Obstructive Sleep Apnoea, Cigarette Smoking and Plasma Orexin-A in a Sleep Clinic Cohort

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Orexin-A is a neuropeptide involved in the regulation of food intake and the sleep–wake cycle. This study investigated plasma orexin-A levels in a sleep clinic cohort, adjusting for smoking habits, in 76 participants comprising 41 with obstructive sleep apnoea (OSA) (apnoea–hypopnoea index [AHI] 44.1 ± 19.1 events/h) and 35 without OSA (AHI 6.3 ± 4.7 events/h). Plasma orexin-A levels were significantly lower in OSA patients (15.0 ± 4.6 ng/ml) compared with those without OSA (31.4 ± 6.5 ng/ml). In non-OSA subjects, there was no significant difference between never smokers and ex/current smokers in plasma orexin-A levels (32.9 ± 9.5 versus 29.7 ± 8.9 ng/ml, respectively) whereas, in the OSA sub-group, orexin-A levels were significantly lower in never smokers than in ex/current smokers (4.0 ± 1.2 versus 21.4 ± 7.0 ng/ml). A significant inverse relationship was found between plasma orexin-A levels and AHI amongst never smokers, but there was no significant relationship amongst ex/current smokers. These results confirm previous studies demonstrating lower levels of plasma orexin-A in OSA patients and indicate that smoking may affect orexin-A levels and AHI.

KEY WORDS: OBSTRUCTIVE SLEEP APNOEA; OREXIN-A; CIGARETTE SMOKING; APNOEA–HYPOPNOEA INDEX

Introduction
Obstructive sleep apnoea (OSA) is a common disorder with a diversity of symptoms¹ and increased cardiovascular morbidity². Underlying mechanisms regarding the pathogenesis as well as the severity of the disease still remain unclear. A neuropeptide, orexin-A (also known as hypocretin-1), has been reported to be involved in the regulation of food intake and the sleep–wake cycle,³,⁴ and some authors have demonstrated low levels of plasma orexin-A in OSA patients,⁵–⁷ while others have found the opposite.⁸ There are also conflicting data regarding the relationship between plasma orexin-A levels and the severity of OSA in...
terms of the apnoea–hypopnoea index (AHI)\(^6\)–\(^8\) as well as the impact of alleviation of OSA by continuous positive airway pressure (CPAP) treatment.\(^7,9\) Results from human trials have suggested that plasma orexin-A levels are negatively correlated with body mass index (BMI)\(^10\) and positively correlated with age.\(^11\)

Cigarette smoking, which is common in clinic populations, has been suggested to be an independent risk factor for OSA by some authors,\(^12,13\) whereas others do not support this.\(^14,15\) Some beneficial effects of nicotine, including protection against OSA, were discussed in the early 1990s,\(^16\) however, overnight transdermal nicotine administration was shown adversely to affect sleep and respiratory parameters in non-smokers\(^17\) and a recent study has revealed that smoking interacts with OSA to increase cardiovascular risk.\(^18\)

The role of nicotine on orexin-A levels has been previously studied in rats, demonstrating suppressed slow-wave sleep (SWS) and rapid eye movement (REM) sleep, and increased wakefulness in a dose-dependent manner.\(^19\) To our knowledge, however, there are no data on the impact of cigarette smoking on plasma orexin-A levels in OSA patients.

The aim of the current study was to address the relationship between levels of plasma orexin-A and OSA, whilst considering cigarette smoking as a confounding factor, in a sleep clinic population.

Patients and methods

STUDY POPULATION

All participants were recruited from subjects who were being investigated in the Sleep Disorders Centre of the Atatürk Chest Diseases and Chest Surgery Education and Research Hospital, Ankara, Turkey, between 1 January 2005 and 31 July 2006, following concerns of snoring or clinical suspicion of OSA.

Subjects were eligible if they were willing to participate in the study, if they did not have cardiovascular disease, diabetes mellitus, renal failure, chronic obstructive pulmonary disease (COPD), asthma or malignancy, if they had not previously been diagnosed or treated for OSA, if they had no history of episodes of cataplexy, and if they were free from hypnotic drugs. Subjects with total sleep time < 240 min and those with signs of central sleep apnoea on polysomnography recordings were excluded. The study protocol was approved by the ethics committee of the Atatürk Chest Diseases and Chest Surgery Education and Research Hospital and both written and verbal informed consent were obtained from each participant prior to study entry.

SMOKING HISTORY AND STUDY ASSESSMENTS

Current smokers were defined as subjects who were smokers at the time of admission to the Sleep Disorders Centre as well as those who had stopped smoking for < 6 months prior to admission. Subjects who had ceased smoking ≥ 6 months prior to admission were defined as ex-smokers. Both current smokers and ex-smokers were questioned about the number of cigarettes that they smoked daily as well as the number of years that they smoked. Assuming that a pack contains 20 cigarettes, pack-years were estimated using the following formula: (cigarettes per day/20) \(\times\) years smoked. Current smokers and ex-smokers were grouped together in the statistical analysis so as to provide sufficient statistical power to evaluate the effect of smoking behaviour on plasma orexin-A levels; subjects who never smoked were grouped separately as non-smokers.
All subjects underwent pulmonary function tests (V_{max} 229; SensorMedics® Corp., Yorba Linda, CA, USA), and forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV_{1}) values and FEV_{1}/FVC ratios were recorded. Subjects with FEV_{1}/FVC < 70% were excluded as they were regarded as having COPD. Body weight and height measurements were assessed and BMI (kg/m^{2}) was computed as the ratio between body weight (kg) and squared height (m). Excessive daytime sleepiness was reported using the Epworth Sleepiness Scale (ESS).20

OVERNIGHT SLEEP STUDIES
All participants underwent full-night polysomnography, using the Compumedics® Voyager Digital Imaging E-series system (Compumedics®, Melbourne, Australia). They were advised not to sleep during the daytime, and not to consume caffeine-containing beverages, food, alcohol, and any medication that might affect their sleep pattern within 12 h prior to the full-night polysomnography.

The polysomnography recording included the following: two electroencephalogram channels according to the 10 – 20 system (C3 – A2 and C4 – A1), two electro-oculograms, bipolar surface electromyograms of the submentalis and bilateral anterior tibialis muscles and position sensors to record body position and movements. Respiratory monitoring consisted of nasal and oral airflow measurements using a thermistor, a tracheal microphone, thoracic and abdominal respiratory effort measurements using piezoelectric belts, finger pulsoximetry using a Monet Porti 5-16/ASD system (Medcare Systems, Buffalo, NY, USA) and an electrocardiogram. Sleep stages were visually scored in 30-s epochs according to the standard criteria of Rechtschaffen and Kales.21 Apnoea was defined as a complete cessation of airflow for ≥ 10 s. Hypopnoea was defined as a reduction in airflow of ≥ 50% accompanied by ≥ 3% desaturation in the preceding 30 s and a reduction in chest wall movement and/or arousal. Data were expressed using the AHI based on the number of apnoea and hypopnoea events per hours slept; AHI ≥ 15 events/h confirmed a positive OSA diagnosis.22

MEASUREMENT OF PLASMA OREXIN-A LEVELS
All venous blood samples were drawn from the participants between 07:00 h and 08:00 h after an overnight fast of approximately 10 h. Current smokers were told to abstain from smoking the night and morning before blood samples were drawn. The samples were collected using vacuum blood-collecting tubes containing ethylenediaminetetraacetic acid, for anticoagulation. To inhibit the activity of proteinases, 1 ml of each blood sample was transferred into centrifuge tubes containing aprotinin, after which plasma was obtained by centrifugation and immediately frozen at –80°C until analysis. The cold chain for the blood samples was adequately maintained at every step.

Plasma concentrations of orexin-A were measured using an enzyme immunoassay (EIA) kit for orexin-A (orexin-A [human, mouse, rat] EIA Kit; Phoenix Pharmaceuticals®, Burlingame, CA, USA) with intra- and inter-assay coefficients of variation of < 5% and < 14%, respectively. The assay sensitivity for orexin-A was 0.37 ng/ml. Specificity of the assay for orexin-A (human, mouse, rat) was 100% with no cross-reactivity with orexin-A 16–33, agouti-related protein 83–132-amide, neuropeptide Y, α-melanocyte stimulating hormone, leptin or hypocretin-1 amide.

Plasma extraction prior to analysis was not performed since this procedure was
optional according to the instructions of the commercial EIA kit.

STATISTICAL ANALYSIS
Continuous variables were expressed as means ± SD except for the plasma orexin-A levels, which were expressed as mean ± SE and categorical variables which were expressed as numbers and percentages. For comparison of the groups, an independent Student's t-test was applied for continuous variables and the χ²-test or Fisher's exact test were used for categorical variables. Correlations between variables were assessed using the Pearson or Spearman's correlation test. All statistical tests were two-sided and a P-value < 0.05 was considered statistically significant. The analyses were assessed with the Statistical Package for Social Sciences (SPSS®) version 11.5 (SPSS Inc, Chicago, IL, USA) for Windows® system.

Results
STUDY SUBJECTS
Of the 81 subjects originally recruited into the study, five were excluded from the final analysis due to considerable clinical evidence of either COPD or asthma. Of the remaining 76 subjects, 41 (54%) had an AHI ≥ 15 events/h revealing an OSA diagnosis, and 35 (46%) were classified as not having OSA. As shown in Table 1, OSA subjects were significantly older than the non-OSA subjects (P < 0.05).

SLEEP OUTCOMES
As expected, ESS was positively correlated with arousal index (r = 0.310, P = 0.006) and AHI (r = 0.350, P = 0.002), and there was a negative relationship between ESS score and REM sleep (r = -0.484, P < 0.001) and SWS (r = -0.243, P = 0.035). There was no relationship between ESS and plasma orexin-A levels.

RELATIONSHIP BETWEEN SMOKING AND OSA
Regarding the smoking habits, 43 of the 76 subjects included in the study were either current smokers (n = 22) or ex-smokers (n = 21). None of the participants was using cigars, snuff, pipes or water-pipes and none

| TABLE 1: Baseline characteristics of the study population (n = 76) |
|------------------------|------------------------|------------------------|
|                        | OSA (n = 41)           | Non-OSA (n = 35)       | Statistical significance |
| Age, years             | 50.6 ± 8.4             | 41.6 ± 9.9             | P < 0.001                |
| Male gender, n (%)     | 30 (73.0)              | 21 (60.0)              | NS                       |
| BMI, kg/m²             | 30.1 ± 4.3             | 28.3 ± 5.9             | NS                       |
| Ex-/current smokers, n (%) | 26 (63.4)       | 17 (48.6)              | NS                       |
| Pack-years of smoking  | 14.3 ± 14.9            | 9.1 ± 12.7             | NS                       |
| FEV1, % predicted      | 91.0 ± 16.2            | 95.9 ± 11.3            | NS                       |
| Epworth Sleepiness Scale | 8.4 ± 4.2               | 5.4 ± 4.0              | P = 0.002                |
| AHI, events/h          | 44.1 ± 19.1            | 6.3 ± 4.7              | P < 0.001                |
| Mean SaO₂ during sleep, % | 90.2 ± 4.7          | 93.4 ± 2.0             | P < 0.001                |
| Minimum SaO₂ during sleep, % | 79.8 ± 10.0        | 89.3 ± 4.0             | P < 0.001                |
| Arousal index, events/h| 46.7 ± 20.1            | 6.8 ± 5.4              | P < 0.001                |

Data are presented as mean ± SD, unless otherwise stated.
OSA, obstructive sleep apnoea; BMI, body mass index; FEV1, forced expiratory volume in 1 s; AHI, apnoea–hypopnoea index; SaO₂, arterial oxygen saturation; NS, not statistically significant (P > 0.05).
had received nicotine replacement treatment within the 6 months prior to the current study. Calculated pack-years of smoking was higher among the ex-smokers (23.8 ± 13.44 years) compared with current smokers (18.4 ± 11.2 years) though this difference was not statistically significant. The proportion of the smokers was higher in the OSA group compared with the non-OSA group (63.4% and 48.6%, respectively) but this difference was not statistically significant (Table 1). The baseline characteristics of the ex/current smokers and never smokers were similar, and AHI and none of the other sleep parameters or the plasma orexin-A level were statistically different between these two groups (Table 2).

**RELATIONSHIP BETWEEN PLASMA OREXIN-A LEVELS AND OSA**

In the whole study population there was no significant relationship between plasma orexin-A levels and age, gender, BMI, smoking habits, AHI and other sleep parameters. However, plasma orexin-A levels were significantly lower in the OSA patients (15.0 ± 4.6 ng/ml) compared with the non-OSA subjects (31.4 ± 6.5 ng/ml) ($P = 0.003$). As illustrated in Fig. 1, orexin-A levels were even lower in the never smoker OSA sub-group compared with the ex/current smoker OSA patients (4.0 ± 1.2 versus 21.4 ± 7.0 ng/ml, respectively; $P = 0.020$). In contrast, in non-OSA subjects, there was no significant difference between never smokers and ex/current smokers in plasma orexin-A levels (32.9 ± 9.5 versus 29.7 ± 8.9 ng/ml, respectively). Amongst never smokers, an inverse relationship between AHI and plasma orexin-A levels was found ($r = -0.361$, $P = 0.039$), but this was not the case for ex/current smokers ($r = -0.092$, $P > 0.05$) (Fig. 2). No relationship was found between pack-years of smoking and plasma orexin-A levels. Excluding 11 outliers (four current smokers, three ex-smokers, four non-smokers) who had plasma orexin-A levels of 100 ng/ml (Fig. 2) did not change the main findings of the study.

### TABLE 2:
Characteristics of the study population according to smoking habits

<table>
<thead>
<tr>
<th></th>
<th>Never smokers ($n = 33$)</th>
<th>Ex/Current smokers ($n = 43$)</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>44.8 ± 10.4</td>
<td>47.7 ± 9.9</td>
<td>NS</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>30.4 ± 5.1</td>
<td>28.5 ± 5.1</td>
<td>NS</td>
</tr>
<tr>
<td>FEV₁, % predicted</td>
<td>95.1 ± 13.4</td>
<td>91.8 ± 14.9</td>
<td>NS</td>
</tr>
<tr>
<td>Epworth Sleepiness Scale</td>
<td>7.6 ± 4.4</td>
<td>6.6 ± 4.2</td>
<td>NS</td>
</tr>
<tr>
<td>Sleep efficiency, %</td>
<td>78.4 ± 10.4</td>
<td>81.2 ± 6.2</td>
<td>NS</td>
</tr>
<tr>
<td>Slow-wave sleep, % total sleep time</td>
<td>15.7 ± 8.6</td>
<td>14.6 ± 8.3</td>
<td>NS</td>
</tr>
<tr>
<td>REM sleep, % total sleep time</td>
<td>15.2 ± 6.4</td>
<td>15.8 ± 5.7</td>
<td>NS</td>
</tr>
<tr>
<td>AHI, events/h</td>
<td>24.6 ± 23.9</td>
<td>28.2 ± 23.7</td>
<td>NS</td>
</tr>
<tr>
<td>Mean Šao₂ during sleep, %</td>
<td>92.6 ± 2.2</td>
<td>91.0 ± 4.9</td>
<td>NS</td>
</tr>
<tr>
<td>Minimum Šao₂ during sleep, %</td>
<td>85.2 ± 7.4</td>
<td>83.4 ± 10.3</td>
<td>NS</td>
</tr>
<tr>
<td>Arousal index, events/h</td>
<td>25.2 ± 24.0</td>
<td>30.8 ± 25.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD, unless otherwise stated. BMI, body mass index; FEV₁, forced expiratory volume in 1 s; REM, rapid eye movement; AHI, apnoea–hypopnoea index; Šao₂, arterial oxygen saturation; NS, not statistically significant ($P > 0.05$).
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FIGURE 1: Box and whisker plots for plasma orexin-A levels in patients with obstructive sleep apnoea (OSA) compared with non-OSA subjects according to the smoking habits, showing that never smoker OSA patients had significantly lower levels of orexin-A compared with ex/current smoker OSA patients ($P = 0.020$), whereas there was no significant difference ($P = 0.810$) among non-OSA subjects (boxes represent 25th – 75th percentiles, the 50th percentile is shown as a solid line within the boxes, ‘whiskers’ represent the minimum and maximum levels excluding outliers, which are indicated with asterisks).

FIGURE 2: Association between plasma orexin-A levels and apnoea–hypopnoea index (AHI) in the whole study population according to smoking habits, showing a statistically significant inverse relationship between plasma orexin-A levels and AHI among subjects who have never smoked ($r = -0.361, P = 0.039$) but no significant association in ex/current smokers ($r = -0.092, P = 0.558$).
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Discussion
The present study demonstrated that lower plasma orexin-A levels occur in OSA patients compared with non-OSA subjects, in accordance with some previous reports.5 – 7 Additionally, the present study showed an inverse relationship between AHI and plasma orexin-A levels only in never smokers.

In recent years, determining the involvement of the orexinergic system in the sleep–wake cycle has been the focus of studies and they suggest that defects in the system might be a potential cause of the pathogenesis of sleep-disordered breathing. Hagan et al.23 have demonstrated that orexin-A activates locus coeruleus cell firing and increases arousal in rats. In other animal studies, it has also been shown that injection of orexin-A into related parts of the central nervous system may suppress REM and SWS, and increase wakefulness in a dose-dependent manner.24 – 26 Clinical trial data, however, have shown abnormally low levels of circulating orexin-A in patients with OSA, independent of the level of somnolence and/or presence of obesity,7 and it was suggested by those authors that the low orexin-A levels might be related to the pathogenesis of OSA.

Orexin-A is involved not only in sleep and arousal regulation but also in regulation of appetite and food intake,27 which is a complex process involving other neuropeptides,28,29 such as leptin.30 It has been shown that leptin and orexin-A concentrations are inversely regulated.31 In the current study, leptin concentrations were not measured but it has been suggested that untreated OSA patients have increased leptin levels.32 There are also data from obesity research showing significantly lower orexin-A levels and higher leptin levels in obese individuals compared with control subjects.10 Bronsky et al.33 have recently demonstrated increasing levels of plasma orexin-A and decreasing levels of leptin during body weight loss in obese children, supporting the hypothesis that orexin-A may be involved in the regulation of nutritional status.

The findings presented here support previous reports showing significantly lower levels of plasma orexin-A in patients with OSA,5 – 7 and levels of the peptide were not related to AHI and other sleep parameters. Confounding factors such as age, gender and BMI were not found to be related to plasma orexin-A levels. Since it is known that plasma orexin-A levels are influenced by creatinine clearance34 as well as by levels of plasma glucose,35 subjects with renal failure or diabetes mellitus were not included in this study. Any subjects with a history of episodes of cataplexy were also excluded since there are many studies validating an orexinergic defect as the underlying mechanism of narcolepsy.36 As patients with COPD are known to have lower orexin-A levels,37 subjects with a known clinical diagnosis of COPD as well as those with impaired lung function tests were also excluded.

The role of nicotine on orexin-A has been previously addressed in rat studies, demonstrating that SWS and REM sleep are suppressed and wakefulness increased in a dose-dependent manner.19 Currently, there are no published data on the impact of cigarette smoking on plasma orexin-A levels in human trials. If nicotine does an acute effect on plasma orexin-A levels, it would be reasonable to expect higher levels of plasma orexin-A in current smokers but not in ex-smokers. Although current smokers in the present study were told to abstain from smoking the night and morning before blood samples were drawn, it was not possible to ensure that they all did so, and this might then have resulted in extremely high values in some of the current smokers. Such values were observed but they were not confined
obligatory to smokers; three to four subjects in all sub-groups exhibited high plasma orexin-A levels regardless of their smoking habits and this suggests that, although smoking might have an influence, it is not necessarily through the acute effects of nicotine.

When considering the mean pack-years of smoking (23.8 years) in the ex smoker group, it may be that feeding behaviour, as well as the effect of neuropeptides in the regulation of food intake, and the sleep–wake cycle play a role. It is also noteworthy that, in the whole study cohort, there was no relationship between smoking and orexin-A levels, but there was a relationship in the sub-group with OSA. Hence, in the absence of OSA, plasma orexin-A levels were comparable in never smokers versus ex/current smokers but, in the OSA group, the levels were significantly lower in never smokers than in ex/current smokers. Plasma orexin-A levels in the ex/current smoker OSA sub-group were even lower than in the non-OSA subjects (regardless of smoking habits), which suggests that OSA plays a major role in the decrease in levels of plasma orexin-A.

As mentioned above, lower plasma levels of orexin-A in OSA patients have been previously reported in three clinical studies. In one there was also an inverse relationship between orexin-A levels and AHl, arousal index and ESS among OSA patients, however this could not be confirmed in a follow-up study performed by the same study group. This may be due to the fact that plasma orexin-A levels are influenced by a wide range of variables and smoking habits might be just one of several confounding factors.

The impact of the treatment of OSA with CPAP for 3 – 6 months on plasma orexin-A levels was investigated by Sakurai et al. in 11 patients with severe OSA (arousal index ≥ 60 events/h), compared with 16 OSA patients with an arousal index < 60 events/h. Compared with baseline values, plasma orexin-A was significantly increased in the severe group but not in the milder group (despite a clinical improvement). As discussed by these authors, if the plasma level of orexin-A reflects neuronal activity of the orexin neurons in the hypothalamus, the plasma level of orexin-A would be a good marker of orexin-A secretion from the hypothalamic orexin neurons. Furthermore, if the plasma level of orexin-A reflects the severity of OSA and correlates with the successful treatment of this disorder, this would be of great value. In this context, the measurement of orexin-A concentrations in the spinal fluid might have been preferable but spinal fluid samples could not be obtained in that study for ethical reasons and this was also why this was not done in the current study.

In summary, the current study confirms and accords with previous studies in demonstrating lower levels of plasma orexin-A in OSA patients. It additionally suggests that smoking habits should be considered as a confounding factor in the evaluation of the plasma orexin-A levels in clinical cohorts of sleep apnoea patients. The fact that plasma orexin-A levels were higher in both ex and current smokers compared with never smokers reflects that smoking might have an influence not necessarily through the acute effects of nicotine. In this context, the feeding behaviour of smokers as well as the effects of other neuropeptides involved in the regulation of food intake and the sleep–wake cycle need to be further explored.

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