Day–night variations in endothelial dysfunction markers and haemostatic factors in sleep apnoea

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ABSTRACT: Patients with sleep apnoea have a significant alteration in the day–night pattern of myocardial infarction and sudden cardiac death observed in the general population. The aim of this study was to investigate the influence of sleep apnoea on the diurnal variations in various haemostatic parameters (factor VII, von Willebrand factor and plasminogen activator inhibitor (PAI)-1) and markers of endothelial dysfunction (asymmetric dimethylarginine (ADMA) and soluble CD40 ligand (sCD40L)).

We studied 26 male patients with obstructive sleep apnoea syndrome (OSAS; 13 patients with severe OSAS (apnoea/hypopnoea index (AHI) >30 events·h⁻¹) and 13 patients with mild-to-moderate OSAS (AHI <30 events·h⁻¹)) and 12 controls of similar body mass index (BMI) and waist circumference. In each subject, six different samples were obtained over 24 h.

Although all the markers values tended to be higher in patients with severe OSAS, differences did not reach statistical significance at any time. PAI-1 levels were significantly related to BMI ($p<0.001$), mean ($p<0.001$) and minimal ($p=0.047$) nocturnal oxygenation saturation. ADMA levels were significantly related to arousal index ($p=0.046$).

The results of this study suggest that day–night variations in factor VII:antigen (Ag), von Willebrand factor:Ag, PAI-1, sCD40L and ADMA levels may be dependent on either the obesity index or metabolic dysfunction rather than on sleep apnoea alone.

KEYWORDS: Endothelial, sleep apnoea

There is evidence that patients with obstructive sleep apnoea syndrome (OSAS) have an increased risk for cardiovascular diseases, including premature death from vascular events [1–5]. In the general population, cardiovascular (CV) events predominantly occur in the first few hours after waking [6–8]. This has been explained on the basis of the circadian variations of heart rate, blood pressure, platelet aggregation and fibrinolytic activity [9–12]. In contrast, patients with OSAS show a marked variation of the day–night pattern of myocardial infarction and sudden cardiac death observed in the general population such that CV events occur preferentially in the middle of the night, while patients are sleeping [13, 14].

It appears that several factors involved in the development of cardiovascular disease are temporally regulated [15–19]. Vascular tone and endothelial function vary according to the time of day and there are differences between healthy individuals and patients with atherosclerotic

disease with respect to the rhythm of nitric oxide (NO) production [20–22].

Circadian periodicity has been observed in components of the haemostatic system (plasminogen activator inhibitor (PAI)-1, factor VII and von Willebrand factor) [19, 23, 24] and markers of endothelial damage (soluble CD40 ligand (sCD40L) and asymmetric dimethylarginine (ADMA)) [21, 25]. Whether an abnormal circadian pattern of endothelial function and thrombotic potential contributes to the altered distribution of CV events in OSAS is poorly understood. Circadian rhythms are likely to be affected by established CV risk factors, including obesity, ageing, diabetes and hypertension, that occur frequently in OSAS [21, 26–29]. A recent study shows that differences in the day–night rhythm of PAI-1 between OSAS patients and non-OSAS controls may not be independent of metabolic factors [30]. The present study was designed to investigate whether the circadian changes in haemostatic and endothelial markers observed in healthy subjects occur in patients with sleep

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apnoea. To address this issue, we compared the diurnal variations in the concentration of various haemostatic parameters (factor VII, von Willebrand factor and PAI-1) and markers of endothelial dysfunction (ADMA and sCD40L) in patients with OSAS and in a control group of similar body mass index (BMI), waist circumference and metabolic profile.

METHODS

Subjects and ethics

We studied 26 male patients with OSAS (13 patients with severe OSAS (apnoea/hypopnoea index (AHI) >30 events·h⁻¹) and 13 patients with mild-to-moderate OSAS (AHI <30 events·h⁻¹). As a reference group, we included 12 controls without OSAS. They had all been referred to the sleep laboratory for snoring or suspected OSAS. Each participant was interviewed and was informed in detail of the purpose of this study. They were matched for BMI (± 3 kg·m⁻²) and waist circumference (± 5 cm). The diagnosis of OSAS was established by full polysomnography (E-Series Compumedics, Abbotsford, Australia), including recording of oronasal flow, thoracoabdominal movements, ECG, submental and pre-tibial electromyography, electro-oculography, electroencephalography and transcutaneous measurement of arterial oxygen saturation. Apnoea was defined by the absence of airflow for >10 s. Hypopnoea was defined as any airflow reduction that lasted >10 s and resulted in arousal or oxygen desaturation. We considered desaturation a decrease in arterial oxygen saturation $>4\%$. The AHI was defined as the sum of the number of apnoeas plus hypopnoeas per hour of sleep.

No participant suffered from any chronic disease (diabetes, systemic hypertension, chronic obstructive pulmonary disease, liver cirrhosis, thyroid dysfunction, rheumatoid arthritis, chronic renal failure and/or psychiatric disorders), or was taking any type of medication. The study was approved by the Ethics Committee of our institution, and all the participants signed their consent after being fully informed of its goal and characteristics.

Protocol

Participants arrived at the sleep unit of our institution (Hospital Universitario Son Espases CIBERES, Palma de Mallorca, Spain) at 21:00 h, after fasting for at least 6 h. A heparinised venous catheter (Introcan Safety®; Braun, Melsungen, Germany) was inserted into an antecubital vein to allow serial blood sampling throughout the night without disturbing sleep [31, 32]. From this catheter, six different samples (20 mL each) were obtained during the next 24 h (22:00, 02:00, 06:00, 10:00, 14:00 and 18:00 h). Blood was collected into tubes containing EDTA (10 mL) and into tubes containing sodium citrate (10 mL). The sample obtained at 10:00 h was followed by an additional one (10 mL) collected into tubes without any anticoagulant for general biochemical assessment. Blood samples were immediately processed and centrifuged during 15 min at $2,500 \times g$ (model CR4 22; Jouan S.A., Saint-Herblain, France). Serum and plasma were frozen at -80°C until analysis.

During the study, participants remained in the hospital. Arterial blood pressure was measured between 08:00 and 10:00 h using a mercury sphygmomanometer after they had

been seated for >5 min, with their arm resting on a standard support. During the day, they were allowed to rest or to just perform low-activity tasks and they ate a standardised three-meal diet.

Haematological and biochemical analysis

Blood cell counting was performed on fresh samples using automatic electronic cell counter (XE 2100, Sysmex Corp, Kobe, Japan). Measurements of glucose, cholesterol, triglycerides, uric acid, creatinine and liver enzymes (alanine transaminase (ALT), aspartate transaminase (AST) and γ -glutamyl transpeptidase (GGT)) were performed using a standard automated enzymatic methods on a Hitachi 917 biochemical analyser (Roche Diagnostics, Indianapolis, IN, USA). High-density lipoprotein cholesterol (HDLc) was measured by a homogeneous, enzymatic colorimetric method using a commercial reagent set (Roche Diagnostics). Low-density lipoprotein cholesterol (LDLc) was calculated using the Friedewald equation.

ELISA assays

Factor VII:antigen (Ag), von Willebrand:Ag, PAI-1, sCD40L and ADMA levels were determined by ELISA using commercially available kits (Diagnostica Stago, Asnieres, France (factor VII:Ag, von Willebrand:Ag and PAI-1); R&D Systems Inc., Minneapolis, MN, USA (sCD40L); and BioMedica Diagnostic systems, Vienna, Austria (ADMA)). Measurements were always performed in duplicate, and mean values were used for analysis. The intra-assay coefficients of variation were 3.5% for factor VII:Ag, 6.1 % for von Willebrand:Ag, 4.4% for PAI-1, 5.1% for sCD40L and 6.3% for ADMA.

Statistical analysis

Results are presented as percentages, median (interquartile range) or mean $\pm$ SD. Between-group comparisons were performed using the Kruskal–Wallis test. The Chi-squared test was used to compare categorical variables.

The distributions of endothelial and haemostatic variables were skewed and therefore the log transformation of these measures was applied before statistical analyses. ANOVA test for repeated measures was performed to compare with and within-group measurements of these variables at different times (22:00, 02:00, 06:00, 10:00, 14:00 and 18:00 h).

Comparison between groups for the concentration during follow-up of each marker was performed by the analysis of the areas under the curves (AUCs) using the Kruskal–Wallis test [33]. Separate intergroup comparisons for night (02:00 and 06:00 h), “morning” (10:00 and 14:00 h) or evening (18:00 and 22:00 h) AUCs were also calculated.

Correlations between the subjects’ characteristics and haemostatic variables were explored using the Spearman rank test. Multiple regression analyses were used to confirm the significant associations detected with adjustment for age, BMI, waist circumference and smoking status.

Statistical significance was defined as $p < 0.05$. All statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Tables 1 and 2 show the main clinical characteristics and haematological and biochemical parameters of the two groups of patients and controls studied. By definition, patients with OSAS showed abnormal sleep parameters, whereas these variables were normal in controls. BMI, systolic and diastolic pressure, and glucose, triglycerides, cholesterol, creatinine, AST, ALT and GGT levels were similar between both groups of patients and controls, although the latter were slightly younger (table 1).

The number of circulating erythrocytes, leukocytes and platelets was similar in all the groups (table 2).

Figure 1 shows the circadian pattern of these variables observed in each group. Although all the markers values tended to be higher in patients with severe OSAS, these differences did not reach statistical significance at any time.

No differences between groups were detected between the AUC for PAI-1 ($p=0.30$), for factor VII:Ag ($p=0.695$), for von Willebrand factor:Ag ($p=0.534$), for sCD40L ($p=0.990$) and for ADMA ($p=0.395$). Moreover, we did not find any differences between the AUC for these variables at three different intervals (morning, afternoon and night) during a 24-h period.

Spearman's correlation analysis showed that PAI-1 levels correlated positively with BMI ($r=0.667$, $p<0.001$) and negatively with the mean ($r=-0.596$, $p<0.001$) and minimal nocturnal oxygenation saturation ($r=-0.333$, $p=0.047$) (fig. 2). The dependency of the association between PAI-1 and oxygenation saturation on measures of adiposity and smoking

TABLE 1 Characteristics of subjects studied

	Severe OSAS	Mild-to-moderate OSAS	Controls
Subjects n	13	13	12
Age yrs	43±2	39±2	37±2
AHI events·h ⁻¹	59±6	20±3	6±1**
Mean Sa _o ₂ %	93±1	95±1	97±1**
Minimum Sa _o ₂ %	80±2	85±1	90±1**
Arousal index	51±7	22±3	8±2**
Epworth scale	9±1	9±1	8±1
TC 90 %	22.3±7.2	2.3±1.2*	1.0±0.5*
TST min	385.5±40.5	335.5±70.5	322.7±50.5
REM sleep %	14.7±6.2	14.3±8.3	16.9±7.7
BMI kg·m ⁻²	28±1	27±1	27±1
Waist circumference cm	100±2	98±3	96±3
Neck circumference cm	41±1	40±1	39±1
SBP mmHg	131±3	123±3	124±4
DBP mmHg	84±2	72±3	74±2
Smokers	3 (23)	4 (31)	5 (41)

Data are presented as mean±SD or n (%), unless otherwise stated. OSAS: obstructive sleep apnoea syndrome; AHI: apnoea/hypopnoea index; Sa_o₂: arterial oxygen saturation; TC 90: % time with Sa_o₂ <90%; TST: total sleep time; REM: rapid eye movement; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure. *: $p<0.05$ versus severe OSAS; **: $p<0.01$ versus OSAS.

TABLE 2 Haematological and biochemical parameters

	Severe OSAS	Mild-to-moderate OSAS	Controls
Subjects n	13	13	12
Glucose mg·dL ⁻¹	97±2	97±3	98±3
Triglycerides mg·dL ⁻¹	155±22	136±21	158±37
Cholesterol mg·dL ⁻¹	200±6	183±11	170±7*
HDLc mg·dL ⁻¹	45±3	46±4	40±4
LDLc mg·dL ⁻¹	128±6	111±11	98±3*
Uric acid mg·dL ⁻¹	6.1±0.4	6.0±0.4	6.0±0.3
Creatinine mg·dL ⁻¹	0.94±0.04	0.93±0.02	0.91±0.02
AST U·L ⁻¹	23±2	23±3	24±2
ALT U·L ⁻¹	28±3	27±2	27±2
GGT U·L ⁻¹	32±3	30±2	30±2
Erythrocytes × 10 ⁶ cells·μL ⁻¹	5.1±0.1	5.0±0.1	5.0±0.1
Leukocytes × 10 ³ cells·μL ⁻¹	8.0±0.6	7.3±0.3	7.8±0.4
Platelets × 10 ³ cells·μL ⁻¹	222±12	228±13	237±19

Data are presented as mean±SD, unless otherwise stated. OSAS: obstructive sleep apnoea syndrome; HDLc: high-density lipoprotein cholesterol; LDLc: low-density lipoprotein cholesterol; AST: aspartate transaminase; ALT: alanine transaminase; GGT: γ-glutamyl transpeptidase. *: $p<0.05$ versus severe OSAS.

status was explored in a multiple regression analysis. Significant associations were diminished, but not eliminated, after adjustment for age, BMI, waist circumference and smoking status (mean oxygenation saturation, $p=0.031$).

ADMA levels were also significantly related to the arousal index ($p=0.046$) (fig. 2).

DISCUSSION

This study shows that day–night variations in factor VII:Ag, von Willebrand factor:Ag, PAI-1, sCD40L and ADMA levels are not significantly different between patients with OSAS and controls without OSAS of similar adiposity and metabolic profile. Our results suggest that sleep apnoea does not have any direct effect on the oscillations of these haemostatic substances. Nevertheless, it is possible that the procoagulant consequences of sleep apnoea may become apparent in the presence of comorbidities, such as the metabolic syndrome.

Coagulation and fibrinolysis may influence cardiovascular risk in OSAS, but the relationship of adiposity with these processes is unclear [34]. Factor VII, von Willebrand factor, representatives of the haemostatic system and PAI-1, as the most important inhibitor of the fibrinolytic system, have been associated with visceral obesity [35–37]. In addition, adipose tissue has emerged as a key secretory organ that may regulate CD40L expression, suggesting a novel mechanism that accounts for the prothrombotic state of obese individuals and patients with metabolic syndrome [38, 39].

In the general population, acute CV events occur frequently in the early morning hours, when there is a marked rise in neural

FIGURE 1. Mean values of a) factor VII: antigen (Ag), b) von Willebrand factor (vWF):Ag, c) plasminogen activator inhibitor (PAI)-1, d) soluble CD40 ligand (sCD40L) and e) asymmetric dimethylarginine (ADMA) at different times during the day in both groups of patients and controls. OSAS: obstructive sleep apnoea syndrome.

and hormonal sympathetic activity, increased platelet activity and hypercoagulability [6, 8, 10, 40]. By contrast, in patients with OSAS, the timing of myocardial infarction and sudden death shifts from the morning hours to the night, while the patient is actually sleeping [13, 14]. The mechanisms by which this occurs are unclear. There is an intimate relationship between the circadian clock, metabolism and obesity [41, 42], and several studies have shown that diabetic patients exhibit a blunted circadian variation in haemostatic and fibrinolytic factors potentially associated with morning peaks of cardiovascular events [26, 29]. The current study assesses the circadian behaviour of these biomarkers in two groups of patients with OSAS and in a control group with a similar degree of obesity. There were no significant differences in the median values of these markers at different intervals between the three groups. Despite the fact that all markers values tended to be higher in patients with severe OSAS, these

differences not reached statistical significance at any time. Nevertheless, the fact that our results suggest that the changes in these patterns may be dependent on the obesity index or metabolic dysfunction rather than on sleep apnoea alone does not mean that they are irrelevant in the pathogenesis of cardiovascular complications in these patients. Metabolic and hormonal aspects should be considered in future studies to test this hypothesis.

Several studies that have assessed haemostasis parameters in healthy subjects and patients with a history of coronary artery disease have shown that, despite the higher activity of PAI-1 in patients, the periodicity of changes was maintained in both groups. In our study, the circadian pattern of PAI-1 found in the controls was still present in the patients, albeit at a higher level. PAI-1 was associated with BMI and mean and minimum nocturnal oxygenation saturation. In a recent study, VON KÄNEL

FIGURE 2. Relationship between plasminogen activator inhibitor (PAI)-1 and a) body mass index (BMI), and b) minimum and c) mean nocturnal arterial oxygen saturation (Sa_{O_2}).

et al. [30] found that the relationship between OSAS and PAI-1 was attenuated after controlling for BMI and mean arterial pressure. We did not include hypertensive subjects and, even though most of this association was explained by central obesity, PAI-1 levels were independently associated with indices of nocturnal hypoxia, even after adjusting for confounders. Furthermore, recent results of the Cleveland Family Study provide evidence for a positive relationship between OSA and PAI-1 levels [43]. These observations suggest a potential role of PAI-1 in the link between obesity, OSAS and CV risk.

Vascular tone and the concentration of NO metabolites in plasma exhibit a circadian variation [21]. Plasma concentrations of ADMA are elevated in several clinical syndromes associated with increased cardiovascular risk [44]. We have previously shown that plasma ADMA levels are elevated in patients with OSAS [45]. However, there is also evidence that ADMA levels are higher in obese and insulin-resistant individuals [46]. In the current study, the ADMA mean values tended to be higher in patients with severe OSAS but these differences did not reach any statistical significance. These results suggest that the concentration of ADMA may vary in OSAS according to the degree of obesity and metabolic disturbances. There was a significant correlation, however, between ADMA levels and the arousal index, suggesting a possible additional mechanism by which OSA may influence ADMA levels and lead to endothelial dysfunction.

One potential limitation of our study is that it included only male subjects. Furthermore, none of the participants described symptoms associated with excessive daytime sleepiness. As a consequence, our data cannot automatically be extrapolated to female patients or patients with excessive daytime sleepiness.

Compared with controls, patients with OSAS showed a higher variability in mean levels of PAI-1 at different times. This implies that other factors, not studied here, may be involved in these changes. Obesity may influence the circadian rhythms of cardiovascular and metabolic markers. The fact that other factors were not related to BMI, in either the patients or the controls, might be explained by the narrow range of BMI in the subjects studied herein. Nevertheless, hormonal aspects should be considered in the future.

Furthermore, although the control group had a very similar metabolic profile to that of the OSAS groups, the sample size limits the conclusions, and large studies should be carried out to evaluate the role of these haemostatic factors in the process of cardiovascular complications in patients with sleep apnoea.

In conclusion, the results of this study indicate that the day-night variations in the levels of several endothelial markers and haemostatic factors are not different between patients with sleep apnoea and controls of a similar weight. It is becoming increasingly clear that the circadian clock and metabolism directly influence one another. The search for additional factors that may contribute to better understanding the links between OSAS, metabolism and cardiovascular disease is highly desirable.

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STATEMENT OF INTEREST

None declared.

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