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S100B in serum and saliva: a valid invasive or non-invasive biomarker in obstructive sleep apnea?

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Abstract. – OBJECTIVE: The aim of this prospective study was to determine whether serum or saliva S100B could be established as an invasive or non-invasive biomarker of cerebrovascular stress due to chronic intermittent hypoxia in obstructive sleep apnea (OSA).

PATIENTS AND METHODS: S100B levels in serum and saliva were measured by an enzyme-linked immunosorbent assay (ELISA) in 40 patients with polysomnographically confirmed OSA (n=34) or ronchopathy (n=6) and 20 control subjects (n=20). We also investigated four healthy volunteers (n=4) to determine whether the S100B levels in serum and saliva are dependent on the time of day.

RESULTS: Serum S100B was significantly higher in OSA than in healthy control subjects (p=0.007), although it was not related to the severity of OSA and was independent of age, sex, and subjective daytime symptoms. Values of S100B in saliva showed a marked scatter, so there was no significant difference between the OSA group and controls (p=0.62). We did not find that S100B levels in either serum or saliva depended on the time of day (p=0.53; p=0.91).

CONCLUSIONS: Serum S100B allows us to discriminate healthy subjects from patients with OSA. However, it does not live up to its promise as a valid invasive predictor of OSA, since it does not correlate with the severity of the disease. Also, S100B in saliva is not suitable for use as a non-invasive biomarker to detect hypoxia-induced cerebrovascular stress in OSA. This finding prevents an S100B saliva-based assessment of risk or possible extent of structural brain damage, ruling out the possibility of non-invasive home monitoring of compliance and therapeutic efficacy in cases of OSA on treatment.

Key Words:
OSA, Obstructive sleep apnea, S100B, Biomarker, Chronic intermittent hypoxia, Saliva.

Introduction

At present, more than 20 small acidic proteins (S100A1-16, S100B, S100G, S100P, S100Z) with a molecular weight of between 9 and 13kDa are known to belong to the S100 family in vertebrates. Moore[1] isolated the first member of this family in 1965. The name ‘S100’ was based on the solubility of these proteins in 100% saturated ammonium sulfate at a neutral pH. Through interactions with numerous target proteins, the individual members of this protein family sometimes show cell-specific expression patterns leading to protein expression in pathological conditions and hence to the regulation of proliferation, cell differentiation, apoptosis, inflammation, and migration. S100B is expressed especially in astrocytes, Schwann cells, melanocytes, chondrocytes, and fat cells. On the one hand, S100B may stimulate proliferation and migration and, on the other, it can act as an apoptosis inhibitor[2]. Depending on its concentration, S100B secreted by astrocytes may have both trophic and toxic effects on neurons, astrocytes, and microglia[3]. For example, S100B concentrations in the nanomolar range stimulate astrocyte proliferation, while higher concentrations in the micromolar range promote inflammatory processes in astrocytes. To date, S100B has been found in human serum, saliva, urine, amniotic fluid, and breast milk[4]. As a clinical biomarker raised serum S100B levels have already been reported in the follow-up testing of malignant melanomas of the skin and central nervous system damage (strokes, subarachnoid hemorrhage, and traumatic brain injury) and seem to correlate with the patient’s outcome[5-7]. The extracranial concentration of S100B (e.g. in the serum) is, however, considerably lower than in nerve tissue or cerebrospinal fluid (CSF)[8]. The most popular explanation is that extracellular S100B passes into the blood circulation following disruption of the blood-brain barrier (e.g. by trauma)[9]. In adults, the serum and CSF concentrations do not seem to be influenced by age or sex[10].

The chronic intermittent hypoxia (CIH) seen with obstructive sleep apnea (OSA) is the reason

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why S100B has become of particular interest in sleep medicine. Various studies[11-16] have already shown a relationship between S100B and OSA, not only *in vitro*, but also *in vivo*. The relationships of OSA with arterial hypertension, heart failure, stroke, and increased mortality, as well as associations with coronary artery disease and cardiac arrhythmias, have already been confirmed[17-19]. Alongside sympathetic activation, the activation of vasoactive substances and inflammatory processes, the induction of oxidative stress, endothelial dysfunction, and the activation of coagulation factors, CIH primarily represents the pathophysiological correlate of these associated comorbidities. Neurological deficits or subtle neuropsychological dysfunction as the manifestations of structural brain damage are common symptoms of chronic intermittent hypoxia in OSA[20]. If an invasive or non-invasive biomarker could be established to detect cerebrovascular stress caused by recurrent nighttime hypoxia, it would allow an early assessment of risk and the possible extent of structural brain damage. It would also be an additional objective parameter to indicate the need for treatment and to monitor the effectiveness of treatment that has already been started. The aim of this study was to evaluate whether the protein S100B in serum or saliva could be established as an invasive or non-invasive biomarker of cerebrovascular stress caused by nighttime hypoxia in obstructive sleep apnea. Regular non-invasive saliva sampling by the patients themselves would allow home monitoring in cases of newly diagnosed obstructive sleep apnea and patients already on treatment.

Patients and Methods

Study Design

The prospective study was carried out in the Department of Otorhinolaryngology, Head & Neck Surgery, Friedrich-Alexander University Erlangen-Nürnberg (FAU) from October 2014 to May 2015 following approval from the Ethics Committee of the Faculty of Medicine. All 60 participants, who were aged between 24 and 77, were recruited through the Department's Sleep Medicine Unit and they gave informed consent before inclusion in the study. The study population consisted of 50 men and 10 women. Besides a standardized sleep interview, patients were examined by an ear, nose and throat (ENT) consultant and underwent endoscopy, while awake, to assess the upper respiratory tract. Cardiorespiratory polysomnography was then carried out in the sleep laboratory for a precise classification of the sleep-related breathing disorder. The severity of the obstructive sleep apnea was ranked as mild (apnea/hypopnea index (AHI) $\geq 5 < 15$), moderate (AHI $\geq 15 < 30$) and severe (AHI ≥ 30) on the basis of the American Academy of Sleep Medicine Task Force criteria[21].

Inclusion and Exclusion Criteria

The inclusion criteria for this study were: men and women aged 20-80 without OSA or with mild, moderate or severe OSA diagnosed on polysomnography. The exclusion criteria were: patients with acute infections, stroke, traumatic brain injury or neurosurgical procedures on the brain in the last three months, neurological or psychiatric diseases, cancer, a positive history of sedative, alcohol or drug misuse, and pregnant women.

Polysomnography

Cardiorespiratory polysomnography (PSG) was performed with the SOMNOscreen diagnostic system (SOMNOmedics, Randersacker, Germany). The technical implementation of the PSG followed the recommendations of the American Academy of Sleep Medicine (AASM) using standardized procedures including an electroencephalogram (EEG; F4-M1, C4-M1, O2-M1), right and left electro-oculograms (EOG), electromyograms (EMG) of the mentalis and tibialis muscles, a nasal respiratory flow sensor (nasal pressure cannula), thoracic and abdominal respiratory effort sensors (induction plethysmography), position sensors, pulse oximetry, snoring microphone, a one-lead ECG, and an infra-red video recording[22]. The results were evaluated according to the AASM criteria (version 2.0, 2012) by a sleep specialist accredited by the German Society of Sleep Medicine (DGSM)[23].

Sampling

Venous blood for the determination of the serum S100B concentration was taken in the morning between 9 and 11 o'clock, after the diagnostic PSG. The blood was then centrifuged (1200 g for 10 minutes) and aliquots of the supernatant (serum) were stored at -80°C.

We obtained saliva samples at the same time as the blood was taken. Without any prior preparation, saliva was obtained by means of a cotton wool roll (Salivette®, Sarstedt, Nümbrecht, Germany), which was inserted into the mouth be-

Table I. Baseline characteristics of individual serum-groups.

	Control	Ronchopathy	OSA
Variables (serum) (n=60)	n=20	n=6	n=34
male (%)	15/20 (75)	4/6 (66.7)	31/34 (91.2)
female (%)	5/20 (25)	2/6 (33.3)	3/34 (8.8)
S100B *	11.4 (7.2; 15.5)	19.4 (14.0; 23.0)	21.1 (14.5; 26.3)
AHI *	-	10.4 (9.1; 11.9)	24.2 (17.3; 44.5)
$tSpO_2$<90% *	-	0.1 (0.0; 0.5)	4.1 (0.6; 12.0)
ESS *	-	5.5 (4.0; 6.0)	10.0 (6.0; 13.0)
age *	30.5 (29.0; 39.0)	53.0 (42.0; 56.0)	46.5 (40.0; 54.0)
BMI *	22.5 (20.9; 24.5)	25.6 (24.3; 29.0)	27.9 (25.6; 32.7)

AHI=Apnea Hypopnea Index/h, $tSpO_2$<90%=time with SpO_2<90% per total sleep time, ESS=Epworth Sleepiness Scale, age in years, BMI=Body Mass Index (kg/m^2), *median (25%, 75% quartile), S100B in pg/mL.

tween the cheek and alveolar ridge or between the tongue and the floor of the mouth and left there for two minutes until it was soaked with saliva. The saliva samples were then stored at -80°C.

To test whether the S100B concentrations depended on the time of day, we took serum and saliva samples from four subjects (n=4), 13 times, at hourly intervals between 8 am and 8 pm. These samples were obtained and stored under the conditions described above.

ELISA

S100B concentrations in the serum and saliva samples were determined with a highly specific ELISA kit (Human S100B ELISA plate, Millipore, Darmstadt, Germany). The sample volume used in each case was 50 µL and the assays were carried out according to the manufacturer's instructions. The approximate range of the system was 2.7-2000 pg/mL with a CV of 3% in intra-assay and 2-4.4% in the inter-assay analysis. All samples were analyzed in duplicate.

Statistical Analysis

The data were analyzed with Statistica (StatSoft, Hamburg, Germany). We determined the relationships between different variables with linear regression analyses. Because the data did not show a normal distribution (Lilliefors test, p>0.05), non-parametric procedures were used for further statistical analysis. These included the Mann-Whitney U test for the comparison of the medians (25% quartile; 75% quartile) of two independent groups, the Kruskal-Wallis ANOVA for the comparison of several independent groups (group comparison by means of multiple comparisons of the mean rank sums, giving the result of the H statistic with the corresponding degree of freedom), and the Wilcoxon test for the comparison of matched pairs.

Results

CIH and OSA

Recurrent nighttime apneas and hypopneas are considered to be a causative factor of CIH in OSA. The percentage of sleep time with an oxygen saturation below 90% as measured by pulse oximetry ($tSpO_2$<90%) represents a CIH equivalent. There was a significant linear correlation (p<0.001) between CIH and the AHI. Also, there was a significant effect in the Kruskal-Wallis ANOVA (p=0.002) (Figure 1). In contrast, we did not establish any correlation between CIH ($tSpO_2$<90%) and the S100B in serum or saliva (p=0.33).

Serum S100B

Table I shows the baseline characteristics.

Comparing the serum S100B values of the ronchopathy (n=6), OSA (n=34), and control groups (n=20), the Kruskal-Wallis ANOVA (Figure 2) showed a significant difference on the S100B concentrations (p=0.0098). Analysis of the individual groups showed significantly higher values in the OSA group (p=0.007) than in the healthy subjects. The serum S100B levels in the ronchopathy group lay between those of the OSA group and control group, without there being a significant difference from either. If, however, the ronchopathy group-which by definition does not meet the diagnostic criteria for OSA-is combined with the control group, there are once again significantly

Table II. Baseline characteristics of OSA-subgroups.

		Mild	Moderate	Severe
Variables (serum) (n=34)		n=5	n=15	n=14
male	(%)	4/5 (80.0)	14/15 (93.3)	13/14 (92.9)
female	(%)	1/5 (20.0)	1/15 (6.7)	1/14 (7.1)
S100B	*	16.0 (13.8; 20.9)	21.6 (16.6; 26.3)	20.9 (11.9; 28.3)
AHI	*	8.4 (6.6; 10.3)	19.3 (17.3; 23.3)	50.8 (34.2; 65.9)
tSpO2<90%	*	0.2 (0.1; 0.7)	3.3 (0.3; 10.0)	10.7 (4.2; 58.3)
ESS	*	11.0 (10.0; 13.0)	8.0 (6.0; 12.0)	10.5 (5.0; 14.0)
age	*	40.0 (38.0; 52.0)	47.0 (42.0; 51.0)	45.5 (41.0; 55.0)
BMI	*	25.6 (25.0; 26.9)	27.0 (24.6; 31.1)	31.6 (28.0; 35.8)

AHI=Apnea Hypopnea Index/h, $tSpO_2$<90%=time with SpO_2<90% per total sleep time, ESS=Epworth Sleepiness Scale, age in years, BMI=Body Mass Index (kg/m^2), *median (25%, 75% quartile), S100B in pg/mL.

lower serum S100B levels (Mann-Whitney U test, *p*=0.006) in comparison with patients with OSA.

Effects of Severity of OSA

Table II shows the baseline characteristics.

The OSA group (n=34) was divided into the subgroups of mild (n=5), moderate (n=15) and severe (n=14), following the AASM Task Force criteria[19]. The Kruskal-Wallis ANOVA likewise showed significant differences between the control group, the ronchopathy group and the OSA subgroups (mild, moderate, and severe) (*p*=0.039) (Figure 2). However, comparison of the individual groups showed a significant increase only in the moderate OSA group about the control group (*p*=0.04). There were no significant differences between the degrees of severity (always *p*>0.05).

Effects of Age and Sex

The serum S100B values found in this study were not dependent on the sex of the patient (Mann-Whitney U test, *p*>0.05).

The serum S100B level did not significantly depend on age in either a linear or non-linear manner (linear regression, *p*=0.12). It should be mentioned, however, that the age of the OSA group was significantly higher (median 46.5 years) than that of the control group, which had a median of 30.5 years (*p*<0.001).

Effects of Daytime Symptoms (ESS)

The daytime symptoms regarding sleepiness recorded with the help of the Epworth Sleepiness Scale (ESS) were significantly less in control subjects than in the patients of the OSA group (*p*=0.03). Nevertheless, no significant linear or non-linear dependency was found between the serum S100B and the ESS score (linear regression, *p*=0.69).

Effects of the BMI

Both the OSA and the control groups were divided into persons of normal weight (BMI < 25 kg/m^2) and those who were overweight (BMI ≥ 25 kg/m^2). This subdivision allows direct individual comparisons using the Mann-Whitney U test

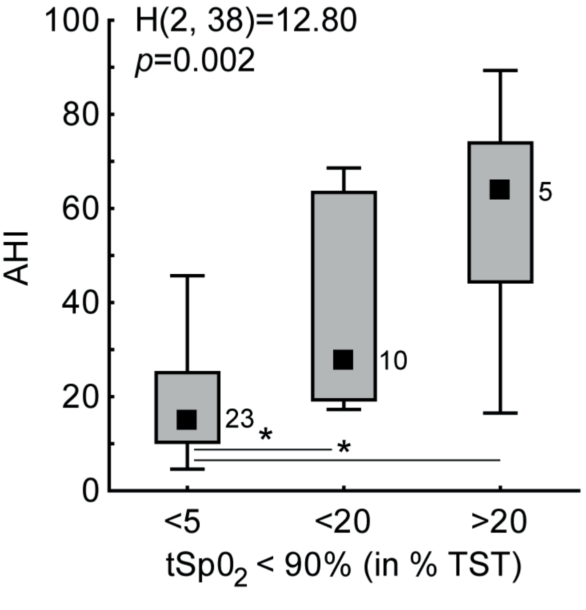

Figure 1. CIH equivalent ($tSpO_2$<90% in %TST) correlated to AHI. CIH=Chronic Intermittent Hypoxia, TST=Total Sleep Time (the total of all REM and non-REM sleep in a sleep episode), AHI=Apnea Hypopnea Index, the respective group sizes (n) are shown next to the boxes on the right side, the classification (<5%, <20%, >20% TST) was made arbitrarily, based on clinical experience; ■ median, □ 25%-75% quartile, ı minimum-maximum.

Table III. Baseline characteristics of the individual saliva-groups.

		Control	Ronchopathy	OSA
Variables (serum) (n=36)		n=20	n=4	n=12
male	(%)	15/20 (75)	2/4 (50)	11/12 (91.7)
female	(%)	5/20 (25)	2/4 (50)	1/12 (8.3)
S100B	*	21.2 (2.1; 58.9)	7.6 (3.5; 122.8)	8.9 (1.4; 43.4)
AHI	*	-	9.8 (6.9; 11.4)	28.1 (17.3; 45.1)
$tSpO_2$<90%	*	-	0.1 (0.0; 1.2)	6.1 (1.0; 10.0)
ESS	*	-	4.5 (2.0; 7.5)	8.0 (3.0; 13.0)
age	*	30.0 (28.5; 35.5)	53.5 (41.5; 57.5)	45.0 (40.5; 51.0)
BMI	*	22.5 (20.9; 24.6)	27.1 (24.7; 31.3)	27.9 (25.6; 31.0)

AHI=Apnea Hypopnea Index/h, $tSpO_2$<90%=time with SpO_2<90% per total sleep time, ESS=Epworth Sleepiness Scale, age in years, BMI=Body Mass Index (kg/m^2), *median (25%, 75% quartile), S100B in pg/mL.

(Figure 3). Taking the individual weight groups into consideration, we found that the serum S100B was significantly higher in the OSA patients of normal weight than in the normal-weight controls (*p*=0.03), while comparison of the two overweight subgroups showed no significant difference. Furthermore, the serum S100B was significantly higher in the overweight control group than in the normal-weight controls (*p*=0.026).

Saliva S100B

Table III shows the baseline characteristics.

The analysis of S100B levels in saliva showed a considerable scatter of the values, not only in the OSA group (min: 0; max: 95.6 pg/mL) but also in the control group (min: 0; max: 567.6 pg/mL). There were no statistically significant differences between the OSA group (n=12), the control group (n=20), and the ronchopathy group (n=4) or the OSA group (n=12) and the combined control/ronchopathy group (n=24) (Kruskal-Wallis ANOVAs, *p*=0.88, and *p*=0.62). In this study, the S100B concentration in saliva was not dependent on the hypoxia-equivalent $tSpO_2$<90% (Kruskal-Wallis ANOVA, *p*>0.05), sex (Mann-Whitney U test, *p*>0.05), age (linear regression, *p*>0.05), BMI (linear regression, *p*>0.05) or daytime symptoms as expressed by the ESS score (Kruskal-Wallis ANOVA, *p*>0.05).

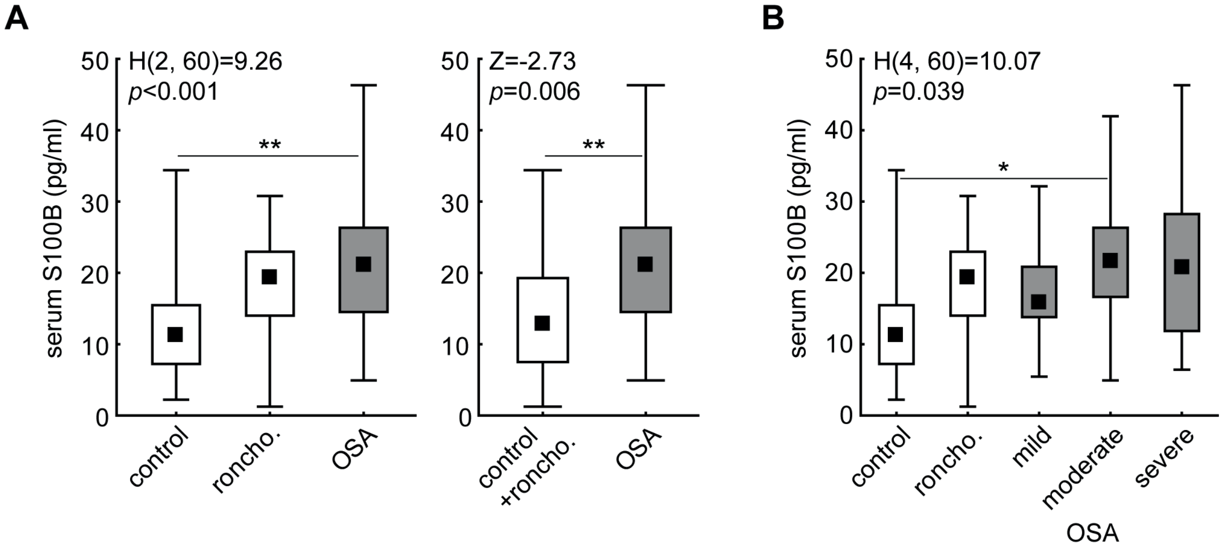

Figure 2. ***A-B***. Serum S100B concentrations in control, ronchopathy and OSA-groups. The respective group sizes (n) are shown in Tables I-III; rhoncho.=snoring ■ median, □ 25%-75% quartile, ı minimum-maximum.

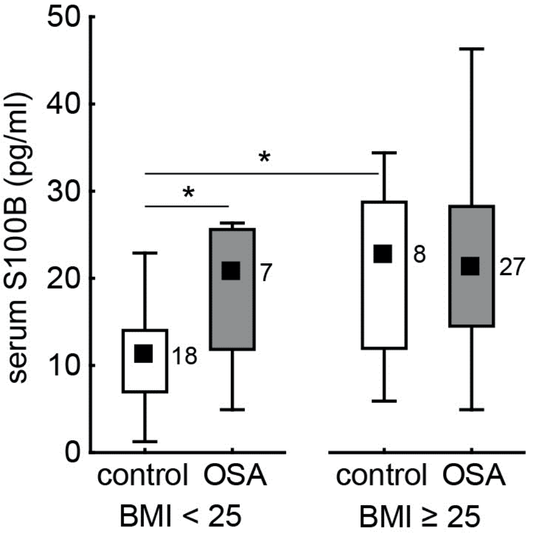

Figure 3. Impact of BMI and OSA on serum S100B. BMI=Body Mass Index (kg/m^2); the respective group sizes (n) are shown next to the boxes on the right side; ■ median, □ 25%-75% quartile, ı minimum-maximum (Mann-Whitney U test).

Dependence on Time of Day

To investigate whether serum and saliva S100B concentrations show a circadian rhythm, we obtained a total of 52 serum and 52 saliva samples from four healthy volunteers. These samples were collected at hourly intervals throughout the day between 8.00 am and 8.00 pm. Of these 52 samples, 43 samples could be examined in each case. Nine serum and nine saliva samples were not suitable for the assay (hemolyzed serum, too little saliva, etc.). The S100B concentrations did not show a significant linear or non-linear dependence on the time of day (Kruskal-Wallis ANOVA) in either serum (*p*=0.53) or saliva (*p*=0.91) (Figure 4A-B).

Discussion

The fact that the duration of nighttime hypoxia (CIH defined as tSpO$_2$<90%) increases significantly as the AHI rises, supports the hypothesis that S100B can be used as a biomarker for cerebrovascular hypoxia-induced stress in obstructive sleep apnea. As some studies have reported previously, we also found a significant increase in the serum S100B levels in adult patients with OSA compared with a healthy control group[11,13,14]. On the effectiveness of nighttime continuous positive airways pressure (nCPAP) in reducing CIH, there is already preliminary evidence that the serum S100B in patients with nCPAP is significantly lower than the pre-treatment levels[24].

The median S100B concentration in our control group (0.01 µg/L) was somewhat lower than the figure for S100B in the blood of healthy adults to be found in the literature (0.05 µg/L)[25]. The results from our OSA group (0.02 µg/L) were also clearly below the reported cut-off limit of 0.10 µg/L in the serum of healthy subjects. The reason for this may be that the hypoxia-induced cerebro-

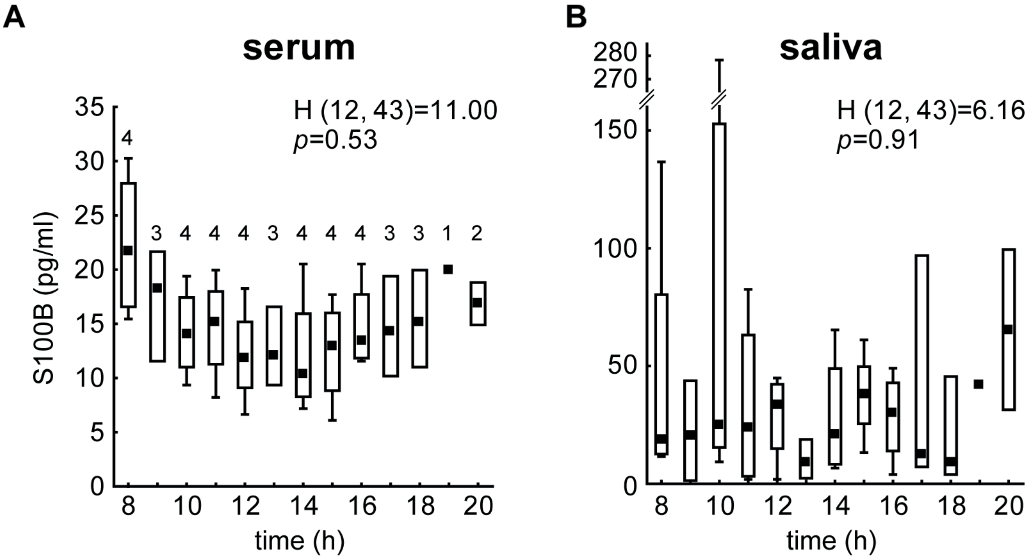

Figure 4. ***A-B.*** Diurnal course of S100B in serum (A) and saliva (B). The respective group sizes (n) are shown above the boxes; ■ median, □ 25%-75% quartile, ı minimum-maximum (Kruskal-Wallis ANOVA).

vascular stress from nighttime apneas/hypopneas does not disrupt the blood-brain barrier to the same extent as traumatic injury to the brain. In addition, studies on the individual assay methods for S100B (ELISA, luminometry, ECLIA, etc.) show only a poor inter-assay agreement, which makes it difficult to make a direct comparison between the results of different test procedures. This sometimes leads to inconsistent data with respect to the validity of S100B as a biomarker in OSA, as seen in the study by Jordan et al [26], who reported that there was an absent or undetectable relationship between OSA and S100B levels in serum.

In agreement with the currently available data, however, it also appears from our study that the serum concentrations of S100B are independent of age or sex[10,14,27]. Exceptions can be found in pediatric populations up to about the age of 20 (excluded from our study), in whom sex- and age-dependent levels of serum S100B have been demonstrated[28]. On the other hand, available data on the relationship between S100B levels and the BMI are controversial. While Steiner et al[29] found the S100B levels in healthy volunteers correlated significantly with the BMI, Duru et al[14] did not confirm these findings in a study on 43 patients with OSA (with a BMI similar to that found in the present study). Our work confirmed the effect (BMI effect) described by Steiner et al[29], although we found that the impact of the BMI on the OSA-associated elevation of S100B (OSA effect) became more pronounced as the BMI increased. In summary, this report basically shows a BMI effect, at least in the overweight groups (OSA *vs*. controls).

The correlation of S100B values to the AHI is similarly controversial[14]. While the individual group comparisons showed a single significant difference between the moderate OSA group (p=0.04) and the controls, there was otherwise no significant correlation to AHI. Another notable fact is that the S100B values in the ronchopathy group tended to lie between those of the control group and those of the OSA group, so that snoring appears to represent a transition stage.

Another potential factor for the use of S100B as a biomarker is its pre-analytical stability. Neither the length nor the temperature of storage seems to have a significant effect on the assay results[30]. Possible circadian fluctuations are also of key interest in the clinical use of biomarkers. The lack of a significant circadian rhythm is an additional argument for the validity of S100B (at least in serum) as a potential biomarker in obstructive sleep apnea[27]. Nevertheless, significant seasonal variations in the serum S100B seem to exist (higher levels in summer than in winter), which may affect the interpretation of measured results in some circumstances[31]. We cannot draw any conclusions about a relevant seasonal effect from the results of our study population, because samples were obtained throughout the year.

The lack of suitability of S100B in saliva as a biomarker, as we found to be the case in this study, may be attributed to a series of problems related to the medium. First of all, the use of saliva (in contrast to serum) must follow a highly standardized procedure. Factors such as the consumption of food or smoking before the sample is taken may be a problem. The salivary flow rate may show considerable variation. Not only the overall composition of saliva, but also its pH and buffering capacity are dependent on the flow rate and thus on the nature and duration of any stimulation[32]. A high proportion of proteases in saliva also lead to a natural instability of the proteome. This may give rise to rapid degradation of the salivary proteins, especially if the sample is stored at temperatures above 4°C prior to being assayed. At the present time, it is impossible to distinguish whether these pre-analytical problems can be considered to be causal factors in the fluctuations seen at different times of the day or whether pronounced primary inter- and intra-individual variations in saliva S100B are responsible for this effect.

Conclusions

Serum S100B is significantly raised in OSA. Despite this fact and its well-characterized properties (pre-analytical stability, lack of circadian rhythm, independence from age or sex) it does not live up to its promise as a valid invasive predictor of obstructive sleep apnea, since it does not correlate with the severity of disease. Also, S100B in saliva is not suitable for use as a non-invasive biomarker to detect hypoxia-induced cerebrovascular stress in OSA. This finding prevents an S100B saliva-based assessment of risk or possible extent of structural brain damage and, at present, rules out the possibility of non-invasive home monitoring of compliance and therapeutic efficacy in patients with OSA on treatment.

Ethical approval

All procedures performed in this study were in accordance with the ethical standards of the institutional Research Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Conflicts of interest

The authors have no funding, financial relationships or conflicts of interest to disclose.

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