or Mann-Whitney U Test. Furthermore, in order to find those factors affecting serum cytokine concentration values, linear univariate and multivariate regression models were considered. A P-value of ≤0.05 was considered as the threshold for statistical significance. All statistical tests were performed using the SPSS 22.0 software (SPSS Inc., Chicago, Illinois, USA).

Results

Nighy-six subjects enrolled in this study, however, eight of them (two in OSA group and six in non- OSA group) withdraw to continue in this study so eighty-eight obese individuals were included that the mean age of them was 55.92 (55.92±7.73) years and female gender was slightly more frequent (46 (52.3%)). Based on study design patients were divid-ed into two groups including OSA (n=46) and non-OSA (n=42). Analysis of basic characteristics data revealed that compared to the non-OSA group, OSA group members

were generally older (me-dian 60 (IQR 53-65.25) vs median 53.5 (IQR 48.75–56.25), P=0.001), heavier (median 97 (IQR 87.75–107.25) vs median 89.25 (IQR 83.25–93.5) Kg, P=0.001) and had higher BMI (median 35.3 (IQR 31.95–40.77) vs median 32.95 (IQR 30–36.05), P<0.001), neck circumference (median 41 (IQR 40–42) vs median 38 (IQR 37–39) cm, P<0.001), systolic blood pressure (median 130 (IQR 130–150) vs median 127.5 (IQR 113.75–136.25) mmHg, P<0.001) and diastolic blood pressure (median 85 (IQR 70–90) vs median 70 (IQR 70–80) mmHg, P=0.003). As these patients were obese and had similar comorbidities, drug use history was comparable in case of all recorded drugs except Rosuvastatin which was more frequent in non-OSA group (5 (12.8) vs 0, P=0.02) (Table-1).

OSA patients had significantly higher blood PCO_2 (median 50 (IQR 43–54.92) vs median 39.5 (IQR 38–46.25) mmHg, P<0.001), HCO_3 (median 29 (IQR 25–31.77) vs median 24.95 (IQR 23.6–28) mEq/L, P<0.001) and higher score in disease indices such as EPWORTH (median 11 (IQR 6.75–18.75) vs

Table 1. Basic Characteristics, Disease and Drug History of Study Groups.

Parameters	non-OSA (n=42)	OSA (n=46)	P value
Gender (male)	19 (45.2)	23 (50)	0.65
***Age**	53.5 (48.75–56.25)	60 (53–65.25)	**0.001
***Height (cm)**	168 (156–170)	162.5 (156–170)	0.98
***Weight (Kg)**	89.25 (83.25–93.5)	97 (87.75–107.25)	**0.001
***BMI**	32.95 (30–36.05)	35.3 (31.95–40.77)	**0.003
***Neck circumference (cm)**	38 (37–39)	41 (40–42)	**<0.001
***BP (systolic) (mmHg)**	127.5 (113.75–136.25)	140 (130–150)	**<0.001
***BP (diastolic) (mmHg)**	70 (70–80)	85 (70–90)	**0.003
Exercise	10 (23.8)	10 (21.7)	0.82
Smoking	16 (38.1)	17 (37)	0.91
Comorbidities			
HLP	9 (23.1)	10 (21.7)	0.88
Hypertension	15 (38.5)	24 (52.2)	0.2
Diabetes	7 (17.9)	9 (19.6)	0.85
BPH	0	2 (4.3)	0.5
Stroke	0	1 (2.2)	1
Drugs			
Atorvastatin	5 (12.8)	10 (21.7)	0.28
Amlodipine	1 (2.6)	2 (4.3)	1
Losartan	13 (33.3)	23 (50)	0.12
Metformin	7 (17.9)	8 (17.4)	0.95
Tamsulosin	0	2 (4.3)	0.5
Insulin	0	1 (2.2)	1
Metoral	1 (2.6)	4 (8.7)	0.37
Rosuvastatin	5 (12.8)	0	**0.02

* Data are presented as Median (IQR)
** Statistical significance
OSA: Obstructive sleep apnea; **BMI**: Body mass index; **BP**: Blood pressure; **HLP**: Hyperlipoproteinaemia; **BPH**: Benign prostatic hyperplasia
Note: Data are presented as n (%)

median 5 (IQR 3–6), P<0.001), STOP_BANG (median 6 (IQR 4.75–6.25) vs medi-an 2 (IQR 2–3), P<0.001) and NOSAS (median 14 (IQR 11–15) vs median 7 (IQR 6.5–7), P<0.001) (Table-2).

Furthermore, laboratory tests revealed that the OSA group had significantly lower HDL values (me-dian 38.5 (IQR 33.75–45.25) vs median 43 (IQR 37–49.25) mg/dL, P=0.03) and higher Hemoglo-bin (median 14.7 (IQR 13–16.02) vs median 13.35 (IQR 12.2–15.4) g/dL, P=0.03) compared to the non-OSA group. Additionally, serum levels of TNF-α and IL-6 were not significantly different be-tween the study groups (median 15.57 (IQR 6.18–43.21) vs median 15.57 (IQR 3.78–50.94) pg/mL, P=0.92 and median 10.65 (IQR 6.7–21.94) vs median 7.36 (IQR 0.7–18.07), P=0.09 pg/mL for IL-6 and TNF-α respectively) (Table-3). Differences in IL-6 and TNF-α serum concentrations between study groups did not reach statistical significance even after subgrouping based on age and BMI indices (Table-4).

In order to find the measured indices effects on the cytokine levels in OSA patients, a series of univariate and multivariate regression analyses were fulfilled. Univariate results revealed that BMI, BP (systolic), PCO_2 and HCO_3 (3.4 (95% CI: 0.1–6.69), P=0.04; 0.2 (95% CI: 0.18–0.21), P<0.001; 2.55 (95% CI: 1.40–3.71), P<0.001; 5.62 (95% CI: 3.75–7.48), P<0.001; for BMI, BP (systolic), PCO_2 and HCO_3 respectively) positively affected the serum TNF-α levels and BP (systolic) and HCO_3 (0.1 (95% CI: 0.02–0.17), P=0.01; 2.96 (95% CI: 0.18–5.74), P=0.04; for BP (systolic) and HCO_3 respectively) positively affected the serum IL-6 levels. As shown in Table-5, all of these re-lations persisted even after the model was adjusted for age and gender in the multivariate analyses. It is worth mentioning that these significant relations were observed in the OSA but not the non-OSA group.

Discussion

In this case -control study, between obese people with or without OSA, we found no significant difference regarding the levels of neither the TNF-α nor the IL-6. Noticeably, the TNF-α serum level was non-significantly higher in the OSA group. Results also revealed an increase in systolic and diastolic blood pressure, CO_2 and HCO_3 levels in OSA patients compared to the non-OSA group.

Evaluation of univariate and multivariate regression models also confirmed the increase in systolic blood pressure, BMI, higher blood CO_2 pressure and HCO_3 level, positively affecting serum TNF-α levels. Furthermore, systolic blood pressure and HCO_3 levels also positively affected the serum IL-6 levels.

The main proposed mechanism responsible

Table 2. Comparison of Disease Specific Indices between Patients in Obstructive Sleep Apnea (OSA) and Non-OSA Groups.

Parameters	non-OSA (n=42)	OSA (n=46)	P value
PH	7.39 (7.39–7.4)	7.39 (7.36–7.4)	0.08
PCO_2 (mmHg)	39.5 (38–46.25)	50 (43–54.92)	*<0.001
HCO_3 (mEq/L)	24.95 (23.6–28)	29 (25–31.77)	*0.004
PSQI	7.5 (5–11)	9 (6.75–13)	0.07
EPWORTH	5 (3–6)	11 (6.75–18.75)	*<0.001
STOP_BANG	2 (2–3)	6 (4.75–6.25)	*<0.001
NOSAS	7 (6.5–7)	14 (11–15)	*<0.001
AHI	0	32.5 (24–53)	*<0.001

* Statistical significance

OSA: Obstructive sleep apnea; **PSQI**: Psychometric properties of the pittsburgh sleep quality index; **EPWORTH**: Epworth sleepiness scale; **STOP_BANG**: STOP_BANG Questionnaire score; **NOSAS**: NOSAS Index score; **AHI**: Apnea-hypopnea index

Note: Data are presented as Median (IQR)

for systemic inflammation includes a disruption in gas exchange and uninterrupted sleep due to OSA. This in turn causes the sympathetic nervous system releases more hormones, which are known to cause insulin resistance and lipolysis. According to in vitro research, intermittent hypoxia can activate hypoxia-Inducible factor-1 (HIF-1) and NF-κB to directly induce inflammation in tissues. In addition to stimulating the HIF-1 pathway and causing an increase in FFA and insulin resistance, sympathetic activation

Table 3. Comparison of Biochemical and Hematological Test Results between Obstructive Sleep Apnea (OSA) and Non-OSA Groups.

Parameters	non-OSA (n=42)	OSA (n=46)	P value
††TNF-α (pg/ml)	7.36 (0.70–18.07)	10.65 (6.70–21.94)	0.09
‡‡IL-6 (pg/ml)	15.57 (3.78–50.94)	15.57 (6.18–43.21)	0.92
FBS (mg/dL)	117 (96–146)	109.5 (98.5–136.25)	0.71
BUN (mg/dL)	19 (17–21)	16.5 (12.75–20.25)	0.06
Creatinine (mg/dL)	1 (0.9–1.12)	1 (0.8–1.12)	0.52
Cholesterol (mg/dL)	208 (197–265.25)	215.5 (193.75–240)	0.93
LDL (mg/dL)	105 (85.25–151.5)	115 (99.75–135)	0.34
HDL (mg/dL)	43 (37–49.25)	38.5 (33.75–45.25)	*0.03
TG (mg/dL)	144 (105.5–187)	164.5 (135.5–213.25)	0.07
AST (U/L)	25 (19.75–30)	24.5 (20.5–31)	0.83
ALT (U/L)	23.5 (19–31)	22 (20–30.5)	0.92
Hb (g/dL)	13.35 (12.2–15.4)	14.7 (13–16.02)	*0.03
PLT ($\times 10^{12}$/L)	269.5 (214.75–310.5)	246 (189.25–300.25)	0.08
ESR	10 (5.75–15)	11 (7–18.25)	0.09
TSH (mU/L)	1.55 (1–2.12)	1.5 (1–2.42)	0.8

†† n=59, ‡‡ n=58

* Statistical significance

OSA: Obstructive sleep apnea; **TNF-α**: Tumor necrosis factor; **IL-6**: Interleukin-6; **FBS**: Fasting blood sugar; **BUN**: Blood urea nitrogen; **LDL**: Low-density lipoprotein; **HDL**: High-density lipoprotein; **TG**: Triglyceride; **AST**: Aspartate aminotransferase; **ALT**: Alanine aminotransferase; **Hb**: Hemoglobin; **PLT**: Platelets; **ESR**: Erythrocyte sedimentation rate; **TSH**: Thyroid-stimulating hormone

Note: Data are presented as Median (IQR)

Table 4. Serum Tumor Necrosis Factor (TNF-α) and Interleukin-6 (IL-6) Differences between Non-Obstructive Sleep Apnea (OSA) and OSA Subjects Based on Age and Body Mass Index (BMI) Subgroups

Parameters	Non-OSA	OSA	P value
TNF-α (Age<55) (n=25)	7.34 (0.7–14.03)	12.21 (6.7–24.59)	0.8
IL-6 (Age<55) (n=24)	12.04 (2–42.66)	15.57 (4.28–43.21)	0.97
TNF-α (Age≥55) (n=34)	7.36 (0.7–20.09)	7.38 (6.36–14.67)	0.07
IL-6 (Age≥55) (n=34)	16.52 (5.26–85.01)	20.10 (7.37–43.29)	0.63
TNF-α (BMI<34) (n=30)	9.37 (5.66–19.37)	14.06 (6.18–24.45)	0.1
IL-6 (BMI<34) (n=30)	17 (6.14–99.77)	16.05 (5.61–47.65)	0.75
TNF-α (BMI≥34) (n=29)	4.58 (0.7–15.52)	9.37 (6.7–20.52)	0.61
IL-6 (BMI≥34) (n=28)	15.09 (2–41.08)	14.11 (4.95–41.05)	0.71

OSA: Obstructive sleep apnea; **TNF- α**: Tumor necrosis factor; **IL-6**: Interleukin-6; **BMI**: Body mass index

Note: Data are presented as Median (IQR)

Table 5. Univariate and Multivariate Regression Models Showcasing the Affecting Factors on Tumor Necrosis Factor (TNF-α) and Interleukin-6 (IL-6) Serum Levels in Obstructive Sleep Apnea (OSA) Patients.

TNF-α	Univariate			Multivariate (adjusted for age and gender)		
	B	P-value	CI 95%	B	P-value	CI 95%
BMI	3.4	0.04	0.1–6.69	4.06	*0.04	0.24–7.88
BP (systolic)	0.2	*<0.001	0.18–0.21	0.2	*<0.001	0.18–0.21
PCO_2	2.55	*<0.001	1.40–3.71	2.5	*<0.001	1.31–3.7
HCO_3	5.62	*<0.001	3.75–7.48	5.49	*<0.001	3.56–7.43
IL-6						
BP (systolic)	0.1	*0.01	0.02–0.17	0.1	*0.01	0.02–0.18
HCO_3	2.96	*0.04	0.18–5.74	3.07	*0.04	0.17–6

* Statistical significance
TNF- α: Tumor Necrosis Factor; **BMI**: Body mass index; **BP**: Blood pressure; **IL-6**: Interleukin-6; **B**: Regression coefficient; **CI**: Confidence interval

of lipolysis also causes an increase in blood pressure and endothelial dysfunction [20]. Increased sympathetic activity due to OSA also causes both peak systolic and chronic hypertension [21].

Blood level of IL-6, have been shown to increase in experimental hypoxia and hypercarbia state [22]. Although this increase in some studies has been associated with the severity of OSA [23], in one of the largest studies, it was unraveled that elevated IL-6 levels are strongly influenced by interfering factors such as obesity [24]. In a recent meta-analysis study by Imani *et al*. on evaluation of serum and plasma IL6 levels in OSA patients, among 63 studies, a significant difference was observed regarding the IL6 plasma level between OSA patients and those of control group with the mean BMI≤30, however, in studies where the mean BMI of control group was >30, no differences were observed [25]. In obese patients with hypoventilation, Roytblat *et al*. reported a 34-fold higher levels of IL6 than in controls, but only 8-fold higher in patients with OSA and no differences were observed between obese OSA and obese non-OSA groups [26]. It is noteworthy that the significant correlation between BMI and IL-6 levels in OSA patients has been verified in some studies [27-29], though not confirmed in others [30, 31].

Regarding the TNF-α enhanced levels among individuals with high BMI, with or without OSA, different results have been reported in previous studies. In a study by Ciftci *et al*. [30] performed on 43 obese patients with OSA and 22 patients with the same BMI without OSA, both the TNF-α and IL-6 were significantly higher in patients with OSA independent of BMI. they also suggested that increased hypoxia and disease severity characterized by a high AHI (Apnea Hypopnea Index) value, is associated with a substantial increase on TNF-α and IL-6 levels [30].

In a study by Vgontzas *et al*. [32] on three groups of patients with either OSA, narcoleptic or idiopathic hypersomnia and a fourth healthy control group, it was concluded that both the TNF-α and IL-6 play substantial role in drowsiness and fatigue in patients with OSA. The authors concluded that obesity and sleep disorders play an important role in the heightened levels of IL-6 and TNF-α respectively [32]. The main differences between that study and the present study include the study population size and a large difference in BMI between the study groups, the OSA group (mean BMI=40.5) and the control group (mean BMI=24.6) in particular. In the current study, although BMI is significantly different between the two study groups (mean BMI=36.56 vs 33.21 for OSA and non-OSA groups respectively), they are relatively close and both over the obesity threshold. Considering the larger sample size

of our study and non-significant differences in measured cytokine levels, although hypercarbia or hypoxia due to sleep disorders are important in the heightened inflammatory state of OSA patients, the obesity may act as an indispensable inflammation regulatory factor.

One of the limitations of the present study is that at the time of sampling, the circadian rhythm of cytokine secretion was not considered. Inflammatory plasma cytokines follow a circadian rhythm, and their peak plasma level is around one to two hours after midnight but in people with OSA, this peak occurs in irregular timeframes [33]. Patient admission was required for sampling during these hours, but in our study, due to limitations, sampling was performed on an outpatient basis. Another limitation was the narrow sample size; in spite of being larger than some of the similar studies, a larger sample size would extraordinarily affect our study's sensitivity especially in the case of TNF-α.

Conclusion

It can be concluded that in OSA, obesity may affect the inflammatory profile of patients and should be considered in interpretations of inflammatory markers for diagnosis, monitoring or therapeutic purposes. Future large-scale studies are needed for in-depth verification and can elucidate the underlying relationship between obesity, hypoxia, inflammation and OSA.

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Conflict of Interest

The authors declare that they have no competing interests.

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