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Increasing levels of saliva alpha amylase in electrohypersensitive (EHS) patients

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Increasing levels of saliva alpha amylase in electrohypersensitive (EHS) patients

Abstract

The purpose: The objective of this study was to assess the level of various salivary and urinary markers of patients with EHS and to compare them with those of the healthy control group.

Materials and methods: We analyzed samples from 30 EHS individuals and a matched control group of 25 individuals (non EHS) aged between 22 and 66. We quantified cortisol both in saliva and urine, alpha amylase (sAA), immunoglobulin A and C Reactive Protein levels in saliva and neopterin in urine (uNeopterin).

Results: sAA was found to be significantly higher ($p < 0.005$) in the EHS group. uNeopterin and sAA analysis showed a significant difference based on the duration of EHS.

Conclusion: Higher level of sAA in EHS participants may suggest that the sympathetic adrenal medullar system is activated. However, most of the analyzed markers of the immune system, sympathetic activity and circadian rhythm did not vary significantly in EHS group. There is a trend to the higher levels of some variables in subgroups according to the EHS duration.

Keywords: IEI-EMF; EMF; alpha amylase; markers; symptoms; electromagnetic fields; environmental illness; saliva

Introduction

A rapid development of technologies has increased the number of electromagnetic field (EMFs) sources. These include extremely low frequencies (ELF) and radiofrequencies (RF). While the debate is still focused on any potential public health impact due to their EMF emissions, some individuals report having symptoms that they relate to EMF exposure (Bergqvist U. and Vogel E. 1997). This sensitivity to EMF has been termed “electromagnetic hypersensitivity” (EHS). These non-specific symptoms include dermatological symptoms (redness, tingling and burning sensations), as well as neurasthenic and vegetative symptoms (fatigue, tiredness, concentration difficulties, dizziness, nausea, heart palpitation and digestive disturbances) (Eltiti et al. 2007). These symptoms are not part of any recognized syndrome. Therefore, and due to the fact that these symptoms are medically unexplained, the world health organization termed this sensitivity as Idiopathic Environmental Intolerance (IEI) attributed to electromagnetic fields (EMF)(Mild KH et al. 2006).

Despite the lack of scientific basis to objectively link EHS symptoms to EMF exposure, these symptoms and associated suffering certainly remain real and can vary widely in term of severity. Whatever the cause, EHS impacts on the quality of life of sufferers. So far, there are no clear diagnostic criteria, and no medical diagnosis has been validated. Therefore, physicians find themselves unarmed when facing patients.

The last decade has also been marked with rare involvement of EHS participants to research in this field. Many studies have suffered from this difficulty of recruitment, and EHS enrollment remains as particular challenge.

In view of the above and despite this challenge of volunteer recruitment, our work aimed to look for biochemical markers in a population suffering from electrohypersensitivity in the hope of bringing new elements that could help diagnosis. In this regard, some variables of the endocrine

and immune systems and of sympathetic activity will be analyzed and compared to a non electrohypersensitive population (control). Our hypothesis is that electrohypersensitive patients might have a different biological profile than the control.

Methods

Participants

After approval from the regional French ethics committee “Comité de protection des personnes (CPP) Nord Ouest CHU Amiens, France (CPP, 2014/8)”, 30 adults reporting EHS across France and 25 control individuals were recruited for this study. Individuals declaring being electrohypersensitive participated in the study. EHS patients (23 females and 7 males) and controls (21 females and 4 males) were aged between 22 and 63 (mean age: 47 ± 9) and 22 to 66 (mean age: 46 ± 10) respectively.

Controls and the EHS group were matched for age, sex and body mass Index (BMI). They were recruited by advertising in self-help blogs and hospital listservs or by traditional word of mouth. The EHS group was selected according to the inclusion criteria from participants who completed a questionnaire of hypersensitivity conducted by the research group before testing.

Inclusion criteria for the EHS group were an attribution of experienced symptom(s) to an electromagnetic source and an absence of acute psychiatric disorders, such as acute depressive or paranoid psychosis. Exclusion criteria for participants were the presence of chronic illness and the consumption of antibiotics, anti-inflammatory agents or any other supplements that may interfere with the biological assessments.

Participants were instructed to abstain from consuming alcohol and coffee for 24 h before and during each experimental session. They were also advised not to brush their teeth and not to exercise for at least an hour before sample collection.

Before entering the study, all participants gave written, informed consent to participate in the study. Used methods were carried out in accordance with the approved guidelines. EHS participants were from different regions of France and were reimbursed for their travel fees, and all participants received financial compensation for their participation. From the questionnaire filled out by the participants, we extracted the duration of their sensitivity.

Experimental protocol

The first part of the experiment took place at the participant's home. Saliva samples were collected with salivettes© tubes and a cotton plug (Sarstedt Nümbrecht, Germany) previously sent to the participants. Instructions were given on how and when to collect the saliva. Samples were self-collected on the day prior the experiment: one sample before bedtime and another one in the morning, just after waking up.

On the day of the experiment, participants came to the laboratory around 09:00 and spent their waking time in the laboratory. Participants were seated in a comfortable armchair inside an electrically shielded room with very low background fields. EMF levels were measured by a personal exposimeter, Satimo EME Spy 140 model. Newspapers, magazines and books were provided. From 10h00 to 11h30 and from 14h00 to 16h30, saliva was collected every 30 min. The last saliva collection (16:30) was performed immediately after recording the electrodermal activity in response to auditory stimuli (results not shown).

In parallel, total urine was collected during the night from 20:00 to wake up time as the night fraction and from wake-up time to 16h30 as the day-time fraction. More details of the study protocol are available in (Andrianome et al. 2016).

Sample collection, immunoassay and enzymatic protocols

After rinsing their mouth with water thoroughly, participants collected saliva for over 3 min with a cotton plugged salivette. Salivettes were immediately kept in an icebox. After transportation to the laboratory, the tubes were centrifuged (2 min, 3000 rpm) and stored without preservatives at -20°C until analysis. Urine fractions were aliquoted in appropriate tubes and stored at -20°C until analysis.

The manufacturer's recommended protocols for saliva were used without modification for saliva cortisol, Immunoglobuline A (sIgA), C-reactive protein (CRP) (Salimetrics, State College, PA) and saliva alpha amylase (sAA) (IBL, Hamburg, Germany). For urine, protocols of neopterin (IBL, Hamburg, Germany), creatinine (Enzo Life Sciences, Framingdate, NY) and cortisol (Arbor assay, MI) were applied.

Analyses were performed on all 12-points for saliva collected for cortisol, IgA and sAA while sCRP analyses were performed only on two points: 10:00 and 16:30.

All samples were tested in duplicate, and the average of duplicates was used as the final value.

Statistical analysis

Data were analyzed with SPSS statistical software, IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp), and plotted by using Graphpad Prism, Version 5 (San Diego, CA). Descriptive results of continuous variables were expressed in mean \pm SD. We applied a two factors ANOVA for assessing time and group effects.

U Mann Whitney or Kruskall Wallis non-parametric tests were used to compare subgroups of EHS in terms of the median duration. Values were considered significantly different when $p < 0.05$. Correlation tests were performed by the Spearman correlation.

Results

Results from EMF recording have showed that levels of EMF for radiofrequency were below the detection limit. The required number of EHS participants for our study was achieved with 30 individuals self-reporting EHS who fully agreed to participate to our study. This number can be considered as satisfactory given the difficulty of patient's enrollment.

Due to aberrant results for biochemical analysis from one EHS male, all data from this participant were not included in statistical analysis, leading to 29 EHS participants. The mean duration of EHS was 7.51 years (range: 1–19 years). Groups did not differ in terms of gender proportion (84 % of female participants in control group versus 79.31 % in the EHS group, $p=0.498$), BMI (22.80 ± 2.60 kg/m² in the control group, and 22.35 ± 3.33 kg/m² in the EHS group, $p=0.268$) and age (46 ± 10 years in the control group, and 47 ± 9 years in the EHS group, $p=0.999$). No significant difference in proportion of smoker were found (24 % in the control group, and 13.8 % in the EHS group, $p=0.307$). In the control group, 24 participants were having an activity (96%), and 24 participants in the EHS group (82.75%), ($p=0.123$).

Salivary biomarkers

The cortisol saliva concentrations in both groups were not significantly different. The cortisol secretion profiles were similar ($p > 0.05$) with the lowest concentration at bedtime (control: mean: 0.04 µg/dL; EHS mean: 0.06 µg/dL) and the highest at waking time (control mean: 0.33 µg/dL; EHS mean: 0.29 µg/dL) (Fig.1).

Figure 1. Saliva level of cortisol in control (black line) and EHS (grey line) groups, data represent mean \pm SEM. Significant time effect ($p < 0.05$) with typical morning peak (waking point). No significant effect of group was observed ($p > 0.05$)

sAA activity was significantly different ($p < 0.0001$) between the control and EHS group. The EHS group showed a significantly higher level of sAA (Fig.2a).

ANOVA analysis showed that the IgA level in saliva was not significantly different between both groups but showed a significant time variation ($p < 0.0001$) (Fig.2b). A peak of sIgA was reached in the control group at awakening ($106.53 \pm 44.04 \mu\text{g/ml}$) with a significant difference when compared to all the other time points. In the EHS group, no difference was disclosed between awakening sIgA levels and those of the other points ($p > 0.05$).

Figure 2. Salivary concentration of alpha amylase (sAA) (a) and immunoglobuline A (IgA) (b) in the control (black line) and EHS group (grey line) during experimentation (from bedtime to 16.30). Data expressed as mean \pm SEM

- Significant difference between groups for sAA ($p < 0.0001$)
- Significant effect of time for sAA ($p = 0.0001$) and IgA ($p < 0.0001$)

sCRP levels showed a significant increase in the morning, when compared to the afternoon levels ($p < 0.0001$). However, no significant difference could be detected between the EHS group and the control group (Fig.3).

Figure 3. Mean \pm SEM of salivary CRP in the control (black bars) and EHS group (grey bars)

Saliva correlation with selected clinical parameters and EHS subgroup analysis

For the EHS group, sAA levels were different between the male and female participants. Male participants expressed higher levels than female participants. sIgA and sCRP were negatively correlated with age at $r = -0.135$, $p = 0.017$ and $r = -0.36$, $p = 0.007$ respectively.

The mean of sIgA showed a tendency to be positively correlated with EHS duration ($r = 0.141$, $p = 0.010$); sAA was also slightly but positively correlated with EHS duration ($r = 0.254$, $p < 0.001$).

According to the median of duration of EHS, which was seven years, there is no subgroup difference in sCRP and sCortisol concentrations (Table 1). sAA activity is significantly higher in patients reporting EHS ≥ 7 years for saliva collected at 10:00. Likewise, sIgA shows a difference between the control and EHS groups at bedtime.

Urinary biomarkers

Urinary cortisol and neopterin concentrations, adjusted to creatinine, did not show any significant difference between the control and EHS groups. Urine neopterin to creatinine concentrations were significantly higher for the morning fraction than for the night fraction for both groups ($p < 0.05$) (Fig. 4).

Figure 4. Mean \pm SEM of urine cortisol:creatinine ratio (a) and neopterin:creatinine ratio (b) in control (black bars) and EHS group (grey bars). No significant effect of group, significant time effect for neopterin:creatinine ratio, $p < 0.05$ (b); abbreviations: UNCR: urinary neopterin to creatinine ratio.

Urine biomarkers and EHS subgroup analysis

According to the median duration of EHS, which is seven years, there is no difference in urinary cortisol levels between participants being EHS less than seven years and those being EHS more than seven years (Table 2). However, in the morning fraction of urine, a significant difference was found for neopterin between the control and EHS group and between the EHS subgroups.

Discussion

This study was designed in an attempt to find out some biological markers that may differentiate EHS individuals from the rest of the population (non-EHS) and explain the occurrence of the atypical symptoms they complain about. In this regard, some markers of the immune, circadian and autonomic nervous systems were explored.

Among the markers analyzed, cortisol is considered as a reliable marker of the endogenous circadian oscillator (Haus E and Touitou Y. 1994) and as a primary biological indicator for stress reactions for the hypothalamic-pituitary-adrenal (HPA) axis (Kirschbaum & Hellhammer 1989; Kirschbaum & Hellhammer 1994). Our present work showed no significant differences in saliva cortisol levels between a group suffering from EHS and a control group. In light of the above, our results are in agreement with the literature and show a peak in the morning immediately after wake up and a trough around bedtime (Krieger 1975; Selmaoui & Touitou 2003). Neither the cortisol peak in the morning nor the lowest values of the day (bedtime) were different between the two groups. This may suggest that the HPA axis is not affected in EHS patients. Additionally, the amount of cortisol in urine did not differ between both groups ($p > 0.05$), confirming the finding of the saliva analysis.

Besides, the salivary enzyme α -amylase has been proposed as a marker for the stress-induced activity of the sympathetic nervous system (SNS), or more precisely of the sympathetic adrenomedullary (SAM) system, which is also involved in the secretion of adrenaline and noradrenaline (Chatterton et al. 1996; Rohleder & Nater 2009). Unlike cortisol, saliva alpha amylase levels were found to be significantly higher in the EHS group, in comparison to the control group. The question that arises here is whether this change in α -amylase activity was a result of outside exposure to EMF or simply a consequence of chronic stress.

The question whether EHS patients are stressed by various environmental/ biological factors other than EMF is at the center of various research. Nowadays, there are only few studies dealing with the EHS population and which are not so far conclusive. Some few studies have tried to find a link with other diseases or other environmental factors such as study by Palmquist et al. (2014) who found an overlap between 4 types of environmental intolerances: exposure to odorous/pungent chemicals, certain buildings, everyday sounds and EMFs. While some other authors (Rubin et al. 2010), proposed the presence of a “nocebo effects”. In our population: no specific questionnaire about environmental stressing factors were presented. On the other hand, levels of cortisol do not indicate the presence of stress.

With regard to the biological stress system, one may wonder why only SAM has been activated and not the HPA axis if there are stress-related changes. It is known that these two physiological systems are independent in terms of neuronal circuits (Ulrich-Lai & Herman 2009) and that they may react differently to stressors. Some results suggested that the responses of the HPA and SAM systems differ depending on the type of stressor (Maruyama et al. 2012). Moreover, salivary cortisol levels increase a few minutes after the initiation of stress (Droste et al. 2008; Takai et al. 2004), meaning that the HPA axis is relatively slower to respond while sAA levels are characterized by a rapid increase after a stress action, followed by a rapid decrease to normal levels after the cessation of the stressor (Speirs et al. 1974; Takai et al. 2004). However, in the present study, sAA levels were shown to be constantly elevated in EHS individuals, even during their stay (from 09h00 to 16h30) in the EMF shielded room in our laboratory. The role of EMF on the increase of AA is still to be proven. However, our results indicated that EHS population seems to have higher levels of sAA than control group. And the cause of this increase is to be yet determined.

Moreover, a significant and positive correlation was shown between sAA levels and the duration of EHS. Indeed, the longer the duration of EHS, the higher the level of sAA. This may suggest

that the longer they are EHS, the more they react. One may speculate that this could be due to a degraded system in patients presenting EHS for a long-time.

Besides, a study has shown that exposure to radiofrequency electromagnetic fields (RF-EMF) emitted by mobile phone base stations affected salivary α -amylase in healthy participants (Augner et al. 2010). In addition, a study of people living next to phone base stations, self-declaring base-station neighbors (distance to base station ≤ 100 meters), had higher levels of α -amylase accompanied by a higher general strain (Augner & Hacker 2009; Augner et al. 2010). This may suggest that people self-reporting EHS could share similarities with this population, but whether this is due to actual exposure to EMF or to other underlying factors is not clear. In our study, unfortunately, recordings of exposure field density before entering the study (outside the experimental lab) were not available.

Moreover, levels of IgA in saliva vary in response to physical and psychological stress through interactions with the autonomic nervous system (Tsujita & Morimoto 1999; Bishop & Gleeson 2009). Saliva IgA concentrations were found to be similar between the EHS population and the matched control group. However, we could observe a significant variation in the pattern of IgA between morning and afternoon. This is in accordance with previous studies showing that IgA patterns follow a circadian rhythm (Casale et al. 1983). Subgroup analysis showed that people declaring longer EHS had higher levels of IgA for bedtime collected saliva.

C reactive protein (CRP) is widely used as an inflammatory marker and could indicate bio inflammation of the body. High levels of serum CRP have been correlated to cardiovascular risks and other inflammatory or immune diseases (Tracy et al. 1997; Du Clos 2003). Recently, elevated levels of CRP and hsp70 have been detected in frequent users of mobile phones (Balakrishnan et al. 2014). In this cohort, concentrations of saliva CRP in the EHS group were

not different from those of the control group, indicating the absence of an acute inflammatory process. Furthermore, highly sensitive CRP was higher in the morning than in the evening. This finding is compatible with results in healthy young adults (Izawa et al. 2013).

Neopterin in urine showed a light-dark variation in both groups, which corroborates previous findings (Garcia-Gonzalez et al. 2006). Surprisingly, subgroups analysis showed a variation according to the duration of the EHS effect. We found, in the day fraction of urine, a lower level of neopterin in patients reporting EHS for more than seven years, compared to those with shorter EHS. Little information is available on immune markers in EHS and on the immune effect of electromagnetic fields. Immune cells or cytokines were not modified under acute exposure to ELF magnetic fields (Selmaoui et al. 1996; Selmaoui et al. 2011) or GSM 900 MHz exposure (Tuschl et al. 2006). In an experiment with exposure to a GSM signal, neopterin was not modified in saliva (Radon et al. 2001). Other studies showed that acute exposure to ELF did not have an effect on blood concentrations of TSH, FSH, LH, testosterone, cortisol or neopterin (Gamberale et al. 1989; Selmaoui et al. 1996).

In our study, we used low tech and bloodless methods to analyze markers in the EHS population. Besides an accepted general method of collection (a cotton swab), all the stress-minimizing methods used here are shown to be appropriate in quantifying such markers.

Regarding the age of the EHS group, variability due to the age widths of participants was reduced by recruiting matched volunteers as far as possible. Besides this age characteristic, for a more powerful analysis and according to the subgroup exploration, a larger number of participants would be required for future studies.

Here, the classification of EHS subgroups according to the duration of their EHS can be a disputable point due to the declarative nature of this information. It could be understood that the

determination of an exact timing of the triggering the syndrome may be an estimate without any known diagnostic. It could correspond here to the development of major symptoms or the occurrence of a special event. Here again, the lack of an existing diagnosis of EHS poses a serious problem.

A retrospective analysis of exposure and recording the lifetime information of participants could be an important issue in understanding EHS that could be coupled with such data. Until now, limited information about the mechanism of EHS is available, and few studies have explored the biological mechanism in EHS, though particular emphasis has been put on “provocation studies,” which were not conclusive. In the absence of a known biological mechanism, the exploration of more markers involved in immunological activity could complete our study, without minimizing the complex interaction among symptoms, health concerns and risk perceptions in this particular population.

Conclusions

In summary, an exploration of selected and measurable markers of EHS was conducted in our study to understand this condition. Our finding indicates that markers of the circadian rhythm and immune system were not affected in EHS compared to non EHS individuals. Our results suggest different profiles of EHS according to the duration of the syndrome. Furthermore, the mechanism underlying the higher expression of α -amylase should be focused on. These findings could be new elements in the understanding, explanation and comprehension of the physiopathology of EHS.

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Declaration of interest statement

The authors report no declarations of interest.

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Figure captions

Figure 1. Saliva level of cortisol in control (black line) and EHS (grey line) groups, data represent mean \pm SEM. Significant time effect ($p < 0.05$) with typical morning peak (waking point). No significant effect of group was observed ($p > 0.05$)

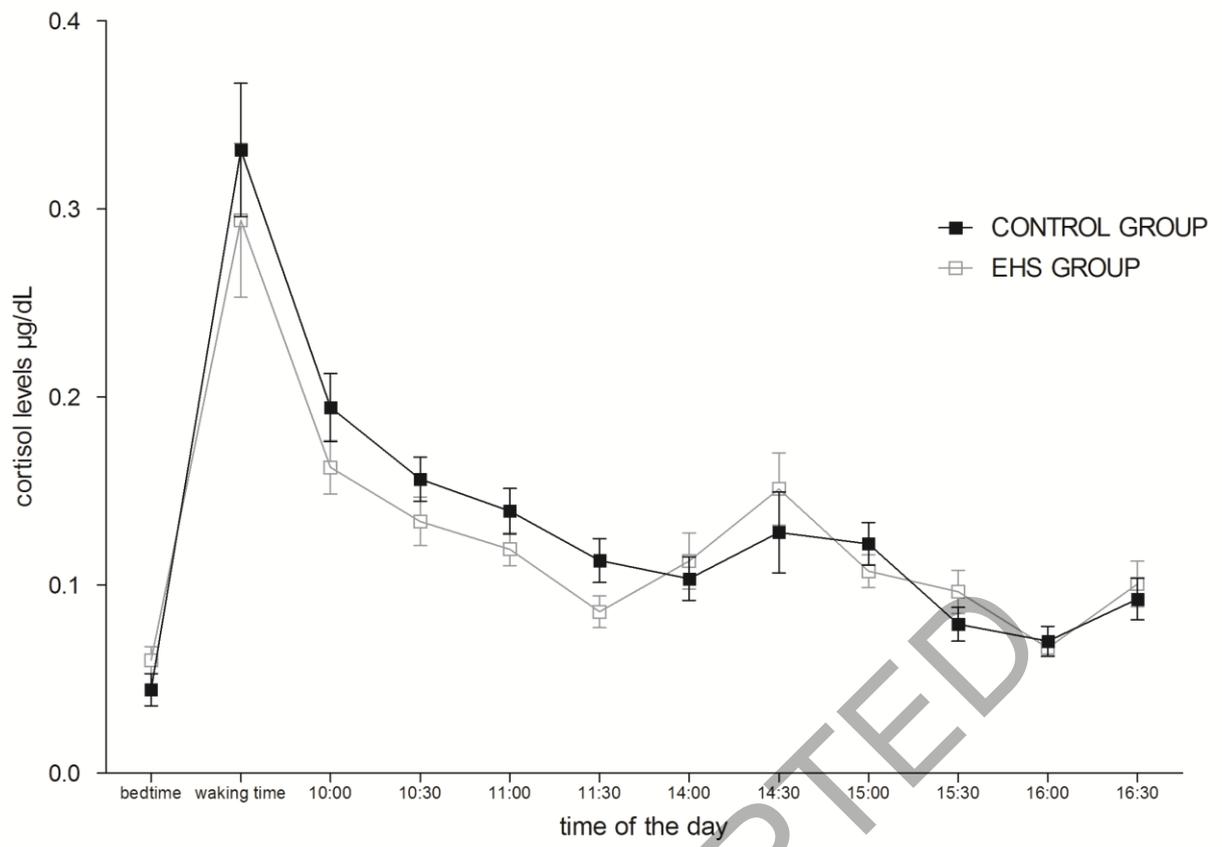
Figure 2. Salivary concentration of α -amylase (a) and IgA (b) in the control (black line) and EHS group (grey line) during experimentation (from bedtime to 16.30). Data expressed as mean \pm SEM

- Significant difference between groups for α -amylase ($p < 0.0001$)

- Significant effect of time for α -amylase ($p = 0.0001$) and IgA ($p < 0.0001$)

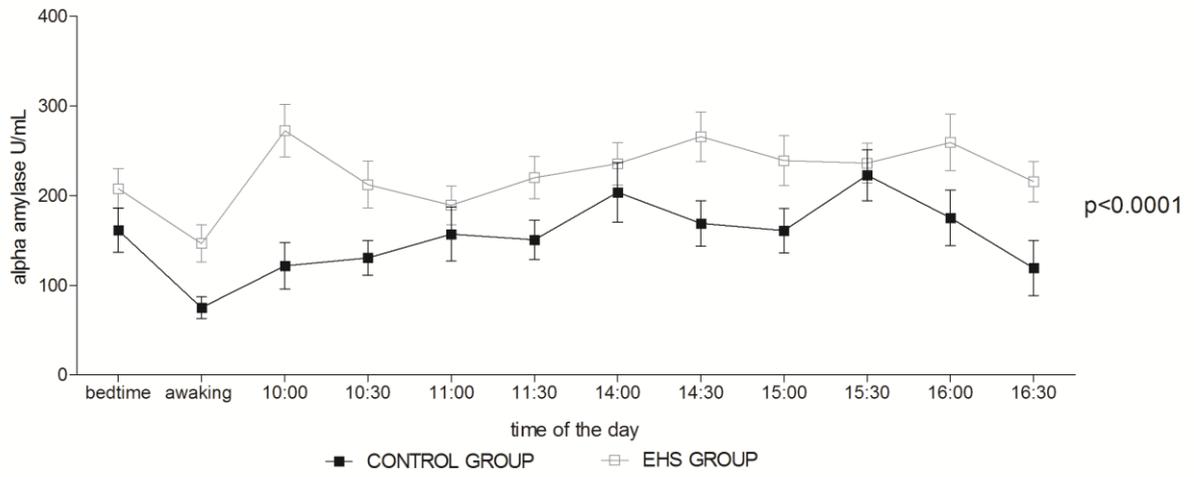
Figure 3. Mean \pm SEM of salivary CRP in the control (black bars) and EHS group (grey bars)

Figure 4. Mean \pm SEM of urine cortisol:creatinine ratio (a) and neopterin:creatinine ratio (b) in control (black bars) and EHS group (grey bars). No significant effect of group, significant time effect for neopterin:creatinine ratio, $p < 0.05$ (b); abbreviations: UNCR: urinary neopterin to creatinine ratio.

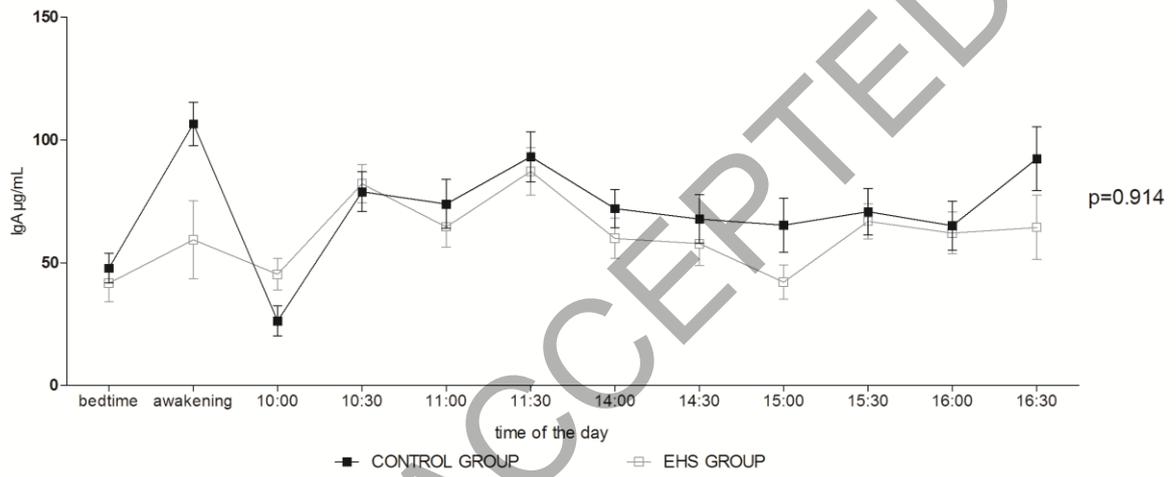


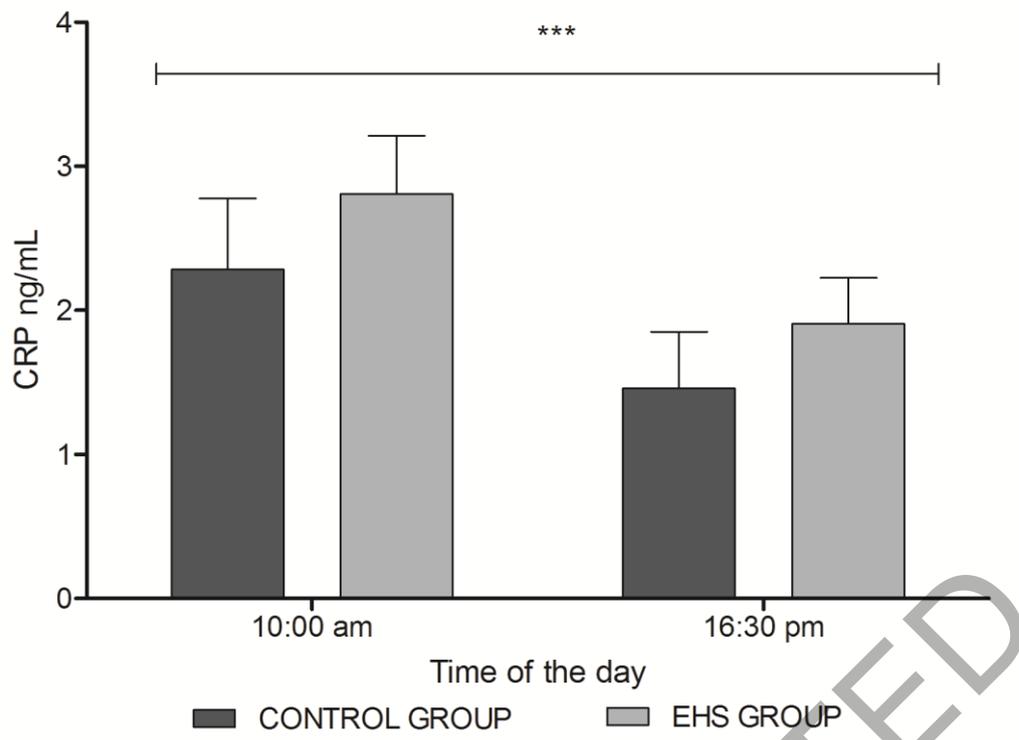
JUST ACCEPTED

2a



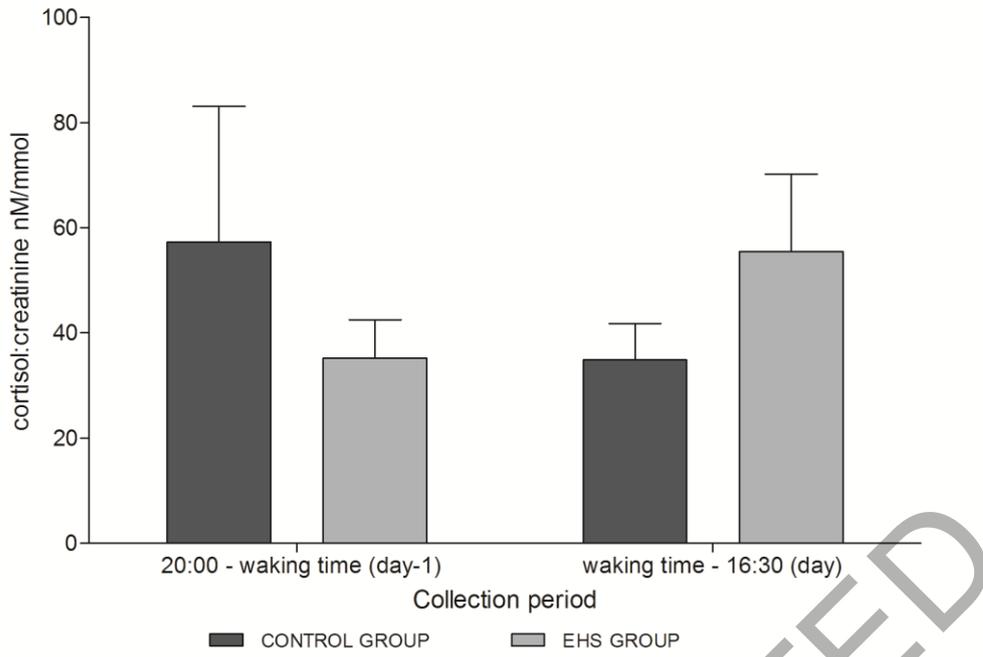
2b





JUST ACCEPTED

4a



4b

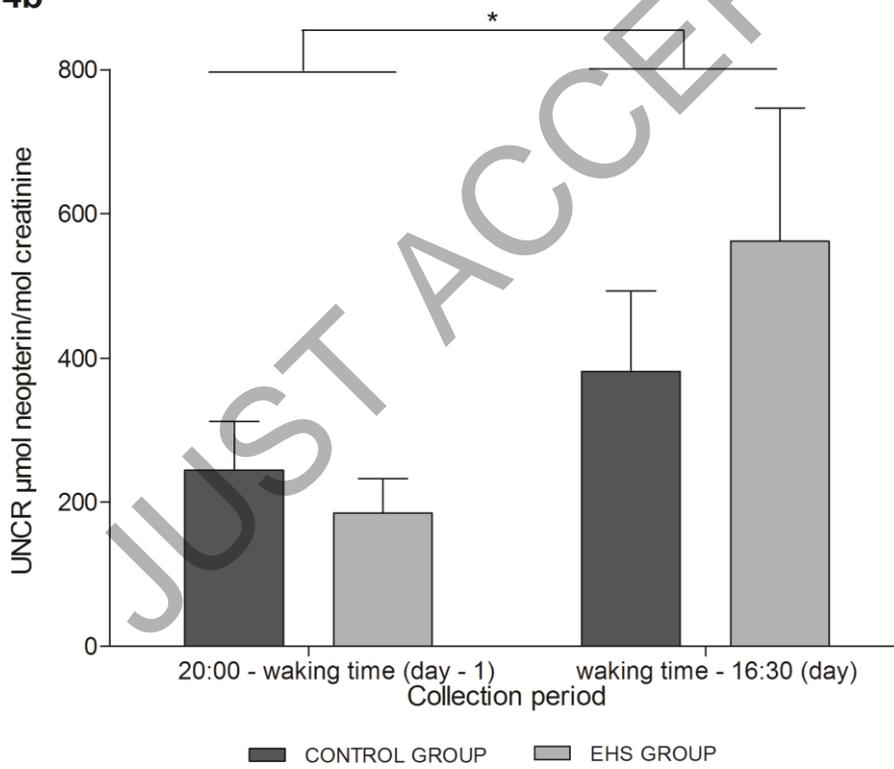


Table 1. Demographic characteristics and levels of markers in control and subgroups of EHS based on EHS duration (median duration = 7 years) in saliva collected at night, wake up, 10:00, and 16:30

*sCRP only analyzed on 10:00 and 16:30 points; Data are quoted as mean \pm SD value for continuous variables, or number;

symbols: ‡ p < 0.05; § p < 0.01

	Control group	EHS duration <7 years (A)	EHS duration \geq 7 years (B)	P-values (A vs. B)	Global P*
n	25	17	12	-	-
Mle	4	3	3	-	-
Female	21	15	8	0.494	-
Age, years	46.24	47.50	44.82	0.674	0.942
BMI, kg/m²	22.80 \pm 2.60	22.11 \pm 2.54	21.91 \pm 3.52	0.707	0.470
sCRP					
*10:00 ng/mL	2.74 \pm 0.90	2.56 \pm 1.11	3.09 \pm 0.88	0.173	0.419
*16:30 ng/mL	1.75 \pm 0.90	1.90 \pm 0.73	2.10 \pm 0.90	0.674	0.489
cortisol					
bedtime μ g/dL	0.041 \pm 0.041	0.061 \pm 0.047	0.049 \pm 0.028	0.777	0.333
awakening μ g/dL	0.331 \pm 0.178	0.279 \pm 0.194	0.299 \pm 0.250	0.698	0.049‡
10:00 μ g/dL	0.187 \pm 0.095	0.152 \pm 0.079	0.186 \pm 0.074	0.227	0.298
16:30 μ g/dL	0.074 \pm 0.058	0.095 \pm 0.075	0.092 \pm 0.052	0.744	0.584
sAA					
bedtime U/mL	161.26 \pm 124.07	182.14 \pm 125.07	240.10 \pm 108.44	0.166	0.124
awakening U/mL	75.12 \pm 60.45	146.29 \pm 140.12	146.82 \pm 62.68	0.501	0.014‡
10:00 U/mL	121.62 \pm 129.84	185.67 \pm 109.54	337.50 \pm 163.42	0.043‡	0.007§
16:30 U/mL	119.20 \pm 152.86	208.79 \pm 142.71	226 \pm 89.71	0.781	0.033‡
IgA					
bedtime μ g/mL	47.74 \pm 30.32	30.41 \pm 37.86	57.46 \pm 39.04	0.03‡	0.034‡
awakening μ g/mL	106.54 \pm 44.04	55.70 \pm 89.12	64.29 \pm 82.56	0.913	0.003§
10:00 μ g/mL	26.20 \pm 31.06	53.57 \pm 33.13	33.38 \pm 34.90	0.073	0.025‡
16:30 μ g/mL	92.27 \pm 64.62	52.74 \pm 36.67	77.90 \pm 96.56	0.781	0.061

Table 2. Biochemical urinary characteristics of the study population: controls (n =25) and the two subgroups (based on the median duration of EHS), data are quoted as mean \pm SD

	<i>Control</i> (n=25)	<i>EHS duration < 7</i> <i>years (A) (n=17)</i>	<i>EHS duration \geq 7</i> <i>years (B) (n=12)</i>	<i>P (A</i> <i>vs. B)</i>	<i>Global P</i>
Night fraction (20:00 to awakening)					
Cortisol:creatinin (nmol/mmol)	54.90 \pm 12.14	38.85 \pm 47.63	34.18 \pm 33.33	0.914	0.989
Neopterin:creatinin (μ mol/mol)	223.82 \pm 314.10	229.40 \pm 322.17	107.15 \pm 81.53	0.377	0.153
Day fraction (awakening to 16:30)					
Cortisol:creatinin (nmol/mmol)	33.51 \pm 33.64	57.10 \pm 10.33	52.47 \pm 53.71	0.603	0.368
Neopterin:creatinin (μ mol/mol)	354.82 \pm 519.18	472.58 \pm 438.35	138.68 \pm 133.43	0.011‡	0.037‡

‡ p < 0.05; § p < 0.01

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