UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA

Impact of obstructive sleep apneas on the immune and metabolic homeostasis.
The influence of the environment

(Impacto das apneias obstrutivas do sono na homeostase imunológica e metabólica. A influência do ambiente)

Richard Staats

Orientadores: Profa. Doutora Cristina Bárbara

Prof. Doutor António Bugalho de Almeida

Tese especialmente elaborada para a obtenção do grau de Doutor em Medicina-Pneumologia

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DECLARAÇÃO

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To the readers:

"§ 1: Et is wie et is (It is as it is); §2: et küt wie et küt (it comes as it comes); § 3 et hät noch emmer jot jejange (in the end everything will go well) From the "Grundgesetz" of Cologne.

The above-mentioned paragraphs of the "Grundgesetz from Cologne" may not be familiar to the reader, in fact, it is possible that many Germans out of the Rhineland area have never heard of it.

While preparing this manuscript I was forced to read some of my early investigations and involuntarily started to reflect about my professional history as it would appear to be natural for a man exceeding the 50th year border.

The reason that allows me to present this manuscript at the Faculty of Medicine of the University of Lisbon is ultimately related to the fact that it rained in Spain on the 2nd of July 1988. At that time, I travelled with a friend via Interrail through Europe after finishing high school. Due to the weather condition in Spain, we made a detour to Portugal, where I met my wife. Thus, rain in Salamanca resulted in a family and my presence in Portugal.

The sometimes not completely understandable present (et is wie et is) that turns into something new and unexpected in the future (et küt wie et küt) with usually a good outcome (et hät noch emmer jot jejange) accompanied me several times in my life. Following the trip to Portugal I started a 20-month term of compulsory community service (instead of going to the army) in the Respiratory Physiology and Allergy Department at the Children's Hospital of Cologne. I had chosen this workplace for two reasons: 1: it was in my favourite city Cologne (and near to home), 2: it was a white-collar job (*et is like et is*). After 5 months of conducting pulmonary function and allergy tests in children I abandoned my idea to follow my parents example to study law and went to medicine (et küt wie et küt) not knowing that this period of community service would help me to conduct respiratory pathophysiology examinations and allergology tests (et hät noch emmer jot jejange). Due to good luck, and some work I studied at the Albert-Ludwig University in Freiburg and since cardiovascular physiology was one of the most interesting pre-clinical fields I started to perform all my electives in Cardiology seeing myself as a future member of the Cardiology Society. However, when searching

for a tutor regarding the necessary thesis to receive the doctor title (Dr.) none of the Cardiologists had either time or adequate proposals for me (et is wie et is). Knocking on the doors of Pneumology I met Professor Heinrich Matthys who recommended me to the respiratory laboratory for basic science at his department run by Werner Luttmann chaired by Prof. Christian Virchow (et is wie et is). Due to mutual sympathy at first glance I joined the laboratory group and changed my plan from a clinical investigation towards basic science (et küt wie et küt). Nowadays this work helps me to conduct some of my investigations and possibly even more importantly permits me to analyse publications in this field (*et hät noch emmer jot jejange*). Finishing medical school, a high percentage of the medical students in my generation went into unemployment due to a surplus of physicians in Germany. I had some offers in Cardiology but all of them came from district hospitals and I did not enjoy the idea of constantly writing discharge letters from patients undergoing coronary artery catheterisation (et is wie et is). Finishing my MD thesis on the laboratory PC of the respiratory laboratory I suddenly asked a passing Neurologist employed as sleep specialist in the respiratory sleep laboratory if she did not have a job for me. To my, and perhaps also her own surprise, she confirmed so, thus I saw myself with the help of Prof. Christina Virchow, suddenly employed in the sleep laboratory of the University Hospital of Freiburg (et küt wie et küt). This did not reflect my expectations to work as a cardiologist or a pneumologist but most of all a clinician. Nevertheless, after some months I got very well adapted. Most of all I discovered that sleep medicine was the medical equivalent of the nineteenth century Belgian Congo, with many more possibilities for investigation compared to e.g. bronchial asthma. My chosen field of sleep medicine was eventually the reason while I managed to change from Germany to Portugal thanks to the support of Profa. Doutora Teresa Paiva and subsequently Prof. Doutor António Bugalho de Almeida and Profa. Doutora Cristina Bárbara, which hopefully will continue for the future (*et hät noch emmer jot jejange)*.

Abbreviations

ABP	Arterial blood pressure
ABPM	Ambulatory blood pressure measurement
AHI	Apnea/hypopnea index
AHT	Arterial Hypertension
AI	Arousal index.
APAP	Automatic positive airway pressure
BP	Blood pressure
$CD3^{+}\gamma\delta$	CD3 positive gamma delta T lymphocytes
Cholesterol tot	Total blood cholesterol
CIH	Chronic intermittent hypoxia
CPAP	Continuous positive airway pressure
CRP	C reactive protein
CTL	Cytotoxic T-lymphocytes
CR-PG	Cardiorespiratory polygraphy
CVD	Cardiovascular disease
d	Cohen´s D or effect size
EEG	Electroencephalography
GrB	Granzyme B
HDL or HDL-C	High-density lipoprotein cholesterol

HP	Hemodynamic parameters
HR	Heart rate
LDL or LDL-C	Low-density lipoprotein cholesterol
MAP	Mean arterial blood pressure
NAFLD	Non-alcoholic fatty liver disease
NK-activity	Natural killer cell activity
NK cells	Natural killer cells ($CD3^{-}CD16/CD56^{+}$)
NKT cells	Natural killer T ($CD3^{+}CD17/CD56^{+}$) Lymphocytes
ODI	Oxygen desaturation index
OSA	Obstructive sleep apnea
PAP	Positive airway pressure
PSG	Polysomnography
Pfr	Perforin
Qt	Quartiles
RDI	Respiratory disturbance index
SBP	Systolic blood pressure
SCB	Standardized correlation coefficient β
SP	Sleep period
SRBD	Sleep related breathing disorders
SV	Stroke volume
TC	Total Cholesterol

TG	Triglycerides
UAR	Upper airway resistance
WP	Wake period

Abstract

Sleep is a condition with reduction or absence of life supporting mechanism like alimentation, self-defence, or reproduction. It occupies about a third of the human life span and has been found in all animals including insects. Despite the intense research in recent years, several mysteries of sleep still need to be revealed. This manuscript aims to increase our knowledge of the effect of obstructive sleep apnea (OSA) on components of human homeostasis.

Study 1:

The stress induced by repetitive sleep apneas is considered one of the main factors of the OSA related morbidity and mortality. A possible explanation is the stress provoked alterations of the immune and inflammatory system. Athletes report after an exhausting exercise an increase in upper respiratory tract infections associated with a decreased natural killer cell activity (NKA). The cytotoxic protein perforin (Pfr) is one of the main contributors of the NKA and therefore important for the virus and tumor defence, but it is also linked to an increased risk of cardiovascular diseases. We investigated if the physical and psychological stress of a sprint triathlon induces changes on perforin positive lymphocytes.

A total of 12 trained male endurance athletes were investigated. A blood sample was collected 168 and 24 hours before and 1, 18 and 48 hours after a sprint triathlon. The athletes were obliged to stop training one week before the sport event. The control group consisted of 10 healthy hospital employees. Peripheral blood mononuclear cells were isolated, and after fixation and perforation stained for lymphocyte membrane markers and the intracellular protein Pfr. The percentage of Pfr positive cells within each lymphocyte subset was investigated by standard flow cytometric analysis. Statistical analysis consisted of the one-way repeated measures analysis of variance (ANOVA) with pairwise student-Newman-Keuls post hoc for the 5 timepoints in athletes. Difference between controls and athletes were investigated via the unpaired Student´s t-test.

We found a decrease of total lymphocytes of 24 before to 1 hour after the exercise. Following 18 hours after the triathlon the value returned baseline. Already the cessation

of training caused a small but significant decline in the percentage of Pfr positive $CD3^+$ ($p<0.01$), $CD4^+$ ($p<0.05$) and $CD8^+$ lymphocytes ($p<0.01$). Physical exercise was related to a further decrease in the Pfr positive $CD3^+$ and $CD8^+$ lymphocytes without any further relevant change in the Pfr positive $CD4^+$ lypmphoyctes. There was no change detected in $CD16^+/CD56^+$ positive natural killer cells (NK). The percentage of perforin positive $CD3^+$ and $CD8^+$ lymphocytes was significantly higher in athletes compared to controls ($p<0.05$).

This study may be helpful to understand the effect of physical and possible competition induced psychological stress on the natural killer cell activity in endurance athletes and other possible stress situations.

Study 2:

OSA is alleged to increase the risk of cardiovascular diseases but also of cancer. Both atherosclerosis and cancer have been linked to alterations in the immune/inflammatory system. The former is characterized by an increased activation of the immune/inflammatory system, while the later was associated with a reduced reaction of the immune system towards tumour cells. In both cases the cytotoxic immune system is of importance.

In this study we included 87 participants divided in 24 controls, 19 patients with respiratory effort related sleep fragmentation without a relevant AHI or oxygen desaturation index (ODI) termed upper airway resistance patients (UAR) and 20 lean OSA (noOSA) patients and 24 obese patients (oOSA) defined as an AHI > 5/h with or without an BMI > 30 kg/m^2. In 20 OSA patients we repeated the analysis after one night of CPAP titration and 3 month of automatic positive airway pressure therapy. In analogy to study 2 a blood sample was prepared from all participants. The percentage of Pfr and granzyme-B (GrB) positive cells was analysed by flowcytometry. Additionally, in a different sample the peripheral blood $\gamma\delta$ T cells of 6 healthy donors were purified by magnetic cell isolation and incubated in a hypoxic chamber with 5 % oxygen content. After hypoxic exposure $\gamma\delta$ T cells were via flowcytometry analysed regarding the presence of the granulation marker CD107a, GrB and Pfr.

The difference between the 4 groups was analysed with the Kruskal-Wallis test with posteriori Wilcoxon rank test for pairwise comparison. Additionally, we examined the association of the Pfr containing γδ T cells and the sleep parameters with the Spearman Correlation Coefficient. For the evaluation under positive airway pressure (PAP) therapy, we analysed repeated measures Anova and the effect size between the time points by Cohen´s d.

There was a significant difference in the percentage of Pfr positive lymphocytes between the groups, specially between controls and OSA patients (p=0.04). We found a negative correlation between the percentage of the Pfr positive γδ T cells and the AHI (coef=-0.36; p=0.001, ODI (coef=-0.34; p=0.002) and the Epworth sleepiness scale (coef=-0.025; p=0.025). Hypoxic provocation decreased the degranulation capacity by a reduced expression of CD107a and a decreased level of GrB, although the result did not reach statistical significance (p=0.065).

Following positive airway pressure therapy, the percentage of perforin positive cells increased in all lymphocyte subset except $CD3^{-}CD8^{+}$ lymphocytes, however only $CD3^{+}CD4^{+}$ lymphocytes reached statistical significance (p=0.025). After a three month PAP therapy, we found a small effect size for the increase in the percentage of Pfr positive lymphocytes (d=0.33); $CD3^{+}CD4^{+}$ (d=0.26), $CD3^{+}CD8^{+}$ (d=0.4); cytotoxic T lymphocytes (d=0.41), and the reduction in Pfr positive $CD3^{-}CD8^{+}$ lymphocytes (d=0.27). The increase in NK cells showed a large effect size (d=0.85).

These results indicate that OSA is associated with a decreased cytotoxic potential in peripheral blood γδ T lymphocytes subset. With the exception of the $CD3^{-}CD8^{+}$ lymphocytes, PAP therapy seems to increase the cytotoxic capability in all other investigated lymphocyte subsets.

Study 3:

Arterial hypertension is the most common comorbidity detected in OSA and has been linked to its increased morbidity and mortality. However, results from recent studies have questioned this concept. In this study we investigated the relevance of OSA for non-dipping blood pressure at sleep onset. A total of 56 participants were included

consisting of 34 controls with an apnea/hypopnea index (AHI) < 15/h and 22 OSA patients with an AHI≥15/h. We used the finger-cuff based Nexfin-HD device for the beat-to-beat measurement of the hemodynamic parameters (HP): heart rate (HR), systolic blood pressure (SBP) and stroke volume (SV). A total time of 5 minutes wakefulness followed of 20 minutes of sleep onset was recorded. Results were compared by paired and unpaired t-test with a significance level of <0.05. Additionally, the effect size was calculated using Cohen's d (d). The consecutive number of each pulse wave was plotted against the measured hemodynamic value. This allowed to investigate the evolution of the HP over the time period by using the standardized coefficient β (SCB). The influence of OSA on SCB was further analysed in a hierarchical block regression analysis including age, body mass index (BMI), AHI and arousal-index (AI).

SBP increased in OSA and decreased in controls (p=0.001; d=0.92) with a similar pattern for SV (p=0.047; d=0.56). Also, short time variation was higher in OSA patients for HR (p<0.001; d= 1.2), SBP (p=0.001; d=0.94) and SV (p=0.005; d=0.82). The hierarchical regression analysis revealed the arousal index as the best predictor of the SBP progression at sleep onset (β: 0.717, p<0.001) and SV (β: 0.469, p=0.003, respectively). We found no statistically significant result for the HR.

This study concludes that OSA effects negatively the short-term evolution and variation of SBP and SV at sleep onset. The arousal index was found the best predictor for the evolution of SBP and SV parameters.

Study 4:

OSA is associated with an increased risk for cardiovascular diseases. Beside the direct effect of repetitive hypoxia there is evidence that OSA interferes with the lipid metabolism. In this study, we investigated the relationship between OSA and plasma lipid concentrations in a cohort from the European Sleep Apnea Database (ESADA).

We included a total of 8592 patients without documented diagnosis of dyslipidaemia or lipid lowering therapy. The relationship between components of the lipid profile and the AHI or ODI was determined by means of a general linear model analysis. Included lipid values were total cholesterol (TC), high-density lipoprotein cholesterol (HDL or HDL-C),

low-density lipoprotein cholesterol (LDL or LDL-C) and triglycerides (TG). Both AHI and ODI were analysed as original values or after splitting in quartiles (Qt) with the lowest quartiles representing the control group. To understand geographical influences, the participating sleep centres were grouped according to the cardinal directions with an additional central region.

We detected a relevant relationship between the AHI and ODI quartiles and the TC. When the AHI of Qt 1 was compared with Qt 2: β: 4.4; p<0.001, Qt 3 vs 1: β 5.1; p<0.001 and Qt 4 vs. 1: β 5.0; p<0.001. For ODI the TC demonstrated pattern with Qt 1 vs. 2: β: 4.2; p=0.001, Qt 3 vs 1: β 5.1; p<0.001 and Qt 4 vs. 1: β 5.4; p<0.001.

HDL correlated negatively with the AHI (r-0.164, p< 0.001) in the univariate analysis. In the adjusted regression analysis mainly the severe OSA patients of the 4th Qt demonstrated a negative relationship (β: -1.99; p<=0.001).

The LDL cholesterol was elevated in patients with severe OSA (Qt 4) with a significant result in the adjusted model for AHI (β: 7.59, p<0.001) and ODI (β: 7.22; p<0.001).

We furthermore detected that AHI and ODI correlated with the TG values (r: 0.127; p<0.001 and r: 0.129; p<0.001, respectively). In the 4th Qt the adjusted regression analyses revealed a highly significant result (β:17.9; p=0.001) for AHI.

There was a clear regional influence on the TC values, with the Northern countries reaching the highest results. When results from the other regions were compared to the one obtained in the Northern centres, the adjusted regression analysis revealed a highly significant result for both AHI and ODI (p<0.001).

These cross-sectional results of a large cohort of OSA patients without known dyslipidaemia demonstrated the positive correlation of several lipid parameters with the AHI and ODI. Also, especially for the highest Qt of AHI and ODI the result remained significant in the adjusted regression analysis. Thus, OSA seems to negatively influence the lipid metabolism with the highest prediction in the northern countries.

Study 5:

To further investigate the relationship between OSA and the lipid metabolism we analysed a cohort of patients from the ESADA database with known hyperlipidaemia defined as a self-reported diagnosis, specific lipid lowering medication or the indication of hyperlipidaemia in the reference letter or hospital charts.

A total of 11892 subjects were included in this study. The definition of the OSA severity was based on the international accepted values of the AHI: no OSA <5/h, mild OSA 5-<15/h, moderate OSA 15-<30/h and severe OSA > 30/h. Additionally the cohort was grouped according to the quartiles (Qt) of both AHI and ODI.

The presence of hyperlipidaemia was reported in 21.7 % of the analysed cohort. The prevalence increased according to the severity of the disease (no OSA: 12.2%, mild OSA 19.3%, moderate 23.2% and severe OSA 27.5%). The odd ratio (OR) for hyperlipidaemia with the correspondent confidence interval (CI) for the three OSA classification was: 1.16 (CI: 0.98-1.38), 1.28 (CI 1.08-1.52) and 1.37 (CI 1.16-1.63). Only the last two reached statistically significance (p: 0.006 and < 0.001, respectively). When splitting the participants into the quartiles of AHI and ODI, we found the ODI in the adjusted model as the best predicter for the presence of hyperlipidaemia. When results were compared to Qt I the odd ratio (OR) increased up to Qt IV (Qt II: OR: 1.3; CI:1.15-1.55; Qt III: OR:1.37; CI: 1.17-1.61, Qt IV: OR: 1.33; CI 1.12-1.58, respectively). All the results reached statistical significance (p<0.001).

We found a regional effect regarding the prevalence of hyperlipidaemia. Although in study four the patients in Northern sleep centres without known dyslipidaemia demonstrated the highest lipid values, this group revealed the lowest prevalence of hyperlipidaemia diagnosis (14.1 %). In the Southern countries, the diagnosis of dyslipidaemia was 27.5 % and reached even a prevalence of 36 % in sleep centres of the central region thus reaching statistical significance.

We could show in this study that the prevalence of the diagnosis of hyperlipidaemia in OSA patients is associated with intermittent hypoxia. There appears to exist a clear underdiagnosis of hyperlipidaemia especially in the Northern sleep centres of the ESADA group. The region may be an important confounder in multicentre multinational studies.

Study 6:

In this study we investigated the prevalence of insomnia and hypersomnolence in OSA patients included in the ESADA database.

A total of 17325 participants were included in this study. Likewise, to study 4 and 5 the sleep centres were divided in regions according to the cardinal directions with an additional central area. Patients in each region were grouped regarding the presence of insomnia and excessive daytime sleepiness (EDS).

We found a highly statistically significant difference ($p<0.001$) between the regions in all investigated anthropometric and sleep recording parameters or the prevalence of comorbidities except for the presence of metabolic disorders ($p=0.015$). For example, the highest percentage of females within the cohort was detected in the Northern and the lowest percentage in the Eastern region (35.6% vs. 26.2%). On the other hand, the highest BMI was encountered in the West and the lowest in the North (34.2 kg/m^2 ± 7.8 vs. 29.8 kg/m^2 ± 6.0). Also, the AHI demonstrated a regional dependency with the highest value in the South and lowest value in the North of the ESADA group (AHI 36.0/h ± 27.3 vs. 15.6/h ± 19.2). The clinical phenotypes of insomnia with or without EDS was predominant in all regions. However, isolated insomnia was more frequent in the Northern region (36.5 %) and lowest in the West (23.0%). Independently from the region, we found an interesting pattern regarding the prevalence of comorbidities. Surprisingly, when compared to isolated EDS patients with insomnia reported a higher percentage of cardiovascular (43.9 % vs. 51.7 %), metabolic (33.6% vs. 34.0%), pulmonary (22.7% vs. 24.3%) and psychiatric disorders (4.8 % vs. 16.5 %).

This study demonstrated significant regional differences in both clinical appearance and apnea testing results within the ESADA cohort. Insomnia patients were associated with the highest number of investigated comorbidities.

Study 7:

In this study we analysed the impact of temperature on OSA in the total ESADA cohort and in different climate zones within the ESADA group. For this purpose, a total of 24

sleep centres from 18 countries were included and grouped according to the Koeppen-Geiger-Climate classification in the following zones: Cfb: Warm temperature, fully humid, warm summer, Csa: Warm temperature, summer dry, hot summer and Dfb: Snow, fully humid, warm summer.

We detected that the prediction of the AHI (β: 0.28), ODI (β: 0.25) and the minimum SpO2 (β: -0.13) increased significantly ($p<0.001$) with the maximum temperature. The results remained significant even after correction for BMI, age, gender and the presence of air conditioning (A/C). In case of T90, the analysis did not reach statistical significance ($p>0.05$). Interestingly, there was a modification due to the climate zone. We found the highest effect of temperature on AHI in the Cfb zone (β: 0.11; $p<0.001$), followed by the Dfb zone (β: 0.08; $p<0.001$). However, the in the warmest climate zone Csa temperature had no significant effect on AHI (β: -0.01; $p=0.386$). Including A/C in a hierarchical regression model demonstrated a significant increase in the prediction with an F change of 44.99 for Cfb and 39.37 for Csa (both $p<0.001$), Nevertheless the prediction of AHI by maximum temperature remained non-significant in the Csa zone. For Cfb a simulation model revealed a constant increase of the AHI from the coldest month in January (3.26ºC; AHI 10.57/h) to the warmest month in July (22.88ºC; AHI: 15.04/h).

We conclude that maximum temperature has a significant effect on the severity of OSA although the clinical importance has still to be proven. This relationship is only visible in the middle or north European countries. At present it remains inconclusive if planned adjustments like the presence of A/C or other e.g. genetical adaption processes are responsible for the differences between the climate zones.

Conclusion:

The studies included in this manuscript demonstrate that obstructive sleep apnea (OSA) negatively influences the cardiovascular system, the cytotoxic immune system and the lipid metabolism. Sleep fragmentation by arousal was found to contribute significantly to the first two findings. The geographical location seems to influence the relationship between OSA and the lipid status, the predominance of clinical phenotypes and the response of OSA towards elevated temperatures.

Keywords: sleep disordered breathing, immune system, lipid metabolism, temperature

Resumo

O sono é uma condição que se caracteriza por redução ou ausência de mecanismos básicos da vida, como alimentação, autodefesa ou reprodução. Ocupa cerca de um terço da vida humana e foi encontrado em animais incluindo os insetos. Embora as últimas décadas tenham sido caracterizadas por uma intensa investigação na medicina do sono, ainda há vários mistérios do sono que precisam ser revelados. Este manuscrito tem como objetivo aumentar o conhecimento do efeito da apneia obstrutiva do sono (AOS) nos componentes da homeostase humana.

Estudo 1:

O stress induzido por apneias do sono repetidas é considerado um dos principais fatores de morbilidade e mortalidade relacionado com a AOS. Uma explicação possível são as alterações do sistema imunológico e inflamatório provocadas pelo stress. Nos atletas verifica-se que após um exercício exaustivo ocorre um aumento das infeções do trato respiratório superior que se relaciona com a diminuição da atividade celular *natural killer* (NKA). A proteína citotóxica perforina (Pfr) é um dos principais contribuintes para a atividade NKA, sendo importante na defesa contra infeções virais e doenças tumorais. Também se encontra associada a um aumento do risco de doenças cardiovasculares. Neste estudo, investigámos o impacto do *stress* físico e psicológico de um triatlo de *sprint,* nos linfócitos do sangue periférico positivos para Pfr.

O estudo incluiu um total de 12 homens, atletas de resistência treinados. Em todos, foi colhida uma amostra de sangue 168 e 24 horas antes e 1, 18 e 48 horas após um triatlo de *sprint*. Os atletas foram obrigados a parar de treinar uma semana antes do evento desportivo. O grupo controlo consistiu em 10 funcionários saudáveis do hospital. As células mononucleares do sangue periférico foram isoladas e, após fixação e perfuração, marcadas para investigar os marcadores da membrana linfocitária e de proteína intracelular Pfr. A percentagem de células positivas para Pfr dentro de cada subpopulação de linfócitos foi investigada por análise de citometria de fluxo padrão. O estudo estatístico consistiu na análise de variância (ANOVA) para medições repetidas com *post-hoc* de *Student-Newman-Keuls* em pares para os 5 pontos no tempo, nos

atletas. A diferença entre os controlos e os atletas foi investigada recorrendo ao teste t de *Student* não emparelhado.

Encontrámos uma diminuição do total de linfócitos de 24 horas antes, para 1 hora após o exercício. Decorridas 18 horas após o triatlo, o valor retornou à linha de base. A interrupção do treino causou um declínio pequeno, mas significativo, na percentagem de células Pfr positivas de $CD3^+$ ($p<0,01$), $CD4^+$ ($p<0,05$) e linfócitos CD8 + ($p<0,01$). O exercício físico resultou numa diminuição adicional dos linfócitos $CD3^+$ e $CD8^+$ Pfr positivos, sem qualquer alteração relevante adicional nos linfócitos $CD4^+$ Pfr positivos. Não foi detetada qualquer alteração nas células positivas para $CD16^+/CD56^+$ (células *natural killer* ou NK). A percentagem de linfócitos $CD3^+$ e $CD8^+$ positivos para Pfr foi significativamente mais elevada nos atletas, em comparação com os controlos ($p<0,05$).

Este estudo pode ser útil para entender o efeito do *stress* físico e do eventual *stress* psicológico induzido pela competição na atividade das células *natural killer* em atletas de resistência, assim como noutras possíveis situações de *stress*.

Estudo 2:

A AOS tem sido associada a um aumento da mortalidade por doenças cardiovasculares e ocorrência mais frequente de cancro. A aterosclerose e o cancro associam-se a alterações no sistema imunológico/inflamatório. A aterosclerose caracteriza-se por uma ativação aumentada do sistema imunológico/inflamatório, enquanto que o cancro tem sido associado a uma reação reduzida do sistema imunológico em relação às células tumorais. Ambas as patologias se associaram a uma alteração no sistema imunológico citotóxico.

Neste estudo, incluímos 87 participantes, divididos em: 24 controlos; 19 doentes com fragmentação do sono relacionada com o esforço respiratório, sem um IAH ou índice de dessaturação de oxigênio (ODI) relevante, classificados como doentes com aumento da resistência das vias aéreas superiores (UAR); 20 doentes não obesos (no OSA); e 24 doentes obesos (oOSA) com AOS definida como IAH>5/h e com ou sem IMC>30 kg/m^2. Em 20 doentes com AOS, repetimos a análise após uma noite de aferição do CPAP e 3 meses de terapêutica com pressão positiva automática nas vias aéreas. Por analogia

com o estudo 2, realizámos uma colheita de sangue em todos os participantes. A percentagem de células positivas para Pfr e granzima-B (GrB) foi analisada por citometria de fluxo. Além disso, numa amostra diferente, as células T do sangue periférico de 6 participantes saudáveis foram purificadas por isolamento magnético de células e incubadas numa câmara hipóxica, com 5% de oxigénio. Após exposição hipóxica, as células T foram analisadas por citometria de fluxo quanto à presença do marcador de granulação CD107a, GrB e Pfr.

A diferença entre os quatro grupos foi analisada pelo teste de *Kruskal-Wallis*, com teste posterior de *Wilcoxon* para a comparação pareada. Além disso, o coeficiente de correlação de *Spearman* foi utilizado para investigar a associação das células T contendo Pfr e os parâmetros do sono. Para a avaliação sob terapêutica com pressão positiva nas vias aéreas (PAP), utilizámos o teste de medidas repetidas ANOVA, com avaliação da dimensão do efeito com o teste *d de Cohen*.

Observou-se uma diferença significativa na percentagem de linfócitos Pfr positivos entre os grupos, especialmente entre os controlos e os doentes obesos com AOS (p=0,04). Encontrámos uma correlação negativa entre a percentagem de células $\gamma\delta$ T Pfr positivas e o IAH (coef = -0,36; p=0,001, ODI (coef = -0,34; p=0,002) e a escala de sonolência de *Epworth* (coef = -0,025 ; p=0,025). A provocação hipóxica diminuiu a capacidade de desgranulação, por uma expressão reduzida de CD107a e um nível diminuído de GrB, embora o resultado não tenha atingido a significância estatística (p=0,065).

Após terapêutica com pressão positiva nas vias aéreas (PAP), a percentagem de células positivas para perforina aumentou em todos os subgrupos de linfócitos, exceto nos linfócitos $CD3^{-}CD8^{+}$, contudo apenas os linfócitos $CD3^{+}CD4^{+}$ atingiram significância estatística (p=0,025). Após três meses de terapêutica com PAP, encontrámos um pequeno dimensão do efeito com aumento da percentagem de todos os linfócitos Pfr positivos (d=0,33); de $CD3^{+}CD4^{+}$ (d=0,26); de $CD3^{+}CD8^{+}$ (d=0,4) e linfócitos T citotóxicos (d=0,41). Adicionalmente, verificou-se uma redução de linfócitos $CD3^{-}CD8^{+}$ Pfr positivos (d=0,27). O aumento das células NK atingiu um tamanho do efeito grande (d=0,85).

Estes resultados indicam que a AOS está associada a um potencial citotóxico diminuído nos linfócitos T $\gamma\delta$ do sangue periférico. Com exceção dos linfócitos $CD3^{-}CD8^{+}$, verificou-se que a terapêutica com PAP aumenta a capacidade citotóxica de todos os outros subgrupos linfocitários estudados.

Estudo 3:

A hipertensão arterial está frequentemente relacionada com a AOS, associando-se também a um aumento da morbilidade e da mortalidade. No entanto, resultados de estudos recentes questionaram esse conceito. Neste estudo, investigámos a relevância da AOS para a falta de redução da tensão arterial no início do sono. Foram incluídos 56 participantes, consistindo em 34 controles com índice de apneia / hipopneia (IAH) <15/h e 22 pacientes com AOS com IAH ≥15/h.

Neste estudo, investigámos 56 participantes, dos quais 34 no grupo controlo com índice de apneia/hipopneia (IAH) <15/h e 22 doentes com AOS com IAH≥15/h. Utilizámos o dispositivo *Nexfin-HD* baseado no *cuff* de dedo para medir os parâmetros hemodinâmicos (PH), batimento a batimento, incluindo: frequência cardíaca (FC), pressão arterial sistólica (PAS) e volume de ejeção (VE). Foi registado um tempo total de 5 minutos de vigília inicial, seguido de 20 minutos de sono. Os resultados foram comparados pelo teste t de *Student* emparelhado e não emparelhado, com nível de significância <0,05. Além disso, o tamanho do efeito foi calculado usando o d de *Cohen*. O número consecutivo de cada onda de pulso foi correlacionado com o valor hemodinâmico correspondente. Isto permitiu investigar a evolução dos PH ao longo do tempo, usando o coeficiente padronizado β (CPB). A influência da AOS no CPB foi analisada numa análise de regressão hierárquica em bloco, incluindo idade, índice de massa corporal (IMC), IAH e índice de microdespertares (IM).

Os doentes com AOS e os controlos demonstraram uma evolução divergente dos parâmetros hemodinâmicos no início do sono. A PAS aumentou na AOS e diminuiu nos controlos (p=0,001; d=0,92), com um padrão semelhante para o VE (p=0,047; d=0,56). Além disso, a variação a curto prazo foi maior nos doentes com AOS, na FC (p<0,001; d=1,2), PAS (p=0,001; d=0,94) e VE (p=0,005; d=0,82). A análise de regressão hierárquica

indicou o índice de despertar como o melhor preditor da progressão da PAS no início do sono (β: 0,717, p<0,001) e VE (β: 0,469, p=0,003). Não encontrámos um resultado estatisticamente significativo para a FC.

Este estudo conclui que a AOS afeta negativamente a evolução a curto prazo e a variação da PAS e VE no início do sono. O índice de despertar foi o melhor preditor para a evolução dos parâmetros PAS e VE.

Estudo 4:

A AOS está associada a um aumento de risco de doenças cardiovasculares. Além do efeito direto da hipóxia repetitiva, existe evidência da uma interferência da AOS com o metabolismo lipídico. Neste estudo, investigámos a relação entre a AOS e as concentrações de lipídios plasmáticos numa coorte da *European Sleep Apnea Database* (ESADA).

Incluímos um total de 8592 doentes sem documentação de dislipidemia ou terapêutica com fármacos antilipídicos. A relação entre componentes do perfil lipídico e o IAH ou ODI foi determinada por análise dos modelos lineares generalizados. Os parâmetros lipídicos incluídos consistiram em colesterol total (CT), colesterol de lipoproteína de alta densidade (HDL ou HDL-C), colesterol de lipoproteína de baixa densidade (LDL ou LDL-C) e triglicéridos (TG). O IAH e o ODI foram analisados como valores originais e distribuídos por quartis (Qt), correspondendo o quartil mais baixo ao grupo controlo. Para entender as influências geográficas, os centros de sono participantes foram agrupados de acordo com os pontos cardeais, tendo sido adicionada uma região central.

Foi detetada uma relação relevante entre os quartis do IAH e ODI e o CT. Quando o IAH do Qt 1 foi comparado com o Qt 2: β: 4,4; p<0,001, Qt3 vs 1: β: 5,1; p<0,001 e Qt4 vs. 1: β 5,0; p <0,001. Para ODI, o CT demonstrou padrão com Qt 1 vs. 2: β: 4,2; p=0,001, Qt3 vs 1: β: 5,1; p <0,001 e Qt4 vs. 1: β: 5,4; p<0,001.

O HDL correlacionou-se negativamente com o IAH (r-0,164, p<0,001) na análise univariada. Na análise de regressão ajustada, foram sobretudo os doentes com AOS grave do 4º quartil que demonstraram uma relação negativa (β: -1,99; p<=0,001).

O LDL estava elevado em doentes com AOS grave (Qt 4), atingindo um resultado significativo no modelo ajustado para o IAH (β: 7,59, p<0,001) e para o ODI (β: 7,22; p<0,001).

Detetámos também, que o IAH e o ODI se correlacionavam com os valores de TG (r:0,127; p<0,001 e r:0,129; p<0,001). Observou-se para o 4º quartil do IAH um resultado altamente significativo na regressão ajustada (β: 17,9; p=0,001).

Verificou-se uma clara influência regional dos valores de CT, com os países do Norte a atingirem os resultados mais elevados. Quando os resultados das outras regiões foram comparados com os resultados dos centros do Norte, a análise de regressão ajustada atingiu um valor significativo para o IAH e o ODI (ambos p<0,001).

Neste estudo transversal, com uma amostra grande de doentes com AOS sem diagnóstico conhecido de dislipidemia, encontrámos uma correlação positiva de vários parâmetros lipídicos com o IAH e o ODI. Além disso, especialmente para o 4º quartil do IAH e ODI, o resultado continuou a ser significativo na análise de regressão ajustada. Concluímos que a AOS parece influenciar negativamente o metabolismo lipídico, com maior expressão nos países do Norte da Europa.

Estudo 5:

Para investigar melhor a relação entre AOS e o metabolismo lipídico, analisámos uma coorte de pacientes da ESADA com hiperlipidemia, sendo esta definida com base no diagnóstico auto-relatado, na terapêutica com antilipídicos ou na indicação de hiperlipidemia na carta de referenciação no processo hospitalar.

Foi incluído neste estudo um total de 11892 indivíduos. A definição da gravidade da AOS baseou-se nos valores internacionalmente aceites do IAH: sem AOS <5/h, AOS ligeira 5-<15/h, AOS moderada 15<30/h e AOS grave >30/h. A *coorte* foi também agrupada de acordo com os quartis (Qt) do IAH e do ODI.

A presença de hiperlipidemia foi relatada em 21,7% da coorte analisada. A prevalência aumentou de acordo com a gravidade da doença (sem AOS 12,2%, AOS ligeiro 19,3%, AOS moderada 23,2% e AOS grave 27,5%). O *odds ratio* (OR) para hiperlipidemia, com o respectivo intervalo de confiança (IC), nos três graus de AOS referidos, foi: 1,16 (IC: 0,98-1,38), 1,28 (IC 1,08-1,52) e 1,37 (IC 1,16-1,63). Apenas os dois últimos atingiram significância estatística (p: 0,006 e <0,001). Ao dividir os participantes nos quartis de IAH e ODI, verificou-se que o ODI no modelo ajustado foi o melhor preditor para a presença de hiperlipidemia. Quando os resultados foram comparados com o 1º Qt, o OR aumentou até ao 4º Qt (2º Qt: OR: 1,3; IC: 1,15-1,55; 3º Qt: OR: 1,37; IC: 1,17-1,61, 4º Qt: OR: 1,33; IC 1,12-1,58). Todos os resultados atingiram significância estatística (p<0,001).

Foi detetado um efeito regional, no que se refere à prevalência de hiperlipidemia. Embora no estudo 4 os doentes dos centros de sono do Norte da Europa sem dislipidemia conhecida tenham demonstrado os maiores valores lipídicos, encontrámos neste grupo a menor prevalência de hiperlipidemia (14,1%). Nos países do Sul, o diagnóstico de dislipidemia foi de 27,5%, tendo alcançado uma prevalência de 36% nos centros de sono da região central com significância estatística.

Este estudo permitiu demonstrar que a prevalência do diagnóstico de hiperlipidemia em doentes com AOS está associada a hipóxia intermitente. Parece existir no grupo da ESADA um claro subdiagnóstico de hiperlipidemia, especialmente nos centros de Norte, por este motivo, a região geográfica pode ser um importante fator confundente em estudos multinacionais multicêntricos.

Estudo 6:

Neste estudo, investigámos a prevalência de insónia e de sonolência excessiva em doentes com AOS incluídos no banco de dados da ESADA.

Foi incluído um total de 17325 participantes. Tal como nos estudos 4 e 5, dividimos os centros de sono em regiões, de acordo com os pontos cardinais, tendo sido incluída adicionalmente uma região central. Os pacientes de cada região foram classificados em grupos, de acordo com a presença de insónia e sonolência diurna excessiva (EDS).

Verificou-se uma diferença estatisticamente significativa (p<0,001) entre regiões, em todos os parâmetros antropométricos e parâmetros do sono, ou presença de comorbilidades, exceto na presença de distúrbios metabólicos, que atingiu um valor de p=0,015. Por exemplo, a maior percentagem de mulheres incluídas na coorte foi detetada no Norte e a percentagem mais baixa na região leste (35,6% vs. 26,2%). Por outro lado, o maior IMC foi registado no Ocidente e o mais baixo no Norte (34,2 kg/m^2 ± 7,8 vs. 29,8 kg/m^2 ± 6,0). Além disso, o IAH demonstrou uma dependência regional, com o valor mais alto no Sul e o mais baixo nos países do Norte do grupo ESADA (IAH 36,0/h ± 27,3 vs. 15,6/h ± 19,2). Os fenótipos clínicos de insónia, com ou sem EDS, foram predominantes em todas as regiões. No entanto, a insónia isolada foi mais frequente na região Norte (36,5%) e menor no Oeste (23,0%). Independentemente da região, encontrámos um padrão particular em relação à prevalência de comorbilidades. Nesta amostra, os doentes com insónia, em comparação com os doentes com EDS, apresentaram uma maior percentagem de distúrbios cardiovasculares (43,9% vs. 51,7%), metabólicos (33,6% vs. 34,0%), pulmonares (22,7% vs. 24,3%) e psiquiátricos (4,8% vs. 16,5%).

Assim, este estudo confirmou que existem, na coorte da ESADA, diferenças regionais significativas, quer no padrão clínico, quer nos resultados dos estudos da apneia do sono. O grupo com insónia apresentou uma percentagem mais elevada de comorbilidades.

Estudo 7:

Neste estudo, analisámos o efeito da temperatura nos valores da AOS na coorte total da ESADA e em diferentes zonas climáticas do grupo. Para tal, um total de 23 centros de sono foram incluídos e agrupados de acordo com a *"Koeppen-Geiger-Climate classification"* nas seguintes zonas: Cfb – temperatura quente, verão húmido e quente; Csa – temperatura quente, verão seco e quente; e Dfb – Neve, verão húmido e quente.

Detetámos que a temperatura máxima aumentou significativamente (p<0,001) a previsão do IAH (β: 0,28), ODI (β: 0,25) e SpO2 mínima (β: -0,13). Os resultados permaneceram significativos mesmo após a correção para o IMC, a idade, o sexo e a presença de ar condicionado (A/C). No caso do T90, a análise não atingiu significância

estatística (p>0,05). Curiosamente, houve uma modificação devido à zona climática. Encontrámos o maior efeito da temperatura no IAH na zona Cfb (β: 0,11; p<0,001), seguida da zona Dfb (β: 0,08; p <0,001). No entanto, na zona climática mais quente, Csa, não encontrámos um efeito significativo da temperatura no IAH (β: -0,01; p = 0,386). A inclusão de A/C num modelo hierárquico de regressão demonstrou um aumento significativo na previsão, com uma mudança F de 44,99 para Cfb e 39,37 para Csa (ambos p<0,001). No entanto, a previsão do IAH por temperatura máxima permaneceu não significativa na zona Csa. Para o Cfb, um modelo de simulação revelou um aumento constante do IAH do mês mais frio, em janeiro (3,26ºC; IAH 10,57/h), para o mês mais quente, em julho (22,88ºC; IAH 15,04/h).

Concluímos que a temperatura máxima tem um efeito significativo na gravidade da AOS, embora a importância clínica ainda necessite ser comprovada. Esta relação verifica-se apenas nos países do centro ou norte da Europa. Atualmente, permanece inconclusivo se os ajustes intencionais, como a presença de A/C ou outros processos de adaptação (p.e. genéticos) são responsáveis pelas diferenças entre as zonas climáticas.

Conclusão:

Os estudos incluídos neste manuscrito demonstram que a apneia obstrutiva do sono (AOS) influencia negativamente o sistema cardiovascular, o sistema imunológico citotóxico e o metabolismo lipídico. Verificou-se que a fragmentação do sono por microdespertares contribui significativamente para os dois primeiros resultados. A localização geográfica parece influenciar a relação entre a AOS e o *status* lipídico, a predominância de fenótipos clínicos e a resposta da AOS a temperaturas elevadas.

Palavras-chave: Distúrbios respiratórios do sono, sistema imunológico, metabolismo lipídico, temperatura

Table of Content

1 Introduction

1.1 Sleep and health

In the last decades sleep medicine and sleep research changed from a small and rather esoteric field ploughed by a small group of scientists to a widespread area exploited by public health administrators, clinical health practitioners of various specialities, basic scientists, mass media and least but not last by the healthcare industry [1]. This increased interest in sleep medicine and sleep research was reflected by an increasing number of publications (according to Pubmed 13047 in 2018), which as a total is overtaking other common medical disorders like coronary heart disease (2018: 10138 publications) or renal failure (2018: 8940 publications).

Sleep maintains its mystery, as the scientific community still discusses the real importance of sleep for the organism. An indicator of the importance of sleep might be assumed by the fact, that in the animal kingdom sleep has been documented across several branches of the phylogenetic tree [2].

While the precise benefit of sleep for humans remains in discussion, there is evidence that sleep disruption has pluripotential effects on various components of the human homeostasis [3]. The direct consequences on the central nervous system of sleepiness and an increased likelihood of accidents are understandable and most people found it comprehensible from their own experience [4-7]. However, other, more subtle consequences of insufficient or inadequate sleep are less obvious and include endocrine or metabolic disorders like diabetes [8-13], dyslipidaemia [14-16] and fatty liver disease [17-20]. The relationship between sleep and the immune system is even more complex since in the brain several inflammatory transmitters are also related to the control of vigilance and sleep [21] (Figures 1).

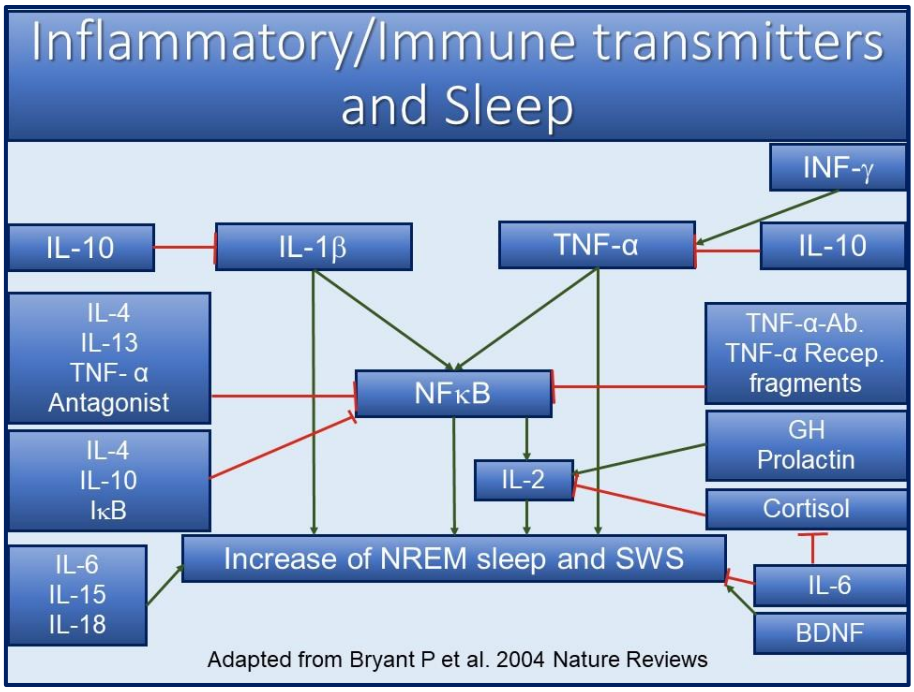


Figure 1: Inflammatory transmitters and the influence on sleep. Pro-inflammatory mediators as Interleukin-1β (IL-1β) or tumour necrosis factor-α (TNF-α) can induce non-REM sleep (NREM) and increase of slow wave sleep (SWS) either directly or over via the activation of nuclear factor light chain enhancer or activated B cells (NFκB) and Interleukin-2 (IL-2). Other pro-inflammatory cytokines like interferon-γ (INF-γ) or the neurotrophin brain-derived neurotrophic factor (BDNF) are also either directly or via TNF-α increasing NREM sleep and SWS. Anti-inflammatory cytokines like IL-10 or the asthma related interleukins IL-4 and IL-13 reduce NREM sleep. The same applies to TNF-α antagonists, TNF-α receptor fragments and IκB Kinase factors that downregulate the inflammatory process.

Already during the antiquity Aristotle described a relationship between sleep and health [22]. However, only modern science manged to reveal some of the interactions between brain, sleep and the immune system that influence the human condition [23]. At present, most known sleep disturbances are proven to influence human health. For example, several studies detected a J-shape curve between the sleep duration and mortality [24-26]. It has been concluded, that either too short or too long sleep is associated with an increase in mortality especially due to cardiovascular events [27]. Insomnia has been linked to depressive disorders [28-30], but there is also some evidence that insomniac patients are at risk of cardiovascular diseases including hypertension, although both the relationship and the mechanism are still under discussion [31-36]. Restless legs syndrome and/or periodic leg movement during sleep (PLMs) are also related to alteration of the cardiovascular system specially hypertension [37-40]. Of all the known sleep disorders the obstructive sleep apnea (OSA) appears to be the most exhaustively investigated disease. However, although the disease is known for centuries so far even modern science has

not managed to respond to all the questions regarding the impact of OSA on health and why the risk is not identical for everybody.

1.2 Obstructive sleep apnea

1.2.1 Definition of obstructive respiratory events during sleep.

At present, the diagnosis of sleep disorders and of sleep related events are defined by two publications of the American Academy of Sleep Medicine (AASM). The third edition of the International classification of Sleep Disorders (ICSD-III) defines the diagnosis criteria for all internationally accepted sleep disorders [41], while the AASM Manual for the Scoring of Sleep and Associated Events defines how to classify sleep and sleep related events by polysomnographic (PSG) sleep recording. This includes arousal, respiratory or movement events and cardiac events which are mainly consist of rhythm disorders [42]. The rules for sleep staging are generally well accepted, however, there is some controversy regarding the scoring of respiratory events during sleep. This is related to the fundamental discussion regarding the importance of hypoxemic events defined as a significant reduction in the peripheral oxygen saturation (SpO_2) and the sleep fragmentation which consists of a brief increase in the electroencephalographic (EEG) frequencies detected via PSG. Obstructive sleep apneas are defined as a complete cessation of naso-oral airflow for at least 10 seconds with the respiratory effort maintained. The definition of hypopneas has changed 3 times within the last 20 years causing confusion within the sleep society and difficulties to compare scientific results (see table 1).

Year	Hypopnea definition (all consider a duration ≥ seconds)	Literature
1999	**1)** A clear decrease (>50%) from baseline amplitude **2)** A clear amplitude reduction but > 50 % associated with a 3 % oxygen desaturation or an arousal	[43]
2007	**A:** 1) Nasal pressure signal excursion drops 30 ≥ of baseline, 2) Presence of a 4% desaturation	[44]

	B: 1) Nasal pressure signal excursion drops 50 ≥ of baseline, 2) Presence of a 3% desaturation or arousal	
2018 (version 2.5)	**1A** recommended: a) peak signal excursion drop by 30 % b) Presence of a 3% desaturation or arousal **1B** acceptable a) peak signal excursion drop by 30 % b) Presence of a 4% desaturation or arousal	[45]

Table 1: Short demonstration of the different hypopnea definition by the American Academy of Sleep Medicine. Of note: The latest hypopnea definition was first stated in the AASM manual version 2.0 and did not foresee the acceptable hypopnea definition 1B.

Beside apneas and hypopneas exists as third sleep related obstructive respiratory event named the respiratory effort related arousals (RERAs). The definition of the event is a sleep fragmentation by an arousal. The arousal itself is related to an increase in the respiratory effort due increased upper airway resistance (UAR) and/or flow limitations. Since this event is sometimes difficult to detect by normal oral-nasal flow analysis RERAs related sleep fragmentation was formerly considered a sleep disorder by itself [46]. Today, the RERAs are classified as a subtype of hypopneas and incorporated in the respiratory disturbance index (RDI), if counted at all [45]. However, to understand the clinical impact of obstructive sleep disordered breathing, it is important to distinguish sleep fragmentation which is considered important for daytime sleepiness from oxygen desaturation which is at present considered more important for cardiovascular diseases [47-51]

1.2.2 Epidemiology of obstructive sleep apnea

Kottow tried in 1980 to classify the definition of a medical disease: “Core disease is defined as a verifiable, self-conscious sensation of dysfunction and/or distress that is felt to be limitless, menacing and aid-requiring. In contrast, conditioned diseases are states labelled as diseases by virtue of consensus on prevalent sociocultural and medical values” [52]. Following this definition OSA would be classified as a conditioned disease, since a high percentage of the patients are not aware of any health problem. Due to this lack of self-consciousness regarding the sleep disturbance an assessment of the relevance, the prevalence and clinical consequences of OSA can only be investigated by

epidemiological studies. In 1993, Terry Young and colleagues published probably the first well-designed study to investigate the prevalence of OSA. The results from the Wisconsin Sleep Cohort Study (WSCS) are still most frequently cited in the epidemiology of sleep medicine. For the minimum criteria of OSA with an AHI > 5/hour sleep and the presence of daytime sleepiness she calculated a prevalence within middle-aged work force of 2 % in women and 4 % in men [53]. OSA was therefore established as a relevant and frequent disease. Other epidemiological studies followed and the Sleep Health Heart Study (SHHS) is possibly one of the more relevant ones. This cohort included patients from nine existing epidemiological studies within the United States of America (USA). A total of 6441 patients were included between the baseline collection in 1995 and the first follow-up visits up to 1998 [54,55]. A moderate OSA defined as an AHI ≥ 15 was found in 24 % of the male and 11 % of the female participants [56]. Other more recent studies estimated the prevalence of OSA of 32,8 % in the general population of São Paulo [57] and the HypnoLaus study detected a respiratory disturbance index (RDI) ≥15/h in 23·4% of the women and 49·7% of the men within the Lausanne area [58]. Although, on the first impression the prevalence of OSA appears to continuously increase between 1993 to 2015, the results cannot be compared uncommented. Methodological differences within the study protocols and definition of OSA are relevant and may be, at least partially, responsible for the increase in the OSA prevalence. Especially the above discussed change in the hypopnea classification is of importance for the higher number of patients with an increase of AHI or RDI. A recent meta-analysis calculated an increase about 20 % regarding the diagnosis of OSA when comparing the 2007 with the 2012 criteria of the AASM [59]. Therefore, the prevalence of OSA within the general populations remains unclear. A recent review of the existing data estimated an OSA prevalence between 9% to 38% of the general population [60]. It is of importance that in this last-mentioned publication, older epidemiologic studies like the WSCS or the SHHS has been excluded and the investigation of the local/ethical impact on SRBD remained limited. In conclusion: although currently the precise prevalence of the OSA is not possible to define, the assembled evidence indicates a high percentage within the general population. It remains a somehow philosophical question if OSA, with a possible prevalence of 49 % within the male population, is still a conditioned disease or if it should be considered normal.

1.2.3 The European Sleep Apnea Database (ESADA)

The European Sleep Apnea Network/European Sleep Apnea Database-ESADA is at present probably the biggest apnea registry worldwide. ESADA started in 2007 as a COST (European Cooperation in Science & Technology) Action B26 project of the European Union [61]. The initial objective was to investigate the cardiovascular risk of OSA in the general population. For this purpose, each sleep centres included their patient´s data including anthropometric values, sleep history, sleep recording results and medication in an online platform. The steering sleep centre is the Sleep Laboratory, Sahlgrenska University Hospital, Gothenburg, Sweden. While ESADA started with 22 sleep centres more and more centres were included the following years. At present (March 2020) ESADA includes 39 sites with a total of 31247 patients included (Figure 2).

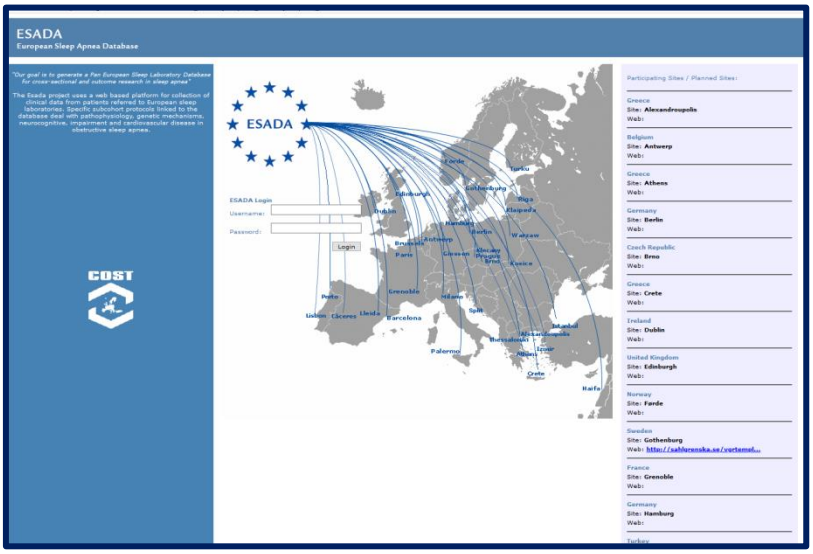


Figure 2: Site of the European Sleep Apnea Database (ESADA); March 2020.

The Lisbon Sleep Laboratory of the Clinica Universitária de Pneumologia, da FMUL/CHLN was the first Portuguese centre invited to become a member of the ESADA group. Since 2012 the Lisbon site includes patients and participates in ESADA projects. The so-called subprotocols are specific clinical questions regarding OSA consequences, that are investigated with the existing database. The proposed project is discussed during the annual meetings of the ESDA group. After a formal consent of the meeting participants a paper-writing-committee of usually less than 10 members are trusted with the manuscript. Only the writing committee appears as authors or co-authors in the

publications, while the otherwise non-involved ESADA participants are mentioned as collaborators. Contrary to the above mention population based epidemiological studies the ESADA project does not embrace a homogenous study protocol. However, what in the beginning was perceived as problem has turned out to be an advantage. The diagnosis of OSA is worldwide heterogenous. Due the high number of included patients in the database, we can not only investigate a specific clinical question, but also analyse which method might be the more adequate for this purpose [62]. The focus of the ESADA based investigation changed from mainly cardiovascular diseases to all aspects of sleep breathing disorders. This includes several common health disorders including renal failure [63], diabetes mellitus [11] and cancer [64]. A list of the publications as collaborator can be found in the reference section.

1.2.4 Impact of obstructive sleep apnea on morbidity and mortality

The impact of OSA on vigilance and health can be appreciated by reading the -in general considered first- description of the disease by Charles Dickens: *"According to Hoyle this hand is called a "full house. Because he had dropped off to sleep, he failed to take advantage of this opportunity. A few days later he entered…hospital"* [65]. Joe the fat boy has been depictured as a young obese man with enlarged legs due to oedema and apparently excessive daytime sleepiness. With few exceptions, there is consensus in the sleep research community that OSA increases morbidity and mortality mainly due to cardiovascular events [66,67]. A recent study based on health register data confirmed in a 10 years follow-up the increased mortality for OSA patients with a hazard ratio of 4, 39 (CI 3,236-5,944) in patients with an age of 40-59 years and 15,81 (CI 11,71-21,345) for patients older than 60 years. CPAP therapy reduced the risk to a hazard ratio of 0,67 compared to non-CPAP patients [68]. Despite the evidence of a cause/effect relationship between OSA and the increased morbidity/mortality the precise mechanisms are still under investigation.

1.2.5 Obstructive sleep apnea and the cardiovascular risk

Since the 1960[ies] the cardio-pulmonary interaction in OSA interested the sleep research community. [69-73]. This was related to the fact that cardiovascular diseases including arterial hypertension, coronary artery disease, atrial fibrillation, heart failure and

pulmonary hypertension were more frequently observed in OSA patients compared to the general population [74]. In 2001 Shahar and colleagues published the cross-sectional results of the sleep health heart study that raised the awareness regarding the impact of even mild sleep disorders on the risk of cardiovascular diseases [75]. Within the group of CVD, the relationship between OSA and hypertension or coronary artery disease are probably the more intensively investigated issues.

1.2.5.1 Obstructive sleep apnea and hypertension

Already early observational studies found a high percentage of patients with OSA in a hypertensive cohort [76]. There exist at present already some very well conducted longitudinal studies regarding the risk factor of OSA for the development of arterial hypertension. Peppard et al. could show in 2000 that an AHI over 15/h was associated with an Odds ratio of 2,89 (95%CI: 1,47-5,69) for the development of hypertension even after adjustment for BMI, waist and neck circumference [77]. Especially the lack of the physiological decrease in arterial blood pressure is a typical feature of OSA patients [78]. The negative impact of non-dipping arterial blood pressure (ABP) on the risk for CVD has been frequently described [79-82]. Interestingly, it has already been suggested that non-dipping ABP predicts OSA [83]. This conclusion is reflected in the actual European hypertension guidelines that specifically refer to OSA as a risk factor for non-dipping ABP [84].

As stated above, the pathomechanism between OSA and CVD remains still under discussion and investigation. This is also true regarding the impact of OSA on arterial hypertension (AHT). At present there are several mechanisms in discussion to explain this effect, but two of them demonstrate up to now the best evidence.

1: Chronic intermittent hypoxia induces increases in blood pressure

Even after a relatively short period of 28 nights hypoxia can cause an increase in the blood pressure values [85]. In a second study, Tamisier et al, confirmed this result. Here, a total of 14 healthy young adults were exposed to nocturnal intermittent hypoxia which resulted in a significant increase of 8 mmHg in the systolic blood pressure (SBP) and of 5 mmHg in the diastolic blood pressure (DBP); an increase in the sympathetic activity and a decrease in the baroreceptor reflex [86]. Nevertheless, the participants remained in

a stable hypoxic condition and reoxygenation was achieved by external oxygen supply over a nasal canula. It is not clear if this study protocol could influence the results of e.g. by canula displacement.

Hypoxia and vascular dilatation and endothelial function

Beside the acute effect of intermittent hypoxia on ABP, several studies investigated the influence on the vascular structure changes mainly the reduced vasodilation. Hypoxia induced oxygen radicals are increased in OSA patients [87]. The presence of the free oxygen radicals interferes with the endothelial-mediated vasodilatation, stiffens conduit arteries and might be involved in the genesis of atherosclerosis [50,88-92].

Beside the direct effect of hypoxia on the endothelial, there is evidence that OSA affects various components of the metabolism and increases the risk and/or gravidity of the metabolic syndrome, which subsequently increased the cardiovascular risk. This includes diseases like diabetes mellitus, increased lipid levels with hypercholesterinaemia and/or hypertriglyceridemia or hepatic alterations, e.g., the non-alcoholic fatty liver disease [8,17,93-95].

1.2.5.2 Obstructive sleep apnea and coronary heart disease

Commonly, the incidence of sudden cardiac death demonstrates a circadian rhythm with a maximum peak of events at 11.00 am [96,97]. In OSA patients this circadian rhythm is changed to an increased risk of acute myocardial infarction between midnight and 6.00 am [98]. Already in 1990 Hung and colleagues could demonstrate an association between OSA and acute myocardial infarction (AMI) [99]. The WSCS detected in OSA patients with a severe disease (AHI > 30/h) a 2,6 fold increased risk for coronary artery disease or heart failure [100]. Several mechanisms have been proposed for the association between OSA and increased acute myocardial infarctions.

Mismatch between oxygen demand and supply

The observation of obstructive sleep apneas can give an impressive demonstration why the oxygen demand/supply mismatch might be of importance for the development of myocardial infarction. This is especially true when hemodynamic recordings are associated to the routine polysomnographic parameters (figure 3).

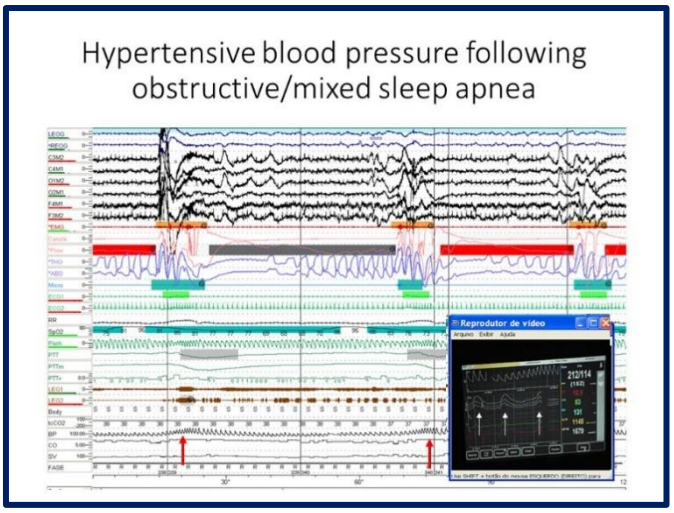


Figure 3: Screenshot of a polysomnographic recording with included hemodynamic measurement. The polysomnography demonstrates repetitive obstructive or mixed sleep apneas followed by the typical arousal reaction and postapneic hyperventilation. The video window displays the screen of the Nexfin-HD device used for non-invasive beat-to-beat measurement of heart rate, systolic and diastolic blood pressure, stroke volume, cardiac output and peripheral vascular resistance. The white arrows in the video window indicate the blood pressure peaks following each obstructive or mixed apnea with the systolic blood pressure values exceeding 200 mmHg. Red arrows demonstrate the recorded blood pressure swings within the polysomnography.

During the cessation of breathing peripheral oxygen SpO_2 decreases sometimes reaching values under 60%. Usually the apnea is finalized by an arousal from sleep in association with an increase in heart rate (HR) and blood pressure. Thus, at the minimum oxygen saturation the workload for the heart muscle is at its maximum. However, the concept of low oxygen supply as one of the important risk factors for acute myocardial infarction has been questioned. Observational studies did not find an increased severity or mortality in AMI patients with known OSA [101,102]. One of the possible mechanisms discussed is the preconditioning of the heart by repetitive hypoxias [103,104]. The fact that OSA is a risk factor for cardiovascular death [105] requires the exploration of other mechanisms than hypoxia alone.

1.2.6 Obstructive sleep apnea and the glucose and lipid metabolism

1.2.6.1 Diabetes mellitus:

Other factors possibly involved in the relationship between OSA and CVD are related to disorders of the glucose and lipid metabolism. The impact of OSA on insulin resistance or diabetes has been discussed for several years [106]. Recent results from epidemiological studies like the SHHS confirm this association but the main mechanism of OSA leading

to the alteration in the glucose metabolism is still not well understood [107]. Other cross-sectional studies detected increased prevalence of diabetes mellitus in the OSA patients with a more severe disease [11,108]. Obesity is the common comorbidity of both diseases and could be a feasible explanation for this relationship. Yet, it has been shown, that OSA patients demonstrated with a Hazzard ratio of 1,71 an increased risk for diabetes mellitus even after adjustment for adiposity [107]. Both human and rodent studies confirmed an altered glucose metabolism due to Intermittent hypoxia [95,109,110]. While the results from Louis and colleagues appear convincing some caution must be kept. He included a total of only 13 young and healthy participants but with overweight. Aside from the small sample size, it is of interest that in obese subjects the influence of hypoxia is more complex. The adipose tissue when in a hypoxic situation induces an inflammatory cascade by stimulating the hypoxia inducible factor-1 (HIF-1) [111]. This fact makes it more difficult to assess if intermittent hypoxemia independent of increased adipose tissue would have the same influence on the glucose tolerance.

1.2.6.2 Dyslipidaemia

Obstructive sleep apnea patients frequently suffer of alterations of the lipid metabolism resulting in a pro-atherogenic situation with a reduced high-density cholesterol (HDL) and an increased low-density cholesterol (LDL) [112]. CPAP therapy results in a reduction of the postprandial hyperlipidaemia found in OSA patients [113]. Also, triglycerides metabolism was found to have decreased in OSA patients with a positive response following CPAP therapy [114]. Interestingly, also the HDL alterations in OSA seem to be sensitive to the therapy with positive pressure devices giving evidence that different parts of the lipid metabolism may be influenced by OSA [115]. As a direct consequence for CVD there exists some evidence that CIH influences the atherosclerotic plaque growth but not its composition. Drager and colleagues describe the OSA-lipid metabolism as one of the possible relevant mechanisms of increased atherosclerosis in OSA patients [116,117]. Nevertheless, this relationship between OSA and atherosclerosis is at present not completely understood and more research is warranted.

1.2.6.3 Non-alcoholic fatty liver disease (NAFLD)

Non-alcoholic fatty liver disease is defined by the American College of Gastroenterology as an accumulation of excess fat in the liver of people that drink little or no alcohol (https://gi.org/topics/fatty-liver-disease-nafld/). NAFLD is considered by some authors part of the metabolic syndrome due to its importance for an altered glucose or lipid metabolism [118,119]. In a mouse model Li and colleagues noted an upregulation of the genes of lipid biosynthesis due to CIH [120]. They suggested that this mechanism might explain the effect of OSA on the non-alcoholic fatty liver disease. Also in humans there is a growing evidence that OSA might increase the risk of NAFLD [17].

1.2.7 Non-hypoxic sleep disturbances and cardiovascular disorders

The section above discussed the relationship between hypoxic events as a possible risk factor for increased CVD based mortality in OSA patients. However, already early studies questioned the limitation of apnea induced blood pressure swings to hypoxic events [121]. New research in the last decades demonstrates that the relationship between sleep duration and cardiovascular events forms a J-curve. Either too little (< 6 hours) or too much sleep (> 9 hours) increases morbidity and mortality [122]. Also, short sleep duration and insomnia have been linked to hypertension (figure 4) [24,31]. Especially short sleep duration, which is not a hallmark sign of OSA, are associated with an increase blood pressure and can cause coronary calcification, dyslipidaemia and diabetes mellitus [123-128]. These results were confirmed in a recent meta-analysis where both increase or decrease of sleep time over 7 hours were associated with an increase in the relative risk for cardiovascular events [27].

Along the same lines to OSA, the pathophysiology of how insomnia and sleep restriction could increase the risk for cardiovascular events is still under investigation [129]. Insomnia patients with objective short sleep duration were found to suffer of a dampened parasympathetic activation and increased sympatho-vagal imbalance [130]. A recent metanalysis described the evidence of an insomnia-cardiovascular relationship as non-conclusive [30]. One of the possible confounding factors to assess the impact of insomnia on the cardiovascular system is the broad definition of insomnia. There is more evidence for the relationship between insomnia and hypertension in the case of patients with a

proven reduction in total sleep time [127]. Moreover, insomnia with a short sleep duration defined as ≤ 5 horas/night revealed a higher risk for diabetes mellitus with a odds ratio of 2,95 (CI 1,2-7). In another longitudinal study, Clark and colleagues observed in patients with disturbed onset of sleep an increased risk of both hypertension and dyslipidaemia [14]. Interruptions in the circadian rhythm as seen in shift workers may increase the risk of diabetes mellitus [131]. Interestingly, a study using microneurography demonstrated that insomnia patients, like OSA patients, suffered of a blunted baroreceptor reflex [132]. Therefore, it is possible that changes the sympathetic activity might contribute to cardiovascular alterations in sleep disturbances not associated with hypoxic events [133].

The presence of CVD in non-hypoxic sleep disorders requires a further investigation of common, not only oxygen related cardiovascular risk factors in patients with disturbed sleep.

1.2.8 Obstructive Sleep Apnea, the immune system and cardiovascular diseases

Already in the 19th century, Rudolf Virchow described that atherosclerotic plaques exhibit an important inflammatory element [134]. In the last years, this inflammatory component of atherosclerosis and coronary artery disease has been intensively investigated. Today is well acknowledged that the immune system is a relevant factor in both cardiovascular morbidity and mortality [135-137]. In fact, one of the best described inflammatory markers is the serum C-reactive protein (CRP) especially the high-sensitive CRP (hsPCR). It can be used to estimate the cardiovascular risk even in a low risk population like women with low LDL cholesterol concentration [138]. Additionally, increased interleukine-6 (IL-6) levels proved to be associated with an elevated cardiovascular risk [139,140]. The acute coronary syndrome as the acute and life-threatening presentation of CVD has also been linked to inflammatory cascades of the immune system. The rationale behind this is based on the fact that the destabilization of atherosclerotic plaques within the coronary arteries might be related to the local presence of inflammatory cells [141,142]. Coronary plaque rupture is a complicated and not yet completely understood process. The concept of a mere “lipid storage disease” in

atherosclerosis has been changed to the recognition that there exists a cholesterol-initiated inflammatory process with both the innate and the adaptive immune system involved [136,143]. The so called "killer cells" have been identified as part of the cellular immune system associated with the plaque ruptures in coronary arteries. They have been intensively studied in both animal models and in in histology samples of sudden cardiac death victims [144-148]. Killer cells or cytotoxic T-lymphocytes (CTL) can be either part of the innate immune system like the natural killer cells (NK cells), be part of the adaptive immune system like the natural killer T lymphocytes (NKT cells) or have demonstrated characteristics of both although mainly innate like the gamma-delta T lymphocytes ($CD3^{+}\gamma\delta$). Killer cells are important in the host defence by neutralizing either infected or tumour cells [149-152]. Of this group the $CD3^{+}\gamma\delta$ T lymphocytes are probably the least investigated T-cell subset so far. It was only in 1986 that Brenner and colleagues published the identification of a second T-cell receptor with a novel T3 associated polypeptide related to the T gamma and delta gene [153]. Nevertheless, there is already some evidence that they are increased in CVD like cardiomyopathy and atherosclerosis [154,155]. All three lymphocyte subsets share the ability to eliminate their target cells via the perforin (Pf) and granzyme-B dependent necrosis and apoptosis pathway.

1.2.8.1 General aspects of perforin and granzyme-B

Since the early 1960^{ies} lymphocyte induced killing of target cells has been described [156]. In 1988, Lichtenheld published the structure and function of perforin (Pfr), a protein stored in the granula of cytotoxic lymphocytes and released after contact with a target cell into the so-called immunological synapse between the two cells [157,158]. About the same time several granzymes belonging to the family of the serine esterase were identified to coexist with perforin in the cytotoxic granules of cytotoxic lymphocytes [159,160]. After release from the granules, Pfr forms in the presence of calcium transmembrane pores on the target cell alike the mechanism of the 9th component of the complement system [161]. This ability to perforate the membrane of the target cell gave the protein its name. There has been some discussion if GrB enters the target cell by passing the perforin created pores, or by endocytosis [162,163]. Although both theories still exist, at present GrB influx into the cell seems more likely to be related to the pores

created by a membrane attack complex and perforin in resemblance to the bacterial cholesterol-dependent cytolysins [164,165]. Up to now five human members of the granzyme family have been detected. Aside from the fact that these cytotoxic proteins are involved in cell death they also modulate the immune system and the inflammatory response [166]. Apoptosis is a programmed cell suicide that is both necessary for the normal development of the organism as well as for the elimination of either infected or neoplastic transformed cells [167]. GrB provides the ability to cleave proteins associated with the apoptosis cascade. Formerly the main action of GrB was considered to be the activation of caspases-3 that promotes the nuclear breakdown [168]. However, as it has turned out that the human GrB interacts with proapoptotic mitochondrial factors like BID, while the mouse GrB cleaves caspase 3/7 and thus confirm differences between the mouse and human granzymes [169]. Therefore, some information obtained, e.g., in knock out mouse models cannot be used thoroughly for the understanding of the human perforin/GrB depending apoptotic pathway. At present GrB appears to be the most powerful pro-apoptotic granzyme [168].

In the mouth model, perforin deficiency increases the risk of viral infections and tumour diseases [170,171]. Both Pfr and GrB are found in a variety of cytotoxic T lymphocytes, including $CD3^{+}\gamma\delta$ T cells [151,172]. The complete absence of perforin in humans is associated with familiar hemophagocytic lymphohistiocytosis [173]. In this rare disease the absence of target cell death ends in a lethal cytokine storm [174]. Lesser forms of perforin deficiencies also termed perforinopathies and are associated with either Pfr mutations or impaired granule exocytosis [175]. The importance of this lack of effective perforin is demonstrated in an increased risk of several mainly hematologic cancer diseases [176]. Although there has been no systematic investigation there exists evidence indicating that environmental factors including heat influence the cytotoxic immune defence including the perforin/granzyme-B system [177].

1.2.8.2 Perforin and Granzyme-B in cardiovascular diseases

Already early studies in heart surgery demonstrated in patients with transplant rejection an increase in the number of perforin and/or granzyme-B positive cytotoxic lymphocytes [178,179] This relationship is already commercially used. Mac and colleagues developed, in

a mouse model, a nanosensor to detect GrB activity and thus incipient transplant rejection processes [180]. While in acute transplant rejection the association of lymphocytes mediated cell death by the perforin/granzyme process is easy to understand increased Pfr and GrB positive lymphocytes have been found in tissues samples of other disorders. Histological samples confirmed a transplant independent association of cytotoxic lymphocytes in atherosclerotic plaques. Coronary artery plaque rupture is a possible life threatening event since highly thrombogenic necrotic core material is suddenly released with the risk of an acute coronary syndrome [181]. The influence of perforin release from cytotoxic lymphocytes has been intensively studied [155]. In atherosclerosis human NK cells demonstrate an increased CD160 expression a protein linked to an enhanced cytotoxic activity [182,183]. Also, in symptomatic atherosclerotic plaques NK subtypes demonstrated an increased presence [184]. There are very few studies investigating the relationship between $CD3^{+}\gamma\delta$ T lymphocytes and atherosclerosis. This is surprising, since the presence of $CD3^{+}\gamma\delta$ T lymphocytes cells within the arteriosclerotic plaque has already been described several years ago [141]. At present there exists more evidence for the active role of $CD8^{+}$ lymphocytes in the genesis of arteriosclerosis and its complications [185-187]. A depletion of Pfr and GrB in $CD8^{+}$ lymphocytes decreased significantly the atherosclerotic lesions indicating a crucial role of the cytotoxic proteins -at least in the mouse model- for the development of the disease [148]. The role of GrB in arteriosclerosis has been summarized by Chamberlain in 2007. More recently Sanad and colleagues detected elevated levels of perforin mRNA within atherosclerotic plaques but not in the peripheral blood [188]. In fact, when comparing coronary artery disease patients with either stable angina pectoris or unstable angina pectoris the mononuclear cells of the later demonstrated an increased production of GrB [189]. The existing evidence of the role of Pfr and GrB in the development of atherosclerotic disease, plaque destabilization and acute coronary syndrome indicates an important role of these proteins in cardiovascular diseases [190]

1.2.8.3 The relationship of obstructive sleep apnea and the inflammatory processes

1.2.8.3.1 OSA and inflammatory serum markers

The described relationship between the immune system and CVD allows to investigate if OSA is a possible moderator or mediator of this relationship. The alleged risk of sleep

apnea on cardiovascular diseases increased the investigation of common molecular risk factors between sleep apnea and arteriosclerosis. As mentioned above, there exists a bidirectional relationship between sleep and inflammation since inflammatory transmitters are used in the brain to moderate sleep (figures 1) and on the other hand sleep disturbances can increase the inflammatory markers [23]. Early research detected that although tumor necrosis factor alpha (TNF- α) induces slow wave sleep it is also increased in patients with OSA [191,192]. OSA patients demonstrated also elevated levels of interleukin-6 (IL-6) in both with and without the coronary heart disease. Thus, there exists a link between OSA and CAD by charring elevated levels of IL-6 and TNF-α [193-195]. Additionally, several studies found that C-reactive protein (CRP) is elevated in OSA and CAD patients when compared to controls [196-198]. Although this common molecular pattern appears convincing, there exists some caution due to the possible influence of the adipose tissue inflammation. Although the interaction of adipose tissue is acknowledged, at present OSA is considered an independent predictor for the increase of inflammatory markers [199-202].

1.2.8.3.2 Obstructive sleep apnea and the cellar inflammatory response

While the interaction between sleep apnea, CVD and the inflammatory markers is still discussed but in general accepted, there is less consensus regarding the impact of obstructive sleep disordered breathing on the cellular immune system. Domagala-Kulawik and colleagues published a study comparing healthy participants and patients with OSA several lymphocyte subtypes. They detected an increased number of several lymphocyte subtypes including NK cell NKT cells and HLADR positive T-cells [203]. However, OSA and controls were very unbalanced in number, age, BMI and gender and therefore the results must be interpreted cautiously. One study group described that sleep apnea influences several components of the cellular immune system including neutrophils, $CD3^{+}\gamma\delta$ lymphocytes, activated CD8 Lymphocytes, [88,204,205]. Especially the relationship between cytotoxic $CD8^{+}$ lymphocyte and OSA was found to be of interest to the sleep society, since it was associated with an increased killer-cell activity that explained possible vascular damage by OSA stimulated lymphocytes [206]. Recently the group of Domagala-Kulawik also described an increase in FAS positive $CD4^{+}$ and $CD8^{+}$ lymphoyctes. FAS or CD95 receptor is a protein belonging to the TNF-α family and is

involved in the caspase 3 induced apoptosis process of the CD95 expressing cells [207]. The authors interpreted their results as an indication for the pro-inflammatory process of OSA. In children with OSA (moderate to severe OSA defined as an AHI > 5/h) Ye and colleagues described an altered relationship between CD17 and T regulator lymphocytes (Treg) [208] indicating a pro-inflammatory activation in paediatric OSA patients. Tan et all also found a predominance of Th1 lymphocytes compared to controls. The T regulatory lymphocytes (Treg) were not significantly different between the two groups, but he found a negative correlation between the AHI and the percentage of Tregs within the $CD4^+$ lymphoyctes [209].

In summary: The existing evidence demonstrates that OSA is increasing, independently of obesity, the inflammation process by increased CRP, IL/6 and TNF/ levels. Also, although, with less evidence, OSA seems to increase pro-inflammatory cells in the peripheral blood.

1.2.9 Obstructive sleep apnea and cancer Incidence, prevalence, and mortality

The increased mortality in OSA patients has been traditionally linked to the described higher risk of cardiovascular events. In fact, the augmented stimulation of the inflammatory system as seen by elevated inflammatory markers like IL-6 and TNF-α or increased cellular immune response seemed to decrease the risk of tumor diseases in OSA patients. This possible anti-cancer effect of OSA was discussed for an unexpected survival advantage in elderly OSA patients when compared to controls [67]. However, this concept had been recently questioned. A possible relationship between sleep disturbances like insomnia or sleep duration and an increased cancer risk was described from both animal models and observational studies [210-212]. Sleep disordered breathing became of interest when the Wisconsin Sleep Cohort demonstrated in a 22 year follow-up an increased risk for cancer in patients with OSA [213]. The possible negative influence of OSA on the cancer prevalence was also detected in an observational study from Spain [214]. The authors of this study found a relationship between the oxygen saturation below 90 % and the increase in cancer risk, although the AHI did not reach a statistically significant result. In another retrospective study, Brenner and colleagues found in a cohort of 5243 subjects an increase for various forms of cancer. This finding was limited

to young participants with a severe OSA (AHI>57/h) [215]. The relationship between the systemic inflammation and risk for cancer is still under investigation. For example, TNF-α is discussed not only to prevent but also to induce cancer. This effective role in carcinogenesis is probably based by up-regulation of other proteins via the NK-KB pathway induced proliferation and morphogenesis [216]. It seems therefore acceptable to investigate, if the increased inflammatory activity in OSA patients is also related to an increase in tumor diseases. Recent studies, especially by Isaac Almendros and colleagues increased the acknowledgement , at least in the mouse model, of OSA as a risk factor for cancer [217,218]. While, most models confirmed an influence of hypoxia on the cellular system and carcinogenesis [210,219,220], there is also evidence in animal models, that sleep fragmentation in OSA increases the probability of cancer [221]. This could be an explanation that other sleep or circadian rhythm disturbance than OSA, e.g., insomnia and shift work are associated with an increased cancer risk [211,212]. Interestingly, a recent meta-analysis demonstrated an increased risk for cancer in very short or very long sleepers [222]. Gozal and colleagues summarized recently the already existing evidence and hypothetical constructs for the relationship between OSA and cancer [223].

1.3 The activation of the sympathetic system common by physical exercise or OSA. A stress model?

One of the common observations in OSA is the increased activity of the sympathetic autonomous nervous system (ANS). Beside results from early microneurography investigation [224] OSA patients also demonstrate increased levels of catecholamines in the peripheral blood. Both values indicate an increased sympathetic ANS activity. Several studies indicate that this increase in the sympathetic activity is normalized to CPAP therapy, although are also contradictory results [225-229]. It is of interest, that increased sympathetic ANS has been also observed in other sleep disturbances including sleep restriction [230], insomnia [132,231], or circadian misalignment [232]. This evidence is against the hypothesis that increased ANS is only related to hypoxic events in OSA [233-235].

Aside from the the well-known impact of increased sympathetic ANS activity on cardiovascular diseases [236,237], this increased activity also influences the cytotoxic

immune system. Already early studies reported an impact of stress (psychological or physical exercise) on circulating immune cells. Schedlowski and colleagues described in 1993, that injection of low doses of adrenalin or noradrenalin increases the $CD16^+$ or $CD56^+$ NK cells and their cytolytic activity in the peripheral blood [238]. Exhausting physical exercise increases by shear stress and/or catecholamine dependent β-2 adrenergic activation all relevant leucocyte groups [239]. Following the exercise, the number and activity of peripheral blood leucocytes decreases with possible clinically relevant lymphopenia of < $1{,}0x10^+$ lymphocytes/ml. This decrease is visible in most lymphocyte populations including NK lymphocytes, $CD3^+\gamma\delta$ lymphocytes or $CD8^+$ cells [240]. Especially the reduced number of NK cells and NK cell activity post exercise have been associated with an increase in of upper airway infections [241,242].

The direct link between exhausting exercise and obstructive sleep apnea appears arbitrary only on the first glance. Aside from the common increase in the sympathetic ANS activity an abrupt change in the stress induced immune condition exists in both. In the case of the athletes, it is the recovery phase after the physical activity. OSA on the other hand can be effectively treated by one night of positive airway pressure therapy and thus significantly decreases the activity of the sympathetic ANS within a few hours. As stated above the relationship between OSA and the increased mortality is not yet completely understood and may be multifactorial. However, the interaction of stress with the immune system plays an important part in the acute coronary disease and may be therefore the most important connection between OSA and CVD related mortality [243].

It is of interest that the relationship between exercise induced immune response/upper airway infection demonstrates a J-curve pattern that is also observed in the correlation between sleep duration and cardiovascular mortality or cancer (figure 4). One could speculate that insufficient or an excess of stress induced by the two important factors of human homeostasis -sleep and physical exercise- compromises the immune system and results in a reduction of general health if not complied in optimal form.

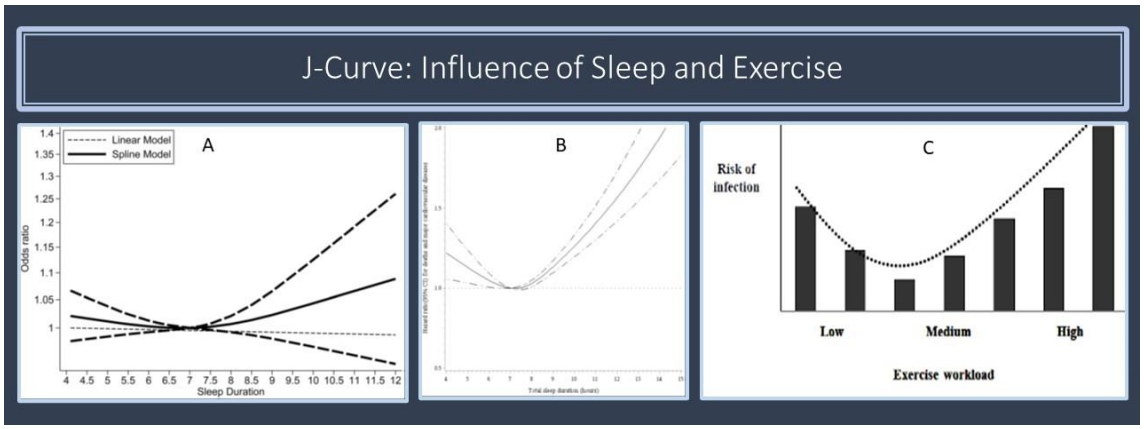


Figure 4: indicating the J-curve like relationship between too little and too much of sleep (A and B) or exercise (C). A describes the increase of cancer in either short and more clear long sleepers [222], B depictures the relationship between sleep restriction/insomnia or excessive sleep and the risk of cardiovascular events [122] and C demonstrates that either sedentary lifestyle or exhaustive physical exercise can increase the risk of upper airway infections [242].

1.4 Geographical and environmental impact on obstructive sleep apnea

In recent years, the increasing number of sleep research results published by either South American or Asian groups opened the discussion regarding a long-neglected problem. Most of the early fundamental works on OSA especially the epidemiological studies, were based on locally well-defined cohorts in either Europe or United States of America including among others the Wisconsin Sleep Cohort Study, the Spanish Cohort and the Sleep Health Heart Study. Although the methods applied were not homogeneous the geographical impact on OSA prevalence and symptoms between the study populations was rather small [244]. Since most cohorts included mainly Caucasian patients this was not surprising, but more recent publications demonstrate study results that indicate the prevalence of OSA in the Chinese population by far higher than expected by the usual anthropometric values, e.g., the BMI [245-247]. Although the impact of obesity on the incidence and prevalence of OSA is not questioned, there is no clear cut-off for all ethnicities and other structural and/or neurological factors must be integrated to explain the impact on OSA prevalence and severity [248,249]

Another factor that influences the universal usage of data obtained from ethnically homogeneous cohorts is the possibility of cultural influences. Alcohol consumption may serve as an easy example. Alcohol consumption is prevalent in the cohorts from Europe,

EUA, Spain and Australia but rare in countries with a predominantly Muslim study population. Even moderate alcohol intake will increase the AHI and influence cardiac parameters [250]. In a sleep laboratory-based study like the WSCS alcohol consumption during PSG recording might be excluded, however, the effect of chronic alcohol abuse with insomnia symptoms is not. Most of the other epidemiologic studies use home sleep recording devices without objective control of alcohol intake before the sleep study. There are several other cultural differences that possibly influence sleep and sleep disordered breathing, e.g., alimentation, physical activity during the day and before bedtime, napping, use of TV and other blue light emitting devices and sleep disruption by religious ceremonies. It is interesting, that although OSA has been recognized in most countries as a health problem there is almost no systematic investigation regarding these influencers. The existing data consists mainly of sleep time and quality but not of sleep disordered breathing [251]. Mindell and colleagues investigated cross cultural sleep patterns by an internet-based questionnaire. The results demonstrated relevant differences between the Asian and Caucasian countries [252,253]. As a possible bias in this study it is important to mention that in this population-based study probably not all possible participants would have had internet access. The European Sleep Apnea Database is within Europe if not worldwide, the cohort with the highest number of OSA patients included. While in ethnical terms it is quite homogeneous since most included participants are Caucasian, there are notable geographical and cultural differences between the participating European countries.

Beside the possible impact of either cultural or ethnical aspects recent studies identify another variable which might influence the presence of sleep disturbances in general and sleep disordered breathing in special. The World Health Organization stated in 2018 that “Climate change is the biggest challenge of the 21th century [254]. Most scientific societies accept the existence of a global warming with rising temperature in both land and ocean (figure 5).

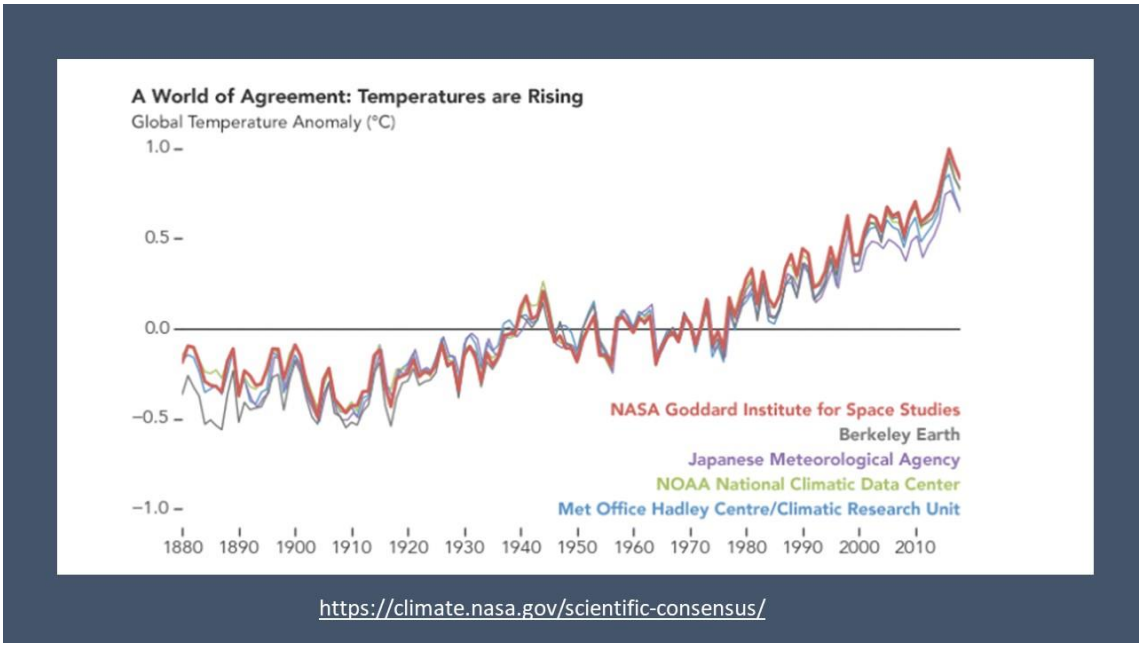


Figure 5: Concordance of increasing temperature of 5 meteorological data bases.

The influence of temperature rise on human health in general and on sleep and sleep disturbances in special is still under investigation. There is a relevant interaction between the human thermoregulatory system and sleep based on core body temperature as well as the proximal and distal skin temperature [255]. In the evening there is a reduction in heart rate and an increase in the peripheral skin temperature leading to a heat loss and ultimately to a reduction in the core body temperature. The proximal skin temperature mirrors at this time the core body temperature resulting in a reduction of the usual diurnal gradient between the peripheral and proximal skin temperature [256]. This process is, in general, associated with an increase in melatonin level and subjective sleepiness [257]. After "lights off" both proximal and peripheral skin temperature increase and core body temperature decreases about 0,3° C [258]. The relationship between temperature and sleep has been intensively investigated, mainly to achieve an improvement of sleep quality. Although the results are conflicting, the evidence indicates that an increase in the core body temperature before bedtime, e.g., with a hot bath might decrease sleep latency and enhance slow wave sleep duration, while constant warm temperature (ambient or electric heat blanket) reduces total sleep time and REM sleep [259,260]. It is of importance that the beginning of an increase in the peripheral skin temperature and decreases in the core temperature occurs in human

hours before sleep onset. Therefore, modern control of bedroom ambience might not be enough to counteract a possible increase in the outdoor temperature. Many studies investigating the relationship between temperature and sleep are under-powered, but in general the results are in favour for a decrease in sleep time and quality in association with higher temperatures [261-263]. Thermoregulation studies involving sleep medicine are already difficult to realise due to the influence of the circadian rhythm [264,265]. Analysing the impact of climate changes on human sleep and sleep disordered breathing is even more complicated since temperature and pollution are influencing each other [266,267]. Authors from the sleep health heart study were probably the first to publish a possible relationship between air pollution by particulate matter with a diameter < 10 μm PM_{10} and OSA [268]. We conducted a pilot study with a small but very well controlled cohort and found also an association between elevated temperature, air pollutants and the severity of OSA [269]. At present results from two very recent epidemiological studies are also indicating a relationship between air pollution and/or temperature and OSA [270,271].

Although we know already for a long time that there exists a complex interaction of the environment and pulmonary health, it is only in the last years that the amount of published investigations have increased substantially. In a recent publication, it was considered that the reduction in the particular matter 2,5 might reduce up to 33 percent of the bronchial asthma in childhood [272]. This suggests that in future investigations of sleep apnea one should not only consider traditionally known confounding factors like age, BMI or neck circumference but also existing environmental data.

2 Aims

The obstructive sleep apnea (OSA) has been intensively investigated with regard to its impact on morbidity and mortality in humans. The contributors to a negative impact of OSA on human health include the increase of stress due to either repetitive hypoxias, sleep fragmentation or both. Investigations regarding the direct e.g. arterial hypertension or indirect e.g. alteration of the immune and/or metabolic homeostasis effect of OSA are continuing. At present it is not clear if and to what extent sleep related breathing disorders are influenced by environmental factors including cultural, temperature or air pollution.

2.1 General objectives of the thesis:

1) Contribute to a greater knowledge about the repercussions of OSA on the immune, metabolic, and cardiovascular system
2) Investigate whether interactions between OSA and the immune and cardiovascular systems may be generalized or, otherwise, are related to regional/local environmental factors.

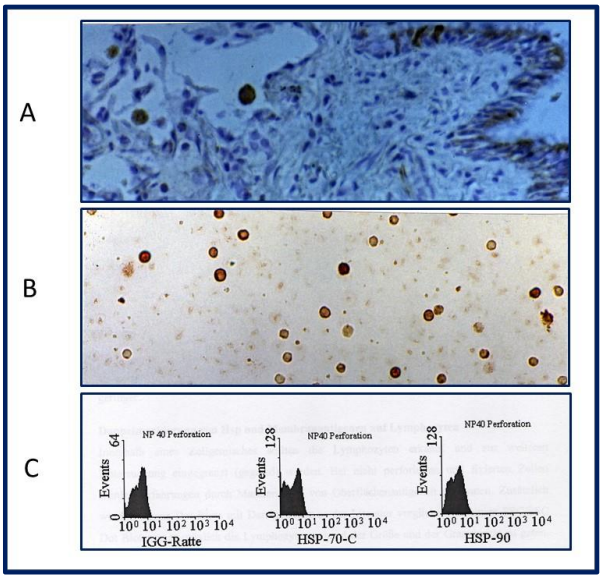
2.2 Specific objectives of the thesis

1) Investigate, if stress induced by exhaustive endurance exercise influence the perforin (Pfr) and granzyme-B (GrB) based cytotoxic immune system and if so, can it be used as a stress model for OSA patients (Study 1).
2) Analyse, if OSA with either hypoxic or non-hypoxic respiratory events have an impact on the Pfr and GrB positive lymphocytes and variables on the metabolic system (study 2).
3) Examine, if OSA changes the evolution of the following hemodynamic parameters: heart rate (HR), systolic blood pressure (SBP) and stroke volume (SV) at sleep onset (study 3).
4) Evaluate, if the impact of OSA on the metabolic system is a general condition or if there are differences within European regions (study 4-6).
5) Assess, if temperature or climate zones represent environmental factors that can influence OSA (study 7).

3 Methods:

3.1 Study 1: Change of perforin positive peripheral blood lymphocytes subpopulations following exercise

We analysed the presence of the cytotoxic protein perforin (P^+) and Granzyme-B (GrB^+) in the peripheral blood lymphocytes of 12 healthy endurance athletes. Prior to this study, the methods of intracellular protein measurement has been established at the laboratory of the department of Pneumology, University Hospital of Freiburg [273]. As a validation of the method we used the investigation of heat-shock proteins depicted in figure 7. The cell membrane perforation was later performed with a 0,1 % saponin solution instead of the initially applied NP-40 detergent.



Figures 7: Assessment of intracellular protein analysis. A: Lung biopsies were stained with anti Hsp90 antibodies to detect the pattern of the heat-shock protein 90 (Hsp90) expression in mononuclear cells. B) Peripheral blood cells were perforated and further on stained with anti-Hsp90 antibodies and either analysed by direct microscopy or C: via flow-cytometry [273].

Exhaustive physical exercise is a model with short term increase in the sympathic autonomous nervous system (ANS) activity followed by a rapid reduction of stress variables after the exercise. We investigated the presence of Pfr^+ peripheral blood lymphocytes in 12 endurance trained athletes before and after a competitional triathlon sprint (400 m swimming, 25 km bicycling and 4 km running). The control group included 10 sedentary hospital employees without any known acute or chronic disease. Blood

was drawn from a peripheral vein for further investigation at 168 and 24 hours before and 1, 18 and 48 hours after the sprint marathon. Beside regular routine blood analysis, peripheral blood mononuclear cells (PBMC) were isolated via a Ficoll gradient. Furthermore, cells were incubated and strained with phycoerythricin (PE) conjugated anti-CD3, anti-CD4, anti-CD8 and anti CD16/CD56 antibodies. Following two steps of washing with PBS, 1%FCS, 0,1%NaN3 the PMNCs were fixed with 4% paraformaldehyde for 15 minutes and after two washings permeabilized with 0,1% saponin. As final step the PMNCs were incubated with fluorescein isothiocyanate (FITC) labelled anti-perforin antibody. After gating the lymphocyte population due do diameter and granula via standard flowcytometric analysis, the percentage of perforin positive cells within each lymphocyte subpopulation was investigated.

3.2 Study 2: Decrease of perforin positive CD3+γδ-T cells in patients with obstructive sleep disordered breathing

For this study 87 subjects were investigated. Participants were consecutively recruited from patients admitted to the sleep laboratory and also included members of the hospital staff or their family. The group was divided in 24 controls, 19 patients with an upper airway resistance syndrome defined by a normal apnea/hypopnea index (AHI) and oxygen desaturation index (ODI) but with a significant sleep fragmentation due to the respiratory effort and therefore with an increased respiratory disturbance index (RDI). There were two different groups of patients with OSA included (AHI >5/h) 1: a lean group with a body mass index (BMI) < 30 kg/m^2 and 2: an obese OSA group with the BMI ≥ 30 kg/m^2. In a nonpublished pilot study, we could demonstrate that the presence and therapy of OSA resulted in changes within the cytotoxic immune system. However, the magnitude of the therapy effect was variable and not only dependent on the traditionally used parameter AHI. (Staats R et al. Poster communication European Respiratory Society Congress 2001; published in the European Respiratory Journal). In the actual study every patient underwent routine blood analysis for the investigation of unknown diseases and evaluation of cardiovascular risk factors like diabetes mellitus or dyslipidaemia. The principal immune-biological methods in this study were derived from study 2. However, some important modifications were implemented in this investigation. By using double surface staining with PE or PE-Cy5 labelled membrane antigens we were able to further distinguish the lymphocyte subsets: CD3+CD4+, CD3+CD8+, CD3-CD8+, CD3+-γδ TCR+ (γδ-T cells), CD3+CD16+/CD56+ (natural killer T cells (NKT) and CD3-CD16+/CD56+ (NK cells). Similar to study 2 the FITC labelling permitted the additional analysis of the intracellular located cytotoxic proteins perforin and this time additionally granzyme-B.

Additionally, in this study human peripheral blood γδ-T cells were isolated and stimulated by hypoxic stress. For this blood from buffy coat units (50–70 mL) were obtained from healthy volunteers and centrifuged in Ficoll-Paque. PMNCs containing interface was collected and washed in PBS. The γδ-T lymphocytes were then incubated with conjugated anti-TCR-γδ monoclonal antibody and labelled with FITC conjugated

monoclonal antibody coupled to magnetic microbeads. The cell suspension was loaded onto a LS magnetic column to aim the selection of γδ-T lymphocytes. Thus, the γδ-T lymphocytes could be subsequently collected from the columns resuspended in serum-free culture medium and supplemented with 5% fetal bovine serum and 2 mM L-glutamine and 70 ng/mL of interleukin-2. This isolated human γδ-T cells population was plated in 96-well plates and incubated at 37 °C, 5% CO2 and 19% O2 (normal condition), in Heracell™ 150i CO2 Incubator or incubated at 37 °C, 5% CO2 and 5% O2 (hypoxic condition), in New Brunswick Scientific Galaxy 14S CO2 Incubator, for 24 h prior to cytokine production analysis and cell surface staining by FACS.

3.3 Study 3: The importance of sleep fragmentation on the hemodynamic dipping in obstructive sleep apnea

In this study, we included a total of 60 participants. The participants included in this study were recruited from patients admitted to the sleep laboratory for to diagnose sleep-related breathing disorders. All performed a complete polysomnography (PSG) with additional beat-to-beat recording of the following hemodynamic parameters by the Nexfin HD device.

Heart Rate (HR)

Systolic blood pressure (SBP)

Stroke volume (SV)

We analysed a period of 25 minutes including 5 minutes before sleep onset (defined as 3 stages of sleep N1 or N2) and 20 minutes after sleep onset. The hemodynamic data recorded by the Nexfin device was exported into an SPSS file for subsequent statistical analysis. Every consecutively recorded pulse wave was numbered and plotted against the corresponding hemodynamic value. Aside from calculating the averages and standard variation, this method allowed the calculation of progression/correlation between anthropometric and polysomnographic parameters and the existence of obstructive sleep apneas and values (see figure 6)

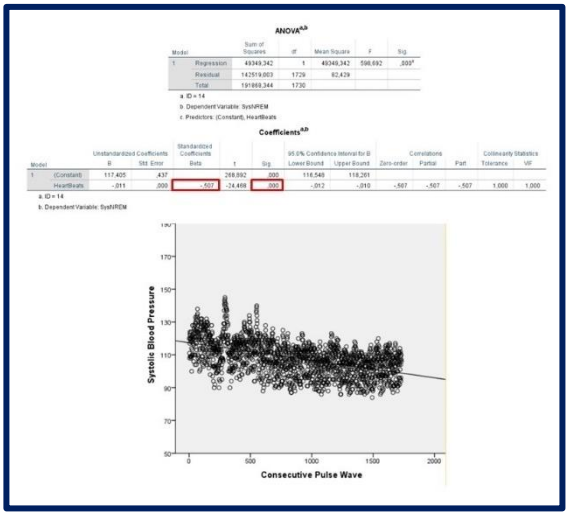


Figure 6: Analysis of the systolic blood pressure evolution at sleep onset. The systolic blood pressure measured at each consecutive pulse wave is demonstrated as a dot-blot graphic. The regression line shows a decline indicating a reduction of the systolic blood pressure at sleep onset. The statistical analysis confirms the observation with a negative standardized coefficient β (SCB). Both model and coefficient reached statistical significance ($p<0.001$).

3.4 Study 4: Obstructive sleep apnoea independently predicts lipid levels: Data from the European Sleep Apnea Database

This subprotocol study of ESADA was conducted by a group of authors actively participating in this project as a so called “writing group” on behalf of all the other ESADA members. For this study, we included a total of 8592 patients without previous diagnosis of hyperlipidaemia or lipid-lowering drugs. The quartiles for the AHI and ODI were computed to generate OSA severity classes with the lowest quartile serving as the control group. Beside total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL) and triglycerides (TG) were included in the analysis. Baseline characteristics across the quartiles were compared using analysis of variance (ANOVA) with post hoc Bonferroni analysis, Kruskal-Wallis and Mann-Whitney U-tests and chi-square tests for parametric, nonparametric and categorical variables, respectively. To further investigate the relationship between the severity of OSA and the lipid levels a factorial analysis of covariance (ANCOVA) was run do produce adjusted mean lipid values for each AHI and ODI quartile. Also, generalized linear regression models (GLM) were created using the variables that achieved statistical significance in the univariate analysis. Adjustments for age, sex, BMI, waist/hip circumference ratio, comorbidities (hypertension, ischemic heart disease, stroke/transient ischemic attack, diabetes) and study site location were done. The last adjustment is based on the fact, that the ESADA cohort contains a high diversity in both geological and social-economically terms. Therefore, participating centres were grouped in either North, South, West, East and Central. The northern centres were considered the reference group when compared to the other four groups. A logistic regression analysis was run to obtain adjusted odds ratios (OR) for TC levels ≥ 200 mg/dL, LDL ≥ 100 mg/dL HDL ≤ 40 mg/dL and TG levels ≥ 150 mg/dL in accordance with the severity quartiles of sleep disordered breathing and for nocturnal hypoxemia. Statistical analysis was accomplished by using the SPSS statistics software version 22.0.

3.5 Study 5: Hyperlipidaemia Prevalence and Cholesterol Control in Obstructive Sleep Apnea: Data from the European Sleep Apnea Database (ESADA)

This study can be considered a further development of study 4. A total 11892 patients were included. Within the ESADA group exist different protocols for the diagnosis of sleep disturbances. Therefore 5996 study participants (50,4 %) were diagnosed via polysomnography (PSG) recording, while the remaining 5896 participants underwent cardio-respiratory polygraphic home monitoring (CR-PG). The ODI was therefore considered the best independent variable to explain possible changes within the lipid metabolism. ODI quartiles were built for each analysis separately. In line with study 4 the baseline anthropometric and sleep data were compared using ANOVA with post hoc Bonferroni analysis, Kruskal-Wallis, Mann-Whitney U-tests and Chi-squared tests for parametric, nonparametric and categorical variables, respectively. A Factorial ANCOVA was run to generate adjusted mean lipid value for each ODI class. After Bonferroni´s post-hoc correction the adjusted means were compared. Additionally, independent predictors and odds ratio for hyperlipemia diagnosis were investigated by GLM with adjustments for age, sex, BMI, waist/hip ratio, comorbidities and the ESADA study region.

3.6 Study 6: Insomnia symptoms combined with nocturnal hypoxia associate with cardiovascular comorbidity in the European Sleep apnea cohort (ESADA)

In this study, we investigated the association of daytime symptoms, sleep study results and the prevalence of cardiovascular diseases in the ESADA cohort.

A total of 17325 patients were classified into four distinct groups according to the prevalence of insomnia at night and excessive daytime sleepiness (EDS). EDS was defined on an Epworth sleepiness scale ≥ 10 points. Insomnia was defined if one or more of the following points were documented: reduced sleep time of < 6 hours sleep; increased sleep latency > 30 minutes; pre-diagnosed insomnia or the use of hypnotics.

Group 1: EDS + no insomnia

Group 2: EDS+ and insomnia +

Group 3: No EDS no insomnia

Group 4: No EDS insomnia +

Furthermore, we divided the total cohort regarding the geographical localization of the participating sleep centre in North (n=4887), West (n=1735), Central (n=2806), East (n=3785) and South (n=4112) (figure 8). To exclude a bias by operating procedures, a small survey was incorporated in the study investigating the local politics of the sleep centres regarding mandatory screening of sleepiness before referral, categorical reasons for denying CPAP treatment or referrals to sleepy studies and other issues which might have an impact on referral patterns.

Comparisons among the groups or phenotypes were performed using ANOVA for the continuous variables, or the chi-square tests for categorical variables. A logistic regression model was run to analyse the impact of anthropometric data, sleep study results, smoking habits, or geographical region on the prevalence of cardiovascular diseases.

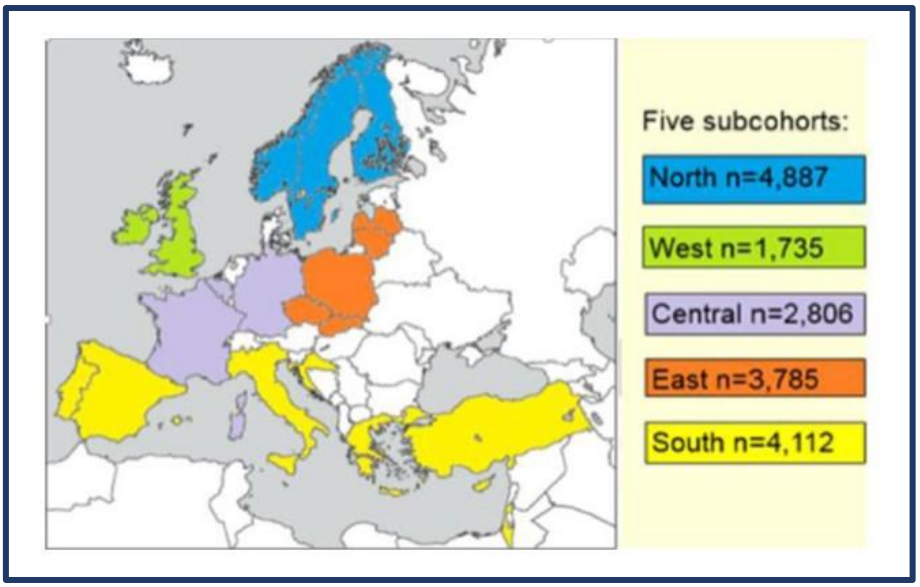


Figure 8. Geographical distribution of the ESADA cohort within Europe

3.7 Study 7: Impact of temperature on obstructive sleep apnea in three different climate zones of Europe. A study of the European Sleep Apnea Database (ESADA)

This study contains data of a manuscript in final preparation for submission. Recent publications indicate a possible impact of environmental factors including pollution and, with less evidence, also environmental temperature on sleep and SRBD. For this purpose, we investigated the impact of temperature and climate zones on the prevalence of OSA.

In this study, the following respiratory parameters during sleep were included: after transformation to the natural logarithm (LN): apnea/hypopnea index (LNAHI), oxygen desaturation index (LNODI), the percentage of study time with a peripheral oxygenation saturation < 90 % (LNT90) and without log transformation: the minimum oxygen saturation (min).

Patient inclusion for this study consisted of a time period between 2007 to 2017. To evaluate environmental variables which could possibly influence the respiratory sleep parameters, we divided the cohort according to the Köppen-Geiger Climate classification. Therefore, the participating sleep centres were grouped according to the main climate, precipitation and temperature (http://koeppen-geiger.vu-wien.ac.at/).

Further investigation was limited to the three climate zones with enough participants to allow a statistical analysis of the seasonal effect.

Cfb: Warm temperature, fully humid, warm summer.
Included study sites: Antwerp, Berlin, Brno, Dublin, Edinburgh, Giessen, Gothenburg, Grenoble, Hamburg, Paris, Prague, Warsaw and Førde(n= 12516)
Csa: Warm temperature, summer dry, hot summer.
Included sites: Alexandropoulis, Athens, Cãceres, Izmir, Lisbon, Palermo and Split (n=4338)
Dfb: Snow, fully humid, warm summer.
Included sites: Klaipeda, Kosice, Turku (n=2439).

3.7.1 Temperature data

For each month of the year the average, minimum and maximum temperature was extracted by the World Bank climate databank (World Bank Group Climate Change Knowledge Portal (CCKP): (URL: http://climateknowledgeportal.worldbank.org/).

For the assessment of a possible impact of temperature and climate zones on the respiratory sleep a categorical variable was developed based on the distribution of 3 intervals measured in Celsius. Low temperature was defined as values up to 5ºC, moderate as temperatures between >5ºC to 15ºC and warm as temperatures > 15ºC. Following this procedure, we computed a linear regression model with the 3 temperature intervals controlling for age, BMI, gender and presence of air conditioner (AC). The same regression analysis was repeated with temperature as a linear variable. Mean temperature and maximum temperature were very related with a r=0.975. For the further analysis we used the maximum temperature as the independent variable.

A hierarchical block regression analysis was run to investigate if temperature remains of significance to explain the variation of AHI when known anthropometric risk factors of OSA like BMI, age and gender are included in the equitation. Since, temperature during the sleep period is often regulated by indoor climate control via air conditioner (A/C) an additional regression analysis included the presence of A/C as indicated by the sleep centres. Thus, the total model included maximum temperature (block I), age, BMI and gender (block II) and further on the presence of air conditioner (Block III).

Descriptive statistics and hypothesis testing were performed using the Statistical Package for Social Science (SPSS) version 24 (SPSS Inc., Chicago, IL, USA) software. A significance level of alpha 5% was used to determine statistical significance.

4 Results

4.1 Study 1

Change of perforin positive peripheral blood lymphocytes subpopulations following exercise

R. Staats, S. Balkow, S. Sorichter, H. Northoff, H. Matthys, W. Luttmann, A. Berg, J. C. Virchow, Clin Exp Immunol; 2000, 120: 434-439

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The author of this manuscript was responsible for the study design, analysis of the data including statistical analysis and main author of the publication.

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Change in perforin-positive peripheral blood lymphocyte (PBL) subpopulations following exercise

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SUMMARY

Perforin, one of the cytotoxic proteins of the immune system, plays a prominent role in protection against viral and bacterial infections. We investigated its expression in PBL and their $CD3^+$, $CD4^+$, $CD8^+$ and $CD16^+$ and/or $CD56^+$ subpopulations in endurance athletes before and after a triathlon. Lymphocyte subpopulations were analysed by flow cytometry following separation of peripheral blood mononuclear cells and staining with antibodies against specific membrane antigens and intracellular perforin. The number of total lymphocytes decreased from $2{\cdot}1 \times 10^3/\mu l$ before the triathlon to $1{\cdot}0 \times 10^3/\mu l$ 1 h after the triathlon ($P < 0{\cdot}01$). Interestingly, there was already a significant spontaneous decline in the percentage of $CD3^+$/$perforin^+$, and in $CD8^+$/$perforin^+$ cells, in the week proceeding the triathlon, when subjects were instructed to refrain from strenuous exercise training. The percentage of $CD3^+$/$perforin^+$, $CD8^+$/$perforin^+$ and $CD16^+$ and/or $CD56^+$/$perforin^+$ cells in each lymphocyte subpopulation decreased 1 h after exercise even further from 14·3% to 5·8% ($P < 0{\cdot}05$), 18·5% to 6·5% ($P < 0{\cdot}05$) and 77·3% to 67·3%, respectively. However, at 18 h and 48 h after exercise the percentage of perforin-expressing $CD3^+$, $CD8^+$ and $CD16^+/56^+$ cells increased again towards baseline levels. Compared with normal controls, baseline perforin co-expression in $CD3^+$ and $CD8^+$ lymphocytes was significantly higher in trained athletes. From our data we conclude that trained athletes have an increased percentage of $perforin^+$ PBL and that following exercise the percentage of $perforin^+$ and therefore potentially cytotoxic lymphocytes transiently decreases in peripheral blood.

Keywords perforin exercise-induced stress $CD3^+$ $CD8^+$ $CD16^+/56^+$

INTRODUCTION

Moderate physical exercise has been reported to stimulate several functions of the immune system such as increasing antibody-dependent cytotoxic activity, natural killer (NK) cell cytotoxic activity (NKCA) and lymphokine-activated killer cell (LAK) cytotoxic activity [1–3]. Clinically, this has been related to a decrease in the number of upper respiratory tract infections [4,5]. Different effects have been reported following high-intensive training [2,6–8], however, and heavy exercise as well as long duration stress have been proposed as models of temporary immunosuppression [9,10]. In the recovery phase after intensive exercise a decrease in the number as well as in the percentage of PBL, and an increase in peripheral blood neutrophils have been observed [11–13]. In addition, a decrease in NK cell and LAK cytotoxic activity has been reported in previous studies [10,14,15]. These findings have been related to the suppressed immunity of high endurance trained athletes.

Perforin is a 60-kD pore-forming protein which is stored intracellulary and secreted by NK cells, cytotoxic $CD8^+$ T lymphocytes and $\gamma\delta$ cells [16–21]. It plays an important role in lymphocyte cytotoxic activity [22]. Bradly *et al.* demonstrated that cytotoxic activity of naive NK cells is primarily perforin-mediated [23] and LAK cytotoxic activity has also been shown to be markedly decreased in perforin knock-out mice. The aim of our study was to investigate whether exercise-induced changes in lymphocyte subpopulations are associated with changes in the expression of intracellular perforin and might serve to explain the depressed NKCA and LAK observed in athletes [3,24].

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SUBJECTS AND METHODS

Subjects and study design

Twelve endurance trained males (average body mass index (BMI) 22·7 kg/m^2, VO_{2max} 59·4 ml/kg; average age 31·9 years) were investigated before and following exhaustive physical stress

(competitional triathlon sprint: 400 m swimming, 25 km bicycling and 4 km running). One week (168 h) and 24 h prior to the exercise and 1, 18 and 48 h following the triathlon the percentage of perforin$^+$ lymphocyte subsets was measured in peripheral blood. Since the activity level differed considerably between the individuals, training intensity was discontinued for about 1 week following the initial blood sample in order to exclude exercise-induced changes in lymphocyte subpopulations due to recent exercise. All athletes kept a regular training diary in which they documented their physical exercise during the trial.

The sedentary control group consisted of 10 hospital employees (six females, four males) without any known acute or chronic disease. Their average age was 30·4 years. None of the study participants had any signs or symptoms of an acute infection or took any regular medication. Written consent was obtained from each participant for this study in accordance with regulations set by the ethics commission of the medical faculty of the University of Freiburg.

Isolation of peripheral blood mononuclear cells

Venous blood (10 ml) was drawn into plastic syringes containing 0·2% EDTA from volunteers at different time points and separated on a Ficoll gradient with a density of 1·077 *g/l* (Seromed, Berlin, Germany) for 20 min at 1330 ***g***. The band of peripheral blood mononuclear cells (PBMC) at the interface was collected, washed twice and stored at 4°C for further analysis.

Simultaneous measurement of intracellular perforin and cell surface antigens

PBMC (2×10^6) were incubated for 30 min at room temperature with PE-conjugated MoAbs specific for CD3 (clone UCHT1; Dako, Hamburg, Germany), CD4 (clone EDU-2; Cymbus Biotechnology, Chandlers Ford, UK), CD8 (clone DK25; Dako), and a combination of CD16 (clone 3G8; Immunotech, Hamburg, Germany), and CD56 (clone B-A19; Diaclone, Besançon, France). After two washes with PBS/1% fetal calf serum (FCS)/0·1% NaN_3 the cells were fixed in 4% paraformaldehyde in PBS for 15 min on ice. After two washes in PBS/1% FCS/0·1% NaN_3 the cells were permeabilized by using 0·1% saponin (Sigma, Deisenhofen, Germany). Thereafter, cells were incubated with a FITC-labelled anti-perforin antibody (clone δG9; Hölzel, Köln, Germany) for 20 min at room temperature in the dark, washed twice with PBS/0·1% saponin. Finally, the cells were resuspended in PBS and analysed by flow cytometry.

Statistical analysis

Statistical analysis was done using Sigma-Stat, Version 2.03 (SPSS Inc., Chicago, IL) After testing for normal distribution, results are expressed as arithmetic mean ± s.e.m. Differences between the individual time points were analysed using the one way ANOVA for repeated measurements with pairwise Student–Newman–Keuls *post hoc* analysis.

Differences of athletes and controls were analysed using the Student's *t*-test.

In both cases differences with P values $< 0{\cdot}05$ were considered significant.

RESULTS

Total number of lymphocytes and lymphocyte subpopulations

A highly significant decline in the mean number of total lymphocytes from $2{\cdot}1 \times 10^3/\mu l$ to $1{\cdot}0 \times 10^3/\mu l$ was observed between 24 h before the exercise provocation and 1 h post exercise. The mean number of lymphocytes increased again to pre-exercise values at 18 h following the triathlon ($2{\cdot}1 \times 10^3/\mu l$) and remained basically unchanged for the rest of the observation period (Fig. 1). As listed in Table 1, similar changes were observed for CD3$^+$, CD4$^+$, CD8$^+$, and CD16$^+$/CD56$^+$ cells. Also, the percentage of each lymphocyte subset within the total lymphocyte population did not change significantly at any point during the trial (Table 2).

Relative distribution of perforin-expressing lymphocyte subpopulations

Perforin expression was detected in each of the lymphocyte subpopulations analysed (Fig. 2a,b,c) although only 7·6% of the perforin-expressing cells were of the CD4$^+$ phenotype (Table 3). Interestingly, within the lymphocyte subpopulations there was already a significant decrease in the mean percentage of perforin$^+$ cells which co-expressed either CD3$^+$ (36·1–14·3%, $P < 0{\cdot}01$), CD4$^+$ (7·6–3·0%, $P < 0{\cdot}05$) or CD8$^+$ (52·3–16·7%, $P < 0{\cdot}01$) when subjects refrained from physical exercise between the first week and 24 h prior to the exercise challenge, although during this period the absolute lymphocyte numbers remained unchanged. This decline in the percentage of perforin$^+$ cells was more steady than the one observed following the exercise. A statistically significant decrease in perforin expression, however, was not observed among CD16$^+$/56$^+$/perforin$^+$ cells (87·4–77·3%), possibly owing to the fact that complete data sets for these cells were obtained for only seven subjects (Fig. 2, Table 3).

In response to the physical stress a further, statistically significant decline in the mean percentage of lymphocytes was observed which co-expressed perforin with CD3 (14·3–5·8%, $P < 0{\cdot}05$) or CD8 (16·7–6·2%, $P < 0{\cdot}05$). Following the exercise this increased again towards baseline levels within 1 h and 18 h post exercise.

We found no significant change following the physical stress in either the CD4$^+$ (3·0–3·4%) or in the CD16 and/or CD56$^+$ (77·3–67·3%) lymphocyte subgroup.

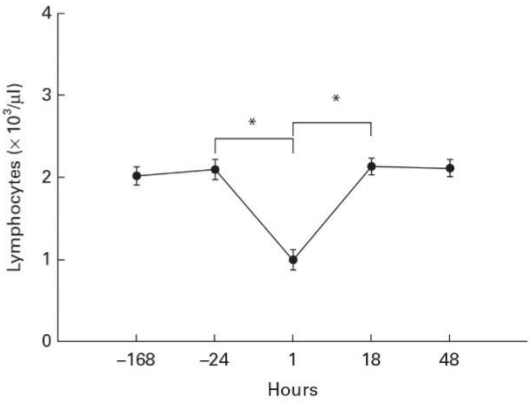

Fig. 1. Total lymphocytes before and after the triathlon. Mean number of PBL ± s.e.m. from participants of the sprint triathlon at: −168 h, −24 h, +1 h, +18 h and +48 h of the trial ($n = 11$). There was a significant decline in the mean number of PBL 1 h after the triathlon (*$P < 0{\cdot}05$), which returned to baseline after 18 h.

Table 1. Total number of lymphocytes and lymphocyte subsets before and after a triathlon (in $10^3/\mu l$)

Hours	Total lymphocytes $n = 11$	$CD3^+$ lymphocytes $n = 11$	$CD4^+$ lymphocytes $n = 11$	$CD8^+$ lymphocytes $n = 11$	$CD16^+/56^+$ lymphocytes $n = 7$
−168	2·0	1·2	0·63	0·51	0·54
−24	2·1 } *	1·4 } *	0·56 } *	0·56 } *	0·66 } *
1	1·0 } *	0·4 } *	0·31 } *	0·11 } *	0·31 } *
18	2·1 } *	1·4	0·54 } *	0·55 } *	0·55 } *
48	2·1	1·3	0·68	0·51	0·58

*$P < 0·05$.

Comparison of perforin expression in lymphocytes of trained and untrained subjects

In order to test our hypothesis that perforin expression in PBL is regulated in an exercise-dependent fashion, we compared our findings in trained athletes with those from an untrained control group. Perforin expression in lymphocytes did not differ between female and male participants in this control group (data not shown).

Co-expression of perforin and CD3 was detected in 36·1% of all $CD3^+$ lymphocytes in athletes during regular training but only in 12·8% of such cells in untrained controls ($P < 0·05$) (Fig. 3). Similar results were obtained for $CD8^+$ lymphocytes, which expressed perforin in 50·1% of the lymphocyte subset from trained athletes, but only in 29·0% of $CD8^+$ lymphocytes from untrained controls ($P < 0·05$). There were no statistically significant differences between the mean percentages of cells expressing $perforin^{+\ and}$ $CD16^+$ and/or $CD56^+$ within the $CD16^+$ and/or $CD56^+$ subgroup (87·4% and 88·7%, respectively) between trained and untrained subjects.

DISCUSSION

Moderate exercise has been reported to positively influence several functions of the immune system, such as an increase in serum immunoglobulin levels, neutrophilia and increased neutrophilic activity [4]. However, there is evidence that, following strenuous exercise, endurance athletes suffer more frequently from infections, mainly of the upper respiratory tract [25–27]. The mechanisms underlying these findings remain at present obscure. Several studies have reported an effect of training and exercise on NK cell cytotoxic activity [28–31] and it has recently been proposed that endurance stress such as running a marathon reduces NK cell activity [29,32]. Since perforin has recently been shown to play a crucial role in NK cell-mediated cytotoxic activity we investigated the effect of exhaustive exercise on the percentage of perforin expression in lymphocyte subsets in endurance athletes prior to and following a triathlon.

In this study we were able to demonstrate that endurance exercise leads to a significant decrease in the total number of lymphocytes and the $CD3^+$, $CD4^+$, $CD8^+$ and $CD16^+/56^+$ lymphocyte subsets following the exercise. These results are consistent with previous findings [30,33–36]. However, in addition we were able to show that this absolute decrease in lymphocytes is accompanied by a further reduction in the percentage of perforin-expressing lymphocytes, suggesting a disproportional decrease in the frequency of $perforin^+/CD3^+$ and $perforin^+/CD8^+$ lymphocytes following exercise, which returned towards baseline within 48 h after exercise.

In addition to these novel findings we could show that not only an acute exercise of physical stress, but also the absence from training was accompanied by a decline in $perforin^+/CD3^+$, $perforin^+/CD8^+$ and $perforin^+/CD16^+/56^+$ lymphocytes, and we provide evidence that perforin expression in lymphocytes of trained endurance athletes is higher compared with normal controls. To our knowledge these findings have not been previously reported in the literature.

Although elevated NK cell activity has previously been reported in trained endurance athletes [3,37], there has been no evidence as to how long this elevated activity remains following cessation of training. Previous studies reported that exhaustive endurance exercise was followed by a diminished NK and LAK cell activity and a higher tendency towards infections of the upper

Table 2. Lymphocyte subsets in percent of the total lymphocyte population

Hours	Percent of $CD3^+$ lymphocytes, $n = 11$	Percent of $CD4^+$ lymphocytes, $n = 11$	Percent of $CD8^+$ lymphocytes, $n = 11$	Percent of $CD16^+/56^+$ lymphocytes, $n = 7$
−168	56·7	33·6	23·5	24·1
−24	64·5	28·5	26·8	28·2
1	57·5	33·8	22·1	28·6
18	66·5	26·9	26·6	24·5
48	59·2	31·9	23·9	25·9

There was no significant change of any subset within the lymphocyte population.

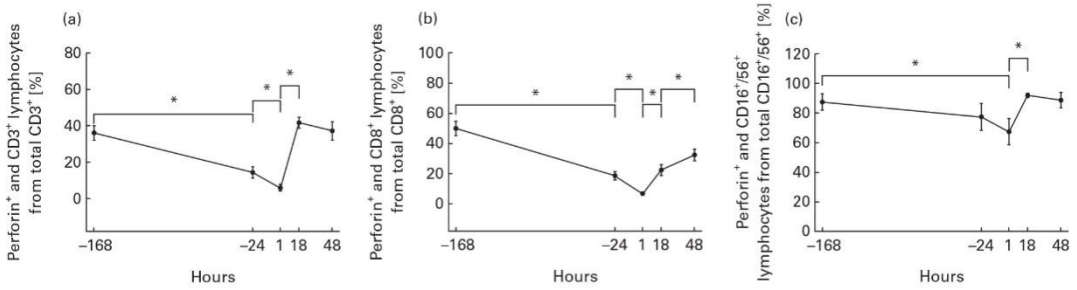

Fig. 2. Percentage of perforin-expressing lymphocytes within lymphocyte subsets. Mean percentage of perforin-expressing PBL subsets ± s.e.m. from participants of the sprint triathlon at: −168 h, −24 h, +1 h, +18 h and +48 h of the trial. (a,b) Mean percentage of perforin$^+$/CD3$^+$ or CD8$^+$ lymphocytes within each subset ± s.e.m. ($n = 11$, $^*P < 0{\cdot}05$). There was a significant decrease in the mean percentage of double-positive lymphocytes after the first week of reduced training, with a further decline 1 h after the physical exercise. Values returned towards baseline levels within 48 h after the triathlon. (c) Mean percentage of perforin$^+$/CD16$^+$ and/or CD56$^+$ lymphocytes ± s.e.m. from the CD16$^+$ and/or CD56$^+$ lymphocyte subset ± s.e.m. during the trial. There was a significant change between the baseline value and 1 h after the exercise ($n = 7$, $^*P < 0{\cdot}05$). Values returned to baseline levels 18 h after the triathlon.

respiratory tract [25,36,38]. Thus, the decrease in perforin$^+$ lymphocytes observed in our study might be causally linked to the decreased NK and LAK activity and the resultant clinical changes observed in these subjects. We also cannot exclude that other factors such as the physical stress induced depression of interferon-gamma (IFN-γ) production [9,39,40] might be involved in the pathogenesis of suppressed immunity of high endurance athletes.

In our study the percentage of NK cells of all lymphocytes was higher than in some other studies [6,36,41,42], possibly due to the fact that in some studies NK cells were detected with specific antibody against either CD16 or CD56 antigens. Thus, our method of combining CD16$^+$ and CD56$^+$ to detect the total NK cell population as CD16$^+$ and/or CD56$^+$ as previously defined [43] might account for these differences.

Similar to another study which found perforin expression in >90% of all NK cells [44], 87·4% of our NK cells expressed perforin. Previous studies which have analysed the effects of training and exercise on NK cell cytotoxic activity have resorted measuring the lysis of target cells by PBMC [28–31]. Although our findings of changes in perforin expression in lymphocytes following exercise do not necessarily reflect cytotoxic activity, our data suggest that cytotoxic activity in PBMC might not be restricted to NK cells but could also be mediated by cytotoxic CD8$^+$ lymphocytes.

It appears unlikely that there is a substantial contribution of CD4$^+$ lymphocytes to perforin-mediated cytotoxic activity. In accordance with previous studies [16,21,45], perforin expression in CD4$^+$ lymphocytes was scarce, while over 50% of all CD8$^+$ lymphocytes expressed perforin. Similar results have been reported elsewhere [44].

Although our findings of a decline in the percentage of perforin expression in PBL is intriguing, it still remains unclear whether there is a causal connection to the clinically observed increased risk of upper respiratory tract infections in athletes. Furthermore, we can only speculate as to whether the observed changes in perforin-containing lymphocytes are due to either a selective migration from the blood or a loss in perforin-containing granula. The reduction in the total number of lymphocytes in peripheral blood, which is accompanied by a further disproportionate decrease in the percentage of perforin$^+$ cells, might be due to a selective sequestration of cells outside of the peripheral circulation.

In conclusion, in this study we report an increase in the percentage of CD3$^+$, CD8$^+$ cells expressing perforin in trained endurance athletes which was significantly elevated compared with normal, untrained controls. Refraining from physical training leads to a significant reduction in perforin co-expression in CD3$^+$, CD4$^+$ and CD8$^+$ lymphocytes. Unlike the decline following the triathlon, this change seems to be more steady. However, as we did not analyse blood samples between 168 h and 24 h before the triathlon we are unable to present clear evidence concerning the rate. In CD3$^+$ and CD8$^+$ cells of endurance athletes the reduction is further enhanced by exhaustive endurance competition. The values return towards baseline levels within 48 h after completion of such an exercise episode. Our findings might be related to the clinically observed abnormal immune responses of high endurance athletes. Additionally, it might contribute to the general understanding about the role of perforin in health and in disease, e.g. asthma (Arnold *et al.* Am J Resp Crit Care Med, 2000; **161**: 182–6).

Table 3. Perforin$^+$/CD4 lymphocytes as percentage of the gated CD4$^+$ lymphocyte subset

Hours	Percent CD4$^+$P$^+$/CD4$^+$ $n = 11$	
−168	7·6	*
−24	3·0	*
1	3·4	*
18	6·6	*
48	6·0	

$^*P < 0{\cdot}05$.

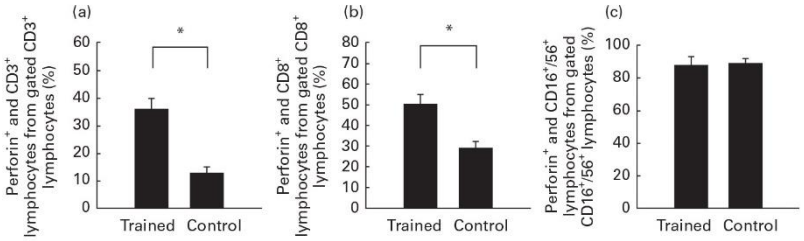

Fig. 3. Comparison of trained athletes and untrained controls. (a) Mean percentage of perforin$^+$/CD3$^+$ lymphocytes ± s.e.m. from the gated CD3$^+$ lymphocyte subset in trained endurance athletes ($n = 11$) and normal, untrained controls ($n = 10$). There was a significant difference between the two groups (*$P < 0{\cdot}05$). (b) Mean percentage of perforin$^+$/CD8$^+$ lymphocytes ± s.e.m. from the CD8$^+$ lymphocyte subset in trained endurance athletes and normal, untrained controls. There was a significant difference between the two groups (*$P < 0{\cdot}05$). (c) Mean percentage of perforin$^+$/CD16$^+$ and/or CD56$^+$ lymphocytes ± s.e.m. from the CD16$^+$ and/or CD56$^+$ lymphocyte subset in trained endurance athletes and normal, untrained controls. There was no statistically significant difference between athletes and the control group.

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4.1.1 Unpublished results investigating the impact of physical exercise on cytotoxic proteins

In a consecutive study we investigated in 11 elite athletes the impact of a I hour run under constant workload.

Methods:

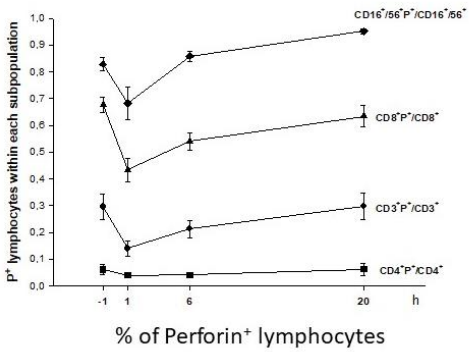
Blood samples were drawn from a cubital vein at -1 hour, + 1; 6 and 20 hours after the exercise. Lymphocytes were isolated and stained according the previously described protocol [274].

Results:

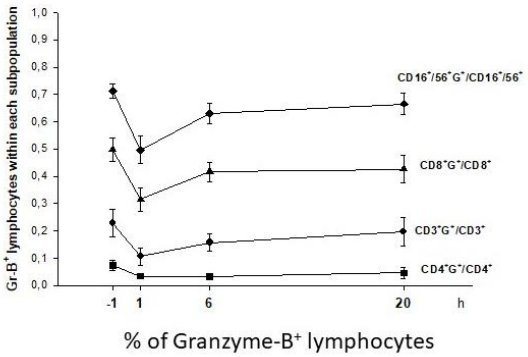
Likewise, to the first study we observed a significant reduction in the percentage of both perforin and granzyme-B positive lymphocytes. Additionally, we detected in this study a significant reduction of the cytotoxic proteins in natural killer cells (NK cells) see table 2.

Lymphocyte subpopulation	**Percentage of change in P^+ cells**	**Percentage of change in GrB^+ cells**
$CD3^+$	-52.5	-56.8
$CD4^+$	-35.4	-53.4
$CD8^+$	-36.8	-36.6
$CD16^+/56^+$	-17.5	-30.3

Table 2: Change of perforin (P+) and granzyme positive lymphocytes following a 60 run under constant workload. All results were significantly positive ($p<0.05$).



% of Perforin$^+$ lymphocytes



% of Granzyme-B$^+$ lymphocytes

Figure 9: Change of perforin and granzyme-B positive lymphocyte subpopulations following a 60 minutes run under constant workload. The percentage of perforin positive cells decreased significantly when directly analysed after the run ($p<0.05$) and returned after 6 hours to the pre-run values.

Conclusion:

This study confirms the previous observation that exhaustive physical exercise reduces the cytotoxic lymphocytes in the investigated cell subsets. In NK cells this effect was more visible in the granzyme positive cell subset.

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4.2 Study 2

Decrease of perforin positive CD3+γδ-T Cells in patients with obstructive sleep disordered breathing

Staats R, Rodrigues R, Barros A, Bacelar-Nicolau L, Aguiar M, Fernandes D, Moreira S, Simões A, Silva-Santos B, Rodrigues JV, Barbara C, de Almeida AB, Moita LF, Sleep Breath. 2018 Mar;22(1):211-221

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The author of this manuscript was responsible for the study design, main analysis of the data and main author of the publication.

Sleep and Breathing
https://doi.org/10.1007/s11325-017-1602-6

BASIC SCIENCE • ORIGINAL ARTICLE

Decrease of perforin positive CD3$^+$γδ-T cells in patients with obstructive sleep disordered breathing

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Abstract

Introduction Sleep related breathing disorders (SRBD) cause sleep fragmentation, intermittent hypoxia or a combination of both leading to homeostasis perturbations, including in the immune system. We investigated whether SRBD patients with or without intermittent hypoxia show substantial differences in perforin and granzyme-B positive peripheral blood lymphocytes.
Methods A total of 87 subjects were included and distributed as follows: 24 controls (C), 19 patients with respiratory effort related arousals due to increased upper airway resistance (UAR) without hypoxic events, 24 obese patients with obstructive sleep apnea (OSA) (oOSA), and 20 without obesity (noOSA). After polysomnographic recording, we analyzed in fasting blood samples routine hematologic and biochemical parameters and the percentage of lymphocytes containing the proteins perforin and granzyme-B (GrB). Kruskal-Wallis tests and a posteriori multiple comparisons were applied for statistical analysis of results.
Results Perforin-positive γδ-cells revealed significant differences between groups ($p = 0.017$), especially between the Control group and the oOSA (p-value = 0.04); the remaining SRBD groups also showed differences from the control (C vs UAR: $p = 0.08$; C vs noOSA = 0.09), but they did not raise to statistical significance. There were no differences among the SRBD groups. Granzyme-B cells were decreased in SRBD patients, but the differences were not statistically significant. No additional statistical significant result was found in the other investigated lymphocyte subsets.
Conclusions Obstructive sleep-disordered breathing is associated with a decrease in perforin-positive CD3$^+$γδ-T cells. Although this finding was detected in lean patients without intermittent hypoxia, the reduction was only statistically significant in obese patients with severe OSA. Because CD3$^+$γδ-T cells play an important role in the control of tumor cells, our findings are directly relevant for the study of the association of OSA and cancer.

Keywords Sleep-related breathing disorders · Obstructive sleep apnea · Perforin and granzyme-B-positive peripheral blood lymphocytes · Cancer · Obesity

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11325-017-1602-6) contains supplementary material, which is available to authorized users.

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Introduction

Sleep is a critical adaptive behavior as it is essential for the maintenance of core homeostatic functions of an organism. A large body of evidence demonstrates the importance of sleep not only for various metabolic and inflammatory pathways, but also for humoral and cellular immune functions [1]. Sleep-related breathing disorders (SRBD) in general and the obstructive sleep apnea (OSA) in particular are extremely prevalent in

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the general population, and therefore constitute a public health problem. OSA is linked to increased mortality mainly due to higher prevalence of cardiovascular events [2]. The underlying mechanism is still under investigation but it is likely multifactorial. In addition to promoting a pro-atherogenic pattern in the peripheral blood [3], OSA increases nuclear factor kappa B (NFκB)-dependent endothelial inflammation [4]. Recent research has linked cytotoxic lymphocytes (CTL) to ateriosclerotic plaque instability and thus the risk of acute cardio-vascular events [5]. The main mechanism of cellular cytotoxicity is based on the secretion of the proteins perforin and granzymes into immunological synapse between a cytotoxic lymphocyte and a target cell [6]. Ultimately, the combination of perforin and GrB induces cell death by activating the caspase cascade leading to nuclear fragmentation and apoptosis [7]. While enhanced activity of cytotoxic lymphocytes constitutes an attractive idea to explain the increased cardiovascular risk in OSA, currently, there is little evidence to support a causal link between OSA in humans and an increased number or activity of perforin positive $CD8^+$ lymphocytes [8]. Chronic inflammation is known to raise the risk of carcinogenesis [9]. Interestingly, OSA has also been linked to carcinogenesis, opening new possibilities to account for the increased mortality in OSA patients [10]. Intermittent hypoxia, sleep fragmentation, and increase in adipose tissue are three typical features of OSA patients. Any of them is known to influence inflammatory cascades and the immune system [11]. In this study, we tested the impact of each component of OSA in the immune response by analyzing perforin and GrB positive lymphocytes in non-obese patients with either intermittent hypoxia (OSA) or non-hypoxic sleep fragmentation.

Material and methods

Subjects

A total of 87 participants with an age between 20 and 59 years were included in this study. A total of 24 controls (C) were recruited from patients admitted to the sleep laboratory without detected sleep disorders, healthy members of the hospital employees or their relatives. All showed in the sleep study a respiratory disturbance index (RDI) and an oxygen desaturation index (ODI) < 5/h. Upper airway resistance (UAR) was defined as sleep fragmentation by mainly respiratory effort related arousals (RERAs). Patients of this group demonstrated a RDI > 5 and an ODI and apnea/hypopnea index (AHI) < 5/h. A total of 19 participants fulfilled the UAR criteria. Patients with obstructive sleep apneas (OSA) were defined by an apnea/hypopnea index (AHI) and ODI > 5/h. A total of 20 lean OSA patients with a body mass index (BMI) < 30 kg/m^2 were detected (non-obese OSA or noOSA). As positive control group 24 obese OSA (oOSA) patients were included. Patients were not instructed to remain on a special diet before the sleep study. Eight oOSA patients were on regular cholesterol or diabetes mellitus therapy. Patients on immune system modulating therapies (e.g., recent vaccines or systemic corticoids) were excluded from this study. This project was approved by the Ethics Committee Review Board of Hospital de Santa Maria (Lisboa, Portugal), and all participants signed informed consent forms.

Polysomnographic recordings

Sleep related events were investigated via standard polysomnography (PSG) Alice 5, Koninklijke Philips N.V. Philips Respironics, Murrysville, USA. The following parameters were recorded: F3; F4; C3; C4; O1; O2, M1, and M2. We used the standard referential montage of scalp electrodes against the contra-lateral mastoid electrode (e.g., C3/M2). Further parameters consisted of submental electrodes, strain gauges to record respiratory movements, EMG at both legs according to standard PSG procedures. Peripheral oxygen saturation was analyzed by pulse oximetry. The scoring of sleep and sleep-related events was based on the recommendation of the American Academy of Sleep Medicine published in 2007 [12].

Evaluation of sleepiness

Sleepiness was evaluated using the Stanford Sleepiness Scale (SSS) and the Epworth Sleepiness Scale (ESS). All patients completed the questionnaires in the morning following the polysomnographic recording.

Positive pressure therapy in OSA patients

All patients with a relevant OSA or UAR were invited to receive continuous positive airway (CPAP) therapy. A total of 42 patients (only with OSA) underwent another full polysomnographic recording with a CPAP titration protocol based on the existing recommendations [13].

Blood analysis

Immediately after the diagnostic and therapeutic polysomnographic study fasting peripheral blood samples were obtained from a cubital vein and further analyzed in the laboratory.

Routine analysis

The following hematologic and biochemical parameters were investigated in all participants following the diagnostic PSG night: full peripheral blood cell count, hepatic enzymes (aspartate transaminase (AST), alanine transaminase (ALT) and gamma-glutamyltransferase (gGT)), renal parameters

(creatinine, blood urea nitrogen (BUN)), standard metabolic parameters (uric acid, glucose, total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL), triglycerides (TG), and C-reactive protein (CRP)).

Analysis of the cytotoxic proteins perforin (P) and granzyme-B (GrB)

Peripheral venous blood sample and Ficoll Ethylenediaminetetraacetic acid (EDTA) anti-coagulated blood was collected directly after the end of the sleep study by peripheral venipuncture. Erythrocytes were lysed by adding 10 ml of FACS lysis buffer (BD Biosciences, Heidelberg, Germany). Peripheral blood mononuclear cells (PMNCs) were isolated by standard Ficoll gradient separation (FICOLL-PAQUE PLUS, GE Healthcare Biosciences).

Monoclonal antibodies Anti-human CD3-phycoerythrine (PE) (clone UCHT1), CD4-PE (clone RPA-T4), CD8-PE (clone HIT8), CD16 indotricarbocyanine dye coupled to PE (PE-CY) (clone 4G8), CD56- PE-CY (cloneB159), perforin-fluorescein isothiocyanate (FITC) (clone γG9) and granzyme-B-FITC (clone GB11) were purchased from BD Biosciences, Heidelberg, Germany. Additionally, we used the CD3-PE-CY- (clone HIT3), and TCT-γδ-PE (clone B1) antibodies manufactured by BioLegend, San Diego, USA.

Cell surface and intracellular staining Peripheral mononuclear cells (pMNCs) were diluted to a concentration of 25×10^3 cells/well and supplemented with phosphate-buffered saline (PBS) and 2% fetal calf serum (PBS/FCS buffer). After twice washing with PBS/FCS buffer and centrifuged at 2000 rpm, cells were incubated with 50 μg of a fluorochrome-labeled anti-human antibody solution followed by washing with the PBS/FCS solution. PMNCs were fixed with 2% paraformaldehyde in PBS for 30 min. After washing with PBS/FCS buffer cells centrifugation at 200 rpm, cells were permeabilized with 150 ml saponin 0.1% in PBS buffer for 10 min. Following washing and centrifugation with 2000 rpm / 3 min, antibodies against intracellular and cytotoxic proteins were added in a 25 μl/well saponin 0.1% solution. Cells were washed with the 0.1% saponin solution followed by two washed steps with PBS/FCS puffer. Membrane and intracellular antigen expression on pMNCs were in the following analyzed by flow cytometry (BD Biosciences, Heidelberg, Germany). Using double surface staining with PE or PE-Cy5labeled membrane antigens we were able to investigate the following lymphocyte subsets: $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^-CD8^+$, $CD3^+$-γδ TCR^+ (γδ-T cells), $CD3^+CD16^+/CD56^+$ (natural killer T cells (NKT)) and $CD3^-CD16^+/CD56^+$ (NK cells). FITC labeling permitted the additional analysis of the intracellular cytotoxic proteins perforin and granzyme-B (Fig. 1).

Isolation of human peripheral blood γδ T cells

Blood from Buffy Coat units (50–70 mL) were obtained from healthy volunteers. Blood was centrifuged in Ficoll-Paque (Histopaque-1077; Sigma-Aldrich) for 35 min at 1.500 rpm and room temperature. The interphase containing peripheral blood mononuclear cells (PBMCs) was collected and washed in PBS. The desired TCR $\gamma\delta^+$ T lymphocytes were labeled by incubation with hapten-conjugated anti-TCRγδ monoclonal antibody (Miltenyi Biotec GmbH), according to the manufacturer's instructions. Further, cells were labeled with FITC-conjugated anti-hapten monoclonal antibody coupled to magnetic microbeads. The cell suspension was loaded onto a LS magnetic column (Miltenyi Biotec) and TCR$\gamma\delta^+$ T lymphocytes were positively selected. $\gamma\delta^+$ T lymphocytes were subsequently collected from the columns, following the manufacturer's instructions, and resuspended in serum-free culture medium (OpTmizer-CTS) supplemented with 5% fetal bovine serum and 2 mM L-glutamine (Thermo Fisher Scientific) and 70 ng/mL of interleukin-2.

Hypoxia induction in human peripheral blood γδ T cells

Isolated human γδ T cells were plated in 96-well plates and incubated at 37 °C, 5% CO_2 and 19% O_2 (normal condition), in Heracell™ 150i CO_2 Incubator (Thermo Fischer), or incubated at 37 °C, 5% CO_2 and 5% O_2 (hypoxic condition), in New Brunswick Scientific - Galaxy 14S CO_2 Incubator (Wolf Laboratories), for 24 h prior to cytokine production analysis and cell surface staining by FACS.

Flow cytometry analysis

Cells were stained with the following antibodies from Biolegend: anti-human CD107a (H4A3), anti-human Granzyme B (GB11), and anti-human Perforin (#353312). Antibodies were coupled with APC and Pacific Blue fluorochromes. For intracellular cytokine production analysis, cells were either stimulated with PMA + Ionomycin + Brefeldin A for 4 h at 37 °C or incubated only with Brefeldin A and further stained with ebioscience IC kit according to the manufacturer's instructions. Flow cytometry acquisition was performed on a LSR Fortessa (BD) and data was analyzed with FlowJo X software (Tree Star).

Statistics

Descriptive statistics and hypothesis testing were performed using the Statistical Package for Social Science (SPSS) version 21 (SPSS Inc., Chicago, IL, USA) software, and multiple regression analysis was performed using R, version 3.4.1. (R Development Core Team, 2008).

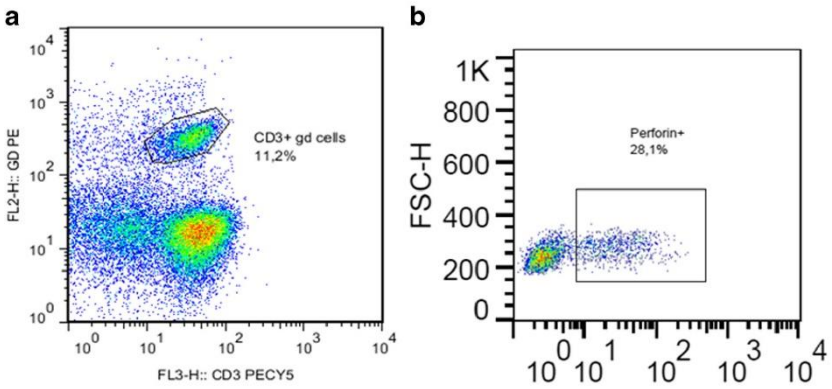

Fig. 1 (A) Plot showing the gated lymphocytes in CD3-PECY5 on the x-axis versus γδ-PE on the y-axis. The graphic demonstrates an unusual high percentage of γδ T cells within the total lymphocytes. (B) Gating and calculating the percentage of the of perforin positive $CD3^+$ γδ T lymphocytes

Anthropometric data results are shown as mean (± SD). Sleep and laboratory results were not normal distributed and are shown as median (interquartile range). When two dependent samples were analyzed, the Wilcoxon signed rank test was applied. To compare between three or more different groups Kruskal-Wallis tests were applied and a posteriori Wilcoxon rank test for pairwise comparisons was performed, using a Holm-Bonferroni adjustment for the obtained *p-values*, due to the increasing probability of false positives inherent to multiple comparisons. A significance level of alpha 5% was used to determine statistical significance.

Results

To investigate the effect of sleep disorders on cytotoxic immune system, we divided the study population into four groups, according to the diagnosis: in 24 of the total 87 included participants we found no sleep related breathing disorders (controls = C). In 19 patients, we detected sleep fragmentation due to increased upper airway resistance (UAR) but an apnea/hypopnea index within the normal range (< 5/H). A total of 20 non-obese and 25 obese patients (noOSA and oOSA, respectively) demonstrated an elevated obstructive sleep apnea/hypopnea (AHI) index (≥ 5/h). The latter three groups were considered to have sleep-related breathing disorders (SRBD).

Anthropometric data

The anthropometric data is presented in Table 1. BMI was higher in the oOSA group ($p < 0.001$) when compared to the three other groups. Both OSA groups were older compared to controls ($p = 0.006$ for noOSA, $p = 0.007$ for oOSA. There were no evidences of any age differences between UAR group and the controls.

Sleepiness evaluation and polysomnographic results

The results of the sleepiness questionnaires and the polysomnographic recordings can be seen in Table 2. The results from the Epworth sleepiness scale (ESS) demonstrated OSA patients significantly sleepier when compared to controls (noOSA $p = 0.008$ and oOSA $p = 0.004$, respectively). Also, the slow wave sleep (N3) was significantly lower for the OSA groups compared to controls (noOSA: $p = 0.008$ e oOSA $p = 0.019$). Sleep fragmentation, defined by the arousal index (AI), was higher in all SRBD groups compared to controls (UAR $p < 0.001$, noOSA $p < 0.001$ and oOSA $p < 0.001$, respectively). Between the three SRBD groups, we detected differences between UAR and OSA groups (noOSA $p = 0.002$, oOSA $p = 0.029$).

Hypoxia-related parameters including the apnea/hypopnea index (AHI), the oxygen desaturation index (ODI) and the percentage of peripheral oxygen saturation < 90% (T90) were significantly higher in both OSA groups when compared to either controls or UAR patients (Table 2). The respiratory disturbance index (RDI) was higher in all SRBD patients compared to controls (UAR $p < 0.001$, noOSA $p < 0.001$ and oOSA $p < 0.001$). Within the SRBD groups the RDI was found significantly higher in both OSA groups compared to UAR (noOSA $p < 0.001$ and controls $p < 0.001$). Following CPAP therapy, all polysomnographic parameters improved significantly ($p < 0.05$) with exception of sleep efficiency, that further decreased, R, and SSS. Results are demonstrated in Table 3 of the online supplementary material.

Routine laboratory analysis

Results for the routine blood analysis are listed in Tables 3 and 4. No significant difference was found in the full blood count analysis. The gGT level was twofold higher in all SRBD groups when compared to the control group (Fig. 2). Interestingly, median values were quite homogeneous between the three SRBD groups although the significance was

Table 3 Routine blood analysis

	Controls	UAR	noOSA	oOSA
Hb[g/dl]	15.70 (15.00–16.10)	15.10 (14.65–15.68)	15.10 (14.78–15.57)	15.00 (14.05–15.95)
HCT[%]	45.30 (41.60–47.40)	44.20 (42.77–45.27)	44.00 (42.20–46.35)	44.20 (41.30–46.95)
Leucocytes [10^9/L]	7.27 (5.75–8.27)	6.20 (5.48–6.90)	7.22 (5.76–9.88)	6.89 (5.85–8.19)
Lymphocytes [10^9/L]	2.44 (1.97–2.82)	2.25 (2.05–2.55)	2.53 (2.01–2.95)	2.79 (2.13–3.02)
Creatinine [mg/dl]	1.02 (0.89–1.12)	1.00 (0.95–1.14)	0.93 (0.88–1.00)	0.95 (0.88–1.06)
BUN [mg/dl]	18.46 (16.59–21.50)	18.69 (16.94–21.03)	17.76 (14.72–19.63)	18.46 (16.24–22.31)
AST [U/L]	23.00 (19.75–25.25)	23.00 (20.25–25.00)	25.00 (21.00–30.50)	27.50 (22.75–29.75)
ALT [U/L]	26.00 # (19.00–29.00)	29.50 (24.25–31.75)	36.00 (22.00–52.00)	38.00 (26.25–54.00)
gGT	20.00 + *# (14.00–27.00)	41.00 (24.75–52.00)	44.00 (29.00–65.00)	40.00 (30.75–64.75)
Glucose [mg/dl]	85.00 # (74.00–89.00)	82.50 § (75.50–86.00)	90.00 (78.50–111.00)	98.00 (89.00–104.00)
Colesterol tot [mg/dl]	177.00 (170.50–192.50)	212.50 (197.00–233.80)	218.00 (198.00–244.00)	197.50 (177.80–217.50)
LDL [mg/dl]	114.00 (105.50–121.50)	142.00 (113.50–153.80)	145.00 (115.00–167.00)	123.50 (105.00–143.00)
HDL [mg/dl]	42.00 (38.00–48.50)	47.50 § (44.00–58.00)	44.00 (37.00–50.00)	40.00 (36.25–44.75)
TG [mg/dl]	101.00 *# (68.50–120.50)	107.00 ^§ (75.25–124.75)	167.00 (152.00–252.00)	163.00 (125.00–221.20)
CRP [mg/dl]	0.04 # (0.04–0.13)	0.05 § (0.04–0.14)	0.12 (0.05–0.51)	0.30 (0.14–0.43)

All values are demonstrated as median (interquartile range). Statistically significant results after adjustment, with a $p < 0.05$ are indicated as: Control vs. UAR: +, Control vs. noOSA: *, Control vs. oOSA: #, UAR vs. noOSA: ^. UAR vs. oOSA: §, noOSA vs. oOSA: °

UAR: Upper Airway Resistance, noOSA: non-obese OSA, oOSA: obese OSA, Hb: hemoglobin, HCT: hematocrite, BUN: Blood Ureia Nitrogen, AST: Apartate Aminotransferase, ALT: Alanine Aminotransferase, gGT:gamma glutamyl transferase, LDL: Low-density lipoprotein Cholesterol, HDL: high-density lipoprotein cholesterol, TG: Triglycerides, CRP: C-reactive Protein

noOSA and in UAR patients compared to the two OSA groups. However, such results were not statistically significant.

Measurement of intracellular perforin and granzyme-B

To study the cytotoxic function, we focused on granzyme B-positive γδ-cells and perforin-positive γδ-cells (Fig. 1). While for granzyme-B cells there were not sufficient evidences among groups, perforin-positive γδ-cells revealed significant differences between groups ($p = 0.01662$, Fig. 3), especially between the control group and the oOSA ($p = 0.04$); the remaining SRBD groups showed differences from the control without reaching statistical significance (control vs UAR: $p = 0.08$; control vs noOSA = 0.09, Fig. 3).

These results are already an indication of an association between sleep-related breathing disorders and the cytotoxicity of the immune system; however, to fully test this association, we performed a correlation analysis between the perforin-positive γδ-cells and sleep-related parameters. Using the non-parametric Spearman Correlation Coefficient approach, we found correlations for AHI (Coef = −0.3568; $p = 0.001$), RDI (Coef = −0.3030; $p = 0.007$), Mean SpO2 (Coef = 0.4750; $p < 0.001$), T90 (Coef = −0.3880; $p < 0.001$), ODI (Coef = −0.3395; $p = 0.002$), SSS (Coef. = −0.2719; $p = 0.022$) and ESS (Coef. = −0.2550; $p = 0.025$).

These results suggest a relationship between sleep breathing-related disorders and the perforin-positive γδ-cells, but because there are other parameters that may influence the cytotoxicity of the immune system—age, BMI, metabolic function (and others)—we further analyzed the data to check

Table 4 Percentage of perforin positive lymphocytes within the lymphocyte subset

	Controls	UAR	OSAS non obese	OSAS obese
Total Perforin	23,60 (20.70–37.00)	19.40 (16.10–27.20)	22.60 (17.65–30.02)	24.90 (17.27–32.75)
$CD3^+P^+/CD3^+$	12.40 (8.49–20.30)	13.75 (8.29–16.40)	14.65 (7.91–24.03)	15.70 (9.32–23.40)
$CD3^+CD4^+P^+/CD3^+CD4^+$	1.60 (0.64–4.19)	2.28 (0.66–5.35)	1.63 (0.84–3.18)	1.60 (0.29–6.82)
$CD3^+CD8^+P^+/CD3^+CD8^+$	28.50 (15.32–51.62)	28.20 (17.10–49.15)	24.80 (18.20–44.00)	30.55 (20.62–48.48)
$CD3^-CD8^+P^+/CD3^-CD8^+$	66.35 (48.50–85.12)	85.80 (71.70–91.10)	79.75 (67.83–92.03)	84.90 (69.25–95.05)
$CD3^+\gamma\delta P^+/ CD3^+gd$	65.10 # (57.90–79.30)	46.20 (36.45–60.10)	45.95 (29.10–62.90)	38.75 (25.20–66.03)
$CTLP^+/CTL$	90.90 (77.90–95.30)	86.15 (81.80–93.55)	86.70 (68.30–94.80)	86.45 (78.90–94.85)
NKP^+/NK	98.20 (94.80–99.10)	96.00 (94.75–98.62)	95.20 (93.00–96.95)	95.75 (92.22–97.55)

All values are demonstrated as median (interquartile range). Statistically significant results after adjustment, with a $p < 0.05$ are indicated as: Control vs. UAR: +, Control vs. noOSA: *, Control vs. oOSA: #, UAR vs. noOSA: ^. UAR vs. oOSA: §, noOSA vs. oOSA: °

Gd cells: $CD3^+$ ©™ T cells, CTL: CD3 + CD16 + CD56+ cell. NK: CD3-CD16 + CD56+ positive cells

whether the identified associations were maintained when such confounding variables are taken into consideration. To this end, we performed a multiple regression analysis, a method that is highly recommended for these situations. The multiple regression analysis results showed that even when several parameters were taken into consideration, SRBD-related variables such as Arousal Index ($p = 0.049$) and Mean SpO2 ($p = 0.050$) were still associated with the perforin-positive γδ-cells. The overall model also demonstrated to be better than a stochastic model ($p = 0.039$).

Measurement of intracellular perforin and granzyme-B following therapy with continuous positive airway pressure (CPAP)

Following CPAP therapy the percentage of perforin positive cells within the lymphocyte subgroups increased with exception of the $CD3^-CD8^+P^+/CD3^-CD8^+$ lymphocytes (Table 4 supplementary material). Only for the $CD3^+CD4^+P^+/CD3^+CD4^+$ lymphocytes there was statistically relevant evidences of differences ($p = 0.022$). We found no statistically

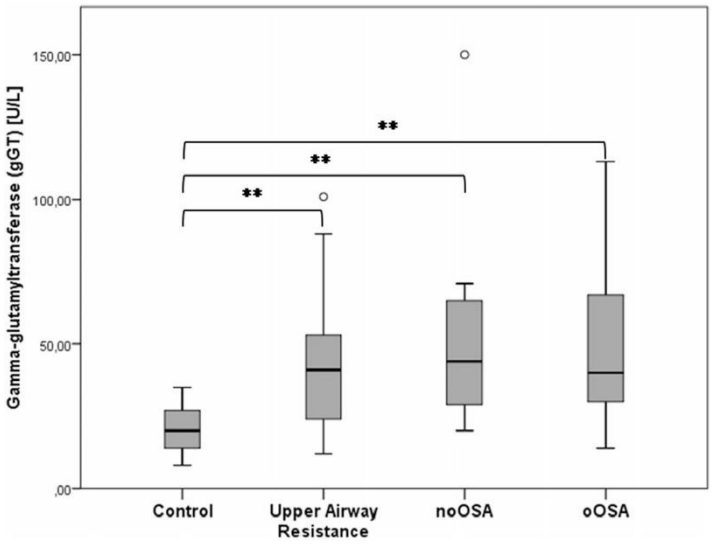

Fig. 2 γGT levels in controls, upper airway resistance (UAR), lean obstructive sleep apnea (noOSA), and obese OSA (oOSA) patients. Statistical significance is indicated by *. Both OSA groups demonstrate a relatively similar pattern

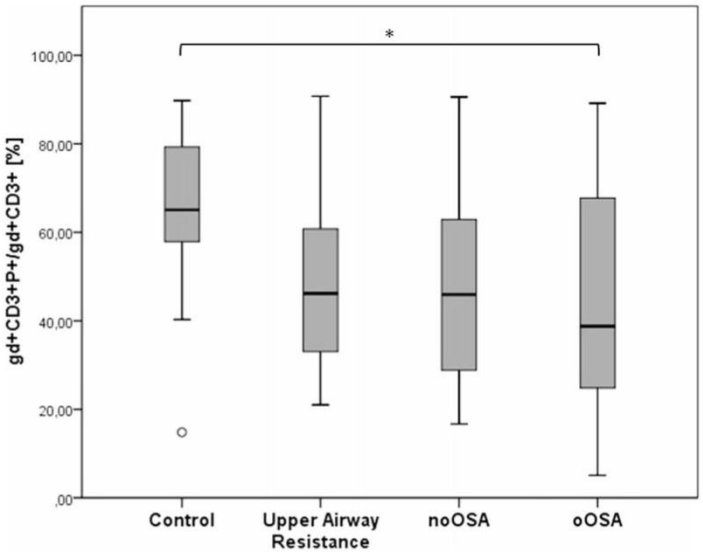

Fig. 3 Perforin-positive CD3$^+$γδ-T lymphocytes. Statistical significant results are indicated by *. All three sleep-disordered breathing groups demonstrate a similar distribution with a significant result in the statistical analysis for the oOSA when compared with the controls

significant impact of the CPAP therapy in the GrB positive lymphocytes.

In vitro measurement of degranulation capacity and intracellular perforin and granzyme-B following hypoxia of human γδ-T cells

To investigate whether intermittent hypoxia seen in sleep apnea patients is causally linked to decreased perforin levels in γδ-T cells, we isolated γδ-T cells from six independent anonymous donors from a blood bank and tested for their degranulation capacity, as measured by CD107a surface expression, perforin and granzyme B, comparing normal oxygen levels and hypoxic conditions. We observed a substantial and statistical significant decrease in the degranulation capacity of γδ-T cells in hypoxic conditions as evidenced by reduced levels of CD107a staining (Fig. 4a). We also found decreased levels of granzyme-B despite not being statistically significant (similarly to our patients, Fig. 4b), but no change in the perforin levels (Fig. 4c).

Discussion

Sleep disturbances, including sleep-related breathing disorders (SRBD) in general and the obstructive sleep apnea (OSA) in particular, are known to contribute human homeostasis disruption [14] but the underlying mechanisms remain poorly understood. In OSA, the intermittent hypoxia due to either apneas or hypopneas and the sleep fragmentation due to the respiratory effort are the most important candidate mechanisms so far implicated.

In this study, we found that obstructive respiratory events including obstructive apnea, obstructive hypopnea, and respiratory effort-related arousals (RERA) are associated with a reduced percentage of perforin positive CD3$^+$γδ T cells. Even in upper airway resistance patients (UAR) who by definition do not exhibit relevant oxygen desaturations, we saw some indications of this reduction, suggesting that sleep fragmentation, not only intermittent hypoxia, can influence the cytotoxic immune system.

Interestingly, therapy of OSA with continuous positive airway pressure (CPAP) increased the percentage of perforin-positive cells in most lymphocyte subpopulations (the differences were statistically significant only for CD3$^+$CD4$^+$ lymphocytes), suggesting that CPAP treatment is able to revert the observed decrease in perforin levels.

We have also investigated whether intermittent hypoxia could be causally linked to the changes observed and sufficient to explain the decrease in perforin levels found in the CD3$^+$γδ T cells of patients. We did find that there are significant differences in the degranulation capacity of γδ T cells subjected to hypoxia as well as decreased levels of granzyme B, but no changes in the levels of perforin. These findings do suggest that hypoxia changes the degranulation capacity of CD3$^+$γδ T cells in patients, but it is not sufficient to change the perforin level, which might be in direct connection to sleep fragmentation as suggested by the in vivo findings (UAR patients, who have no intermittent hypoxia, also have decreased perforin levels). The fact that we did not find changes in the levels of perforin might also reflect experimental

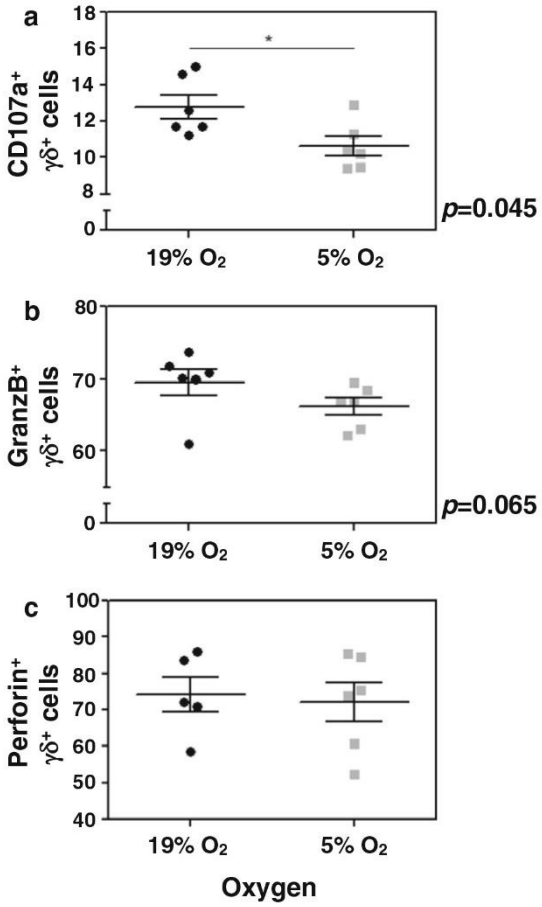

Fig. 4 In vitro analysis of degranulation capacity (A), granzyme B, (B) and perforin (C) $CD3^+\gamma\delta$-T lymphocytes, comparing normal levels of oxygen and hypoxia

limitations as it was not possible to mimic intermittent hypoxia and we were limited to continuous hypoxia. In addition, because we got samples from anonymous donors of a blood bank, we cannot exclude that some of the samples came from sleep apnea patients, as we know that the prevalence is very high in the general population [15]. By the current epidemiological data, it is possible that up to two out of the six samples came from donors with some level of SRBD. If this was the case, our in vitro experiment underestimated the real impact of hypoxia on the degranulation capacity of $CD3^+\gamma\delta$ T cells.

There have been few studies investigating the influence of OSA on the cellular immune system. Dyugovskaya and colleagues found an increased cytotoxic activity against endothelial cells for both γδ T cells and $CD8^+$ lymphocyte in OSA patients [16, 17]. Although, at least at first glance, these results appear contradictory to ours, in fact they are not mutually exclusive. The described increased cytotoxicity of γδ T cells in OSA patients was TNF-α dependent and not based on the perforin/granzyme B pathway which is in general considered the faster and most effective mechanism of lymphocyte mediated cytotoxicity [6]. In our study, the percentage of perforin-positive $CD8^+$ lymphocytes not expressing CD3 was higher in all three SRBD groups. This $CD8^+$ lymphocyte subset is considered the most cytotoxic cells within all CD8 lymphocytes, especially when expressing CD56 [18]. Following one night of CPAP therapy, the percentage of perforin positive $CD3^-CD8^+$ lymphocytes decreased although the result was not statistically significant. Thus, in this small subset we observed the same trend as Dyugovskaya and colleagues.

It is currently unclear if the cytotoxic defense in OSA is increased [17], normal [19] or even decreased. Recently, Gaoatswe and colleagues demonstrated that circulating invariant natural killer T cells (iNKT) are reduced in OSA patients [20]. The more severe patients revealed the lowest number of iNKT lymphocytes. Incubation in a hypoxic environment increased apoptosis and decreased cytotoxicity of iNKT lymphocytes. Recent research provided evidence that OSA might increase the risk of cancer (reviewed by Gozal et al. [21]). Since invariant NKT cells are important for the anti-tumor response, Gaoatswe and colleagues suggested their results might contribute to explain the possible relationship between OSA and cancer. The γδ T cells are a critical component of the anti-tumor capabilities of the human immune response [22]. Therefore, our results constitute an important contribution to mechanistically explain the epidemiological relation between OSA and tumor diseases. The percentage of γδ T cells in the peripheral blood is small and usually less than 5%. However, γδ T cells demonstrate a high migratory capability with relevant accumulation in specific tissues [23, 24]. The decrease of perforin-positive γδ T cells in the peripheral blood that we describe might reflect a more important depression of the cytotoxic γδ T cells within epithelial tissues [25]. Interestingly, in a recent study Akparpour and colleagues showed that both intermittent hypoxia and sleep fragmentation reduces GrB^+ CD8 lymphocytes within the tumor environment, in a mouse model of OSA [26].

Until now the epidemiological data for a possible OSA-cancer relation found mainly an association between nocturnal hypoxemia and cancer mortality [27]. The evidence regarding non-hypoxic sleep disturbances and tumor diseases is less established and mostly related to sleep restriction, insomnia, or shift work investigations with inconsistent results. Perhaps these conclusions can be explained by the lack of objective sleep data and abundance of subjective data based on questionnaires and sleep diaries. In the better-controlled animal studies both sleep fragmentation and intermittent hypoxia affected various components of the tumor progression including tumor growth or metastasis [28], which is in excellent agreement with the findings that we now report. The influence of

obstructive sleep disordered breathing on tumor diseases was not the subject of the current study but our data suggests that both sleep fragmentation and hypoxia influence the cytotoxic immune defense independently and cumulatively.

Our study protocol did not include special diet recommendations prior to the sleep study, nor did we control for glucose or cholesterol affecting medication as we did not set out to investigate if RERAs are capable to influence the metabolic system. Therefore, the results regarding the glucose, cholesterol, and lipoprotein analysis should be interpreted cautiously. However, it appears noteworthy to mention that the gGT was significantly higher in all SRBD groups without any relevant difference between each other. To our knowledge, there is no evidence regarding the impact of respiratory effort related sleep fragmentation on the liver enzymes and this result deserves further investigation. CRP was higher in other oOSA groups when compared to either UAR patients or controls, confirming previous reports [29]. Also, it is of interest that controls and upper airway resistance patients had a significantly lower triglyceride level when compared to both OSA groups, although none of them was on regular medication. With the reservations mentioned, it is possible that apneas and hypopneas with relevant oxygen desaturation increase, independently from obesity, the pro-atherogenic role cholesterol and lipoprotein metabolism [30].

There are some limitations to the interpretation of the statistical results. The mean age was higher in the OSA groups when compared to controls. However, UAR patients had no significantly different age when compared to the other three investigated groups. In fact, these patients would be considered controls if less attention had been attributed to the RERAs classification. Any significant result in UAR patients compared to controls or OSA patients must be therefore considered significant. The immunosenescence described for the percentage of natural killer cells and the perforin related cytotoxicity might be of some concern [31]. Nevertheless, most evidence has been found in patients with an age above 60 years [32]. In our study, the inclusion was limited to an age below 60 years with a mean age of 47 years. It is thus questionable if imunosenescence already has any effect. Also of importance is the fact that there are only a small number of patients included in each group. However, with a total number of 87 included patients the study population is higher than most other studies investigating the relationship between SRBD and the immune system. In the future, to fully test the validity of our conclusions, a study on a more homogeneous group could be more effective.

In conclusion, this study analyzed the effect of hypoxic and non-hypoxic respiratory events on the perforin and granzyme-B positive lymphocytes. We found a decreased percentage of perforin positive $CD3^{+}\gamma\delta$ lymphocytes for the oOSA patients. Our results suggest that the sleep fragmentation and intermittent hypoxia observed in OSA cause changes in the cytotoxic potential of $CD3^{+}\gamma\delta$ lymphocytes which might be causally related to the increased cancer risk in these patients.

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Compliance with ethical standards

Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the Ethics Committee Review Board of Hospital de Santa Maria (Lisboa, Portugal).

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

Abbreviations AHI, Apneia/hypopneia index; ALT, Alanine transaminase; AST, Aspartate transaminase; CTL:, Cytotoxic T lymphocytes (CD3 + CD16 + CD56+); CRP, C-reactive protein; ESS, Epworth Sleepiness Scale; γδ T cells, Gamma-delta T lymphocytes; gGT, Gamma-glutamyltranspeptidase; GrB, Granzyme-B; HDL, High-density lipoprotein; LDL, Low-density lipoprotein; NK, Natural killer cells ($CD3^{-}CD16^{+}CD56^{+}$); NKT, Natural killer T cells ($CD3^{+}CD16^{+}CD56^{+}$); ODI, Oxygen desaturation index; P, Perforin; PBS, Phosphate-buffered saline; PSG, Polysomnography; RDI, Respiratory disturbance index; RERAs, Respiratory effort-related arousals; SRBD, Sleep-related breathing disorders; SSS, Stanford sleepiness scale; TG, Triglycerides

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4.2.1 Role of CD3+γδ-T cells in the association of obstructive sleep-disordered breathing and cancer.

Post Publication Discussion: Invited Letter to the Editor

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SLEEP BREATHING PHYSIOLOGY AND DISORDERS • LETTER TO THE EDITORS (INVITED)

Role of CD3$^+$γδ-T cells in the association of obstructive sleep-disordered breathing and cancer

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Dear Editor,

We wish to thank Dr. Tomoyuki Kawada for his interest in our report and for bringing to our attention previous epidemiological studies that have explored the association of obstructive sleep apnea (OSA) and cancer, including the meta-analysis that directly supports our statement that OSA affects cancer incidence [1] and another study that finds overall elevated cancer burden in OSA patients [2], but decreased in some cancer types when data is stratified [2]. While the majority of the available reports show an increased incidence of cancer in OSA, including [3–7], a few studies have either failed to identify a significant correlation [8], or even saw a negative association for specific types of cancer [2]. This is where the finding that the numbers of perforin-positive CD3$^+$γδ-T cells are significantly decreased in the peripheral blood of OSA patients becomes even more interesting. In fact, it has recently become clear that CD3$^+$γδ-T cells do not always eliminate tumors, but in some tumor types, CD3$^+$γδ-T cells actually promote tumor progression and metastasis [9–15]. We would like to stress that, contrary to that stated in the comment by Dr. Kawada, we have not investigated the tumor burden in our patients nor does our study directly support a positive association between OSA and cancer incidence. What we did was to identify a substantial and statistically significant decrease in a population of perforin-positive CD3$^+$γδ-T cells in patients with obstructive sleep-disordered breathing [16], which led us to speculate that this might be a previously unappreciated reason for the effect of OSA in cancer incidence. Interestingly, we have also found strong correlations between this population and polysomnographic parameters relevant for the characterization of intermittent hypoxia, including AHI, RDI, mean SpO_2, and ODI [16]. We certainly agree with the main message of Dr. Kawada's comment, supporting the need for further epidemiological and laboratory studies to explore the nature of the association between OSA and specific types of cancer incidence—this seems to be a universal truth as no study is definitive in science.

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4.2.2 Obstructive sleep apnea and the cytotoxic immune system unpublished results.

The effect of positive airway pressure on the cytotoxic protein perforin

This study was a continuation of the published study described above. The aim of this study was to investigate the effect of continuous positive pressure therapy on perforin positive peripheral blood lymphocytes.

Methods:

In a subgroup of 32 participants with obstructive sleep apnea (OSA) the impact of positive airway pressure (PAP) therapy was investigated after 1 night of therapy (CPAP titration) and 3 months of therapy with automatic positive airway pressure (APAP). All patients underwent full polysomnographic (PSG) recordings. In the morning after each recording a fasting blood sample was obtained from a cubital vein. 6 patients did not participate in one of the follow up investigations. Also 6 samples were excluded due to technical problems that provoked a prolonged storage of the blood which may modify in blood lymphocytes the expression of cytotoxic proteins. Therefore, 20 participants remained in the total cohort. Although most parameters demonstrated a normal distribution by the Sharpiro-Wilk analysis due to the small sample size the results were analysed in the non-parametric Friedman's two-way analysis for related samples with a significance leve (alpha) of < 0.05. Additionally, the effect size between the 3 time points (Cohen´s d) was calculated. A value of 0.2 was considered a small effect size, 0.5 a moderate effect size and ≥ 0.8 a large effect size.

Results

The mean age of the group was 48.19 years (standard variation ± 9.77) and the mean BMI was 22.66 (4.07).

The main results from the polysomnographic study is displayed in table 3.

Time points	SE	N3	R	Arousal Index	RDI	ODI	T90	ESS
1	86.70 (19.65)	5.80 (20.35) *	10.80 (14.45)	52.20 (20.80) * #	39.80 (35.55) *, #	30.70 (31.00) * #	2.60 (7.05) * #	9.00 (8.50) #
2	80.90 (24.75)	20.20 (23.75)	12.80 (7.55)	26.80 (29.50) +	11.70 (9.85)	7.10 (7.80)	0.0 (0.40)	5.00 (6.50)
3	86.90 (7.25)	19.90 (16.20	17.90 (5.95)	21.90 (11.30)	2.90 (4.20)	2.10 (4.40)	0.0 (0.10)	5.00 (5.00)

Table 3: Median (interquartile range) of 20 patients completing the 3 measurement points at 1: after diagnostic polysomnography, 2: after 1 night of CPAP, 3: after 3 months of APAP. Significant results (alpha level of 0.05) are indicated: *: time point 1 vs. time point 2; # time point 1 vs. time point 3; +: time point 2 vs. time point 3. Abbreviations: SE: sleep efficiency; N3 sleep stage 3 (slow wave sleep); R: REM sleep; RDI: respiratory disturbance index; ODI oxygen desaturation index; T90: percentage of SpO2<90 %; ESS: Epworth sleepiness scale.

At the first night of positive airway pressure (PAP) therapy we detected a significant increase in the slow wave sleep (N3) and a significant decrease in all three respiratory parameters (RDI, ODI and T90). Following three month of therapy the respiratory parameters remained stable but without a further significant improvement. The arousal index decreased significantly in the first night of PAP therapy but contrary to the respiratory values, continued to improve when measured after three month reaching statistical significance. Sleepiness measured by the Epworth sleepiness scale was significantly lower only after three months of PAP therapy.

Table 2 contains the results regarding the perforin positive cells at the three time points as median (interquartile difference). We found after the first therapy night an increase in the $CD3^{+}CD4^{+}$ lymphocytes that did not reached statistical significance (p=0.058). After 3 months the percentage of perforin positive $CD4^{+}$ lymphocytes increased further and reached now a significant result (p=0.025). The percentage of all perforin positive cells increased within total lymphocytes population and within each investigated subpopulation except in $CD3^{-}CD8^{+}$ lymphocytes where the percentage of perforin

positive cells decreased. However, these results did not reach statistical significance (table 4).

Time Point	Total Pfr	$CD3^+$ P^+	$CD3^+$ $CD4^+P^+$	$CD3^+$ $CD8^+P^+$	$CD3^-$ $CD8^+P^+$	CTL	γδ-T cells	NK cells
1 (n=20)	19.95 (17.30)	10.02 (16.52)	1.59 (7.24)	31.70 (30.15)	76.75 (27.58)	85.65 (29.48)	32.15 (34.85)	93.70 (5.68)
2 (n=20)	27.95 (16.78)	13.25 (20.74)	2.05 (7.72)	37.75 (35.58)	76.05 (35.03)	89.05 (27.75)	34.90 (35.18)	95.95 (6.13)
3 (n=20)	29.85 (14.38)	18.15 (18.93)	3.83 (6.42)	45.80 (35.10)	72.75 (35.90)	92.50 (10.18)	42.55 (28.13)	96.90 (3.95)

Table 4: Median (interquartile range) of the 20 patients completing the 3 measurement points at 1: after diagnostic polysomnography; 2: after 1 night of CPAP; 3: after 3 months of APAP. Only in CD3+CD4+ lymphocytes the result reached statistical significance when timepoint 1 was compared to timepoint 3. Abbreviations: CTL: cytotoxic T-lymphocytes (CD3+CD16+CD56+), NK cells: natural killer cells (CD3-CD16+CD56+).

Due to the small sample we further investigated a possible relevant change of perforin positive lymphocytes by calculating the effect size (Cohen´s d) between all three measurements. Mean results and the Cohen´s d values are demonstrated in table 5.

	1 (n=20)	2 (n=20)	3 (n=20)	d: 1 vs. 2	d: 2 vs. 3	d: 1 vs. 3
Total P^+ cells	24.53 (10.16)	26.84 (9.53)	27,98 (9.84)	0.23	0.11	0.33
$CD3^+$ P^+ [%]	18.07 (18.34)	19.39 (16.97	17.28 (9.94)	0.07	0.05	0.05
$CD3^+CD4^+$ P^+ [%]	3.55 (4.04	5.02 (5.30)	4.65 (4.39)	0.26	0.07	0.26
$CD3^+CD8^+$ P^+ [%]	33.23 (20.32)	38.31 (20.96)	41.45 (19.78)	0.25	0.15	0.40
$CD3^-CD8^+$ P^+ [%]	76.97 (17.07)	70.25 (26.40)	72.02 (19.43)	0.30	0.08	0.27
CTL P^+ [%]	75.77 (24.68)	80.39 (21.98)	85.39 (22.47)	0.20	0.22	0.41
γδ-T cells P^+ [%]	37.28 (20.86)	40.45 (21.83)	45.77 (17.89)	0.15	0.27	0.44
NK P^+ [%]	93.06 (5.19)	94.54 (4.65)	96.45 (2.27)	0.30	0.52	0.85

Table 5: Mean (standard deviation) of the percentage of perforin positive cells of the total lymphocyte population and within each lymphocyte subset. Cohen´s d (d) was calculated for each combination of time

points. Abbreviations: CTL: cytotoxic T-lymphocytes ($CD3^+CD16^+CD56^+$), NK cells: natural killer cells ($CD3^-CD16^+CD56^+$).

In general, a d value of 0.2 is considered a mild effect size. Therefore, one night of PAP therapy resulted in a relevant increase of the perforin positive cells within the total lymphocytes population. This was related to the increase in most subsets with exception of the $CD3^-CD8^+$ lymphocytes that demonstrated a relevant decrease after one night of PAP therapy. Interestingly, the highest effect size was observed within the natural killer cells, although it remains discussable if an increase of 1.48 % is of any clinical relevance. After 3 months of PAP therapy the percentage of perforin positive CD3 and CD4 cells decreased slightly, but without relevant effect size between the first therapy night and the 3 months control. Also, Cohen´s D remained relevant when comparing the first and the third measurements.

Interestingly the lymphocyte subsets with natural killer cell activity ($\gamma\delta$-T cells, CTL and NK cells) still demonstrated a relevant increase with an effect size of > 0.2 when comparing the second with the third analysis and a moderate ($\gamma\delta$-T, cells, CTL) to large (NK cells) when comparing the three months values with the initial results.

In conclusion: With exception of $CD3^-CD8^+$ lymphocytes all investigated groups demonstrated an increase in the percentage of perforin positive cells after one night of PAP therapy that did not reach statistical significance. However, we found a relevant effect size in all lymphocyte subsets after one night of therapy and for the lymphocyte subsets with natural killer cell activity a further relevant increase after three months.

4.3 Study 3

The importance of sleep fragmentation on the hemodynamic dipping in obstructive sleep apnea

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The Importance of Sleep Fragmentation on the Hemodynamic Dipping in Obstructive Sleep Apnea Patients

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Introduction: Obstructive sleep apnea (OSA) has been associated with non-dipping blood pressure (BP). The precise mechanism is still under investigation, but repetitive oxygen desaturation and arousal induced sleep fragmentation are considered the main contributors.

Methods: We analyzed beat-to-beat measurements of hemodynamic parameters (HPs) during a 25-min period of wake–sleep transition. Differences in the mean HP values for heart rate (HR), systolic BP (SBP), and stroke volume (SV) during wake and sleep and their standard deviations (SDs) were compared between 34 controls (C) and 22 OSA patients. The Student's *t*-test for independent samples and the effect size by Cohen's *d* (d) were calculated. HP evolution was investigated by plotting the measured HP values against each consecutive pulse wave. After a simple regression analysis, the calculated coefficient beta (SCB) was used to indicate the HP evolution. We furthermore explored by a hierarchical block regression which variables increased the prediction for the SCB: model 1 BMI and age, model 2 + apnea/hypopnea index (AHI), and model 3 + arousal index (AI).

Results: Between the two groups, the SBP increased in OSA and decreased in C resulting in a significant difference ($p = 0.001$; $d = 0.92$). The SV demonstrated a similar development ($p = 0.047$; $d = 0.56$). The wake/sleep variation of the HP measured by the SD was higher in the OSA group—HR: $p < 0.001$; $d = 1.2$; SBP: $p = 0.001$; $d = 0.94$; and SV: $p = 0.005$; $d = 0.82$. The hierarchical regression analysis of the SCB demonstrated in SBP that the addition of AI to AHI resulted in ΔR^2: +0.163 and $\Delta F + 13.257$ ($p = 0.001$) and for SV ΔR^2: +0.07 and ΔF 4.83 ($p = 0.003$). The AI but not the AHI remained statistically significant in the regression analysis model 3—SBP: $\beta = 0.717$, $p = 0.001$; SV: $\beta = 0.469$, $p = 0.033$.

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Conclusion: In this study, we demonstrated that in OSA, the physiological dipping in SBP and SV decreased, and the variation of all investigated parameters increased. Hierarchical regression analysis indicates that the addition of the AI to BMI, age, and AHI increases the prediction of the HP evolution following sleep onset for both SBP and SV and may be the most important variable.

Keywords: sleep disordered breathing, cardiovascular risk, sleep disturbance, arterial blood pressure, stroke volume

INTRODUCTION

Sleep and sleep disturbances affect various components of the human homeostasis including the cardiovascular system (Muller et al., 1989; Silvani, 2019). In the last decades, the relationship between obstructive sleep apnea (OSA) and cardiovascular diseases (CVDs) has been intensively investigated by both basic science and clinical researchers (Floras, 2018). The assembled scientific evidence indicates a relevant negative impact of OSA on several components of the cardiovascular system, including an increased risk for arterial hypertension (AHT) (Javaheri et al., 2017). However, the precise mechanism of this interaction remains unclear, and the effect of OSA therapy on AHT is inconsistent. Although several longitudinal studies confirmed OSA as a risk factor for AHT and a benefit from therapy with continuous positive airway pressure (CPAP) (Peppard et al., 2000; Marin et al., 2012; Mokhlesi et al., 2014), others were inconclusive (McEvoy et al., 2016). The importance to use the correct methods for OSA diagnostic to avoid false-negative results has been recently underlined (Parati et al., 2019).

Sleep onset is usually accompanied by a physiological reduction or dipping of the blood pressure (BP) reaching about 10–20% of the values recorded during daytime (Staessen et al., 1997). A decrease in this physiological BP change is alleged to induce relevant health consequences due to an increased risk for CVD (Cuspidi et al., 2018). Compared to the daytime BP, the nocturnal BP appears more relevant for the prediction of cardiovascular risk (Fagard et al., 2008; Investigators et al., 2014). Nocturnal dipping can be reduced, absent, or even inversed by various internal and external factors including sleep disorders, obesity, high salt intake, chronic kidney disease, diabetic neuropathy, and old age (Parati et al., 2014). Although the presence and extent of dipping is very variable, OSA has been identified in several studies as a risk factor for non-dipping BP (Suzuki et al., 1996; Hla et al., 2008; McEvoy et al., 2016). Recently, the European Society of Cardiology/European Society of Hypertension considered the suspicion of nocturnal hypertension in OSA patients an indication for ambulatory BP measurement (ABPM) rather than home BP measurement (HBPM) (Williams et al., 2018). However, during the nocturnal period, the ABPM devices commonly measure the BP in fixed intervals of 30 min. Thus, the critical change of BP reduction at the wake/sleep transition is frequently missed. In fact, ABPM recordings give little detailed information over the night-time period, since both micro- and macrostructure of the sleep are not recorded. This is of clinical relevance since respiratory events during REM sleep are associated with higher BP surges (Sasaki et al., 2018) and are thought to be especially relevant for the cardiovascular risk of OSA patients (Mokhlesi et al., 2015; Mokhlesi and Varga, 2018; Varga and Mokhlesi, 2019). Missing BP recordings during REM periods due to large intervals might therefore generate nocturnal BP values that might not reflect reality. A more detailed measurement of BP values in association with sleep recordings appears therefore important to objectively assess the relationship between AHT and OSA.

At present, repetitive oxygen desaturations and activation of the sympathetic nervous system are considered the main pathomechanisms in the development of AHT in OSA (Lesske et al., 1997; Iturriaga et al., 2017). However, AHT is also linked to other sleep disorders without effect on the arterial oxygen saturation (Gottlieb et al., 2006; Abbott et al., 2019). A possible explanation for this is the complex interaction between the baroreceptor reflex and sleep fragmentation by micro-arousals (Silvani et al., 2015). Taylor et al. (2016) reported recently an increase in the daytime sympathetic activity due to sleep fragmentation that did not depend on the presence of obstructive apneas/hypopneas.

In this study, we investigated in a detailed beat-to-beat analysis the influence of either OSAs or sleep fragmentation on the evolution of hemodynamic parameters (HPs) including heart rate (HR), systolic BP, and stroke volume (SV).

MATERIALS AND METHODS

Patients

A total of 60 participants were included. All of them were subsequently admitted to the sleep laboratory for the investigation of sleep-related breathing disorders. None of the participants was clinically unstable or referred a relevant not controlled disease. The use of beta-blockers was an exclusion criterion, while there was no further restriction regarding antihypertensive drugs including diuretics, angiotensin converting enzyme inhibitors, or angiotensin-II receptor blocker. Four patients were excluded due to major body movement during the wake/sleep transition, which did not allow enough quality in the hemodynamic data analysis. The final cohort consisted of

Abbreviations: ABP, arterial blood pressure; ABPM, ambulatory blood pressure measurement; AHI, apnea/hypopnea index; AHT, arterial hypertension; AI, arousal index; BP, blood pressure; CVD, cardiovascular disease; d, Cohen's *D* or effect size; HP, hemodynamic parameters; HR, heart rate; MAP, mean arterial blood pressure; ODI, oxygen desaturation index; OSA, obstructive sleep apnea; RDI, respiratory disturbance index; SBP, systolic blood pressure; SCB, standardized correlation coefficient β; SP, sleep period; SRBD, sleep-related breathing disorders; SV, stroke volume; WP, wake period.

23 females and 33 males. Likewise, to other studies, OSA was considered present if the apnea/hypopnea index (AHI) was equal or higher than 15/h (Martinez-Garcia et al., 2013). Participants with an increased percentage of central sleep apneas (≥35%) or periodic leg movement (>15/h) were excluded.

The study was approved by the local Ethical Committee and informed consent was obtained from all participants.

Polysomnographic Sleep Study

Sleep and sleep-related events were investigated via standard polysomnography (PSG) by Alice 5 (Koninklijke Philips N.V. Philips Respironics, Murrysville, PA, United States). The following parameters were recorded: F3, F4, C3, C4, O1, O2, M1, and M2. We used the standard referential montage of scalp electrodes against the contralateral mastoid electrode (e.g., C3/M2). Further parameters consisted of submental electrodes, thermistor and nasal pressure transducer for flow analysis, strain gauges to record respiratory movements, and EMG at both legs according to standard PSG procedures. Peripheral oxygen saturation was analyzed by pulse oximetry. The scoring of sleep and sleep-related events was based on the recommendation of the American Academy of Sleep Medicine from 2012 (Berry et al., 2015). Hypopneas were defined as a 30% decrease in nasal flow during 10 s with a 4% oxygen desaturation (acceptable hypopnea criteria according to the manual of American Academy of Sleep Medicine). This classification results in very high correlation between the AHI and the oxygen desaturation index (ODI), which are therefore interchangeable. Also, the respiratory effort related arousals (RERAs) were scored allowing to distinguish between hypoxic obstructive respiratory events alone and the total amount of obstructive respiratory events including the non-hypoxic one. Additionally, by applying the acceptable hypopnea classification, the correlation between AI and AHI decreases, thus allowing the use of both variables independently in the statistical analysis.

Sleep onset was defined as three subsequent epochs of stable sleep (in all recordings sleep stage N1 or N2). The 5 min of wakefulness prior to the first sleep epoch was considered as the wake period (WP). The following 20 min of sleep after sleep onset was considered as sleep period (SP).

Measurement of Hemodynamic Parameters

The Nexfin HD^{TM} monitor (BMEYE, Netherlands) has been developed to investigate non-invasively arterial BP (ABP), SV, cardiac output (CO), and peripheral vascular resistance (PVR). The method and its advantage against the non-invasive ambulatory ABP measurement (ABPM) in fixed intervals have been previously described (Wesseling, 1996). In short, the arterial pressure is measured in the finger arteries and consecutively reconstructed to brachial artery values. This methodology builds on the volume clamp technique as proposed by Penáz and the physiological criteria of Wesseling to calibrate the ABP measurement (Penaz et al., 1976; Wesseling, 1996). CO and SV are calculated by analyzing the pressure wave via a specific algorithm (de Jong et al., 2009). Frank and colleagues described the original concept already in 1899, which was further developed by Sagawa et al. (1990), Wesseling et al. (1993). This methodology allows a beat-to-beat analysis of the HPs described above. We used the heart reference system of the device to minimalize the impact of postural changes on the HP. In this study, the analyzed HPs were limited to HR, systolic BP (SBP), and SV.

Statistical Analysis of Hemodynamic Changes During the Wake/Sleep Transition

Data from the Nexfin-HD device was exported and further analyzed with the SPSS Statistics software (v.24, IBM Corp., Armonk, NY, United States). Values for HR, SBP, and SV were continuously recorded over the analyzed 25-min period. For each consecutive pulse wave, the corresponding hemodynamic value was plotted in a dot blot graphic (**Figure 1**). A regression analysis was run with each hemodynamic value as the dependent variable and the consecutive number of the pulse wave as the independent variable. This allowed to calculate the scope of the HPs during the time window with a positive value indicating a rise and a negative value a decrease in either HR, BP, or SV. For further statistical analysis of the hemodynamic evolution at sleep onset, we utilized the standardized coefficient β (SCB).

Differences of the hemodynamic variables between wake and sleep were computed by subtraction of the average value during the WP from the average value after falling asleep. To analyze the short-term variance of the HPs, the standard deviation (SD) of each HP during sleep was subtracted from the corresponding value during wakefulness. After controlling for the normal distribution with the Shapiro–Wilk's test ($p > 0.05$), the statistical significance between the control and the OSA group was calculated by the Student's t-test for independent samples and the change of wake–sleep transition by the paired sample Student's t-test. In case of a non-normal distribution of a variable, we applied the non-parametric Mann–Whitney U-test for independent samples or the related samples Wilcoxon signed-rank test, respectively.

A receiver operator characteristic (ROC) analysis was computed to investigate if the SBP values during wakefulness (WP) and sleep (SP) and the mean difference between the two periods can discriminate between the presence or absence of OSA (AHI > 15/h) or sleep fragmentation [arousal index (AI) > 15/h].

Additionally, a simple linear regression analysis was run between the consecutive heart beats or better pulse waves as the independent variable and each corresponding HP as dependent variables. The SCB was calculated and used to analyze the development of the HP during the observation period. A negative SCB is equivalent to a decrease in the investigated parameter, while a positive result indicates an increase during the SP (**Figure 1**).

Furthermore, a hierarchical block multiple linear regression analysis was applied to explore the impact of age, BMI, AHI, and AI during the investigated wake/sleep period, on the linear development of the HPs (increase or decrease) characterized by the SCB.

Sleep efficiency was not included since we found no significant impact on any of the investigated HPs in the initial simple regression analysis (**Table 4**). Model 1 consisted of age and BMI, model 2 added the AHI, and model 3 added the AI to model 2 variables. We choose to add sleep fragmentation after the AHI to investigate a possible increase in the prediction of the

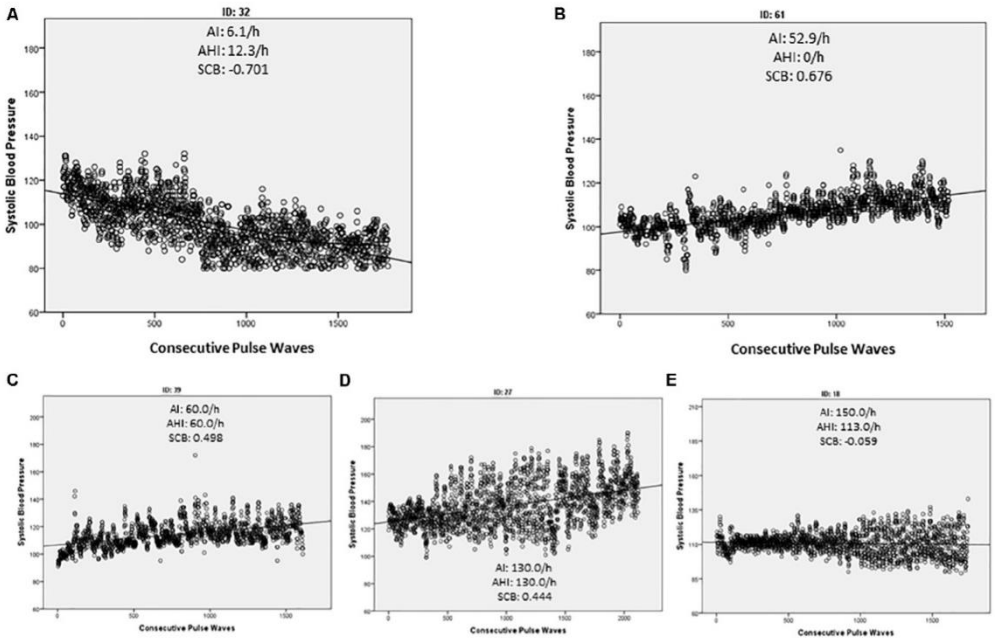

FIGURE 1 | Simple dot blot of each measured pulse wave against the corresponding systolic blood pressure (SBP). The regression line indicates the general direction of the SBP during the observed time period. Arousal index (AI), apnea/hypopnea index, and the calculated standardized coefficient β (SCB) of each demonstrated example are indicated. In controls we found mainly a negative SCB indicating a decrease in the SBP **(A)** while patients with OSA tended to increase the SCB **(C,D)**. However, an increase in the SBP was also detected in the absence of a significant OSA (AHI > 15/h) if sleep fragmentation was present **(B)**. In some OSA patients, the SCB kept stable and only the variance of the SBP increased **(E)**.

hemodynamic values. As stated above, the AHI and ODI were highly correlated and thus only AHI was used in this model.

A binominal logistic regression model was performed to test the prediction quality of a model including age, BMI, and AI for a non-dipping of the HP values at sleep onset. Non-dipping was classified as a SCB ≥ 0 for all three HP variables. A ROC curve was plotted to calculate the overall measure of discrimination [area under the curve (AUC)].

RESULTS

We found in 22 participants an AHI $\geq$ 15 (OSA) and in 34 an AHI < 15/h (controls). Anthropometric and polysomnographic values are listed in **Table 1**. Between controls and OSA, we found no statistically significant difference in either age or body mass index (BMI) ($p > 0.05$). Sleep efficiency, percentage of slow wave sleep (N3), and mean peripheral oxygen saturation (SpO2) were significantly higher in controls (both $p < 0.05$), while the AI, the AHI, and the ODI were significantly increased in the OSA group (all $p < 0.001$). There was no statistically significant difference in the percentage of oxygen saturation under 90% (T90) ($p > 0.05$). Also, no statistically significant gender difference was detected for either AHI or ODI ($p > 0.05$). Within the total study population, we found both AHI and ODI highly correlated ($r = 0.90$; $p < 0.001$). Also, for AI versus AHI ($r = 0.80$; $p < 0.001$) or AI versus ODI ($r = 0.74$; $p < 0.001$), a positive correlation was detected, although to a lower agreement compared to AHI/ODI. Thus, all three parameters were correlated with each other, but AHI and ODI could be considered almost interchangeable.

During the wake-to-sleep transition, almost all HPs changed significantly. The major results are listed in **Table 2**. Besides indicating significant results with an alpha level of 0.05, we also calculated the effect size (Cohen's d) to increase the visibility of possible relevant outcomes. Controls HR and SBP decreased significantly from wakefulness to sleep, but not SV. Contrary to this result, in the OSA group, sleep onset was not associated with any significant change in HP. In both wakefulness and sleep, we detected a significantly higher HR in the OSA group. Interestingly, during wakefulness, neither the SBP nor the SV differed between the two groups. The wake–sleep transition revealed in both groups a significant change for SBP and SV. While in controls both parameters decreased, the evolution was inverse in OSA patients. The effect size of this divergent HP development was high for SBP ($d = 0.92$) and moderate for SV ($d = 0.56$). Thus, only during sleep a highly relevant difference could be seen. Contrary to this, we did not find significant results for HR ($p = 0.49$),

TABLE 1 | Anthropometric and polysomnographic data.

	Age [years]	BMI [kg/m^2]	Sleep-E [%]	N1/N2 [%]	N3 [%]	AI [/h]	AHI [/h]	ODI [/h]	Mean SpO_2	T 90 [%]
Controls *n*: 34	44.91 (±10.0)	36.75 (±9.46)	72.6 (±17.6) *	87. 53 (±19.46) *	12.46 (±19.46) *	21.51 (±20.56) **	3.08 (±5.06) **	5.76 (±14.30) **	94.85 (±1.91) *	1.65 (±6.29)
OSA *n*:22	50.41 (±10.0)	40.92 (±8.18)	62.48 (±13.06)	98.19 (±6.38)	1.80 (±6.38)	75.06 (±36.82)	66.0 (32.67)	76.35 (±34.04)	93.09 (±2.78)	11.78 (22.54)

*Mean values (±standard deviation) of the anthropometric and polysomnographic data. Significant results ($p < 0.05$) are indicated by simple * and highly significant results ($p < 0.001$) with a double **. There was no statistically significant difference in age, BMI, and T 90. BMI, body mass index; Sleep-E, sleep efficiency; N1, N2 sleep stage NI or N2 or light sleep; N3, sleep stage NIII, slow wave sleep; AI, arousal index; AHI, apnea/hypopnea index; ODI, oxygen desaturation index; SpO_2, peripheral saturation of oxygen; T90, percentage of $SpO_2 < 90\%$.*

TABLE 2 | Student's *t*-test and Cohen's *d*: Hemodynamic parameters and its variation during wakefulness and sleep in controls and OSA patients.

	Controls (*SD*) (*n* = 34)	OSA (*SD*) (*n* = 22)	Δ Controls-OSAS (*SD*) significance; effect size
HR wake [/min]	67.51 (10.60)	76.93 (13.12)	−9.42 (−2.52) $p = 0.005$; $d = 0.79$
HR sleep [/min]	66.21 (11.11)	76.22 (12.01)	−10.01 (−0.90) $P = 0.002$; $d = 0.86$
Δ HR wake–sleep [/min] (*SD*); significance; effect size	1.30 (2.97); $p = 0.016$); $d = 0.11$	0.71 (3.27) $p = 0.32$; $d = 0.07$	0.59 (−0.30) $p = 0.49$, $d = 0.19$
SBP wake [mmHg]	116.82 (14.84)	123.51 (19.35)	−6.69 (−4.51) p = 0.150; d = 0.39
SBP sleep [mmHg]	113.09 (15.40)	126.78 (18.80)	−13.69 (−3.4) $p = 0.004$; $d = 0.80$
Δ SBP wake–sleep [mmHg] (*SD*); significance, effect size	3.74 (7.50); $p = 0.007$; $d = 0.25$	−3.26 (7.62) $p = 0.058$); $d = 0.02$	7.0 (−0.12) $p = 0.001$; $d = 0.92$
SV wake [ml]	100.23 (14.41)	99.20 (25.67)	1.03 (−11.26) $p = 0.85$; $d = 0$ 0.05
SV sleep [ml]	98.86 (14.65)	100.75 (24.38)	−1.89 (−9.73) $p = 0.72$; $d = 0.09$
Δ SV wake–sleep [ml] (*SD*); significance, effect size	1.37 (5.52) $p = 0.158$; $d = 0.09$	−1.55 (4.78) $p = 0.14$; $d = 0.06$	2.92 (0.74) $p = 0.047$; $d = 0.56$
HR SD wake [/min]	6.40 (3.69)	5.97 (3.80)	0.43 (−0.11) $p = 0.681$; $d = 0.11$
HR SD sleep [/min]	4.69 (2.05)	7.27 (3.59)	−2.58 (−1.54) $p < 0,001$; $d = 0.88$
Δ HR SD wake–sleep [/min] (*SD*); significance; effect size	1.70 (3.07) $p = 0.003$; $d = 0.57$	−1.75 (2.67) $p = 0.006$; $d = 0.35$	3.45 (0.4) $p < 0,001$; $d = 1.20$
SBP SD wake [mmHg]	8.20 (3.26)	9.04 (2.63)	−0.83 (0.63) $p = 0.32$; $d = 0.28$
SBP SD sleep [mmHg]	7.83 (2.35)	11.73 (3.48)	−3.90 (1.13) $p < 0.001$; $d = 1.31$
Δ SBP wake–sleep [mmHg] (SD); significance; effect size	0.373 (2.89) $p = 0.46$); $d = 0.13$	−2.69 (3.51) $p = 0.002$; $d = 0.87$	3.06 (−1.86) $p = 0.001$; $d = 0.94$
SV SD wake [ml]	8.62 (4.54)	13.97 (21.53)	−5.35 (−16.99) $p = 0.16$; $d = 0.34$
SV SD sleep [ml]	7.04 (2.77)	15.32 (21.61)	−15.28 (−18.84) $p = 0.03$; $d = 0.54$

(Continued)

TABLE 2 | Continued

	Controls (*SD*) (*n* = 34)	OSA (*SD*) (*n* = 22)	Δ Controls-OSAS (*SD*) significance; effect size
Δ SV wake–sleep SD [ml] (*SD*); significance, effect size	1.58 (3.92) $p = 0.025$; $d = 0.42$	−1.34 (3.15) $p = 0.058$; $d = 0.06$	2.92 (0.77) $p = 0.005$; $d = 0.82$

Average (±standard deviation) of the hemodynamic values and the mean standard deviation (SD) in controls and OSA patients during wakefulness (wake) and sleep. Significance values and the effect size (Cohen's d) were calculated between wakefulness and sleep for each group and between controls and OSA. Δ, difference (either wake/sleep or controls/OSA); SD, standard deviation; d, Cohen's d; HR, heart rate; SBP, systolic blood pressure; SV, stroke volume.

and the effect size between the two groups was very small ($d = 0.19$).

The assessment of the HP variability was performed by analyzing SDs of the mean values during wakefulness and sleep. In controls, sleep onset was not accompanied by any significant change in the SBP variance ($p > 0.05$). Contrary to this, we observed a significant decrease in the SD of HR and SV ($p = 0.003$ and 0.025, respectively) with a moderate effect size ($d = 0.57$ and 0.42, respectively). This reflects the physiological stabilization of the HP during sleep. In the OSA group, SD increased significantly in both HR and SBP ($p = 0.006$ and $p = 0.002$, respectively) but not in SV ($p = 0.058$). The effect size was very small for SV ($d = 0.06$), small to moderate for HR ($d = 0.35$), and high in SBP ($d = 0.87$). In the comparison of changes in the HP during sleep onset, statistically significant results were detected for all three HPs. The calculated effect size was huge for the HR exceeding one SD ($d = 1.20$) and large for SBP ($d = 0.94$) and SV ($d = 0.82$). Therefore, analyzing each group isolated, sleep onset caused only a mild to moderate change in the HP. However, since the direction was opposed (decrease in controls and increase in OSA), we found highly significant results when comparing the two groups.

To further investigate the relationship between OSA and HP during sleep, we run a receiver operator curve (ROC) analysis. SBP values discriminated between the presence and absence of OSA during sleep with an AUC of 0.745 (95% CI, 0.616–0.873; $p < 0.001$) with an overall model accuracy of 0.62. However, during wakefulness, the AUC for the SBP value was 0.596 (95% CI, 0.442–0.751), and the result did not reach statistical significance ($p = 0.222$). Since SBP during sleep revealed a discriminative capability for OSA versus non-OSA, we furthermore investigated the relevance of the change in SBP values (wake SBP values minus sleep SBV values). The AUC was 0.74 (95% CI, 0.61–0.87), indicating an acceptable discrimination. The result reached statistical significance ($p < 0.001$) with an overall model quality of 0.61. When the same analysis was run for the presence of sleep fragmentation defined as an AI > 15/h, the AUC for the SBP during sleep was 0.79 (95% CI, 0.64–0.94). On the other hand, during wakefulness, the result was not significant. The AUC for the wake–sleep difference of the SBP was 0.79 (95% CI 0.67–0.91; $p < 0.001$) and therefore good discrimination with an overall model accuracy of 0.67. The paired sample of the wake–sleep area difference under the ROC curves of the SBP reached a z score of −3.17 with an AUC difference of −0.154 (CI, −2.49 – 0.59), which reached statistical significance ($p = 0.002$). These results indicate that SBP values and the change of the SBP in the first 20 min of sleep discriminate between the presence or absence of OSA. This was even more evident for the presence or absence of sleep fragmentation.

Each measured pulse wave was plotted beat-to-beat against the value of the investigated HP (**Figure 1**). As described in the "Materials and Methods" section, the SCB was calculated to assess the continuous evaluation of the HPs during the investigated period. Controls demonstrated a negative SCB and thus a reduction in all HPs (**Table 3** and **Figure 1A**). The effect was more visible in the SBP values compared to HR or SV. In the OSA group, the decline of the BP values was attenuated or inverted, although this was also visible in patients with sleep fragmentation without significant OSA (**Figure 1B**). These results reached statistical significance for SBP ($p = 0.031$) with a small to moderate effect size ($d = 0.32$) and SV ($p = 0.033$) with a medium effect size ($d = 0.62$). Results are listed in **Table 3**. It is of interest that we found considerable variation of the HP changes at sleep onset even in individuals with severe OSA. In **Figure 1C**, wake–sleep transition is accompanied by an increase in the SBP but almost without any change in variability. The participant in **Figure 1D** reveals an increase in both SBP and its variability. In **Figure 1E**, only SBP variability increases while SCB indicates a small decrease in SBP.

To investigate the effect of anthropometric and sleep variables on the progression of the HPs, we performed a simple regression analysis with HR, SBP, and SV as dependent variables and age, BMI, AHI, AI, and sleep efficiency (Sleep-E) as independent variables. The main results are illustrated in **Table 4**. Sleep-E was ruled out for further analysis since no significant result was detected. Age reached statistical significance for HR and just exceeded the alpha level for SBP ($p = 0.073$). BMI did not reach statistical significance for any of the three investigated HPs, but due to its correlation with the AHI (r:0.256; $p = 0.057$), it was kept for further analysis.

A hierarchical multiple regression analysis was run to investigate if prediction of hemodynamic development measured by the SCB was improved by adding to age and weight (model 1)

TABLE 3 | Standardized coefficient β of consecutive pulse waves versus hemodynamic parameter.

	Controls *n*: 34 Average (±SD)	OSA *n*:22 Average (±SD)	*P*-value	*d*
HR SCB	−0.01 (0.28)	−0.06 (0.23)	0.483	0.19
SBP SCB	−0.16 (0.38±)	0.05 (±0.29)	0.031	0.32
SV SCB	−0.07 (±0.29)	0.09 (0.22)	0.033	0.62

Average (standard deviation) of the standardized coefficient β (SCB), which was calculated by regression analysis of the measured hemodynamic value against each consecutive pulse wave. Controls demonstrated a negative SCB and thus decline of heart rate (HR), systolic blood pressure (SBP), and stroke volume (SV) during the 25-min period. In the OSA group, this decrease was attenuated or in case of stroke volume even inverted. The detected significance values and the effect size (d = Cohen's d) are listed in the last two columns.

TABLE 4 | Simple regression analysis of anthropometric and sleep variables on the standardized coefficient β (SCB) of the hemodynamic parameters.

	SCB HR			SCB SBP			SCB SV		
	B (CI 95)	**β**	***P***	**B (CI 95)**	**β**	***P***	**B (CI 95)**	**β**	***P***
Age [years]	−0.007 (−0.014 to 0.000)*	−0.268	0.046	0.008 (−0.001 to 0.018)	0.242	n.s.	0.002 (−0.006 to 0.009)	0.07	n.s.
BMI [kg/m^2]	−0.001 (−0.008 to 0.008)	−0.021	n.s.	−0.005 (−0.016 to 0.005)	−0.147	n.s.	−0.005 (−0.013 to 0.003)	0.162	n.s.
AHI [/h)	−0.001 (−0.003 to 0.001)	−0.194	n.s.	0.003 (0.00 to 0.005)	0.296	0.027	0.003 (0.001–0.005)	0.347	0.005
AI [/h)	−0.001 (−0.003 to 0.001)	−0.106	n.s.	0.004 (0.002 to 0.007)	0.476	0.007	0.003 (0.002 to 0.005)	0.451	<0.001
Sleep-E [%]	0.001 (−0.003 to 0.005)	0.064	n.s.	−0.002 (−0.008 to 0.004)	−0.079	n.s.	0.000 (−0.004 to 0.005)	0.023	n.s.

Simple correlation body mass index (BMI); apnea/hypopnea index (AHI), arousal index (AI), and sleep efficiency (Sleep-E) with standardized coefficient β (SCB) of heart rate (HR), systolic blood pressure (SBP), and stroke volume (SV). The p-values for the statistically significant results are displayed in the third column of each hemodynamic parameter. Age negatively correlated with the HR evolution, but statistical significance was low. Both AHI and AI were positively correlated with an increase in the SBP and SV.

TABLE 5 | Hierarchical regression analysis of anthropometric and sleep variables for the hemodynamic standardized coefficient β prediction of heart rate.

	Standardized coefficient β (SCB) of Heart Rate (HR)					
Variable	**Model 1**		**Model 2**		**Model 3**	
	B (95% CI)	**β**	**B (95% CI)**	**β**	**B (95% CI)**	**β**
Constant	0.237 (−0.143 to 0.617)		0.202 (−0.081 to 0.585)		0.166 (−0.239 to 0.571)	
Age	−0.008 (−0.015 to 0.000)	−0.271	−0.007 (−0.014 to 0.00)	−0.271	−0.007 (−0.014 to 0.001)	0.264
BMI	0.002 (0.006 to 0.011)	0.083	−0.001 (−0.005 to 0.00)	0.115	0.004 (−0.005 to 0.012)	0.124
AHI			−0.001 (−0.003 to 0.001)	−0.162	−0.002 (−0.005 to 0.001)	0.138
AI					0.001 (−0.002 to 0.004)	0.138
	Model 1		**Model 2**		**Model 3**	
R^2	0.078		0.319		0.328	
F	2.236 (n.s.)		1.962 (n.s.)		1.54 (n.s.)	
ΔR^2			0.024		0.006	
ΔF			1.383 (n.s.)		0.348 (n.s.)	

Hierarchical regression analysis of the SCB from heart rate. None of the models reached statistical significance. BMI, body mass index; AHI, apnea/hypopnea index; AI, arousal index; Sleep-E, sleep efficiency.

TABLE 6 | Hierarchical regression analysis of anthropometric and sleep variables for the hemodynamic standardized coefficient β prediction of systolic blood pressure.

	Standardized coefficient β (SCB) of Systolic Blood Pressure (SBP)					
Variable	**Model 1**		**Model 2**		**Model 3**	
	B (95% CI)	**β**	**B (95% CI)**	**β**	**B (95% CI)**	**β**
Constant	−0.229 (−0.737 to 0.280)		−0.136 (−0.628 to 0.357)		−0.392 (−0.857 to 0.073)	
Age	0.012 (0.002 to 0.021) *	0.334	0.010 (0.001-0.019	0.284	0.011 (0.003 to 0.020) *	0.321
BMI	−0.010 (−0.021 to 0.000)	−0.264	−0.013 (−0.024 to −0.002) *	−0.327	−0.011 (−0.021 to −0.001) *	−0.279
AHI			0.003 (0.001 to 0.006) *	0.314	−0.003 (−0.007 to 0.001)	−0.295
AI					0.007 (0.003 to 0.010) *	0.717
	Model 1		**Model 2**		**Model 3**	
R^2	0.345		0.458		0.373	
F	3.59 (p = 0.034)		4,595 (p = 0.006)		7.572 (p < 0.001)	
ΔR^2			0.09		0.163	
ΔF			5.935 (p = 0.018)		13.257 (p = 0.001)	

*Hierarchical regression analysis of the SCB of systolic blood pressure. In model 1 age reached statistical significance (p = 0.019). In model 2 age (p = 0.038), BMI (p = 0.018), and AHI (p = 0.018) were statistically significant. There was a significant change compared to model 1. In model 3, age (p = 0.010), BMI (p = 0.026), and arousal index (p = 0.001) were statistically significant. R^2 $F(1.51)$ increased significantly from model 2 to model 3. *p < 0.05.*

TABLE 7 | Hierarchical regression analysis of anthropometric and sleep variables for the hemodynamic standardized coefficient β prediction of stroke volume.

	Standardized coefficient β (SCB) of Stroke Volume (SV)					
Variable	**Model 1**		**Model 2**		**Model 3**	
	B (95% CI)	**β**	**B (95% CI)**	**β**	**B (95% CI)**	**β**
Constant	0.58 (−0.352 to 0.469)		0.151 (−0.235 to 0.536)		0.021 (−0.37 to 0.412)	
Age	0.004 (−0.004 to 0.012)	0.144	0.002 (−0.005 to 0.010)	0.081	0.003 (−0.004 to 0.01)	0.105
BMI	−0.007 (−0.015 to 0.002)	−0.213	−0.009 (−0.017 to −0.001) *	−0.294	−0.008 (−0.016 to 0.00)	−0.263
AHI			0.003 (0.001 to 0.005) *	0.404	0.0004 (−0.003 to 0.003)	0.005
AI					0.003 (0.00 to 0.007) *	0.469
	Model 1		**Model 2**		**Model 3**	
R^2	0.045		0.194		0.263	
F	1.240 (n.s.)		4.161 (p = 0.01)		4.558 (p = 0.003)	
ΔR^2			0.149		0.07	
ΔF			9.599 (p = 0.003)		4.83 (p = 0.033)	

*Hierarchical regression analysis of the SCB of stroke volume. Both BMI and AHI reached statistical significance in model 2 (p = 0.035 and p = 0.003, respectively). R^2 F(1.52) changed significantly when compared to model 1. In model 3, only the arousal index remained statistically significant (p = 0.033), and we found a significant increase in R^2 F(1,51) when compared to model 2. *$p < 0.05$.*

the AHI (model 2) and the AI (model 3). There was independence of residuals with the Durbin–Watson statistic of 2.337 for HR, 2.00 for SBP, and 1.60 for SV, respectively. Multicollinearity was ruled out as assessed by tolerance values above 0.2 (with a minimum of 0.297 for AHI).

For HR, none of the models increased significantly the prediction of the SCB. When analyzing the SCB of SBP, all three models reached statistical significance in the regression ANOVA analysis. However, the full model 3 of age, BMI, AHI, and AI eventually was the most robust one with R^2 = 0.373; $F(4.51) = 7.572$, $p < 0.001$; adjusted R^2 = 0.323. The addition of the AI to the prediction model 2 (age, BMI, and AHI) resulted in a significant increase in R^2 by 0.163 and $F(1.51)$ of 13.257 ($p = 0.001$). For SV, we found models 2 and 3 reaching statistical significance. For model 2 with an R^2 = 0.194; $F(3.52)$ = 4.16, p = 0.01; adjusted R^2 = 0.147, the R^2 increased 0.149; $F(1/52)$ 9.599. In model 3, R^2 reached a value of 0.206; $F(4.51)$ 4.56, p = 0.003 and adjusted R^2 = 0.206. R^2 increased by 0.07 with $F(1.51)$ 4.83, p = 0.033. Thus, the impact of the AI on the SCB of the SV was less clear compared to the results seen for SBP. The main results are listed in **Tables 5**–**7**.

To confirm our assumption that AHI and ODI are interchangeable in this analysis, we recalculated model 3 with either the respiratory disturbance index (RDI) or ODI instead of AHI. As shown in **Supplementary Table 1**, in both cases, only the AI was significant.

Furthermore, a binominal logistic regression analysis was run to investigate the capability of a model with age, BMI, AHI, and AI to predict non-dipping of HP, defined as SCB $\geq$ 0. We found no statistically significant results for HR. Non-dipping in SBP was detected in 46% of the study population and therefore higher than the overall percentage of OSA patients (39.28%). The regression model was statistically significant [$\chi^2(4) = 29.91$; $p < 0.001$; Nagelkerke R^2 = 0.54]. All four parameters reached significant results with lower values for BMI ($B = -0.103$, SE = 0.52; p = 0.045) and AHI ($B = -0.048$, SE = 0.02; P = 0.03) and higher for age (B = 0.170, SE = 0.05; p = 0.001) and AI (B = 0.042; SE = 0.02; p = 0.010). The odds ratio was 1.19 (95% CI: 1.07–1.31) for age, 0.90 for BMI (95% CI: 0.81–1.00), 1.073 for AI (95% CI: 1.02–1.12), and 0.95 for AHI (95% CI: 0.91–0.99), respectively. The overall correct prediction for the non-dipping of SBP was 78.6% with a sensitivity to identify non-dipper of 76.9% and a specificity of 80%. The positive predictive value was 66.7% with a negative predictive value of 76.9%. The ROC curve for the total model demonstrated an excellent AUC of 0.88 (95% CI: 0.78–0.97). When running the analysis only with the AI, the AUC reached a value of 0.72 (95% CI: 0.59–0.86), which can be considered an acceptable discrimination (**Figure 2**).

Regarding SV, we that found that, in 51.8% of the total population, the SCB of SV was equal to or superior to 0, indicating non-dipping. The regression model for non-dipping in SV reached statistical significance [$\chi^2(4) = 11.19$; $p < 0.025$, Nagelkerke R^2 = 0.242]. However, only the BMI was a statistically significant (p = 0.048) predictor in the model.

DISCUSSION

In this study, we investigated the impact of OSA on the HPs during the wake–sleep transition. Conventionally, changes of BP from wake to sleep are investigated comparing the averages of selected periods within each state. We used this method to confirm our study protocol, but our main objective was to evaluate the impact of respiratory events and sleep fragmentation on the evolution of the recorded HP at sleep onset. This approach allows a more precise knowledge of short-term hemodynamic consequences of OSA at sleep onset and the effect of sleep disruption by respiratory events. As a major finding, we could detect that sleep fragmentation by arousals correlated better with the SBP evolution at sleep onset than the AHI.

Khatri and Freis. (1967) were one of the early groups to describe a decrease in ABP at sleep onset and a reduction in the

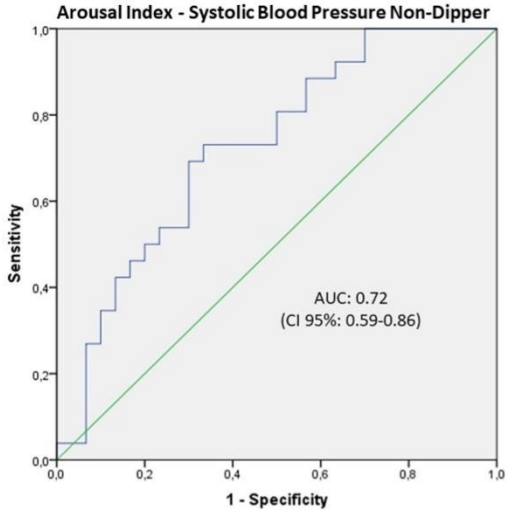

FIGURE 2 | ROC curve of the arousal index to separate dipping and non-dipping systolic blood pressure (SBP). There was an acceptable discrimination by the AUC of 0.72 (95%CI: 0.59–0.86), which indicates that sleep fragmentation can be used to identify patients with non-dipping blood pressure and therefore cardiovascular risk.

CO due to a decreased HR. However, in this study, ABP was investigated by an intra-arterial catheter. Due to its invasiveness, this method has been, in general, replaced by a beat-to-beat measurement via the volume clamp methodology (Marrone and Bonsignore, 2018). We used the Nexfin-HD monitor in this study, which has been thoroughly investigated in several clinical settings (Fischer et al., 2012; Bubenek-Turconi et al., 2013; Pouwels et al., 2017).

When compared to the study of Khatri and Freis. (1967), our control group demonstrated a similar development of the HP. The averages between wake and sleep period demonstrated a significant decrease in HR, while the SV remained unchanged. The reduction in the SBP has been previously described by other groups and is linked to a reduction in the sympathetic-nerve activity (Somers et al., 1993). It is notable, that in our control group neither HR nor SBP reached the described reduction between 10 to 20% compared to wakefulness indicated by several authors (Silvani and Dampney, 2013 #4419; Cuspidi et al., 2018 #4505) including a joint recommendation of the International Society for Chronobiology (ISC), American Association of Medical Chronobiology and Chronotherapeutics (AAMCC), Spanish Society of Applied Chronobiology, Chronotherapy, and Vascular Risk (SECAC), Spanish Society of Atherosclerosis (SEA), and Romanian Society of Internal Medicine (RSIM) (International Society for Chronobiology et al., 2013 #4522). However, in Khatri's study, the mean arterial pressure also reached a maximum reduction of only 8.5% when analyzed during slow-wave sleep (Khatri and Freis., 1967). Positional changes of the arm might be considered a risk factor for unreliable ABP measurement by the volume-clamp method. Leroy and colleagues found in a study with a Finapres volume-clamp device that, in fact, hand movements had little relevance on the final ABP results (Leroy et al., 1996). The more modern Nexfin-HD monitor automatically smoothens the impact of hydrostatic variation in the ABP pressure due to positional changes by means of an integrated heart reference system. Nevertheless, we excluded four participants due to large body movements and therefore a decrease in the reliability of the ABP measurement. The measured HP averages in our control group at wake–sleep transition are in accordance with other studies supporting the adequacy of our method.

A blunted hemodynamic response to sleep onset is considered a risk factor for CVDs (Hermida et al., 2013). We could show that no significant wake–sleep variation of HR, SBP, or SV occurred in the OSA group. The relevance of OSA for the existence of non-dipping ABP was recently described in the Wisconsin Sleep Cohort Study. Mokhlesi et al. (2015) found in OSA patients an increased risk of 2.84 (95% CI 1.10–7.29) for non-dipping ABP in REM sleep when compared to the control group. Our study was not designed to evaluate the overall dipping of the hemodynamic values during sleep. Nevertheless, it might be of interest that the detected effect size of OSA was moderate in case of the SV and large for SBP. These results support therefore the observation that the presence of OSA has an important impact on the non-dipping of HP during sleep. In this study, the ROC curve analysis revealed that SBP values during sleep and the wake–sleep difference of the mean SBP had acceptable discriminating capability to identify OSA patients. However, SBP was an even better discriminator for the presence of sleep fragmentation even at a relatively low value of 15 arousals/h of sleep. A missing reduction of the SBP is associated with an increase in the total cardiovascular risk (Seif et al., 2014) and can cause preclinical cardiac damage (Cuspidi et al., 2018). Therefore, our study underlines the importance to diagnose OSA in an early state of the disease to avoid possible cardiovascular side effects, which can be prevented by adequate therapy (Sapina-Beltran et al., 2019).

An increased ABP variability is considered a risk for target organ damage (Stamatelopoulos et al., 2010). In an early study, Leroy et al. (1996) demonstrated a higher ABP variation in OSA patients when compared to controls. Narkiewicz et al. (1998) reproduced this result even during wakefulness. In our study population, ABP variation during wakefulness did not differ statistically significantly between the two investigated groups. Contrary to this, with sleep onset, the variation of all HP values decreased in the control group, while OSA patients manifested the opposite reaction. Besides reaching statistical significance, the calculated effect size between the two groups was either large (SV) or very large (HR and SBP) confirming the relevance of this result.

It is difficult to compare the results between beat-to-beat and ambulatory BP measurements (ABPMs). Evidence indicates that besides the increased short-term BP variability, the midterm ABP variability investigated by ABPM is also increased in OSA (Martynowicz et al., 2016). Ke et al. (2017) associated OSA-related enhanced SBP variability in ABPM recordings with an increased risk of CVDs. In our study, the short-term HP variability was the most significant difference between controls

and OSA patients. Indeed, in some OSA patients, the HP variability was the only value that changed following sleep onset. It can be concluded that OSA has, at least during sleep, a negative impact on the variation of HP. Nevertheless, a cause–effect relationship regarding CVDs needs to be further established.

To our knowledge, we are the first to investigate the influence of sleep on the HP progress by the SCB. The advantage of this method is that a successive evolution of the HP is investigated and not only a selected representative episode. Hence, the physiological progress of the HP at sleep onset is better reflected, and short periods of wakefulness or body movement have, if at all, only little influence on the results. The SCB mirrored the results detected from the sleep/wake period analysis. Controls displayed negative SCB values corresponding to the physiological decrease of HP during sleep. The OSA group exhibited an attenuation of the SCB value for SBP and reached a positive value for the SV. This reflects the impact of OSAs on the two HPs. The HR was, in agreement with the previously discussed results, not significantly different between the two groups.

Interestingly, both the simple and the hierarchical regression analyses revealed for sleep fragmentation a higher relevance concerning the ABP evolution than the AHI itself. The impact of arousals on ABP changes has been intensively investigated in previous studies. Already Somers et al. (1995) showed that OSA-induced arousals caused significant changes in the ABP. Morgan et al. (1996) found that cortical arousals induced by auditory stimuli induces a cardiovascular response leading to an increase in the ABP and HR and a decrease in the CO. Several other forms of arousals without relevant flow limitation and/or oxygen desaturations can induce transitory ABP swings (Ali et al., 1991; Davies et al., 1993; Lofaso et al., 1998). Thus, the stimulation of the central nervous system visible as acceleration in the EEG frequency induces a direct and instantaneous reaction of the cardiovascular system. Ringler et al. (1990) described that the mean arterial BP (MAP) reacted equally to hypoxic apneas as to apneas without desaturations due to oxygen supplementation. Also, acoustic arousals triggered the same hemodynamic response as hypoxic apneas, while chronic hypoxia at 80% did not induce any change in the MAP (Ringler et al., 1990). At present, it is still under discussion if arousals without respiratory events especially without relevant change in the oxygen tension can provoke a longer lasting increase in the SBP. Morrell et al. (2000) described in a cross-sectional analysis of the Wisconsin Sleep Cohort study that non-apneic participants with increased sleep fragmentation index revealed an elevated ABP when compared to controls. Stradling et al. (2000) investigated if sleep variables could predict the evening and morning ABP difference. In this study, only the ODI and the respiratory effort demonstrated a relevant independent impact on the decrease in the evening/morning ABP difference. ABP arousals measured by the pulse transient time (PTT) did not reach statistical significance, while the respiratory effort and ODI together explained 7–10% of the ABP variation (Stradling et al., 2000). We investigated the SCB of the HP instead of the evening/morning difference over a shorter time period but with continuous measurement. Nevertheless, model 2 of the hierarchical regression analysis including age, BMI, and AHI accounted for 21% variation of the systolic ABP SCB variation. By addition of the AI, we found a significant increase in the predictive value of the model to 37.3% (**Table 6**). In accordance to our results, Carrington and colleagues could show that artificial sleep fragmentation during 20 min after sleep onset reduced the physiological ABP decrease (Carrington and Trinder, 2008). In another study, Taylor et al. (2016) investigated the impact of several sleep parameters on the daytime sympathetic activity. They found that the sympathetic discharges correlated best with the AI followed by the ODI and discussed if sleep fragmentation by any cause could increase the probability of cardiovascular events (Taylor et al., 2016). Noda et al. (2000) detected in an ABPM study that movement arousals were the most important factor for elevated 24-h ABP values followed by the ODI. It is of importance that our study was designed to use the AHI practically interchangeable with the ODI. We controlled this assumption by using the ODI instead of the AHI in the statistical analyses and found an identical outcome. Taken alone, the AHI increases significantly the predictive value compared to age and BMI alone. Only when the AI was joined in the model 3 AHI lost its relevance in the regression model (**Table 6**). Sleep fragmentation due to increased respiratory effort seems unlikely to explain this result, since the substitution of RDI instead of AHI did not reveal a different result.

The relationship between SBP and sleep fragmentation was also confirmed in the ROC analysis of the SBP during the wake and sleep period. The mean SBP values during the SP and the difference of the mean SBP during the wake and sleep period demonstrated a good discrimination accuracy for the presence of sleep fragmentation. Recently, Chenini et al. (2019) described for a group of restless legs patients that the leg movement arousals were better associated with the presence of non-dipping BP than the restless legs index by itself. This study emphasizes that arousals induced by sleep-related respiratory or movement events are important for the investigation of non-dipping BP.

Interestingly, sleep efficiency had no impact at all in our model of the HP analysis. There is some evidence that both short sleep duration and insomnia might decrease cardiovascular health especially due to AHT (Anttalainen et al., 2018; Jarrin et al., 2018). However, our protocol only analyzed the percentage of wakefulness in the first 20 min of sleep. It is possible that the investigated period was too short to cause any important effect on the nocturnal HP.

There are some limitations in our study. First, the study population is relatively small. However, most of the studies using the beat-to-beat measurement technique and those cited above included a substantial smaller number of participants than ours. At present, only ABPM studies manage recruitments of large cohorts to investigate the relationship between OSA and nocturnal BP. Another possible confounding factor consists in the fact that the inflated cuff of the Nexfin/HD monitor could be responsible for sleep fragmentation. This might be particularly true in patients with a low arousal threshold, which are more commonly found in the control group. However, we cannot exclude the possibility that discomfort increased the BP or induced the artificial sleep fragmentation. The overall HP development in the control group is not in accordance to this

hypothesis. The volume clamp method has been successfully tested in several sleep studies, and in our experience, the complaints about the cuff pressure started after a measurement period exceeding the one used in this study protocol. The definition of OSA in this study was an AHI of ≥15/h. We cannot rule out that in the control group, there exists a clinically relevant OSA with an AHI between 5 and 15 events/h sleep. Nevertheless, the aim of this study was to investigate which parameters influence the development of the HP at sleep onset, not OSA as a disease. Therefore, like in other studies, the criterion C of the international classification of sleep disorders was applied (American Academy of Medicine,, 2014; Genta-Pereira et al., 2018).

CONCLUSION

In this study, we investigated in patients with OSA the evolution of three HPs: SBP, SV, and HR during sleep onset. In the OSA group, both SBP and SV increased during sleep resulting in a significant distinction compared to the control group. This finding reached a moderate to high effect size confirming the relevance of the results. HP short-term variation of the three parameters was significantly higher in OSA patients with either a large (SBP and SV) or very large (HR) effect size. In the ROC curve analysis, nocturnal SBP and wake–sleep BP difference demonstrated a higher AUC for sleep fragmentation than for OSA. Analysis of the HP evolution at sleep onset by means of the standardized correlation coefficient β (SCB) confirmed an opposed evolution pattern between controls and OSA patients with increasing SBP and SV in the latter group. The AI as a marker of sleep fragmentation was identified as the major contributor for HP evolution and the development of the non-dipping pattern of the SBP in OSA patients.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Comissão de Ética do Centro Académico de Medicina de Lisboa (CAML), Lisbon, Portugal. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RS was the project leader and main author. IB analyzed the data. DF and DG were involved in the data acquisition. CR, FM, and JV reviewed the data. JM gave support in statistical analysis. AA and CB were project supervisors and were involved in the interpretation of the data. All the listed authors contributed substantially to this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00104/full#supplementary-material

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4.4 Study 4

Obstructive sleep apnoea independently predicts lipid levels: Data from the European Sleep Apnea Database.

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ORIGINAL ARTICLE

Obstructive sleep apnoea independently predicts lipid levels: Data from the European Sleep Apnea Database

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ABSTRACT

Background and objective: **Obstructive sleep apnoea (OSA) and dyslipidaemia are independent risk factors for cardiovascular disease. This study investigates the association between OSA and plasma lipid concentrations in patients enrolled in the European Sleep Apnea Database (ESADA) cohort.**

Methods: **The cross-sectional analysis included 8592 patients without physician-diagnosed hyperlipidaemia or reported intake of a lipid-lowering drug (age 50.1 ± 12.7 years, 69.1% male, BMI: 30.8 ± 6.6 kg/m^2, mean apnoea–hypopnoea index (AHI): 25.7 ± 25.9 events/h). The independent relationship between measures of OSA (AHI, oxygen desaturation index (ODI), mean and lowest oxygen saturation) and lipid profile (total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and fasting triglycerides (TG)) was determined by means of general linear model analysis.**

Results: **There was a dose response relationship between TC and ODI (mean ± SE (mg/dL): 180.33 ± 2.46, 184.59 ± 2.42, 185.44 ± 2.42 and 185.73 ± 2.44; $P < 0.001$ across ODI quartiles I–IV). TG and LDL concentrations were better predicted by AHI than by ODI. HDL-C was significantly reduced in the highest AHI quartile (mean ± SE (mg/dL): 48.8 ± 1.49 vs 46.50 ± 1.48; $P = 0.002$, AHI quartile I vs IV). Morbid obesity was associated with lower TC and higher HDL-C values. Lipid status was influenced by geographical location with the highest TC concentration recorded in Northern Europe.**

Conclusion: **OSA severity was independently associated with cholesterol and TG concentrations.**

Key words: cholesterol, dyslipidemia, hypoxia, obesity, sleep apnoea.

SUMMARY AT A GLANCE

The present study examines the association of obstructive sleep apnoea (OSA) and dyslipidaemia in the large multicentre European Sleep Apnea Database cohort. There was an independent relationship between OSA severity and lipid concentrations which was influenced by geographical region and measures of central obesity.

Abbreviations: , AHI, apnoea–hypopnoea index; CIH, chronic intermittent hypoxia; CPAP, continuous positive airway pressure; ESADA, European Sleep Apnea Database; ESS, Epworth Sleepiness Scale; GLM, generalized linear regression model; HDL-C, high-density lipoprotein cholesterol; IHD, ischaemic heart disease; LDL-C, low-density lipoprotein cholesterol; ODI, oxygen desaturation index; OSA, obstructive sleep apnoea; PG, polygraphy; PSG, polysomnography; SDB, sleep disordered breathing; SpO_2, arterial oxygen saturation measured by pulse oximetry; TC, total cholesterol; TG, triglyceride; TIA, transient ischaemic attack; WHR, waist-to-hip ratio.

INTRODUCTION

Obstructive sleep apnoea (OSA) is characterized by repeated episodes of apnoeas and hypopnoeas during sleep affecting at least 20% of male and 10% of female

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adults in a clinically relevant manner.[1,2] OSA is more prevalent in patients with cardiovascular co-morbidity, in particular systemic hypertension and coronary heart disease.[3,4] Previous studies have emphasized metabolic dysfunction as an important driver of cardiovascular disease in subjects with OSA. Independent associations between OSA and impaired glycaemic health as well as insulin resistance were reported.[5,6] A role of OSA in the development of dyslipidaemia has been implied in rodent models and cohort clinical studies but the existence of an independent relationship beyond the effects of obesity remains unclear.[7–9]

Sleep fragmentation and chronic intermittent hypoxia (CIH) in OSA cause activation of the sympathetic nervous system, increased oxidative stress and systemic inflammation,[10] mechanisms that may promote dyslipidaemia. A recent animal study suggested that CIH stimulates the generation of sterol regulatory element binding protein-1 and stearoyl coenzyme A desaturase-1, and is associated with increased total cholesterol (TC) concentration.[11] Furthermore, the population-based Sleep Heart Health Study cohort demonstrated the association of severe OSA with higher TC and triglyceride (TG) concentration, particularly in males aged 65 years and below.[8] A relationship between the severity of CIH, a higher TG concentration and a lower high-density lipoprotein cholesterol (HDL-C) concentration has also been described in a large prospective OSA cohort.[12] In contrast, other systematic reviews[9,13] have not provided definite support for an independent association between OSA and lipid concentration.

Both OSA and dyslipidaemia are associated with measures of central obesity and in OSA, the association is positive and linear. However, lower levels of TC have been reported in patients with morbid obesity compared with less obese individuals[14] and the term 'obesity paradox' has been used to describe this phenomenon.[15] Body weight or BMI as continuous variables in a regression analysis may not accurately reflect a possible non-linear relationship between obesity and cholesterol concentration. Differences in regional dietary habits or genetics may also influence the variability in cholesterol concentration, independent of OSA severity. Indeed, prevalence data of hypercholesterolaemia in Europe and Asia suggest significant regional differences[16] which may explain the conflicting results of previous studies addressing the association between OSA and dyslipidaemia.

The European Sleep Apnea Database (ESADA) cohort study is a multicentre, multinational study which prospectively recruits patients investigated for suspected OSA in European sleep laboratories. The aim of the current analysis was to examine the relationship between lipid profile and severity measures of OSA in this population. It was hypothesized that severity of OSA is associated with plasma lipid concentration and that measures of central obesity as well as regional differences may affect the association between OSA and lipid concentration.

METHODS

The ESADA cohort has been described elsewhere in detail.[17] Briefly, the ESADA is comprised of data provided by 30 sleep centres distributed across 20 countries in Europe and Israel. In total, 24 centres from 18 countries contributed to the current analysis. Either cardiorespiratory polygraphy (PG) or full polysomnography (PSG) were performed (according to the American Academy of Sleep Medicine criteria)[18,19] in accordance with local practice at each participating centre and were used in the assessment of OSA severity.[20] The ESADA protocol was approved by the local research ethics committee at each of the participating centre and informed consent is obtained from all included patients. The details of the ESADA, sleep study, selection criteria on different lipid profile (according to the National Cholesterol Education Program Adult Treatment Panel III criteria)[21] and assessment of anthropometric measures can be found in Appendix S2 (Supplementary Information).

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 22.0 (IBM Corp., Armonk, NY, USA). Severity of OSA was measured categorically according to apnoea–hypopnoea index (AHI) and oxygen desaturation index (ODI) quartiles for each lipid parameter separately, with subjects in the first quartile having the lowest AHI/ODI and serving as a reference category in the analysis. Baseline patient characteristics across quartiles were compared using analysis of variance (ANOVA) with post hoc Bonferroni analysis, Kruskal–Wallis and Mann–Whitney U-tests and chi-square tests for parametric, nonparametric and categorical variables, respectively. To address the relationship between lipid concentrations with OSA severity, correlations of patient TC, TG, low-density lipoprotein cholesterol (LDL-C) (Friedewald formula[22]) and HDL-C levels with OSA severity measures (AHI and ODI), nocturnal oxygenation (mean nocturnal arterial oxygen saturation measured by pulse oximetry (SpO_2) and lowest nocturnal SpO_2), demographic and anthropometric measures, clinical variables and Epworth Sleepiness Scale (ESS) scores were assessed by Pearson's or Spearman's correlation coefficients according to distribution. To further explore the relationship between lipid levels and OSA severity, factorial analysis of covariance (ANCOVA) was performed to generate adjusted mean lipid value for each AHI class or ODI quartile. This analysis was adjusted for study site (classification of regions, distribution of centres and sample contribution by site listed in Table S14, Supplementary Information) and demographic, anthropometric and clinical variables. Adjusted means were compared following Bonferroni's post hoc correction. Generalized linear regression models (GLM) were built using variables with significant univariate relationships with each lipid parameter. Adjustments for age, sex, BMI-classes, waist to hip (W/H) circumference ratio, co-morbidities (hypertension, ischaemic heart disease, stroke/transient ischaemic attack and diabetes) and study site location according to the five European regions were performed. Finally, logistic regression analysis was performed to generate adjusted odds ratios (OR) for TC levels ≥200 mg/dL, TG levels ≥150 mg/dL, LDL levels ≥100 mg/dL and HDL-C levels <40 mg/dL according to severity quartiles of Sleep Disordered Breathing (SDB) and for nocturnal hypoxaemia.

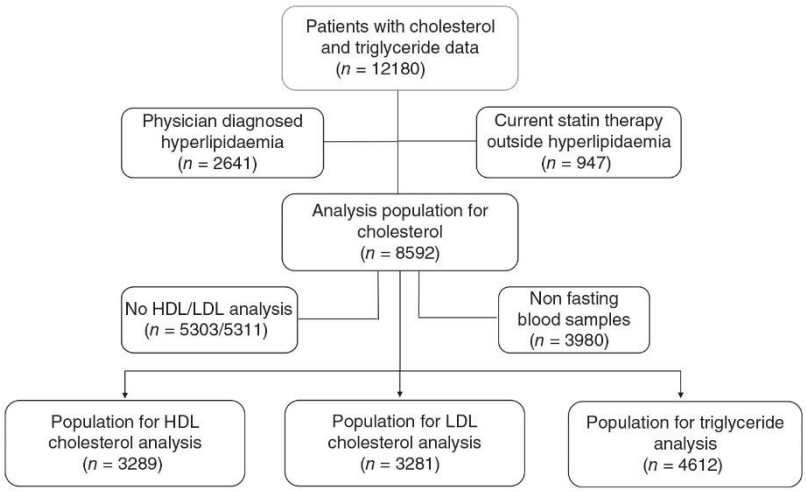

Figure 1 Study flow diagram. HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Several sensitivity analyses were performed using AHI classes defined by clinical cut-off values for severity (AHI: 5 to <15, 15 to <30 and ≥30) as well as for the individuals studied with PG and PSG separately. All tests were two-tailed and statistical significance was taken at $P = 0.05$.

RESULTS

Anthropometric data

A total of 12 180 subjects with data fulfilling the purpose of the current study were identified. A subgroup of 8592 patients without physician-diagnosed

Table 1 Patient characteristics according to sleep apnoea severity

		AHI quartiles				
	Total n = 8592	<5 n = 2152	5.51–16.5 n = 2146	16.51–38.77 n = 2146	38.78–188 n = 2148	*P*-value
Age (years)	50.1 ± 12.7	45.0 ± 12.9	50.6 ± 12.2	52.6 ± 12.0	52.1 ± 12.2	<0.001
BMI (kg/m^2)	30.8 ± 6.6	27.9 ± 5.2	29.7 ± 5.8	31.1 ± 6.0	34.7 ± 7.4	<0.001
Systolic blood pressure (mm Hg)	133.2 ± 17.6	129.2 ± 17.4	132.6 ± 17.7	134.3 ± 17.1	136.6 ± 17.3	<0.001
Diastolic blood pressure (mm Hg)	82.2 ± 11.7	80.4 ± 11.2	82.0 ± 11.6	82.4 ± 11.8	83.8 ± 12.1	<0.001
Heart rate (bpm)	73.9 ± 12.8	71.0 ± 12.0	73.0 ± 12.5	73.6 ± 12.2	78.1 ± 13.4	<0.001
Waist (cm)	105.7 ± 15.8	97.5 ± 13.9	102.6 ± 13.6	106.9 ± 13.9	115.8 ± 15.7	<0.001
Hip (cm)	109.6 ± 12.9	105.3 ± 10.7	107.6 ± 11.3	109.8 ± 12.0	115.6 ± 14.7	<0.001
W/H ratio	0.96 ± 0.09	0.92 ± 0.09	0.95 ± 0.08	0.97 ± 0.08	1.00 ± 0.08	<0.001
Neck circumference (cm)	40.8 ± 4.3	38.6 ± 3.8	39.9 ± 3.9	41.2 ± 3.8	43.5 ± 4.2	<0.001
Gender (female, %)	30.9	44	34.4	25.9	19.3	<0.001
Diabetes mellitus (%)	7.7	3.6	5.8	8.6	12.8	<0.001
Systemic hypertension (%)	31.1	15.8	29.0	36.3	43.5	<0.001
Ischaemic heart disease (%)	3.4	1.2	3.0	3.7	5.5	<0.001
Chronic heart failure (%)	1.4	0.5	1.4	1.6	2.2	<0.001
COPD (%)	4.5	4.1	4.2	4.1	5.6	0.06
Smokers (%)	24.6	25.5	23.1	22.2	27.5	0.004
ESS score	10.1 ± 5.2	9.6 ± 5.2	9.5 ± 4.9	9.9 ± 5.0	11.2 ± 5.4	<0.001
AHI (events/h)	25.7 ± 25.9	2.3 ± 1.6	10.6 ± 3.1	26.2 ± 6.3	63.9 ± 19.8	—
ODI (≥4% events/h)	22.3 ± 25.3	2.7 ± 4.3	8.7 ± 6.6	21.1 ± 11.8	56.9 ± 24.2	<0.001
Mean SpO_2 (%)	93.4 ± 3.4	94.9 ± 1.8	94.2 ± 2.2	93.6 ± 2.2	90.8 ± 4.7	<0.001
Lowest SpO_2 (%)	81.2 ± 9.9	88.0 ± 5.1	84.3 ± 6.0	80.7 ± 7.3	71.6 ± 11.3	<0.001
HbA1c (%)	5.5 ± 1.0	5.2 ± 0.8	5.4 ± 0.8	5.6 ± 1.0	5.9 ± 1.1	<0.001
Cholesterol (mg/dL)	202.8 ± 39.7	202.9 ± 38.0	205.2 ± 40.1	203.5 ± 41.6	199.4 ± 39.0	0.017
Triglyceride (mg/dL)*	148.8 ± 87.1	132.1 ± 74.3	145.9 ± 82.9	150.7 ± 83.3	166.3 ± 102.2	0.241
HDL (mg/dL)*	47.3 ± 13.4	49.0 ± 14.2	48.8 ± 14.1	48.0 ± 13.6	44.5 ± 11.8	<0.001
LDL (mg/dL)*	124.6 ± 35.0	128.4 ± 34.1	125.7 ± 34.0	125.0 ± 35.9	121.2 ± 35.1	<0.001

*, reduced numbers according to Figure 1.

AHI, apnoea–hypopnoea index; BMI, body mass index; COPD, chronic obstructive pulmonary disease; ESS, Epworth Sleepiness Scale; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ODI, oxygen desaturation index; SpO_2, arterial oxygen saturation measured by pulse oximetry; W/H, waist to hip.

Table 2 Independent predictors of cholesterol levels: GLM analysis results

	Variables reflecting sleep-disordered breathing							
	AHI (*n*/h) (*n* = 8592)		ODI (*n*/h) (*n* = 8302)		Mean SpO_2% (*n* = 8355)		Lowest SpO_2% (*n* = 8286)	
	β	*P*-value	β	*P*-value	β	*P*-value	β	*P*-value
Quartile 2 vs 1	4.4	<0.001	4.2	0.001	6.0	<0.001	1.9	0.122
Quartile 3 vs 1	5.2	<0.001	5.1	<0.001	6.0	<0.001	1.9	0.121
Quartile 4 vs 1	5.0	<0.001	5.4	<0.001	4.2	0.003	3.9	0.004
Factors known to confound cholesterol levels								
Age	0.339	<0.001	0.335	<0.001	0.325	<0.001	0.351	<0.001
Males	−7.0	<0.001	−6.9	<0.001	−7.0	<0.001	−6.9	<0.001
Ref: BMI <25								
Overweight	5.9	<0.001	5.9	<0.001	5.7	<0.001	6.4	<0.001
Obesity	4.2	0.004	3.6	0.014	3.8	0.010	4.4	0.002
Morbid obesity	−4.2	0.010	−4.5	0.007	−4.2	0.011	−3.9	0.019
W/H ratio (10%)	2.42	<0.001	2.34	<0.001	2.41	<0.001	2.66	<0.001
Hypertension	−3.1	0.002	−3.4	0.001	−3.1	0.002	−3.2	0.002
IHD	−13.9	<0.001	−13.1	<0.001	−13.2	<0.001	−13.3	<0.001
TIA/stroke	−7.2	0.053	−7.6	0.049	−6.9	0.064	−7.2	0.053
Diabetes	−13.9	<0.001	−13.9	<0.001	−13.1	<0.001	−13.7	<0.001
Influence of geographical regions, Northern Europe used as reference in geographical analysis								
Central	−9.0	<0.001	−6.2	<0.001	−5.8	<0.001	−6.1	<0.001
South	−10.2	<0.001	−8.0	<0.001	−7.3	<0.001	−7.5	<0.001
East	−17.5	<0.001	−14.9	<0.001	−14.7	<0.001	−14.6	<0.001
West	−17.0	<0.001	−16.2	<0.001	−15.7	<0.001	−15.4	<0.001

AHI quartiles: quartile 1: 0–5.5, AHI quartile 2: 5.51–16.5, AHI quartile 3: 16.51–38.77, AHI quartile 4: >38.77; ODI quartiles: quartile 1: 0–3.60, quartile 2: 3.61–12.0, quartile 3: 12.01–32.90, quartile 4: >32.90; Mean SpO_2 quartiles: quartile 1: >95.19, quartile 2: 94.01–95.19, quartile 3: 92.3–94.0, quartile 4: <92.3; Lowest SpO_2 quartiles: quartile 1: >87.99, quartile 2: 84–87.99, quartile 3: 77–83.99, quartile 4: <77. Overweight, BMI 25-<30, obesity BMI30-<35, morbid obesity, BMI =>35 kg/m^2.

AHI, apnoea–hypopnoea index; BMI, body mass index; GLM, generalized linear regression model; IHD, ischaemic heart disease; ODI, oxygen desaturation index; SpO_2, arterial oxygen saturation measured by pulse oximetry; TIA, transient ischaemic attack; W/H, waist to hip.

hyperlipidaemia or reported intake of lipid-lowering drugs were included in the analysis of TC. The analysis of TG included 4612 subjects with data on fasting TG; 3289 subjects were identified for the HDL-C analysis and 3281 subjects were identified for the LDL-C analysis (Fig. 1). Descriptive characteristics of the study population, stratified according to AHI quartiles, are shown in Table 1. Subjects with severe OSA were more likely to be male, more obese and to have co-morbidities including dyslipidaemia.

Total cholesterol

In the univariate analysis, TC correlated negatively with AHI and ODI (Table S1, Supplementary Information). AHI, ODI, mean nocturnal SpO_2 and the fourth quartile of lowest nocturnal SpO_2 independently predicted TC in the adjusted model (Table 2). TC levels were lower in males and in subjects with cardiovascular co-morbidities. There was an inverse U-shaped relationship between BMI and cholesterol concentration. Obese and overweight patients had higher cholesterol levels than patients with morbid obesity. Waist-to-hip ratio (WHR) as an indicator of abdominal obesity presented a significant association with TC levels. TC levels differed significantly between European regions, with the highest TC values in the North, followed by the Central, Southern, Eastern and Western regions (mean ± SE (mg/dL): 209.23 ± 0.66 vs 192.13 ± 1.62, $P < 0.001$, for Northern and Western regions, respectively, Fig. S1 (Supplementary Information)). Mean TC concentration, adjusted for demographic, anthropometric and clinical variables, was found to gradually increase across the quartiles of AHI, ODI, mean and lowest saturation (Figs 2A,S2 (Supplementary Information)). When using the clinical cut-off values for apnoea severity classification, the results were comparable (TC in severe OSA (AHI ≥ 30) was 5.24 mg/dL higher than in patients with AHI < 5). The sleep diagnostic test method used was almost equally distributed in the overall study sample (polysomnography: 47%, polygraphy: 53%). However, the distribution differed among European regions and polygraphy was the prominent sleep test used in North and West European regions, whereas polysomnography was most frequently used in Eastern, Central and Southern Europe (Table S2, Supplementary Information). Nonetheless, TC concentration was independently associated with AHI irrespective of the sleep diagnostic test method used (PSG/PG, Tables S3–S6, Supplementary Information).

Sleep apnoea measures and mean nocturnal oxygenation independently predicted a higher TC concentration (≥200 mg/dL). The OR was 1.31 (1.15–1.49), 1.30 (1.13–1.50) and 1.31 (1.13–1.53) in ODI quartiles II–IV

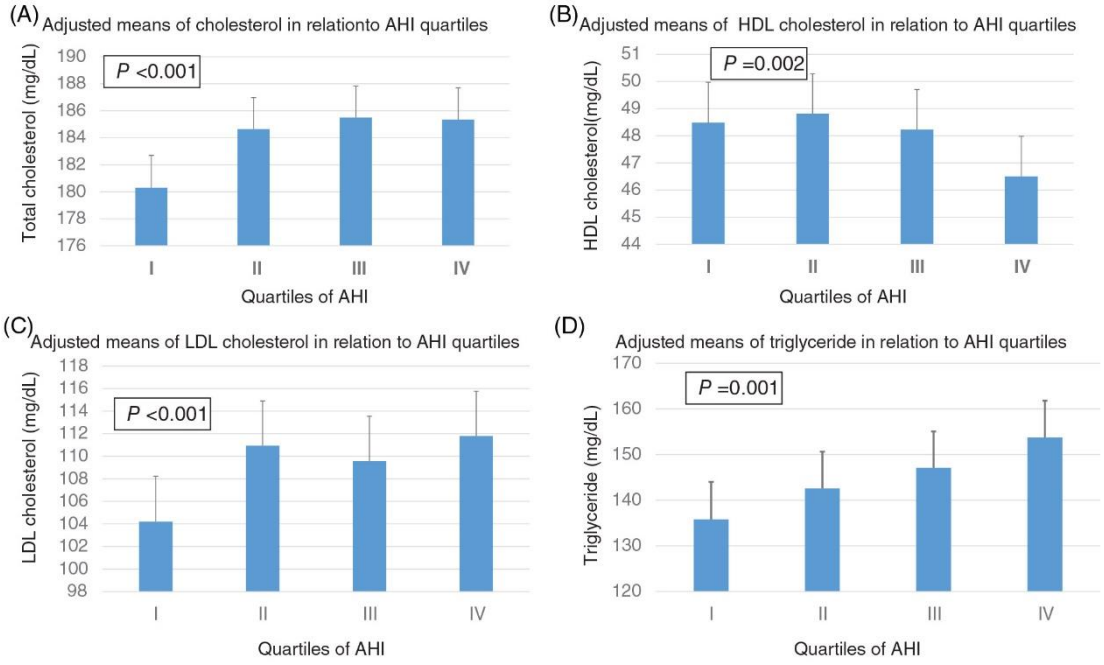

Figure 2 Adjusted means of (A) total cholesterol, (B) high-density lipoprotein cholesterol, (C) low-density lipoprotein cholesterol and (D) triglycerides in relation to apnoea–hypopnoea index quartiles.

compared with the lowest quartile ($P < 0.001$, Table S7, Supplementary Information) after adjustment for age, BMI, WHR and co-morbidities.

HDL cholesterol

HDL-C correlated negatively with AHI (Pearson r = –0.164, $P < 0.001$) and ODI (Pearson r = –0.173, $P < 0.001$) in the univariate analysis (Table S8, Supplementary Information). In the adjusted model, HDL-C was significantly reduced in patients with severe OSA (AHI quartile IV, $\beta = -1.99$, $P = 0.008$ and ODI quartile IV, $\beta = -2.45$, $P = 0.001$). Other predictors for a lower HDL concentration included male gender, smoking, diabetes and ischaemic heart disease. Obesity and WHR were associated with a lower HDL concentration (Table 3). Mean HDL-C was lower in patients with a high AHI, ODI and in those within the lower mean nocturnal SpO_2 quartiles (Figs 2B,S3 (Supplementary Information)). The OR for a low HDL concentration (<40 mg/dL) was 1.48 (1.11–1.97, $P = 0.008$) in patients belonging to the highest ODI quartile (Table S9, Supplementary Information).

LDL cholesterol

LDL-C correlated negatively with AHI (Pearson r = –0.069, $P < 0.001$) and ODI (Pearson r = –0.072, $P < 0.001$) in the univariate analysis (Table S10, Supplementary Information). In the adjusted model, LDL-C was significantly increased in patients with severe OSA (AHI quartile IV, $\beta = 7.59$, $P < 0.001$ and ODI quartile IV, $\beta = 7.22$, $P < 0.001$). Other predictors for a higher LDL concentration included male gender, diabetes, a cardiovascular co-morbidity and overweight (Table 4). Mean LDL-C was elevated in patients with increased OSA severity (upper AHI and ODI quartiles) (Figs 2C,S4 (Supplementary Information)). The OR for an abnormal LDL concentration (≥100 mg/dL) was 1.40 (1.05–1.87, $P = 0.024$) and 1.41 (1.05–1.90, $P = 0.021$) in patients within the highest AHI/ODI quartile compared with the lowest AHI/ODI quartile, respectively (Table S11, Supplementary Information).

Triglycerides

TG concentration correlated with AHI (Pearson r = 0.127, $P < 0.001$) and ODI (Pearson r = 0.129, p < 0.001) (Table S12, Supplementary Information). AHI severity independently predicted TG concentration ($\beta = 6.76$, $P = 0.070$ in the second; $\beta = 11.26$, $P = 0.005$ in the third and $\beta = 17.90$, $P = 0.001$ in the fourth compared with the first AHI quartile; Table 5). ODI and mean SpO_2 also predicted TG concentration. Adjusted means of TG were linearly associated with AHI and ODI quartiles (Figs 2D, S5A (Supplementary Information)). The OR for a high TG concentration (≥150 mg/dL) was 1.26 (1.08–1.46, $P = 0.003$), 1.42 (1.21–1.67, $P < 0.001$) and 1.42 (1.19–1.68, $P < 0.001$) in AHI quartiles II–IV compared with the lowest quartile, respectively (Table S13, Supplementary Information).

DISCUSSION

In this cross-sectional analysis of data collected from a prospective observational cohort, addressing the largest

Table 3 Independent predictors of HDL-C: GLM analysis results

	Variables reflecting sleep-disordered breathing							
	AHI (*n*/h) (*n* = 3139)		ODI (*n*/h) (*n* = 3105)		Mean SpO_2% (*n* = 3115)		Lowest SpO_2% (*n* = 3095)	
	β	*P*-value	β	*P*-value	β	*P*-value	β	*P*-value
Quartile 2 vs 1	0.32	0.615	−0.23	0.721	−0.86	0.173	−0.61	0.321
Quartile 3 vs 1	−0.26	0.071	−1.03	0.136	−1.03	0.080	−0.56	0.380
Quartile 4 vs 1	−1.99	0.008	−2.45	0.001	−2.49	<0.001	−1.04	0.134
Factors known to confound HDL-C levels								
Age	0.17	<0.001	0.17	<0.001	0.18	<0.001	0.17	<0.001
Males	−7.29	<0.001	−7.26	<0.001	−7.43	<0.001	−7.46	<0.001
Ref: BMI <25								
Overweight	−4.15	<0.001	−4.14	<0.001	−4.17	<0.001	−4.07	<0.001
Obesity	−7.18	<0.001	−7.12	<0.001	−7.18	<0.001	−7.28	<0.001
Morbid obesity	−7.12	<0.001	−6.85	<0.001	−6.90	<0.001	−7.29	<0.001
W/H ratio (10%)	−1.87	<0.001	−1.85	<0.001	−1.79	<0.001	−1.98	<0.001
Smokers	−2.66	<0.001	−2.57	<0.001	−2.51	<0.001	−2.68	<0.001
Alcohol (unit)	0.24	<0.001	0.24	<0.001	0.24	<0.001	0.25	<0.001
Hypertension	0.00	0.998	−0.02	0.971	0.04	0.939	0.11	0.830
IHD	−2.51	0.026	−2.56	0.023	−2.68	0.018	−2.62	0.021
TIA/stroke	2.42	0.353	2.16	0.406	2.38	0.359	2.54	0.329
Diabetes	−2.90	<0.001	−2.85	<0.001	−2.79	<0.001	−2.87	<0.001
Influence of geographical regions, Northern Europe used as reference in geographical analysis								
Central	4.41	<0.001	4.52	<0.001	3.83	<0.001	4.12	<0.001
South	5.11	<0.001	4.90	<0.001	4.47	<0.001	4.44	<0.001
East	0.00	0.997	0.14	0.885	−0.64	0.517	−0.22	0.822
West	2.02	0.001	2.10	<0.001	1.50	0.006	1.60	0.003

AHI quartiles: quartile 1: 0–7.75, quartile 2: 7.76–22.6, quartile 3: 22.61–47, quartile 4: >47; ODI quartiles: quartile 1: 0–5.40, quartile 2: 5.41–17.30, quartile 3: 17.31–43, quartile 4: >43; Mean SpO_2 quartiles: quartile 1: >94.99, quartile 2: 94–94.99, quartile 3: 92–93.99, quartile 4: <92; Lowest SpO_2 quartiles: quartile 1: >87.99, quartile 2: 83–87.99, SpO_2 quartile 3: 75–82.99, quartile 4: <75. Overweight, BMI 25-<30, obesity BMI30-<35, morbid obesity, BMI =>35 kg/m^2.

AHI, apnoea–hypopnoea index; BMI, body mass index; GLM, generalized linear regression model; HDL-C, high-density lipoprotein cholesterol; IHD, ischaemic heart disease; ODI, oxygen desaturation index; SpO_2, arterial oxygen saturation measured by pulse oximetry; TIA, transient ischaemic attack; W/H, waist to hip.

patient sample on this topic to date, OSA was identified as an independent predictor of potentially harmful lipid levels. The analysis was conducted in an OSA population without physician-diagnosed hyperlipidaemia or reported intake of a lipid-lowering drug. We identified a strong association between higher TC, higher LDL-C, elevated TG, lower HDL-C and several measures of OSA severity. There was a strong linear association between obesity and TG concentration, whereas morbid obesity was associated with a reduced TC and a tendency towards higher HDL-C concentration. Finally, we observed a strong geographical influence (European regions) on lipid profiles.

The influence of OSA on lipid status has not been conclusively established in previous studies. SDB severity was associated with higher TG and lower HDL-C concentration in only 3 out of 13 cross-sectional studies in a recent meta-analysis.[9] The Sleep Heart Health Study[8] reported a significant correlation between OSA severity and TC concentration in younger males and HDL-C and TG concentrations in women. Börgel *et al.*[23] found only an association between AHI and HDL-C in their sample of OSA patients. Lam *et al.*[24] reported no association between OSA and HDL-C or TG concentration in a Chinese population of 255 subjects and Togeiro *et al.*[25] found a weak independent association between OSA and TG. The present study, by contrast, identified a strong linear association between AHI severity and lipid concentration in a large sample of patients with OSA.

The influence of CIH on lipid status has been addressed in both animal and human studies. CIH induced hyperlipidaemia and triggered upregulation of genes associated with hepatic lipid biosynthesis in an animal model.[11] Drager *et al.* demonstrated that CIH promoted atherosclerosis and inhibited the clearance of TG-rich lipoproteins in mice.[26,27] Togeiro *et al.*[25] reported an independent association between CIH and elevated TG concentration and suggested CIH as a better marker than AHI for dyslipidaemia. Similarly, in a large cross-sectional study, nocturnal CIH was associated with high TG and low HDL-C concentration[12] while neither OSA severity nor degree of hypoxia was associated with lipoprotein concentration in a smaller study,. However, insulin resistance acted as a strong independent predictor of lipid/lipoprotein abnormalities.[28] In our study, ODI, as a measure of CIH, had a slightly more pronounced influence on TC concentration than AHI. However, TG concentrations showed a strong linear association with both AHI and ODI. Unexpectedly, our study found lower levels of TC in patients with established cardiovascular disease such as hypertension or coronary artery disease. It may

Table 4 Independent predictors of LDL-C levels: GLM analysis results

	Variables reflecting sleep-disordered breathing							
	AHI (*n*/h) (*n* = 3281)		ODI (*n*/h) (*n* = 3234)		Mean SpO_2% (*n* = 3248)		Lowest SpO_2% (*n* = 3228)	
	β	*P*-value	β	*P*-value	β	*P*-value	β	*P*-value
Quartile 2 vs 1	6.734	<0.001	3.78	0.031	3.34	0.054	3.51	0.036
Quartile 3 vs 1	5.37	0.005	6.44	0.001	5.00	0.002	3.64	0.037
Quartile 4 vs 1	7.59	<0.001	7.22	<0.001	2.37	0.191	3.40	0.073
Factors known to confound LDL-C levels								
Age	0.22	<0.001	0.22	<0.001	0.22	<0.001	0.23	<0.001
Males	−4.16	0.007	−3.87	0.013	−3.47	0.025	−3.59	0.021
Ref: BMI <25								
Overweight	6.25	0.002	6.62	0.001	6.27	0.002	6.63	0.001
Obesity	3.23	0.135	2.68	0.226	3.32	0.130	3.59	0.102
Morbid obesity	−2.91	0.218	−3.14	0.195	−2.14	0.372	−1.88	0.433
W/H ratio (10%)	15.26	0.092	1.92	0.160	14.94	0.101	15.04	0.101
Smokers	1.89	0.164	−2.03	0.150	1.79	0.190	1.93	0.156
Hypertension	−2.07	0.139	−9.87	0.001	−1.63	0.245	−1.74	0.215
IHD	−9.94	0.001	−6.46	0.365	−9.50	0.002	−9.56	0.002
TIA/stroke	−7.65	0.273	−11.74	<0.001	−7.77	0.265	−7.93	0.255
Diabetes	−11.31	<0.001	1.92	0.160	−11.65	<0.001	−11.66	<0.001
Influence of geographical regions, Northern Europe used as reference in geographical analysis								
Central	−25.54	<0.001	−25.77	<0.001	−22.88	<0.001	−23.38	<0.001
South	−12.79	<0.001	−11.98	<0.001	−10.43	<0.001	−10.37	<0.001
East	−22.16	<0.001	−21.91	<0.001	−19.62	<0.001	−20.07	<0.001
West	−13.43	<0.001	−13.23	<0.001	−10.78	<0.001	−11.52	<0.001

AHI quartiles: quartile 1: 0–7.69, quartile 2: 7.70–22.54, quartile 3: 22.55–46.99, quartile 4: >46.99; ODI quartiles: quartile 1: 0–5.39, quartile 2: 5.40–17.29, quartile 3: 17.30–42.99, quartile 4: >42.99; Mean SpO_2 quartiles: quartile 1: >94.99, quartile 2: 94–94.99, quartile 3: 92–93.99, quartile 4: <92; Lowest SpO_2 quartiles: quartile 1: >87.99, quartile 2: 83–87.99, SpO_2 quartile 3: 75–82.99, quartile 4: <75. Overweight, BMI 25-<30, obesity BMI30-<35, morbid obesity, BMI =>35 kg/m^2.

AHI, apnoea–hypopnoea index; BMI, body mass index; GLM, generalized linear regression model; IHD, ischaemic heart disease; LDL-C, low-density lipoprotein cholesterol; ODI, oxygen desaturation index; SpO_2, arterial oxygen saturation measured by pulse oximetry; TIA, transient ischaemic attack; W/H, waist to hip.

be speculated that patients with identified cardiovascular disease are referred to sleep centres as part of an overall CV risk management. Lifestyle measures such as regular exercise, smoking cessation or dietary counselling are known to reduce TC levels and may have already been implemented in these patients. In addition, GLM data demonstrated an unexpected association between high cholesterol and female, but not male gender, which may also be attributed to pre-selection bias in a male-dominant sleep apnoea patient cohort and a potential oversampling of post-menopausal women.

Randomized controlled trials addressing the effect of OSA therapy by continuous positive airway pressure (CPAP) in patients with co-morbid dyslipidaemia provide important information to unravel a potential causal relationship between OSA and dyslipidaemia. A meta-regression analysis evaluating 1958 OSA subjects from 29 studies demonstrated a mild positive impact of CPAP on cholesterol, but not on TG levels.[29] However, there was considerable inconsistency between the studies as some showed a positive result, whereas others found no effect of CPAP on lipid status.[23,30–34]

Obesity constitutes the most important confounding factor for the association between OSA and dyslipidaemia. However, several studies also suggest that this association is independent of BMI.[8,23] In a study addressing a non-obese male population, the association persisted independent of measures of visceral fat.[35] Another study reported an association between OSA and the metabolic syndrome independent of obesity.[36] Conversely, McArdle *et al.*[37] demonstrated increased levels of TC and LDL-C in OSA subjects mainly related to the confounding influence of central obesity.

Our study, which controlled for both abdominal obesity and BMI category, found a strong non-linear association between BMI and TC. This finding suggests that categorical classification of BMI, rather than using BMI as a continuous variable, may provide more reliable results on the influence of obesity on lipid status. Abdominal obesity is widely defined as a major risk factor for cardiovascular disease and this association is attributed to the visceral adipose tissue promoting insulin resistance, dyslipidaemia and hypertension.[38] WHR, described as the primary anthropometric measure of abdominal obesity, has been defined as a stronger predictor for cardiovascular diseases compared with BMI.[39] Thus, in our study, the possible confounding influence of WHR in the regression models was considered. Our study was not designed to analyse the exact mechanisms linking OSA and lipid concentrations, but the so-called

Table 5 Independent predictors of plasma TG concentrations: GLM analysis results

	Variables reflecting sleep-disordered breathing							
	AHI (n = 4369)		ODI (n = 4318)		Mean SpO_2 (n = 4310)		Lowest SpO_2 (n = 4284)	
	β	*P*-value	β	*P*-value	β	*P*-value	β	*P*-value
Quartile 2 vs 1	6.76	0.070	5.42	0.141	0.38	0.921	3.29	0.359
Quartile 3 vs 1	11.26	0.005	10.34	0.010	7.14	0.056	4.76	0.178
Quartile 4 vs 1	17.90	0.001	15.05	0.001	9.82	0.019	8.29	0.035
Factors known to confound TG levels								
Age	−0.39	0.001	−0.40	0.001	−0.41	0.001	−0.34	0.003
Males	8.90	0.008	9.19	0.006	9.93	0.003	10.11	0.002
Ref: BMI < 25								
Overweight	23.43	0.001	23.05	0.001	24.16	0.001	23.20	0.001
Obesity	40.60	0.001	40.96	0.001	41.96	0.001	42.02	0.001
Morbid obesity	33.20	0.001	33.36	0.001	34.59	0.001	35.03	0.001
W/H ratio(10%)	12.58	0.001	12.25	0.001	12.51	0.001	13.31	0.001
Smokers	13.29	0.001	13.79	0.001	12.78	0.001	14.49	0.001
Alcohol (unit)	0.71	0.002	0.60	0.007	0.70	0.002	0.59	0.007
Hypertension	−2.58	0.406	−3.40	0.274	−2.31	0.461	−4.84	0.116
IHD	−7.26	0.319	−6.63	0.359	−6.34	0.389	−6.07	0.396
TIA/stroke	−6.92	0.609	−5.62	0.675	−6.91	0.610	−6.75	0.610
Diabetes	23.02	0.001	23.19	0.001	22.79	0.001	24.12	0.001
Influence of geographical regions, Northern Europe used as reference in geographical analysis								
Central	−11.97	0.009	−9.35	0.039	−6.70	0.126	−6.65	0.125
South	−8.27	0.034	−3.70	0.322	−3.24	0.392	−2.26	0.539
East	−22.57	0.001	−20.04	0.002	−17.42	0.007	−16.59	0.008
West	−15.33	0.001	−13.02	0.001	−10.39	0.002	−9.69	0.003

AHI quartiles: quartile 1: 0–5.6, AHI quartile 2: 5.61–18, AHI quartile 3: 18.01–40.97, AHI quartile 4: >40.97; ODI quartiles: quartile 1: 0–3.60, quartile 2: 3.61–12.60, quartile 3: 12.61–35.75, quartile 4: >35.75; Mean SpO_2 quartiles: quartile 1: >95.2, quartile 2: 94.01–95.20, quartile 3: 92.01–94.0, quartile 4: <92.01; Lowest SpO_2 quartiles: quartile 1: >87.9, quartile 2: 84–87.99, quartile 3: 77–83.99, quartile 4: <77.

AHI, apnoea–hypopnoea index; GLM, generalized linear regression model; IHD, ischaemic heart disease; ODI, oxygen desaturation index; SpO_2, arterial oxygen saturation measured by pulse oximetry; TG, triglyceride; TIA, transient ischaemic attack; W/H, waist/hip.

obesity paradox should be considered in this context as it defines the inverse U-shaped association between BMI and mortality.[15] Genetic and biological differences originating from the variances between intra-abdominal visceral fat and peripheral subcutaneous fat rather than only BMI may have an influence concordantly.[40] Interestingly, Ozeke *et al.*[41] hypothesized that OSA may provide one such underlying mechanism that contributes to the obesity paradox by a cardioprotective effect induced by CIH. Our data also demonstrate a lower frequency of dyslipidaemia in morbidly obese OSA patients.

The prevalence of an elevated TC concentration was high (54% for both sexes) in Europe according to the World Health Organization. There are consistent reports suggesting considerable regional differences whereby elevated cholesterol was more prevalent in Northern European countries and less prevalent in the South.[16,42] Potential explanations for these regional differences include previously identified genetic and dietary factors.[21] Our study included geographical origin in the analysis and we could confirm a strong influence of region. However, geographical differences alone did not explain the influence of OSA on lipid concentration in our current analysis.

Important strengths and limitations of our study need to be considered. The multinational, multicentre study design and the large sample size of OSA patients clearly increase the generalizability of our results. However, potential referral bias cannot be excluded for this clinical cohort and the results are therefore not necessarily applicable to community populations. The study controlled for a number of confounders and excluded subjects with previously identified dyslipidaemia, as well as those with lipid-lowering medication. It may be argued that these exclusion criteria may have acted to underestimate the influence of OSA on lipid status. Our study also analysed LDL concentrations which are of particular interest in cardiovascular disease prevention. Previous knowledge about LDL in large OSA patient populations is limited. Important limitations include the cross-sectional design as no conclusions on the possible causal relationship between OSA and dyslipidaemia may be drawn. Large number of patients without fasting lipid data had to be excluded for TG analysis. Moreover, the ESADA includes data retrieved from a sleep analysis performed by either PG or PSG and this will clearly influence the reported AHI and ODI values. This particular aspect of the ESADA has been extensively discussed elsewhere[20] and, in order to address this limitation, we performed a separate sensitivity analysis for TC addressing the PG and PSG cohorts separately. In fact, this subanalysis confirmed

the results seen for the entire population. For example, the increase of TC in the fourth versus the first AHI quartile was 5.1 mg in the PSG group and 5.8 mg in the PG group (Tables S3,S4 (Supplementary Information), P = 0.01 and 0.004, respectively). The corresponding value was 5.0 mg for the entire study cohort (Table 2, $P < 0.001$). Important to note, the current data set provided no information on physical exercise or fitness which may have influenced in particular the HDL-C data. Patients with OSA are generally known to have a sedentary lifestyle as shown in several previous studies.[43] A prospective analysis examining the effects of CPAP therapy on the lipid profile in OSA patients included in the ESADA cohort is warranted in order to address some of these study limitations.

As a clinical implication of our findings, clinicians should be aware of the association between OSA and dyslipidaemia. Indeed co-morbid dyslipidaemia, a well-recognized cardiovascular risk factor, is highly likely to affect the overall cardiovascular consequences and prognosis in OSA. Therefore, sleep physicians may systematically assess the coexistence of sleep apnoea and traditional CV risk factors including smoking, hypertension and dyslipidaemia. Finally, the impact of OSA treatment on lipid status still needs to be further evaluated.

In conclusion, OSA was independently associated with dyslipidaemia. CIH was slightly more strongly associated with cholesterol concentration than the severity metric of AHI. New important confounders such as the inverse effect of morbid obesity and the influence of different European regions on lipid status in an OSA patient population were identified.

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Supplementary Information

Additional supplementary information can be accessed via the *html* version of this article at the publisher's website.

Appendix S1 Collaborators in the ESADA project (alphabetical order).
Appendix S2 Supplementary methods.
Figure S1 Mean concentration of TC in European Regions within the ESADA study.
Figure S2 Adjusted means of cholesterol in relation to quartiles of ODI, mean SaO_2 and lowest SpO_2.
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Table S11 Adjusted OR of LDL-C level ≥ 100 mg/dL across quartiles of OSA syndrome severity and oxygenation indices.
Table S12 Univariate correlations of TG with demographic, clinical and anthropomorphic factors, and indices of OSA syndrome severity and nocturnal hypoxaemia.
Table S13 Adjusted OR of TG level ≥ 150 mg/dL across quartiles of OSA syndrome severity and oxygenation indices.
Table S14 The classification of European regions and ESADA study centres.

4.5 Study 5

Hyperlipidaemia prevalence and cholesterol control in obstructive sleep apnoea: Data from the European sleep apnea database (ESADA).

Gunduz C, Kacmaz Basoglu O, Hedner J, Bonsignore MR, Hein H, Staats R, Bouloukaki I, Roisman G, Pataka A, Sliwinski P, Ludka O, Pepin JL, Grote L, European Sleep Apnoea Database c. J Intern Med. 2019

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Hyperlipidaemia prevalence and cholesterol control in obstructive sleep apnoea: Data from the European sleep apnea database (ESADA)

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Abstract. Gunduz C, Basoglu OK, Hedner J, et al; on behalf of the European Sleep Apnoea Database collaborators (Biruni University; Ege University; Gothenburg University; Sahlgrenska University Hospital; University of Palermo; CNR Institute of Biomedicine and Molecular Immunology; St. Adolf Stift; Hospital de Santa Maria; University of Crete; Antoine-Beclere Hospital; G. Papanikolaou Hospital; Institute of Tuberculosis and Lung Diseases; University Hospital Brno; St. Ann's University Hospital; Université Grenoble Alpes) Hyperlipidaemia prevalence and cholesterol control in obstructive sleep apnoea: Data from the European sleep apnea database (ESADA). *J Intern Med.* 2019;https://doi.org/10.1111/joim.12952

Background and objective. Obstructive sleep apnoea (OSA) and hyperlipidaemia are independent risk factors for cardiovascular disease. This study investigates the association between OSA and prevalence of hyperlipidaemia in patients of the European Sleep Apnea Database (ESADA) cohort.

Methods. The cross-sectional analysis included 11 892 patients (age 51.9 ± 12.5 years, 70% male, body mass index (BMI) 31.3 ± 6.6 kg/m^2, mean oxygen desaturation index (ODI) 23.7 ± 25.5 events/h) investigated for OSA. The independent odds ratio (OR) for hyperlipidaemia in relation to measures of OSA (ODI, apnoea-hypopnoea index, mean and lowest oxygen saturation) was determined by means of general linear model analysis with adjustment for important confounders such as age, BMI, comorbidities and study site.

Results. Hyperlipidaemia prevalence increased from 15.1% in subjects without OSA to 26.1% in those with severe OSA, $P < 0.001$. Corresponding numbers in patients with diabetes were 8.5% and 41.5%, $P < 0.001$. Compared with ODI quartile I, patients in ODI quartiles II-IV had an adjusted OR (95% CI) of 1.33 (1.15–1.55), 1.37 (1.17–1.61) and 1.33 (1.12–1.58) ($P < 0.001$), respectively, for hyperlipidaemia. Obesity was defined as a significant risk factor for hyperlipidaemia. Subgroups of OSA patients with cardio-metabolic comorbidities demonstrated higher prevalence of HL. In addition, differences in hyperlipidaemia prevalence were reported in European geographical regions with the highest prevalence in Central Europe.

Conclusion. Obstructive sleep apnoea, in particular intermittent hypoxia, was independently associated with the prevalence of hyperlipidaemia diagnosis.

Keywords: cholesterol, hyperlipidaemia, hypoxia, obesity, sleep apnoea.

[†]For a list of the ESADA collaborators and their affiliations, see Appendix 11.

Introduction

Obstructive sleep apnoea (OSA) is a common disorder characterized by repeated episodes of apnoeas and hypopnoeas during sleep affecting at least 20% of male and 10% of female adults in the general population [1,2]. OSA is an independent risk factor for the incidence of cardiovascular disease [1,3,4]. Metabolic dysfunction also increases the risk of cardiovascular morbidities. Likewise, independent associations between OSA and impaired glycaemic health and insulin resistance have also been reported [5–8]. However, both the underlying mechanisms and the existence of an independent relationship between OSA and hyperlipidaemia remain unclear [9–11].

Experimental data suggested a potential causal role of OSA for the incidence of hyperlipidaemia through pathophysiological mechanisms such as intermittent hypoxia (IH) together with elevated sympathetic activity, oxidative stress, systemic inflammation and sleep fragmentation in patients with OSA [8,12,13]. Indeed, in the current study cohort we identified an independent correlation between cholesterol levels and OSA severity indices in individuals without a known history of hyperlipidaemia [14]. However, systematic reviews summarizing the current epidemiological evidence came up with inconclusive results for an independent association between OSA and a hyperlipidaemia diagnosis [11,15].

The European Sleep Apnea Database Cohort (ESADA) study is a multicentre, multinational study which prospectively recruits patients investigated for suspected OSA in European sleep laboratories. The aim of the current analysis was to examine the relationship between the prevalence of diagnosed hyperlipidaemia and the severity of OSA. It was hypothesized that OSA is associated with the diagnosis of hyperlipidaemia and that control of cholesterol in these patients despite pharmacological treatment is worse in patients with concomitant OSA. We addressed measures of central obesity, and the ESADA study design allowed us to study potential geographical influences on the prevalence of hyperlipidaemia in OSA patients.

Methods

The European Sleep Apnea Database (ESADA)

The ESADA has been described elsewhere in detail [16]. Shortly, the ESADA is comprised of data provided by predominantly academic sleep centres distributed across Europe. Data from 24 centres in 18 countries contributed to the current analysis. Patients eligible for the inclusion in the cohort were aged between 18 and 80 years and underwent a sleep study for suspected OSA. Data collected in the ESADA include anthropometrics, daytime symptoms, smoking, alcohol consumption, blood test data, medical history and medication. Patient and physician-reported comorbidities such as cardiovascular disease, and metabolic diseases such as diabetes mellitus, hyperlipidaemia and hyperuricaemia are captured in detail. Daytime sleepiness is quantified by the Epworth sleepiness scale (ESS) score [17]. Coded data are entered, reported via a web-based system and stored in a central database. The ESADA protocol was approved by the local research ethics committee at each of the participating centre, and informed consent is obtained from all included patients.

Definition of hyperlipidaemia

The diagnosis of hyperlipidaemia is based on the sleep physicians' decision at the time of the diagnostic workup. The information is based on different sources including patients' self-report, information about concomitant medication, the referral letter and/or the hospital charts. In addition, cholesterol levels are assessed in conjunction with the sleep apnoea evaluation visit. The lipid analysis was performed at each study centre. Patients using lipid-modifying agents were identified when concomitant medication with ATC code C10 ('lipid-modifying agents') was reported. Control of hyperlipidaemia was defined using the National Cholesterol Education Program Adult Treatment Panel III criteria [18]: total cholesterol (TC) <200 mg/dL.

Sleep study

A total of 5996 subjects (50.4%) underwent polysomnography (PSG) and the remainder cardiorespiratory polygraphy (n = 5896) sleep studies for the diagnosis of OSA in accordance with local practice at each participating centre. Data were edited manually before entry according to protocol definitions. Scoring of PG and PSG studies in the ESADA was performed according to AASM criteria [19], and the procedures are described in detail [20]. Severity of sleep-disordered breathing (SDB) was assessed by calculation of the apnoea-hypopnoea index (AHI) and the oxygen desaturation index (ODI).

AHI was defined as the mean number of apnoeas/hypopnoeas and ODI as the number of transient desaturations (≥4%) per hour of sleep (PSG) or per hour of analysed time (PG recordings) [19].

Diagnosis of obstructive sleep apnoea

Diagnosis and severity of OSA were established according to the AHI cut-off values of ≥5, 5–<15, 15–<30 and ≥30 events/hour. In accordance with previous clinical and population-based studies [10,21], quartiles of sleep-disordered breathing (AHI, ODI, mean and lowest SpO_2) were calculated for regression analysis where the first quartiles were representative of subjects without sleep apnoea. Thus, predictors of HL were aimed to be identified in subjects with increasing burden of sleep-disordered breathing compared to subjects without OSA.

Assessment of anthropometric measures

Weight and height were measured with light clothing and without shoes. BMI was defined as the body mass (kilograms) divided by the square of the body height (metres), expressed in units of kg/m^2. Neck, waist and hip circumferences were measured, and the waist-to-hip ratio (WHR) was calculated.

Statistics

Statistical analyses were performed using IBM SPSS Statistics 22.0 (Armonk, NY, USA: IBM Corp.). In order to minimize incompatibilities due to the use of different sleep methodologies as well as variabilities in ESADA centres, ODI data were used to characterize the severity of OSA in the present study. ODI quartiles were built for each analysis separately with subjects in the first quartile having the lowest ODI and serving as a reference category in the analysis. Baseline patient characteristics across quartiles were compared using ANOVA with post hoc Bonferroni analysis, Kruskal–Wallis and Mann–Whitney *U*-tests, and chi-squared tests for parametric, non-parametric and categorical variables, respectively. Factorial ANCOVA was performed to generate adjusted mean lipid value for each ODI class. Adjusted means were compared following Bonferroni's post hoc correction. Generalized linear regression models (GLMs) were used to identify independent predictors and odd ratios for hyperlipidaemia diagnosis. Adjustments for age, sex, BMI, waist-to-hip circumference ratio, comorbidities (hypertension, ischaemic heart disease, stroke/transient ischaemic attack, diabetes) and European study sites were performed in the analyses described above. All tests were two-tailed, and statistical significance was defined at $P < 0.05$.

Results

Anthropometric data

Amongst 18 542 subjects enrolled in ESADA, 11 892 subjects were included in the current study. Reasons for study exclusion were lack of information on cholesterol levels ($n = 5062$), sleep study results ($n = 979$) and hyperlipidaemia diagnosis ($n = 470$). There were no clinically meaningful differences between included and excluded patients (age 51.9 ± 12.5 vs 52.7 ± 13.2, BMI 31.3 ± 6.6 vs 31.5 ± 6.7, male gender 69.9% vs 72.1%, ODI 23.7 ± 25.5 vs 22.9 ± 24.8, respectively). Descriptive characteristics of the final study population, stratified according to ODI quartiles, are shown in Table 1. Subjects with severe OSA were more likely to be male, more obese and to have comorbidities.

Prevalence of hyperlipidaemia in the ESADA Cohort

The prevalence of hyperlipidaemia increased significantly along with different measures of OSA severity. In the entire cohort, 21.7% ($n = 2657$) had reported hyperlipidaemia and the prevalence increased from 12.2% in subjects without OSA (AHI < 5) to 19.3%, 23.2% and 27.5% in patients with mild (AHI 5–<15), moderate (AHI 15–<30) and severe OSA (AHI ≥ 30), respectively, $P < 0.001$. The corresponding numbers for ODI quartiles (95% CI) ranged from 15.1% (13.9–16.4) in ODI quartile I to 26.1% (24.6–27.7) in ODI quartile IV, $P < 0.001$ (Table 1, Figure 1). Between-group comparison demonstrated a significant difference in the prevalence rates of HL in ODI quartile I vs quartiles II-IV (Figure 1). Lipid-lowering medication usage amongst subjects with hyperlipidaemia diagnosis increased across ODI classes. Patients with diabetes mellitus, hypertension and ischaemic heart disease had higher prevalence of hyperlipidaemia, and the prevalence increased significantly with OSA severity (Table 1). The prevalence of hyperlipidaemia also increased across BMI classes (13.8%, 20.1%, 24.9% and 27.3%; BMI classes I-IV, respectively, $P < 0.001$).

Table 1 *Patient characteristics (n = 11 892) according to ODI quartiles*

ODI quartiles						
	Total $n = 11\,892$	$<4.50 n = 2964$	$4.50–13.69 n = 2966$	$13.70–35.89 n = 2984$	$>35.89 n = 2978$	*P* value
Anthropometric data						
Age	51.9 ± 12.5	46.9 ± 12.9	52.6 ± 11.9	54.5 ± 11.8	53.5 ± 11.8	<0.001
Gender (female)	30.1%	41.0%	32.4%	26.0%	21.2%	<0.001
BMI (kg/cm^2)	31.3 ± 6.6	27.6 ± 4.8	30.2 ± 5.5	31.7 ± 5.8	35.6 ± 7.2	<0.001
Systolic blood pressure (mmHg)	133.9 ± 17.8	129.0 ± 17.4	134.0 ± 17.8	135.6 ± 17.2	136.9 ± 17.7	<0.001
Diastolic blood pressure (mmHg)	82.0 ± 11.7	79.8 ± 11.2	82.1 ± 11.3	82.7 ± 11.7	83.5 ± 12.3	<0.001
Pulse pressure	51.8 ± 13.9	49.1 ± 12.9	52.0 ± 14.0	52.9 ± 13.9	53.3 ± 14.3	<0.001
Waist (cm)	107.1 ± 15.6	97.0 ± 12.8	104.3 ± 13.1	109.1 ± 13.2	117.9 ± 15.4	<0.001
Hip (cm)	110.2 ± 12.7	104.7 ± 9.9	108.2 ± 10.7	110.9 ± 12.0	117.0 ± 14.5	<0.001
W/H ratio	0.97 ± 0.08	0.93 ± 0.08	0.96 ± 0.08	0.99 ± 0.08	1.01 ± 0.08	<0.001
Neck (cm)	41.2 ± 4.3	38.6 ± 3.7	40.4 ± 3.8	41.8 ± 3.8	43.9 ± 4.3	<0.001
BMI categories						
Normal weight	14.2%	30.6%	15.1%	7.9%	3.2%	<0.001
Overweight	33.9%	44.1%	39.5%	35.2%	16.8%	<0.001
Obesity	28.6%	17.9%	29.3%	33.3%	33.7%	<0.001
Morbid obesity	23.3%	7.4%	16.0%	23.6%	46.3%	<0.001
Smoking	24.1%	25.9%	22.5%	22.4%	25.6%	<0.001
Comorbidities						
Hypertension	39.9%	21.8%	38.6%	45.6%	53.6%	<0.001
Ischaemic heart disease	8.4%	4.6%	7.7%	10.2%	11.1%	<0.001
TIA/stroke	2.4%	1.2%	2.8%	3.0%	2.5%	<0.001
Diabetes	12.7%	4.8%	10.2%	15.4%	20.4%	<0.001
AHI	27.5 ± 26.1	5.7 ± 8.4	13.4 ± 10.2	27.8 ± 12.6	62.8 ± 21.6	<0.001
Mean SpO_2 (%)	93.2 ± 3.4	95.1 ± 1.7	94.1 ± 1.8s	93.3 ± 2.1	90.3 ± 4.7	<0.001
Lowest SpO_2 (%)	80.8 ± 9.8	88.2 ± 4.9	84.0 ± 5.2	80.0 ± 6.7	70.5 ± 10.8	<0.001
Hyperlipidaemia diagnosis						
Hyperlipidaemia	21.9%	15.1%	21.7%	24.4%	26.1%	<0.001
Treated hyperlipidaemia	12.2%	7.3%	13.0%	14.5%	14.2%	<0.001
Hyperlipidaemia in hypertension	4749	13.1%	23.7%	28.6%	34.5%	<0.001
Hyperlipidaemia in ischaemic heart disease	997	11.7%	23.7%	31.2%	33.3%	0.005
Hyperlipidaemia in diabetes mellitus	1511	8.5%	20.2%	29.9%	41.5%	<0.001

BMI, body mass index classes; ODI, oxygen desaturation index; SpO_2, arterial oxygen saturation measured by pulse oximetry; TIA, transient ischaemic attack; W/H, waist-to-hip.

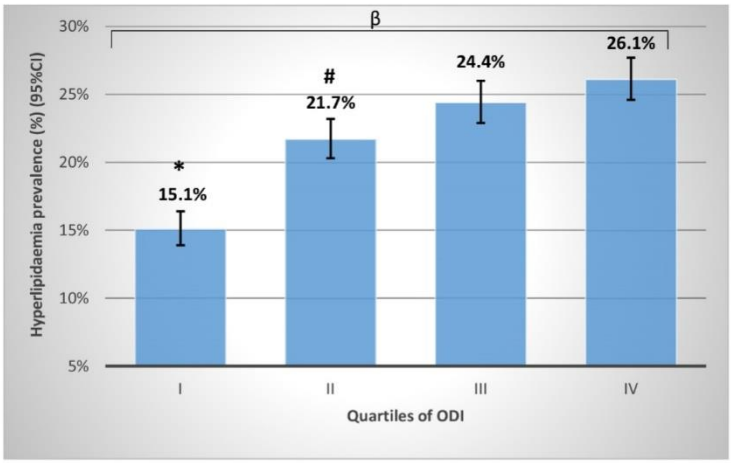

Fig. 1 *Hyperlipidaemia prevalence rates (95% CI) in OSA increases across quartiles of ODI (15.1%, 21.7%, 24.4% and 26.1%, respectively;* P < *0.001, between groups). Significant differences in HL prevalences between groups were reported for quartile I versus quartiles II-IV and for quartile II vs quartile IV (*P < *0.05 both, within groups). 95% confidence intervals were demonstrated by bars.* $^{\beta}$P < *0.001 between groups I-IV.* *P < *0.05 within groups I vs II, III and IV.* $^{\#}$P < *0.05 within groups II vs IV.*

Measures of sleep apnoea event frequency (AHI and ODI) as well as of nocturnal oxygenation independently predicted the likelihood of a reported hyperlipidaemia diagnosis. In the unadjusted model, all measures of sleep-disordered breathing predicted HL significantly and the ORs increased linearly across the quartiles of OSA severity (Table 2). In the adjusted model, in comparison with subjects in ODI quartile I, patients in ODI quartiles II-IV had an OR (95% CI) of 1.33 (1.15–1.55), 1.37 (1.17–1.61) and 1.33 (1.12–1.58) ($P < 0.001$), respectively, for a hyperlipidaemia diagnosis. The fourth quartile of AHI and second and third quartiles of mean and lowest SpO_2 significantly predicted hyperlipidaemia diagnosis after adjustment for confounders (Table 3). Hyperlipidaemia prevalence was also significantly predicted by cardiovascular comorbidities. There was an inverse *U*-shaped relationship between BMI and hyperlipidaemia prevalence. Obese and overweight patients were associated with higher hyperlipidaemia prevalence than patients with morbid obesity or normal weight in the GLM. Hyperlipidaemia prevalence was significantly influenced by study sites and geographical regions with the highest prevalence in the Central region and the lowest in Northern Europe (Figure 2). Finally, when using the clinical AHI cut-off for OSA severity (5–<15, 15–<30, ≥30), adjusted ORs for hyperlipidaemia diagnosis were 1.16 (0.98–1.38), 1.28 (1.08–1.52) and 1.37 (1.16–1.63), $P = 0.078$, 0.006 and <0.001, respectively.

Control of cholesterol levels in treated and untreated hyperlipidaemia

The adjusted mean cholesterol concentrations in subjects without hyperlipidaemia and in hyperlipidaemia with and without lipid-lowering treatment (ATC code C 10) were 172.9 ± 2.1 mg/dL, 179.4 ± 4.1 and 207.5 ± 7.17 mg/dL, respectively ($P < 0.001$) (Figure 3). In patients with a known hyperlipidaemia diagnosis (with and without lipid-lowering treatment), we could not identify a dose–response relationship between lipid level (TC, HDL and LDL cholesterol or triglycerides) and the degree of sleep apnoea severity classified as AHI or ODI quartile (data not shown).

Table 2 *Predictors of hyperlipidaemia diagnosis across quartiles of SDB severity measures (unadjusted model)*

	ODI (n = 11 892)		Mean SpO_2 (n = 11 730)		Lowest SpO_2 (n = 11 859)		AHI (n = 11 892)	
	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
Quartile 2 vs quartile 1	1.56 (1.36–1.78)	<0.001	1.41 (1.24–1.60)	<0.001	1.37 (1.24–1.56)	<0.001	1.64 (1.43–1.88)	<0.001
Quartile 3 vs quartile 1	1.82 (1.59–2.07)	<0.001	1.60 (1.43–1.80)	<0.001	1.72 (1.51–1.96)	<0.001	2.05 (1.79–2.34)	<0.001
Quartile 4 vs quartile 1	1.99 (1.12–1.58)	<0.001	1.79 (1.74–2.26)	<0.001	1.62 (1.42–1.84)	<0.001	2.45 (2.15–2.79)	<0.001

AHI, apnoea-hypopnoea index; ODI, oxygen desaturation index; OR, odds ratio; SpO_2, arterial oxygen saturation measured by pulse oximetry

Table 3 *Independent predictors of hyperlipidaemia diagnosis across quartiles of SDB severity measures*[*]

	ODI (n = 11 892)		Mean SpO_2 (n = 11 730)		Lowest SpO_2 (n = 11 859)		AHI (n = 11 892)	
	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
Quartile 2 vs quartile 1	1.34 (1.15–1.56)	<0.001	1.28 (1.11–1.48)	0.001	1.22 (1.05–1.41)	0.008	1.14 (0.98–1.33)	0.095
Quartile 3 vs quartile 1	1.37 (1.17–1.61)	<0.001	1.27 (1.10–1.46)	0.001	1.29 (1.11–1.51)	0.001	1.15 (0.98–1.35)	0.083
Quartile 4 vs quartile 1	1.33 (1.12–1.58)	0.001	1.12 (0.96–1.31)	0.147	1.09 (0.93–1.28)	0.298	1.28 (1.08–1.50)	0.004

ODI quartiles (n/h): quartile 1: 0–4.49, quartile 2: 4.50–13.69, quartile 3: 13.70–35.89 and quartile 4: >35.89; mean SpO_2 quartiles (%): quartile 1: >94.99, quartile 2: 94–94.99, quartile 3: 92–93.99 and quartile 4: <92; lowest SpO_2 quartiles (%): quartile 1: >87.99, quartile 2: 83–87.99, quartile 3: 77–82.99 and quartile 4: <77; AHI quartiles (n/h): quartile 1: 0–6.79, AHI quartile 2: 6.80–18.99, AHI quartile 3: 19.10–41.99 and AHI quartile 4: >41.99. AHI, apnoea-hypopnoea index; ODI, oxygen desaturation index; OR, odds ratio; SpO_2, arterial oxygen saturation measured by pulse oximetry.
*The values were adjusted for age, gender, body mass index classes, waist-to-hip ratio, smoking, hypertension, ischaemic heart disease, transient ischaemic attack/stroke, diabetes and study sites.

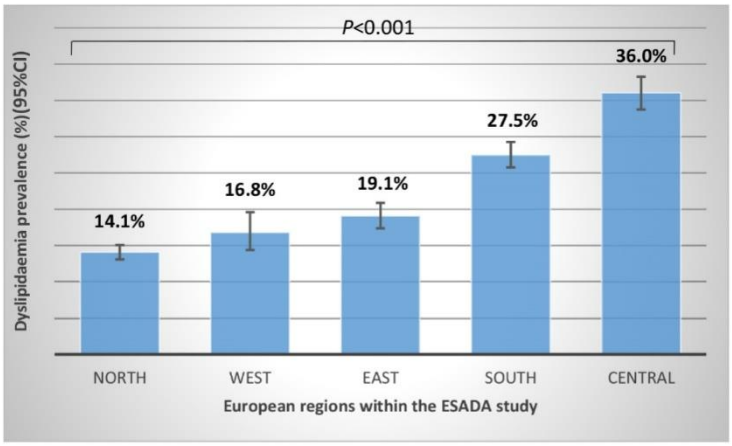

Fig. 2 *Reported hyperlipidaemia prevalence (95% CI) differed amongst five ESADA study geographical regions (P < 0.001, between groups). Lowest prevalence was demonstrated in North (14.1%) and highest in Central (36.4%) regions. 95% confidence intervals were demonstrated by bars.*

Discussion

This cross-sectional study, including the largest patient sample on this topic to date, demonstrated that intermittent hypoxia during sleep is an independent predictor of hyperlipidaemia diagnosis. Hyperlipidaemia prevalence increased from 15.1% in subjects without OSA to 26.1% in those with severe OSA. Hyperlipidaemia prevalence was higher in subgroups of OSA subjects with coexisting cardiovascular comorbidities, particularly in severe OSA subjects. Obesity was identified as a significant predictor of hyperlipidaemia. Differences in prevalence rates of hyperlipidaemia were recorded amongst geographical European regions.

Hyperlipidaemia in OSA – epidemiological evidence

The influence of OSA on hyperlipidaemia has been examined predominantly through lipid concentrations rather than reported hyperlipidaemia diagnosis. In the large Sleep Heart Health Study cohort [10], OSA severity was associated with TC concentration in younger males and with HDL-C and triglyceride concentrations in women. In a meta-analysis of 13 cross-sectional studies, OSA severity demonstrated a significant relationship with lipid concentrations in only three studies. Additionally, several studies either reported a weak association or no relationship at all. [11,22–24] Chou et al. [25] reported a very high hypercholesterolaemia prevalence of 61.1% in 236 male, mostly obese OSA subjects. Kono et al. [26] demonstrated an association between OSA and components of metabolic syndrome including hypertension, dyslipidaemia and hyperglycaemia in a nonobese male population. In the study of Guan et al. [27], a nonlinear dose–effect relationship between dyslipidaemia and OSA severity has been reported. In the Hypnolaus study investigating the prevalence of OSA in a general population with a mean BMI of 25.6 kg/m^2, a 30% (n = 641) prevalence of metabolic syndrome as well as independent association between OSA and metabolic syndrome has been reported [1]. In our previous study from the ESADA cohort, we determined a strong linear association between OSA severity and several lipid concentration (total cholesterol, HDL and LDL cholesterol, and triglycerides) in OSA patients without a reported diagnosis of hyperlipidaemia or use of lipid-lowering medication [14]. In the current study, these findings were substantially confirmed by the identification of an association of OSA with a reported hyperlipidaemia diagnosis. On the other hand, although the prevalence of elevated cholesterol blood levels in our previous study was 51%, the prevalence for reported hyperlipidaemia in the current study was 21.9%. Despite differences in the populations actually studied, the low prevalence of a recognized hyperlipidaemia diagnosis reported in the current study suggests a significant under-recognition of hyperlipidaemia in OSA

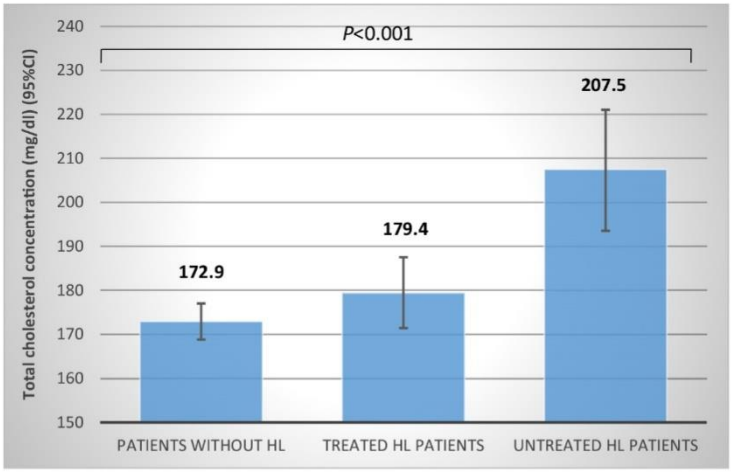

Fig. 3 *Adjusted means of cholesterol (95% CI) in subjects without HL diagnosis and in treated and untreated patients with a HL diagnosis demonstrated a significant difference between groups (P < 0.001). Subjects without HL diagnosis had the lowest cholesterol level (172.9 mg/dL), and untreated HL subjects had the highest (207.5 mg/dL). 95% confidence intervals were demonstrated by bars.*

subjects. This finding is supported by data from the Hypnolaus study. Despite a lower BMI compared to our subjects, the rate of metabolic syndrome was higher (30%) in this population-based cohort supporting a potential under-recognition of HL in our OSA patient population [1]. In the present study, we analysed several measures of OSA severity in predicting hyperlipidaemia in the adjusted model. Measures of intermittent and sustained hypoxia, such as all ODI quartiles II-IV, mean and lowest saturation quartiles II and III, were defined as significant predictors of HL, but only the fourth quartile of AHI demonstrated an independent association with HL. Differences in sleep study methodology (PG/PSG) may partially explain these findings since ODI is less sensitive to the between-centre variability in recording and analysis method used. Thereby, our data suggest that ODI as a measure of intermittent hypoxia is a stronger predictor of HL in OSA subjects when compared with AHI as a measure of OSA event frequency.

Studies examining the relationship of OSA and dyslipidaemia have also investigated the influence of obesity. As some studies claim that there is not a true relationship beyond the effects of obesity [28], some suggest the existence of a strong association between OSA and hyperlipidaemia even independent of BMI [10,22,26,29]. Despite obesity being a strong risk factor for cardiovascular diseases, a phenomenon called obesity paradox, in which overweight or obese people with cardiovascular diseases have a better prognosis than lean subjects, has been described [30]. Recently, the term 'adiposopathy', described as the primary cause of adiposity-related metabolic disease and elevated risk of cardiovascular diseases, is being referred for elucidating the obesity paradox [31]. These findings are in line with the data in our study, showing that morbid obesity did not have an influence on the association of OSA and hyperlipidaemia, whereas an independent risk of hyperlipidaemia has been established for overweighted and obese patients (BMI categories 25–<30 kg/m^2 and 30–<35 kg/m^2, respectively). In this context, the effects of central obesity and peripheral subcutaneous fat on the development of manifest hyperlipidaemia may be different [32]. However, a specific focus on diet and increases in physical activity in the morbid obesity group may also be a potential reason for the nonlinear association seen between body weight and hyperlipidaemia diagnosis, often referred to as 'reversed causality' in j- or u-shaped cross-sectional association studies.

Whilst we observed a cross-sectional association between OSA and hyperlipidaemia prevalence, a potential causative role for OSA in driving the

development of hyperlipidaemia is suggested by clinical trials of continuous positive airway pressure (CPAP) therapy. In the meta-analysis of Li et al., 6 RCTs with 348 patients and 351 controls were analysed and a modest but significant effect of CPAP on the decrease in total cholesterol levels was reported [33]. A further study examining the effect of CPAP on lipid profiles in ESADA cohort is warranted.

There are consistent reports suggesting considerable regional differences in lipid control. In particular, elevated cholesterol was more prevalent in Northern European countries and less prevalent in the Southern regions [34,35]. In a recent study from the Multi-Ethnic Study of Atherosclerosis (MESA) cohort, race/ethnicity has been demonstrated as a risk factor for cardiovascular disease and increased OSA severity amongst four different race groups in United States of America [36]. The reflections of regional varieties such as different body fat distribution patterns, previously identified genetic and dietary factors, may account for the differences in hyperlipidaemia prevalence amongst study sites [18]. Our data confirm those previously mentioned interactions by demonstrating a strong influence of different European sites on the hyperlipidaemia prevalence. Nonetheless, certain disparities regarding the prevalence of impaired lipid metabolism in our data set were noted. For instance, patients in Northern region, where highest cholesterol concentrations were reported in our previous study, demonstrated the lowest prevalence of a hyperlipidaemia diagnosis in the current study [14]. Potential explanations of this finding include regional differences in genetic predisposition to hyperlipidaemia, diet and awareness of the medical profession to lipid status. Thus, further studies providing insight regarding regional/ethnical disparities as well as treatment strategies in the lipid metabolisms of European OSA populations are needed.

Strengths and limitations

There are several strengths and limitations of our study. The generalizability of the results originating from the multinational and multicentre study design as well as the large cohort constitutes a major strength. On the other hand, a trend for clinical referral bias is present in the current cohort which constitutes data from academic sleep centres. Since ESADA collaborator institutions are mostly tertiary hospitals, patient referrals from primary and secondary hospitals generate a potential clinical referral bias for the studies. Thus, the present results may be representative for European OSA patients but not for the general population. Our study results may be also affected by clinical referral bias. In fact, the design of our study does not allow us to identify the actual cause of referral for each individual patient. However, according to clinical guidelines, nocturnal symptoms, excessive daytime sleepiness or the existence of comorbid diseases such as hypertension, ischaemic heart disease, diabetes or stroke are the most common reasons for an evaluation of suspected sleep apnoea. The latter named comorbidities are likely to be associated with an elevated prevalence of a HL diagnosis and may constitute a referral bias which may affect the association between OSA severity and the prevalence of a HL diagnosis in our study. Indeed, our data clearly showed stronger associations between OSA severity and HL in the subgroups with comorbidities (Table 1, lower part). Our study evaluated the influence of OSA on hyperlipidaemia as a clinical diagnosis but controlled also for actual drug treatment on cholesterol levels in hyperlipidaemia. However, the cross-sectional design of our study does not allow to determine any causal relationship between OSA and hyperlipidaemia. Besides, we could not evaluate the precise effect of OSA on the control of HL since the treatment of HL varies depending on the risk assessment of the physician and is not limited to medication. In addition, patient adherence to prescribed lipid-lowering medication was not monitored in our study. Some important confounders that could influence the association between OSA and HL such as family history of lipid disorders, diet and exercise could not be controlled in the present study. Sleep test methodology was either PG or PSG, which influences AHI and ODI values substantially, a detailed analysis of the sleep analysis performed in the ESADA study can be found elsewhere [20]. In the current analysis, we therefore focused on ODI quartiles as a measure for OSA severity as this parameter has been demonstrated to be less sensitive to methodological differences when compared with the AHI [20]. Lastly, despite studies report that patients with OSA tend to be sedentary [37], we could not examine the potential influence of physical exercise in our study cohort. The prospective evaluation of CPAP therapy on lipid status in the ESADA cohort may overcome at least some of the study limitations.

Conclusion

Obstructive sleep apnoea was independently associated with the diagnosis of hyperlipidaemia, and the link was particularly strong with intermittent hypoxia. Meanwhile, hyperlipidaemia was notably under-recognized in OSA subjects. The geographical impact of different European sites was identified and defined as a new confounder. Further studies elucidating the effect of CPAP therapy on lipid status in the ESADA cohort are of importance.

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Conflict of interest

Amongst the authors of the present manuscript, Dr. Pepin reports grants from Air Liquide Foundation, Fisher and Paykel, Mutualia and Vitalaire; grants and personal fees from Agiradom, Philips, ResMed and AstraZeneca; and personal fees from Boehringer Ingelheim, Jazz pharmaceutical, Night Balance and Sefam, outside the submitted work. Dr. Grote reports grants from ResMed Foundation, Respironics Foundation and European Respiratory Society; nonfinancial support from European Sleep Research Society, during the conduct of the study; personal fees and nonfinancial support from Itamar Medical; and personal fees from ResMed and Philips, outside the submitted work. In addition, Dr. Grote has a patent in pharmacological therapy of OSA licensed. Dr. Hedner reports grants from ResMed, and Philips Respironics during the conduct of the study; and personal fees from ResMed, Philips, Itamar Medical, Astra Zeneca, Bayer, Takeda, Bresotec and Desitin, outside the submitted work. The remaining authors have no conflict of interest to declare.

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APPENDIX

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4.6 Study 6

Insomnia symptoms combined with nocturnal hypoxia associate with cardiovascular comorbidity in the European Sleep Apnea cohort (ESADA)

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SLEEP BREATHING PHYSIOLOGY AND DISORDERS • ORIGINAL ARTICLE

Insomnia symptoms combined with nocturnal hypoxia associate with cardiovascular comorbidity in the European sleep apnea cohort (ESADA)

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Abstract

Purpose The aim of the current study was to further investigate the concept of previously reported high occurrence of comorbidities in obstructive sleep patients (OSA) with insomnia-like symptoms. We hypothesized that this finding at least partly is mediated by nocturnal hypoxia. Moreover, we speculated that the spectrum of the clinical OSA phenotypes differs between European geographical regions.

Methods Cohort of the European Sleep Apnea Database ($n = 17{,}325$; 29.9% females) was divided into five subcohorts according to geographical region (North, East, South, West, Central) and further into four clinical presentation phenotypes based on daytime symptoms (EDS) and characteristics suggestive of insomnia.

Results The insomnia phenotype (alone or together with EDS) dominated in all European regions. Isolated insomnia, however, was less common in the West. Insomnia phenotype was associated with the highest proportion of cardiovascular comorbidity (51.7% in the insomnia vs. 43.9% in the EDS type). Measures of nocturnal hypoxemia were independently associated with cardiovascular comorbidity in phenotypes with insomnia-like symptoms. The burden of comorbidities was high across all geographical regions and clinical phenotypes. Regional differences were clinically relevant for age (48 vs. 54 years), BMI (29 vs. 34 kg/m^2), and ODI (15 vs. 32/h).

Conclusion High prevalence of particularly cardiovascular comorbidity among patients with insomnia-like symptoms was linked to nocturnal hypoxemia. Considerable differences in clinical presentation were found among OSA patients across Europe. Our data underline that physicians should ask their patients with suspected OSA also for insomnia symptoms. It remains to be explored if a reduction of nocturnal hypoxemia predicts the improvement of insomnia symptoms.

Keywords Cardiovascular disease · Comorbidity · Hypoxemia · Insomnia · Sleep apnea · Phenotype

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Introduction

Obstructive sleep apnea (OSA) is a heterogeneous, complex disorder encompassing a wide variety of symptoms and disorders. A good understanding of the comorbidities and therapeutic outcome in various clinical phenotypes improves the possibility to provide a targeted therapy in each individual patient. We have previously reported on four clinical phenotypes defined by daytime and nighttime symptoms that differ in terms of burden of comorbidity [1]. A phenotype associated with insomnia symptoms was linked to a higher prevalence of cardiovascular diseases (CVD) and other common disorders [1, 2] in a manner that was not explained by the severity of OSA.

The underlying mechanism behind the increased prevalence of cardiovascular comorbidity in the insomnia-like OSA phenotype remains unexplained but some observations have been reported. For instance, patients of the insomnia phenotype were generally older [1]. There are also data suggesting a higher sympathetic activity [3] and association of cardiovascular comorbidity in non-sleepy OSA [4]. Severity of nocturnal hypoxemia could be another explanation. Nocturnal intermittent hypoxia in the current ESADA cohort predicted prevalent hypertension [5] and impaired ventricular relaxation during diastole in another study [6]. Proportion of sleep time spent at an oxygen saturation below 90% was independently associated with an increased risk of hypertension [7] or a higher systolic blood pressure during both sleep and awake in OSA patients [8]. Further, in community-dwelling elderly with OSA, hypoxia was associated with insomnia only in those with CVD [9].

Prevalence estimations of physical and mental disease vary between countries and regions and may impact on comorbidity among OSA phenotypes. For instance, the age-standardized CVD prevalence rates are relatively high in Eastern and Central European countries and lower in Western, Northern, and Southern Europe [10]. Other disorders like chronic depression and insomnia also differ by region [11]. Finally, perception of OSA among health care providers and lay people will determine the characteristics of the patients referred for sleep studies. Therefore, referral patterns among geographical regions are likely to differ and may result in distinct distribution of clinical phenotypes of OSA.

The aim of the current study was to further investigate the concept of different OSA phenotypes. We hypothesized that the previously reported high occurrence of comorbidities in OSA patients with an insomnia phenotype at least in part is mediated by nocturnal hypoxia. Moreover, we speculated that the spectrum of the clinical OSA phenotypes differs between European geographical regions.

Methods

The European Sleep Apnea Database (ESADA) has prospectively collected data from unselected adult patients aged 18 to 80 years referred to several European sleep centers with a history of snoring and other symptoms suggesting OSA like witnessed apneas or increased daytime sleepiness [12]. Comorbidities like cardiovascular, pulmonary, metabolic, and psychiatric diseases based on medical records were also reported to the ESADA database. The study protocol was reviewed and approved by a local ethics committee at each participating center. All patients gave their written, informed consent. Patient data were coded and de-linked before entry into the central database. Data recorded between March 2007 and May 2016 were submitted for analyses. The cohort comprised 19,556 adult patients of which 17,325 (29.9% females) had complete data. The cohort was divided into five subcohorts according to geographical region (North, East, South, West, and Central) (Fig. 1).

The influence of region on clinical patient characteristics (phenotype) was studied after adjustment for age, gender, and BMI. Four clinical phenotypes were defined according to daytime symptoms (EDS) and characteristics suggestive of insomnia (self-reported sleep duration, sleep latency, diagnosed insomnia, or hypnotic use defined by ATC code N05) as reported previously [1]. ATC code N05 includes antipsychotics, anxiolytics, hypnotics, and sedatives. In brief, the criteria for the subgroups were as follows: (1) EDS (daytime+/nighttime−), (2) EDS/insomnia (daytime+/nighttime+), (3) non-EDS/non-insomnia (daytime−/nighttime−), and (4) insomnia (daytime −/nighttime+). Daytime+ indicates that the patient had daytime sleepiness defined by ESS score > 10 and daytime− that ESS score was ≤ 10. Criteria for nighttime+ included at least one of the following: diagnosis of insomnia, self-reported sleep latency ≥ 30 min, self-reported sleep duration ≤ 6 h, or use of

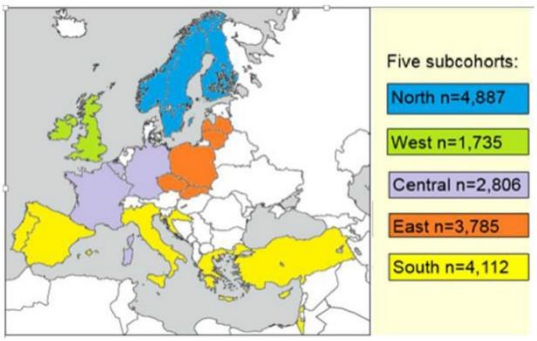

Fig. 1 Subcohorts applied in the current study defined by geographical region. *North:* Finland, Norway, Sweden, *East:* Czech Republic, Latvia, Lithuania, Poland, Slovakia, *South:* Greece, Croatia, Israel, Italy, Portugal, Spain, Turkey, *West:* Ireland, UK, *Central:* Belgium, France, Germany

hypnotics defined by ATC code N05. Nighttime− referred to situation where none of the nighttime+ criteria were fulfilled. The scoring methods used for polysomnography (PSG) or level 3 cardiorespiratory polygraphy (PG) have been reported previously [1, 12, 13]. A survey was made among participating centers to evaluate possible differences in standard operating procedures (SOPS) of handling referrals or other factors influencing referral patterns. The survey included four questions: possible mandatory screening of sleepiness before referral, categorical reasons for denying CPAP treatment or referrals to sleep studies and finally, which issues may impact on referral patterns for a sleep study.

Statistical methods

Data are presented as mean ± standard deviation or as frequency (%). Comparisons among the groups or phenotypes were performed using ANOVA for continuous variables, or the chi-square tests for categorical variables. Impact of age, gender, body mass index (BMI), current smoking, nocturnal hypoxia (mean oxygen saturation (SaO_2)), minimum SaO_2, oxygen desaturation index (ODI of 4%), apnea hypopnea index (AHI), and geographical region on prevalence of cardiovascular diseases among clinical presentation phenotypes was analyzed by logistic regression. Statistical analyses were performed using IBM SPSS Statistics 22.0 (Armonk, NY, USA: IBM Corp.). P value < 0.05 was considered statistically significant. All tests were two-sided.

Results

Anthropometric measures, comorbidities, and sleep apnea activity in relation to region and clinical phenotypes

Regional differences were clinically relevant for age (minimum 48 vs. maximum 54 years), BMI (29 vs. 34 kg/m^2), and ODI (15 vs. 32/h) (Table 1, Online Resource 1). The highest proportion of females was reported in the North region. The youngest, but most obese and sleepy patients, were found in the West, the most severe OSA in the South and the mildest degree of the disorder in the North region. In addition, the prevalence of the four defined clinical phenotypes varied between the European regions (Table 2, Fig. 2). The insomnia phenotype (alone or together with EDS) was the dominant phenotype in all regions. Isolated insomnia, however, was less common in the West.

Clinical phenotypes differed in terms of comorbidity profile. Sleep apnea appeared to be more severe in the EDS group but less severe among those characterized with insomnia ($P < 0.001$). Conversely, cardiovascular morbidity was most prevalent among those with insomnia. A metabolic condition and/or a pulmonary disorder was more common in those with EDS combined with insomnia while the highest prevalence of a psychiatric disorder was found in those with insomnia or insomnia with EDS (Table 3, Fig. 3, Online Resource 2). Insomnia and EDS-insomnia phenotypes were more prevalent among women than men (Table 4).

Table 1 Basic characteristics of patients by region

Characteristic	Total	Region					*P* value
		North	East	South	West	Central	
Female gender (%)	29.9	35.6	26.2	28.5	30.3	26.9	< 0.001
Age (years)	52.2 ± 12.6	51.3 ± 13.2	53.6 ± 11.8	53.1 ± 12.5	48.4 ± 12.0	52.9 ± 12.4	< 0.001
BMI (kg/m^2)	31.3 ± 6.6	29.8 ± 6.0	32.0 ± 6.2	32.4 ± 6.9	34.2 ± 7.8	29.9 ± 5.9	< 0.001
Current smoker (%)	24.2	21.7	23.3	26.9	26.7	24.3	< 0.001
AHI/h	27.0 ± 25.5	15.6 ± 19.2	31.1. ± 26.2	36.0 ± 27.3	23.4 ± 24.6	31.2 ± 24.7	< 0.001
ESS	9.8 ± 5.3	9.8 ± 5.0	9.3 ± 5.5	9.9 ± 5.3	11.2 ± 5.6	9.6 ± 5.2	< 0.001
ODI/h	23.5 ± 25.3	14.7 ± 18.6	29.0 ± 26.6	32.2 ± 28.3	19.7 ± 24.4	20.2 ± 22.9	< 0.001
Mean SaO_2 (%)	93.1 ± 3.4	93.8 ± 2.4	92.2 ± 4.3	92.6 ± 4.2	93.7 ± 2.1	93.4 ± 2.6	< 0.001
Min SaO_2 (%)	80.6 ± 10.0	83.3 ± 7.6	78.1 ± 11.3	78.8 ± 11.3	81.6 ± 8.9	80.5 ± 9.2	< 0.001
CVD (%)	48.5	41.6	59.4	51.0	34.6	51.2	< 0.001
Metabolic (%)	34.6	26.5	31.6	42.9	24.6	46.7	< 0.001
Pulmonary (%)	23.4	26.4	25.8	13.8	42.9	28.8	< 0.001
Psychiatric (%)	11.4	11.8	10.5	11.1	14.2	10.9	0.001

P value across regions defined by ANOVA except for current smoker, female gender, and proportion of comorbidities with chi-square test. *AHI*, apnea-hypopnea index; *BMI*, body mass index; *CVD*, cardiovascular disease; *ESS*, Epworth Sleepiness Scale; *ODI*, oxygen desaturation index; *SaO_2*, oxyhemoglobin saturation

Table 3 Basic characteristics by clinical phenotype

Characteristic		Clinical phenotype				*P* value
	Total	EDS	EDS-ins	Non-EDS non-insomnia	Insomnia	
Female gender (%)	29.9	26.0	32.6	21.3	36.0	< 0.001
Age (years)	52.2 ± 12.6	50.4 ± 12.5	51.6 ± 12.1	52.0 ± 12.8	53.8 ± 12.7	< 0.001
BMI (kg/m^2)	31.3 ± 6.6	31.8 ± 6.6	32.4 ± 7.0	30.7 ± 6.1	30.8 ± 6.5	< 0.001
Current smoker (%)	24.2	24.3	26.2	22.0	24.1	< 0.001
ESS	9.8 ± 5.3	14.8 ± 3.2	14.8 ± 3.1	6.1 ± 2.7	5.8 ± 2.9	< 0.001
AHI/h	27.0 ± 25.5	31.2 ± 27.8	30.1 ± 27.6	26.4 ± 24.3	22.9 ± 22.7	< 0.001
ODI/h	23.5 ± 25.3	27.7 ± 28.0	27.3 ± 27.6	21.6 ± 23.8	19.5 ± 22.0	< 0.001
Mean SaO_2 (%)	93.1 ± 3.4	92.7 ± 4.0	92.8 ± 3.6	93.4 ± 3.1	93.4 ± 3.0	< 0.001
Min SaO_2 (%)	80.6 ± 10.0	78.9 ± 11.3	79.7 ± 10.4	81.2 ± 9.4	81.8 ± 8.9	< 0.001
CVD (%)	48.5	43.9	48.5	47.7	51.7	< 0.001
Metabolic (%)	34.6	33.6	36.6	34.2	34.0	0.015
Pulmonary (%)	23.4	22.7	26.8	18.9	24.3	< 0.001
Psychiatric (%)	11.4	4.8	17.2	3.4	16.5	< 0.001

P value defined by ANOVA except for current smoker and female gender and proportion of comorbidities with chi-square test. *AHI*, apnea-hypopnea index; *BMI*, body mass index; *CVD*, cardiovascular disease; *EDS*, excessive daytime sleepiness; *ESS*, Epworth Sleepiness Scale; *ODI*, oxygen desaturation index; *SaO_2*, oxyhemoglobin saturation

independently associated with CVD comorbidity in phenotypes with insomnia-like symptoms. Third, single clinical characteristics and clinical OSA phenotypes differed among European geographical regions. However, the burden of comorbidities was high across all geographical regions and clinical phenotypes.

Association between phenotypes and comorbidities

Although the presence of comorbid insomnia [14, 15] has been recognized in OSA, the endeavor of phenotyping OSA is quite recent [1, 16–18]. The clinical characteristics (ESS score, subjective sleep duration and sleep latency, physician-diagnosed sleep disorder, use of hypnotics) that were applied to phenotype patients in the present study are readily available to clinicians treating patients with OSA. Insomnia-like phenotypes (EDS-insomnia and insomnia) were identified in more than 50% of patients, thereby confirming previous findings [1, 19]. Cardiovascular, pulmonary, and psychiatric comorbidities were more prevalent in phenotypes with insomnia-like symptoms. These results are in line with the findings in an Icelandic OSA cohort using quite similar phenotyping criteria [18] and our previous report [1].

Traditional risk factors such as age, gender, BMI, or smoking explained partly the comorbidity burden among distinct clinical phenotypes in our study. It has been suggested

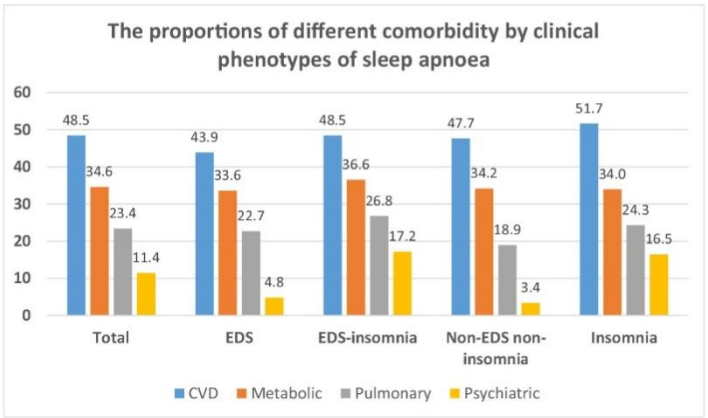

Fig. 3 The proportions of cardiovascular, metabolic, pulmonary, and psychiatric comorbidity by four clinical phenotypes of sleep apnea. *P* value (ANOVA) for trend was 0.015 for metabolic disease and < 0.001 for other comorbidities

Table 4 Gender differences in clinical phenotypes

Phenotype	Female, *n* (%)	Male, *n* (%)	Total, *n* (%)	*P* value
EDS	844 (16.2)	2402 (19.7)	3246 (18.7)	< 0.001
EDS-insomnia	1414 (27.2)	2927 (24.1)	4341 (25.0)	< 0.001
Non-EDS non-ins	837 (16.1)	3100 (25.5)	3937 (22.7)	< 0.001
Insomnia	2102 (40.4)	3739 (30.7)	5841 (33.6)	< 0.001

P value defined by chi-square test. *EDS*, excessive daytime sleepiness

that an increased time lag from the start of the disease to final diagnosis and treatment in these OSA patients with rather atypical symptoms allowed for a higher exposure to harmful OSA-induced cardiovascular consequences [18]. Unfortunately, our database does not allow an assessment of the duration from the first OSA symptoms to diagnosis.

The higher prevalence of cardiovascular comorbidity in phenotypes with insomnia-like symptoms could also be explained by elevated adrenergically mediated alertness manifested as long sleep latency or short self-reported sleep duration. This "hyperarousal"-hypothesis is supported by observations of higher sympathetic activity in non-sleepy patients with severe OSA compared with the sleepy ones [3] as well as in primary insomniacs compared with good sleepers [20]. In fact, previous data has linked cardiovascular comorbidity to non-sleepy OSA in patients with peripheral arterial disease [4]. Epidemiological studies have also confirmed high cardiovascular comorbidity in depressive disorders [21], a condition with frequent symptoms of insomnia. Our data are also in concordance with the literature suggesting that the EDS plus insomnia group is more obese. Insomnia is a potential factor behind weight gain, an observation that has been explained by altered leptin and ghrelin activity and higher glucose and insulin levels as well as an increased appetite [22].

An important novel finding of our study was that nocturnal hypoxia, particularly nadir SaO_2, was independently associated with higher prevalence of CVD in both phenotypes with insomnia-like symptoms. In the current analysis, three measures of hypoxia were included (ODI, mean, and lowest saturation) in the model and this modifies the statistical power of the single variable AHI or ODI. In fact, when taken as single OSA variable in the analysis, ODI increases the risk for CVD in all phenotype classes. However, it is important to highlight that the nadir of nocturnal hypoxia, a value which is not often in the focus of clinical sleep medicine, was a predictor of CV disease in the insomnia phenotypes. Indeed, recent studies demonstrated that subjects with chronic insomnia have increased sympathetic activity, an impaired sympathetic baroreflex function, and an augmented neural cardiovascular responsiveness to stress [23]. Further, in a Swedish study of community-dwelling elderly subjects with OSA, there was an association between spending more than 1.5% of the sleep time with at a $SaO_2 < 90\%$ and insomnia in those with a CVD [9]. In summary, our data point towards a potential cumulative risk of insomnia and hypoxemia in OSA patients.

Geographical differences

Our study is the first to address geographical differences in the distribution of the clinical presentation phenotypes in European OSA patients. One of our key findings was that single clinical characteristics and the proportions of distinct clinical presentation phenotypes in the ESADA cohort

Table 5 Risk for CVD among clinical phenotypes when adjusted for measures of nocturnal hypoxemia, age, gender, BMI, smoking, and geographical region

Phenotype Adjusted for	HR	EDS 95% CI (*P* value)	HR	EDS-insomnia 95% CI (*P* value)	HR	Non-EDS non-insomnia 95% CI (*P* value)	HR	Insomnia 95% CI (*P* value)
Mean SaO_2	1.014	0.983–1.045 (0.390)	1.015	0.984–1.047 (0.348)	0.987	0.950–1.024 (0.481)	1.001	0.970–1.033 (0.966)
Nadir SaO_2	0.999	0.987–1.011 (0.840)	*0.984*	*0.974–0.995 (0.005)*	0.994	0.982–1.007 (0.378)	*0.988*	*0.978–0.999 (0.035)*
ODI	1.001	0.997–1.006 (0.547)	1.001	0.997–1.005 (0.485)	1.001	0.996–1.006 (0.698)	1.000	0.996–1.004 (0.924)
Age	*1.083*	*1.074–1.092 (< 0.001)*	*1.080*	*1.072–1.088 (< 0.001)*	*1.087*	*1.079–1.095 (< 0.001)*	*1.088*	*1.081–1.095 (< 0.001)*
Male gender	*1.323*	*1.089–1.608 (0.005)*	*1.309*	*1.115–1.536 (0.001)*	1.201	0.995–1.450 (0.056)	*1.402*	*1.221–1.610 (< 0.001)*
BMI	*1.084*	*1.066–1.102 (< 0.001)*	*1.074*	*1.060–1.088 (< 0.001)*	*1.088*	*1.071–1.105 (< 0.001)*	*1.096*	*1.082–1.109 (< 0.001)*
Smoking	*1.279*	*1.046–1.563 (0.016)*	1.005	0.848–1.192 (0.952)	1.078	0.892–1.304 (0.435)	1.158	0.994–1.349 (0.060)
West	1.000		1.000		1.000		1.000	
North	*1.543*	*1.155–2.073 (0.003)*	*1.381*	*1.075–1.774 (0.011)*	*1.733*	*1.283–2.342 (< 0.001)*	*1.862*	*1.417–12.448 (< 0.001)*
East	*2.675*	*1.982–3.611 (< 0.001)*	*2.247*	*1.698–2.972 (< 0.001)*	*3.052*	*2.241–4.156 (< 0.001)*	*2.936*	*2.195–3.927 (< 0.001)*
South	*1.811*	*1.341–2.447 (< 0.001)*	*1.555*	*1.205–2.006 (0.001)*	*1.452*	*1.065–1.981 (< 0.018)*	*2.120*	*1.601–2.808 (< 0.001)*
Central	*1.972*	*1.341–2.447 (< 0.001)*	*1.963*	*1.466–2.629 (< 0.001)*	*1.782*	*1.2941.981 (< 0.001)*	*2.571*	*1.884–3.508 (< 0.001)*

BMI, body mass index; *CVD*, cardiovascular disease; *EDS*, excessive daytime sleepiness; *ODI*, oxygen desaturation index; *SaO_2*, oxyhemoglobin saturation

The significant findings are in italic

differed across geographical regions. The finding that insomnia phenotypes are less frequent in Western and Central Europe is in concordance with the report of Dregan and coworkers [24]. In middle-aged and elderly population, they found that insomnia was less common in the western and central part of Europe at least with regard to Ireland and Germany. In the UK, insomnia was similar to Belgium, France, and Bulgaria but higher than in Germany or in northern countries.

The prevalence of comorbidities including cardiovascular disease [25, 26], metabolic syndrome [27], mood disorders [28], and obstructive lung disease [29] was, as expected, high in the ESADA cohort. CVD was most prevalent in the East, metabolic comorbidity in the Central region, and pulmonary and psychiatric comorbidity in the West region, the associations with region and comorbidity being in some cases quite high. The observed differences may at least in part reflect geographical differences in comorbidity according to EU statistics [10, 30, 31]. Also, lifestyle factors such as smoking or physical activity may explain differences [30]. Patients with chronic depression frequently suffer from insomnia and the proportion of the population reporting chronic depression is higher in the northern, western, and central Europe compared to southern and eastern Europe [31]. Particularly high prevalence rates of insomnia have been reported in Poland, Hungary, Estonia, Germany, France, and Portugal but much lower rates in Denmark, Italy, and the Netherlands [11]. In summary, data from this largest cohort or European sleep apnea patients point towards substantial geographical differences in comorbidity and clinical phenotype which may explain the heterogeneity of OSA management between countries. Our data also promote further research to gain deeper insight and to allow better interpretation of the regional effects and risk of comorbidities.

Referral routines

The management of OSA in Europe is variable [32] and cultural factors, general public, and medical awareness of OSA as well as available diagnostic facilities and treatment options might explain the observed differences. It is also likely that the perception of what constitutes a "typical OSA" patient varies considerably among health care providers in different regions. Although the prevalence rates of EDS and insomnia linked with distinct clinical phenotypes varied considerably, we need to acknowledge that cultural differences in attitudes to sleep may have an impact on how respondents in different countries interpret sleep problems and subsequently rate their sleep [33].

It might be argued that the differences seen in the clinical phenotypes between regions result from referral bias. However, thorough investigation of SOPS to handle referrals did not explain regional differences in clinical phenotypes. On the other hand, for example, media campaigns of awareness of sleep apnea were reported to influence referral patterns in all regions. Further population-based epidemiological studies in the different European regions may help to identify the role of referral bias as the underlying cause for the observed differences in our study.

Strengths and limitations

The ESADA cohort provides a unique opportunity to explore the real-life clinical practice and characteristics of OSA in different parts of Europe. Although, the ESADA does not reflect the individuals in the general population, they are part of a referral bias which in part may reflect the observed regional differences in clinical phenotypes in Europe. The wide age range and notable female representation in the cohort also allow a consideration of age and gender-related issues. The centralized data monitoring and web-based report format ensure uniformity in the reported data sets. In the ESADA protocol, apneas and hypopneas were significant respiratory events and RERA (respiratory effort related arousals) or RDI (respiratory disturbance index) were not scored. However, phenotypes were not defined based on sleep apnea data. Therefore, the conclusions in terms of regional distribution or comorbidity burden related to phenotypes are considered not to be affected by lack of RERA or RDI. The locally used diagnostic routines applied at participating centers provide a specific methodological challenge (for example, lack of comprehensive pulmonary function tests), which may contribute to differences in the reported comorbidities. A major limitation in our study was the broad definition of insomnia, which did not comply with the ICD or DSM criteria and therefore may lead to overestimation of the prevalence of "real" insomnia. Until now there is no special classification of insomnia with regard to severity but there are first ideas to cluster patients by polysomnography [34] or by the history of disease. However, the finding that even symptoms of insomnia in OSA patients are associated with increased comorbidity is of importance. Although the database does not allow for comprehensive analysis of the effects of sociodemographic factors (for example, socio-economic status, degree of physical exercise, marital status, or caffeine intake) on referral patterns or comorbidity of OSA, it represents the by far most comprehensive description of clinical characteristics in European OSA patients and has revealed a novel finding of link between nocturnal hypoxemia and cardiovascular comorbidity in OSA phenotypes with insomnia-like symptoms. Finally, our finding does not suggest a bias related to SOPs of handling referrals, since the finding was independent of geographical region.

Clinical implications

Interestingly, comorbid insomnia or insomnia-like symptoms may aggravate the burden of OSA with respect to cognitive function and vigilance. Identifying those patients has

implications for personalized treatment. Insomnia-like symptoms have been associated with lower adherence to continuous positive airway pressure (CPAP) therapy [1, 2]. Moreover, treatment effects on outcomes like blood pressure, prevention of cardiovascular events, traffic accident rate, or mood disturbance are likely to differ depending on OSA phenotype. A recent study has demonstrated that cognitive behavioral therapy (CBTi) is effective also in patients with comorbid insomnia and OSA [35]. Combining CBTi with CPAP treatment might improve adherence to CPAP therapy and improve morning restfulness and daytime alertness in patients with OSA [14] and possibly protect from CVD events.

Conclusions

High prevalence of particularly cardiovascular comorbidity among patients with insomnia-like symptoms was linked to nocturnal hypoxemia. Characteristics of patients referred for suspected OSA differed between European sleep centers independently of confounders like age, gender, and obesity. Considerable differences in clinical presentation were found among OSA patients across Europe. In consideration of the wide generalized spread of patients in the Pan-European database, the ESADA database may turn to be particularly useful for the analysis on how clinical phenotypes may influence treatment outcomes in OSA.

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Compliance with ethical standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare that they have no conflict of interest.

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4.6.1 Invited Editorial Sleep Apnea Screening Tools is there an impact of the geographical location

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PULMONOLOGY

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EDITORIAL

Sleep apnea screening tools. Is there an impact of the geographical location?

In this edition of Pulmonology, José Coutinho Costa and colleagues are validating the NoSAS score (neck, obesity, snoring, age, sex) as a screening tool for the diagnosis of obstructive sleep apnea (OSA).[1] The authors investigated the accuracy of the NoSAS in a population consisting of patients that were referred to the sleep centre by general practitioners. The relevance of methods other than sleep recordings -including questionnaires- for the diagnosis of OSA have recently been discussed in this journal and therefore will not be subject of this editorial.[2] Also, the general pros and cons of the NoSAS were earlier presented by Walter McNicholas in the editorial to the original validation study and his comments do also apply to the present study.[3]

In 2016 the NoSAS was validated in a Swiss population.[4] The methods used in this study differ in several aspects from the original one. In this study, an in-laboratory polysomnography (PSG) setting was preferred, while the HypnoLaus study investigated the population with an ambulatory PSG protocol.[4] This difference might be only minor; however, the selection of the study cohort was also different, and this is of relevance. Marti-Soler and colleagues[4] used a general population-based study, while in José Coutinho Costa's study[1] the patients were pre-selected by the general practitioners. Each choice of the study population has its advantages or disadvantages[3] but they are not identical. A direct comparison of the NoSAS results from both studies is therefore rather complex. However the present study is very important for anybody using the NoSAS questionnaire in Portugal.

Table 1 demonstrates a selection of published studies validating the NoSAS. It is interesting that between the four studies the concordance probability or area under the curve (AUC) is quite similar, each showing an acceptable discrimination by the NoSAS model. However, the OSA prevalence in each study population, the collected anthropometric data and the comparable cut off value for the presence of OSA are not interchangeable.

This would therefore indicate that the NoSAS questionnaire is a reliable instrument to predict OSA within a given local population even if it is multi-ethnic as in the study from Tan and colleagues (Chinese, Indian and Malays).[6] If so, it raises questions regarding the absolute values as sleep apnea indicators. It is surprising, that in the study cohort from Coutinho Costa et al.[1] the OSA/Non-OSA discrimination calculated by the AUC equals the result from the study of Peng and colleagues[5] that demonstrated a similar study design. Surprisingly, because the average neck-circumference and body mass index were clearly higher in the Coutinho Costa study.[1] Results from the European Sleep Apnea Database (ESADA) demonstrated, that geographical localization (ethnicity was not a problem) of a sleep apnea cohort influences clinical presentation, biochemical results and probably cardiovascular outcome of OSA patients .[7–10] Likewise, differences can be seen in the NoSAS data. Coutinho Costa et al.[1] found 19.1% of the control group with a neck circumference over 40 cm, while Tan and colleagues[6] detected a prevalence of only 15.7% within a group of patients identified by the NoSAS score as high risk for OSA. Thus, the clinical implication of a 40 cm neck circumference or BMI class might differ depending on the location and/or consistence of the study population.

In conclusion: The aim of any diagnostic test in medicine is to distinguish between healthy and not healthy persons. The results from Coutinho Costa and colleagues[1] will be of importance to predict the presence of OSA in the Portuguese population. However, it was investigated in preselected patients and therefore the results need to be validated in a general population cohort. The implication of geographical localization on predictors for OSA, sleep study results and the prognosis of patients with OSA will need further investigation.

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Table 1 Comparison of anthropometric data, OSA prevalence and calculated AUC for NoSAS in four different studies.

Country	General population study	AHI used to define OSA	OSA prevalence	Mean Neck circumference (SD) [cm]	Mean BMI (SD) [kg/m^2]	Age [years]	AUC	Publication
Switzerland	Yes	20/h	26%	36.9 (3.9)	25.6 (4.1)	59 (11)	0.74	(4)
China	No	≥15/h	78.1%	36.9 (4.1)	25.9 (4.3)	48.9 (14.4)	0.731	(5)
Singapore	Yes	≥20/h	28.1%	36.4 (4.1)	No mean	48.3 (14.0)	0.738	(6)
Portugal	No	≥15/h	34.8%	41.0 (3.6)	30.8 (5.1)	53.5 (12.1)	0.77	(1)

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4.7 Study 7

Impact of temperature on obstructive sleep apnea in three different climate zones of Europe. A study of the European Sleep Apnea Database (ESADA)

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4.7.1 Anthropometric and sleep recording data of the total ESADA Cohort and within climate zones.

A total of 19293 patients from 23 sleep centres were included in this study. To harmonize the general environmental characteristics the participating centres were grouped according to the Koeppen-Geiger Climate classification.

The recruitment of participants for each month of the year varied between the three climate zones. The Cfb zone demonstrated a minimum inclusion during July (n=747) and a maximum in January (n=1178). For the Csa climate zone a nadir of included patients was observed during August (215 patients) and the maximum recruitment was in June (426 patients). In Dfb we detected the lowest inclusion number in June (n=166) and the highest number of patients included in July (n=262). The participating sleep centres reported no possible bias such as a preferred selection of clinically severe patients during the period of the summer vacation.

The ESADA sleep centres and corresponding climate zones in Europe are shown in figure 10.

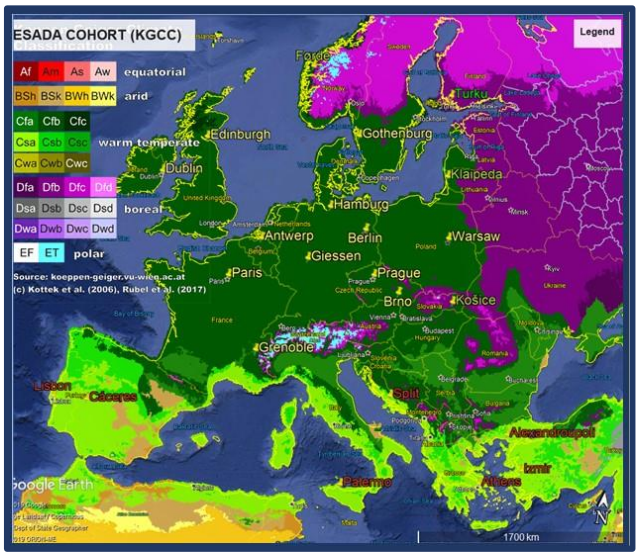

Figure 10: ESADA cohort according to the Köppen-Geiger Climate Classification. A KMZ file downloaded from http://koeppen-geiger.vu-wien.ac.at/present.htm and imported into Google Earth Pro with indication of the participating sleep centres. Three study sites demonstrate a borderline situation between Csb and Dfb (Førde, Klaipeda, Kosice). Førde was at characterized as Csb while Klaipeda, Kosice remained Dfb.

Results for the analysis of the anthropometric data and sleep recordings are listed in table 6. The one-way analysis of variance (ANOVA) with post hoc Bonferroni multiple comparison analysis revealed a statistically significant differences between the climate zones. This concerned all investigated variables except for BMI and the nadir of SpO2 when comparing Cfa with Dfb.

	Total Cohort (CI)	Cfb (n=12516) (CI)	Csa (n=4338) (CI)	Dfb (n=2439) (CI)
Age [years]	52.33 (52.15-52.51)	52.22 (51.99-52.44)	52.79 (52.42-53.16	52.09 (52.15-52.51)
BMI [kg/m^2]	31.19 (31.00-31.28)	30.79 (30.68-30.90)	31.98 (31.77-32.17)	31.57 (31.57-21.11)
Male/Female [%]	70.25/29.75	71.01/28.99	71.9/28.1	63.47/36.53
AHI [/h]	26.76 (26.41-27.11)	24.50 (24.10-24.91)	34.15 (33.36-34.93)	25.19 (24.13-26.26)
ODI [/h]	23.10 (22.74-23.45)	20.99 (20.58-21.40)	30.11 (29.29-30.92)	21.13 (20.13-22.14)
T90 [minutes]	39.61 (38.11-41.12)	38.40 (36.45-40.34)	41.80 (39.30-44.30)	38.50 (31.10-45.91)
Lowest SpO2 [%]	80.48 (80.34-80.63)	80.91 (80.74-81.08	78.69 (78.35-79.03)	81.77 81.33-82.22)
PSG [%]	50.55	41.4	71.1	62.8

Table 6: Anthropometric and sleep recording data of the total ESADA cohort and the three investigated climate zones Cfb, Csa, Dfb. Results are displayed as mean values with 95 % confidence interval (CI) and percentage for the male/female ratio. The one-way Anova analysis indicated significant results for age: $F(2,19290)=3,78$; $p=0.02$, BMI: $F(2,19218)=68.20$; $p<0.001$, AHI: $F(2,19290)=257.95$; $p<0.001$, ODI:$F(2,18263)=229.43$; $p<0.001$ and lowest SpO2: $F(2,18407)=97.62$; $p<0.001$) T90: $F(2,9488)=2.25$; $p=1.05$. Statistically significant results were detected for age between Cfb and Csa ($p=0.032$) and BMI for Cfb versus Dfb ($p<0.001$). The AHI and ODI were significantly different between Csa and the two other climate zones ($p<0.001$). We found no significant result when comparing Cfb versus Dfb. The lowest oxygen saturation reached significance in the multiple comparison analysis with Cfb versus Csa $p<0.00.1$; Cfb versus Dfb $p=0.001$ and Csa versus Dfb $p<0.001$ respectively. There was no significant result in the analysis of T90. Annotation: AHI: Apnea/hypopnea index, ODI: Oxygen desaturation index, T90 time of oxygen saturation < 90 %, PSG=polysomnography

4.7.2 Analysis of apnea/hypopnea index, desaturation index and percent<90 % oxygen saturation in cold, mild and warm temperature environment of the total cohort

When analysing the effect of temperature on the respiratory parameters (natural logarithm of AHI=LNAHI) or ODI=LNODI) we detected a small but significant increase in the AHI in warm temperature when compared with moderate or cold environment with

F(2,18617) of 127,49 (p<0.001. The maximum effect size defined by Cohen´s d was 0.33 when comparing cold with warm temperature. This result corresponds a difference for LN apnea of 0,4 or $e^{0,4}$ =1,49 indicating that sleep studies performed in an environment of > 15ºC show an increase of the AHI by 1,49 events/hour. The correlation between the AHI and the ODI was 0,858 and therefore both variables show the same relationship towards the elevated temperature. In the T90 analysis we found a significant increase when comparing cold and the mild temperature, but the effect size was very small (d=0.11). There was no further significant change of the oxygen saturation under 90 % when analysing mild versus warm temperature environment (p>0.05 and Cohen´s d: 0.01). Regarding the minimum oxygen saturation results resembled the AHI and ODI since values deteriorated from cold to warm temperature. All main results are listed in table 7. When controlling the results of either mild or warm temperature for age, BMI and gender results remained statistically significant with cold vs. mild reaching a d of 0.05, p<0.001 and cold vs. warm with a d of 0.17, p< 0.001.

	Cold (CI) [Cº]	Mild (CI) [Cº]	Warm (CI) [Cº]	Cold vs. Mild	Mild vs. Warm	Cold vs. Warm
LN AHI [/h]	2,.56 (2.50-2.58)	2.64 (2.61-2.67)	2.96 (2.93-2.99)	p<0.001 d=0.06	P<0.001 d=0.25	p<0.001 d=0.33
LN ODI [/h]	2.29 (2.25-2.33)	2.36 (2.33-2.39)	2.70 (2.66-2.74)	p=0.018 d=0.04	p<0.001 d=0.24	p<0.001 d=0.29
LN T90 [%]	2.09 (1.99-2.19)	2.35 (2.28-.42)	2.33 (25-2.41)	p<0.001 d=0.11	p>0.05 d=0.01	P=0.001 d=0.11
Lowest SpO_2 [%]	81.20 (80.94-81.47)	80.66 (80.45-80.87	79.52 (79.23-79.81)	p=0.007 d=0.05	p<0.001 d=0.11	P<0.001 d=0.17

Table 7: Mean values and 95 % confidence interval (CI) of the natural logarithm (LN) of the apneia/hypopneia index (AHI), desaturation index (ODI); percentage of SpO2 < 90% (T90) and lowest oxygen saturation (SpO_2) during cold, mild and warm temperature. The one-way Anova analysis demonstrated a significant difference between the groups (p<0.001). The post-hoc Bonferroni multiple comparison analysis revealed statistically significant results between all groups except when T90 was compared in mild vs. warm temperature environment. LNT90 did not reach a relevant effect size. All other respiratory values reached a mild to moderate (LNAHI) effect size when cold and warm environment was compared and in case of LNAHI and LNODI also between mild and warm temperatures.

The analysis was repeated with maximum temperature (MaxTemp) as independent continuous variable (model 1). The results were controlled for gender, age and BMI (model 2) and gender, age, BMI and presence of air conditioner (A/C) model 3.

Model 1 reached a small R^2 of 0.08. Nevertheless, the result reached statistical significance ($p<0.001$), When including age, BMI and gender the prediction model demonstrated a reasonable R^2 of 0.28. The control for A/C in model 3 did not change the results. The standardized coefficients β for each model and investigated respiratory parameter are listed in table 8. For LNAHI and LNODI the standardized coefficient β demonstrated acceptable values that reached statistical significance ($p<0.001$). In case of the lowest oxygen saturation we detected also statistically significant result, although with a lower standardized coefficient β. In contrast to this we found no statistically significant result for T90.

	Model 1		Model 2		Model 3	
	β	P value	β	P value	β	P value
LN AHI	0.28	<0.001	0.24	<0.001	0.25	<0.001
LN ODI	0.25	<0.001	0.22	<0.001	0.21	<0.001
LN T90	0.03	0.051	0.04	0.05	0.03	0.19
Min. SpO_2	-0.13	<0.001	-0.12	<0.001	-0.11	<0.001

Table 8 Linear regression analysis with the standardized coefficient β of the maximum temperature (TempMax) adjusted for age, BMI, gender and presence of air conditioner. Model 1: Maximum temperature (TempMax), model 2: MaxTemp, gender, BMI, Age and model 3: Maximum temperature (TempMax), gender, BMI, Age, air conditioning. MaxTemp reached statistically significant results for all respiratory parameters except of T90. Abbreviations: LN: natural logarithm, AHI: apnea/hypopnea index; ODI: oxygen desaturation index, T90: time of oxygen saturation < 90 %, Min. SpO_2: Lowest oxygen saturation

Table 9 demonstrates the results for all the variables in the equitation of model 3. The BMI demonstrated the highest standardized coefficients β, while the results of maximum temperature, gender and age were almost interchangeable. The effect of air conditioner was low but almost reached statistical significance.

These results indicate the existence of a small but statistically significant effect of temperature on the respiratory parameters during sleep except for the recorded oxygen saturation under a value of 90 %.

	LN AHI		LN ODI		LN T90		Min. SpO_2	
	β	P value	β	P value	β	P value	β	P value
MaxTemp	0.25	<0.001	0.21	<0.001	0.03	0.194	-0.11	<0.001
Gender	0.24	<0.001	0.22	<0.001	0.13	<0.001	-0.12	<0.001
BMI	0.32	<0.001	0.41	<0.001	0.38	<0.001	-0.41	<0.001
Age	0.25	<0.001	0.26	<0.001	0.21	<0.001	-0.17	<0.001

A/C	-0.03	0.05	0.03	0.097	0.052	0.091	-0.01	0.39

Table 9 Linear regression analysis of model 3 with the standardized coefficient beta (β) of all variables in the equitation. Except for the presence of air conditioner (A/C) all other variables reached statistical significance for LNAHI, LNODI and minimum SpO2 value. The correlation coefficient maximal temperature (MaxTemp) did not reach statistical significance in LNT90. Abbreviations: LN: natural logarithm, AHI: apnea/hypopnea index; ODI: oxygen desaturation index, T90: time of oxygen saturation < 90 %, Min. SpO_2: Lowest oxygen saturation.

4.7.3 The impact of temperature on respiratory parameters during sleep in different climate zones.

We divided the cohort for further analysis into the climate zones suggested by the Koeppen-Geiger climate classification. The annual temperature varied between the three climate zones as depictured in figure 11.

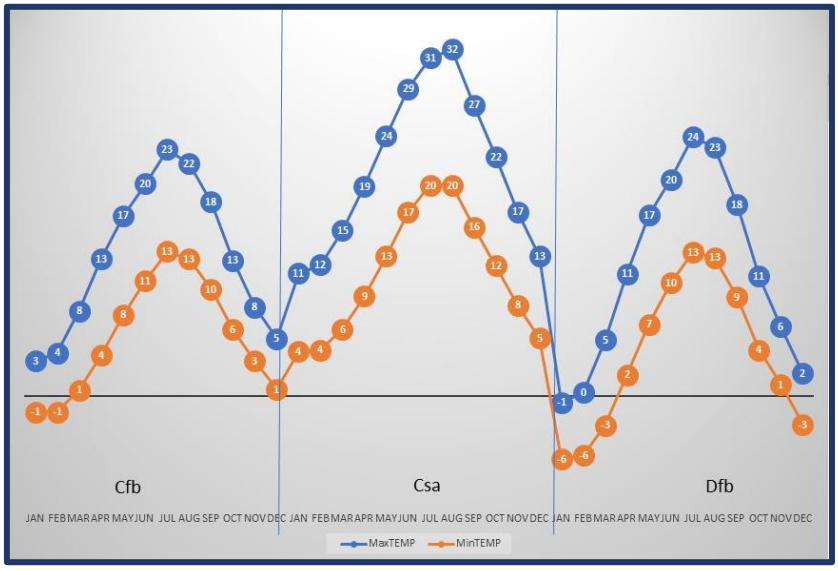

Figure11: Mean values of the annual maximum and minimum temperature recorded in the analysed time period. The Cfb climate zone demonstrated the lowest amplitude between highest temperature in summer and coldest temperature in winter, while Csa showed the highest amplitude.

A multiple block regression analysis was run for each climate zone of this study. Model I included maximum temperature, model II BMI, Age and gender and for model 3 we added as independent variable the presence of A/C during the sleep study. Main results for LNAHI and LNODI are shown in in table 10 and table 11.

					Maximum Temperature	
Cfb	Block	R^2	F change	Significance of F change	Standardized coefficient β	Significance β
	I	0.013	164.31	<0.001	0.11	<0.001

	II	0.233	1173.77	<0.001	0.09	<0.001
	III	0.235	44.99	<0.001	0.09	<0.001
Csa	I	0.000	0,75	0,38	-0.01	0.386
	II	0.194	345.54	<0.001	-0.01	0.335
	III	0.202	39.37	<0.001	-0.02	0.127
Dfb	I	0.006	13.90	<0.001	0.08	<0.001
	II	0.28	291.44	<0.001	0.05	=0.003

Table 10: Hierarchical block regression analysis to investigate the impact of maximum temperature on LNAHI. Results are depictured for the three climates zones Cfb, Csa and Dfb. Block I: maximum temperature, Block II: +age, BMI and gender and Block III + presence of air conditioner. Maximum temperature demonstrated a very small but significant effect in the Cfb and Dfb climate zones while in Csa there was no effect visible. The impact of air conditioner was small but significant (R^2 chang=0.003 in Cfb and 0.007 in Csa). There were no sleep studies with air conditioner in the Dfb climate zones.

					Maximum Temperature	
Cfb	Block	R^2	F change	Significance of F change	Standardized coefficient β	Significance β
	I	0.008	97.43	<0.001	0.09	<0.001
	II	0.273	1379.29	<0.001	0.07	<0.001
	III	0.277	62.81	<0.001	0.06	<0.001
Csa	I	0.000	0.60	0.439	-0.01	0.439
	II	0.259	480.55	<0.001	-0.01	0.453
	III	0.270	59.80	<0.001	-0.02	0.124
Dfb	I	0.004	9.33	=0.002	0.06	=0.002
	II	0.342	391.83	<0.001	0.04	<0.001

Table 11: Hierarchical block regression analysis to investigate the impact of maximum temperature on LNODI for the three climates zones Cfb, Csa and Dfb. Block I: maximum temperature, Block II: +age, BMI and gender and Block III + presence of air conditioner. In resemblance to the results of the LNAHI analysis there are differences between the three climate zones Maximum temperature demonstrated a very small but significant effect in the Cfb and Dfb climate zones while in Csa there was no effect visible. The inclusion of air

Interestingly, we found a modulating effect by the climate zones. While in Cfb and Dfb the maximum temperature in block I resulted in a small but significant increase of the prediction model, we detected no visible effect for the Csa climate zone. Figure 12 is demonstrating the relationship between maximum temperature and LNAHI for each climate zone with both variables standardized.

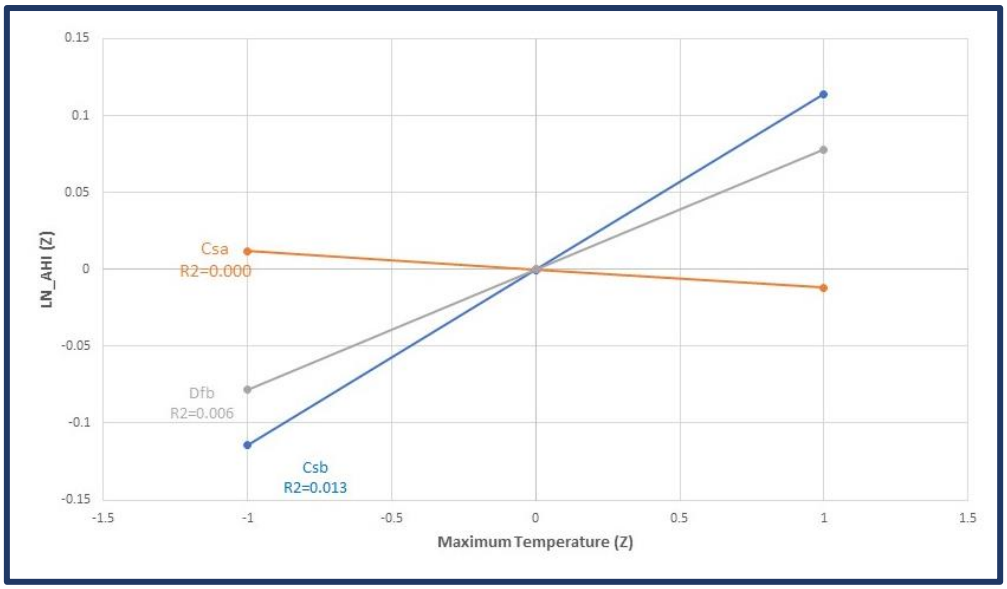

Figure 12: Standardized natural logarithm of the apnea/hypopnea index (LNAHI) was plotted against standardized maximum temperature values. The Cfb climate zone demonstrated the lowest values of the AHI with a clear temperature dependent increase. This result was also detected in the Dfb climate zone although the effect of temperature was lesser compared to Cfb. For the Csa climate zone we found no relevant effect of the temperature on the LNAHI.

However, even for Cfb and Dfb the capability of maximum temperature to explain the variance in LNAHI and LNODI was more than tenfold smaller compared to the anthropometric variables age, BMI and gender. Even so, temperature is of interest for the respiratory parameters during sleep in clinical terms as the simulation in table 7 demonstrates.

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
(Constant)	1	1	1	1	1	1	1	1	1	1	1	1
MaxTEMP	3.26	4.02	7.91	12.69	16.75	19.68	22.88	21.57	18.01	12.59	8.25	5.34
Gender	1	1	1	1	1	1	1	1	1	1	1	1
Age	50	50	50	50	50	50	50	50	50	50	50	50
BMI	28	28	28	28	28	28	28	28	28	28	28	28
LN_Apeneia	2.36	2.37	2.44	2.53	2.60	2.65	2.71	2.69	2.62	2.52	2.44	2.39
Apneia	10.57	10.71	11.49	12.52	13.47	14.20	15.04	14.69	13.78	12.50	11.56	10.97

Table 12 Simulation of the relationship between maximum temperature during each month and the AHI for a male with an age of 50 years and an BMI of 28 kg/m^2. The AHI reflected the evolution of the temperature during the year resulting in an increase of 4.47 events per hour when lowest temperature in January was compared to highest temperature in July.

The results for the impact of maximum temperature on the lowest oxygen saturation were alike the findings from the LNAHI and LNODI analysis. The maximum temperature demonstrated for Cfb in block I an R^2 of 0.004 and for Dfb 0.002. In both cases the F

change of 34,71 and 6.46 were very small but reached statistical significance (p<0.001 and p=0.002, respectively). For Csa the R^2 of maximum temperature was 0.002 with an F change of 6.46 which contrary to the results in LNAHI and LNODI was statistically significant (p=0.01). The standardized coefficient β demonstrated for the nadir of the oxygen saturation an inversed direction compared to LNAHI and LNODI. This indicates that for Cfb and Dfb higher temperatures resulted in a lower minimum oxygen saturation. Interestingly, compared to the former two groups higher temperature provoked a lower oxygen drop in the Csa group. For Cfb the standardized coefficient β was -0.06 (p<0.0.01) and for Dfb -0.05 (p=0.002). In Csa the standardized coefficient β was 0.039 reaching statistical significance (p=0.011).

The relationship between maximum temperature and the LNT90 was less clear compared to LNAHI and LNODI. Within the Cfb climate zone maximum temperature showed in the block I analysis an R^2 of 0.001 with a F change of 6.86 (p=0.009) and the standardized coefficient β was 0.05 (p=0.003). For Csa the R^2 change was also 0.001 with a F change of 3.07 almost reaching statistical significance (p=0.08). For the Dfb climate zone we found no significant R^2 change (p=0.57). In both Csa and Dfb the standardized coefficient did not reach statistical significance (p=0.08 and p=0.83, respectively). Nevertheless, it is notable, that in the Csa climate zone the addition of air conditioner in the block III analysis resulted in a significant R^2 change of 0.006 with a F change of 20.15 (p<0.001). The unstandardized coefficient β in CSA was -0.03 and approximated statistical significance (p=0.08).

4.7.3.1 Effect of air conditioner.

Since the measured outdoor temperature does not necessarily reflect the indoor environment, we added the presence of air conditioner (A/C) for each sleep centre at the time of the analysis. The Dfb group was excluded since no sleep laboratory indicated at the time of this analysis the presence of a A/C. While A/C was existing in only 3.9 % of the Cfb climate zone we found an estimation of 69.3% of the sleep studies withing Csa group with A/C. It is notable, that although the effect of the air-conditioner was very small it significantly increased the prediction of the model for all respiratory parameters in both climate zones except for LNT90 in Cfb and lowest SpO_2 in Csa. Within the Csa the standardized coefficient β of maximum temperature demonstrated a more negative

direction for LNAHI, LNODI and T90 but reached statistically significance only for the lowest oxygen saturation.

5 Discussion

5.1 General considerations

Sleep medicine and sleep science is covered by a variety of medical specialities, e.g. Neurology, Pneumology, Psychiatry, ENT surgeons and specialists in paediatric medicine. This plurality sometimes causes frictions regarding the correct diagnosis and treatment of sleep disorders. Nevertheless, there exists consensus that the impact of obstructive sleep apnea (OSA) goes beyond daytime symptoms like excessive daytime sleepiness, but also influences other components of the human equilibrium. Depending on the definition, it might even fulfil the criteria for a systemic disease [276,277]. Although OSA has been studied for decades there still does not exists a general accepted concept regarding the pathophysiology behind the interaction of OSA with the human body. It even remains for some members of the sleep community an open question if OSA induces measurable changes in the cardiovascular system at all, and if so whether the immune or metabolic system is involved [74].

This manuscript provides some novel information about the influence of obstructive sleep apnea (OSA) on components of the human homeostasis. Additionally, this work demonstrates that results might be influenced by the geographical location of the cohort investigated.

To obtain this information two established study concepts were combined:

1. Experimental study design: The study cohorts investigated are well controlled regarding the anthropometric values. Usually, data is generated by special equipment or laboratory methods.
2. Real life studies with data from a large cohort for that permits more advanced statistical analysis. In this manuscript this refers to the cohort of the European Sleep Apnea Database (ESADA).

Both approaches have their positive or negative points. Experimental studies are commonly used to prove new concepts or techniques. Although experimental studies are essential, they are often costly since they require specific procedures and are

therefore underpowered. Beside this, the results obtained under controlled conditions may be questioned, since they do not reflect real life conditions. This might apply even to large but very well controlled trials, e.g. pharmacological studies [278]. Large cohorts with an open or "real life" study design may not suffer of this limitation, but they are prone to other possible confounders. In multicentre and multinational trials, the clinical procedures of each centre might be influenced by the local regulations and the need to comply with local reimbursement policies. This might cause relevant differences due to cultural and geographical influencers [279]. This fact is rarely discussed but might be of utter importance since the outcome in some multicentre studies did not reflect previous observations within smaller studies. In sleep medicine, the most recent example is the SAVE study published in 2016. In this publication, the authors did not find any positive impact of continuous positive airway pressure (CPAP) on cardiovascular diseases which was contradictory to many studies previously published. Several critics of the study identified the multicentre/multi-ethical design as a major cause for this negative result due to a high number of included patients from China [280-282].

This manuscript developed from a controlled study design to a real-life observation. The included multicentre and multinational studies from the European Sleep Apnea Database (ESADA) helped to understand data previously acquired by the experimental study design. But the ESADA studies also opened new questions since we found several unexpected regional differences in the ESADA group regarding both the OSA phenotype and secondary effects of OSA. Therefore, some aspects of the environmental or regional influences are also included in this work.

In the following the results will be discussed considering the above-mentioned observations.

5.2 Change of perforin positive peripheral blood lymphocytes subpopulations following exercise

In this study we used exhausting endurance exercise as a model to investigate the impact of physical and psychological stress during a sprint triathlon competition on the percentage of cytotoxic proteins positive peripheral lymphocytes. Intensive sport is

associated with an increase in the catecholamine levels in both trained athletes and less trained normal individuals [283]. Even minor changes of the catecholamine levels effect the natural killer cell activity [284]. We detected in our study, that already the absence of regular training for one week reduced the percentage of perforin (Pfr) or granzyme B (GrB) positive lymphocytes with a further decrease following the triathlon. Several values only returned to baseline one week after the competition. The immunological depression detected in elite athletes after exhaustive physical exercise has been subject for intensive investigation. Shephard and colleagues found a reduction in the natural killer cell activity (NKCA) following strenuous exercise [285]. However, the link to the cytotoxic protein Pfr was first detected by us. Clinically this reduction of the NKCA was associated with an increased risk of upper airway infections. This fact is apparently contradicting the general opinion that exercise even if exhaustive positively influences general health [286] Thus, the "open window" theory was created to explain the increased risk of infections following exercise. The effect of sportive activity was also described in form of a J-curve were the two extremes of too little and too much physical activity increases the risk of infections [242,287]. While most publications during the 90th of last century indicated a migration of the lymphocytic defence cells into the muscular tissue this concept has been questioned since health problems in elite athletes have been more associated with nutritional misalignment and low energy availability and, possibly even more important, due to sleep disruptions [288] or mere psychological stress [289].

The above mentioned factors are related to a raise in the catecholamine levels in intensive exercise which can change natural killer cell activity [290]. OSA is associated with an increased activity of the sympathetic nervous system and our study may serve as a model for a possible immunological change induced by the presence of obstructive sleep apneas. Recent studies revealed that both atherosclerosis [291] and atherosclerotic plaque disruption [155] are not only a cholesterol storage disease but are also related to inflammatory and immune processes involving cytotoxic lymphocytes. Stress, even if psychological has been found to activate inflammatory cascades and increase natural killer cell activity (NKCA) [292]. Catecholamine levels have been found elevated in OSA with a clear reduction following effective positive airway pressure (PAP) therapy [293].

Therefore, the better cardiovascular outcome in treated OSA patients may be associated with the stress release and its associated reduction in the cytotoxic killer cell activity.

5.3 Decrease of perforin positive CD3+γδ-T Cells in patients with obstructive sleep disordered breathing

This study aimed to investigate the impact of OSA on cytotoxic lymphocytes including gamma-delta T lymphocytes. As important subprotocol we tried to separate between the effect of hypoxic and non-hypoxic respiratory events

Even after almost forty years of thorough investigation the scientific society remains indecisive regarding the possible impact of OSA on the immune system. Results are often controversial even in concepts investigated for several years like the increase of Interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) in OSA patients [294].

In our study we found a significantly lower percentage of perforin positive $CD3^{+}\gamma\delta-T$ cells in obese patients with OSA when compared to controls. Additionally, sleep fragmentation due obstructive respiratory events but without intermittent desaturation was already associated with a reduced percentage of perforin positive $CD3^{+}\gamma\delta-T$ cells, although the result did not reach statistical significance. It is notable, that in the correlation analysis between the percentage of perforin positive $CD3^{+}\gamma\delta-T$ and the sleep parameters most respiratory values like AHI or ODI but also sleepiness assessments like the Epworth Sleepiness Score (ESS) or the Stanford Sleepiness Scale (SSS) reached statistical significance. Nevertheless, after controlling for the anthropometric values only the arousal index remained significant. Exposing $CD3^{+}\gamma\delta-T$ lymphocytes to severe hypoxia reduced the marker for degranulation capacity and percentage of GrB^{+} cells, but without changing the percentage of $perforin^{+}$ cells. It is of note that the complexity of this last investigation allowed only a limited number of participants. There are only a few studies investigating the impact of respiratory sleep disorder on the cellular immune system and even less for the cytotoxic immune system. Dyugovskaya and colleagues demonstrated an increased cytotoxicity in a subgroup of $\gamma\delta-$cells [205]. They found an increased number of γδ-cells expressing the natural killer (NK) cell receptor NKB1 in OSA patients, while other typical NK membrane markers like CD16 or CD56 cells were not significantly different between the groups. Moreover, the $\gamma\delta-T$ lymphocytes in the OSA group demonstrated an increased cytotoxicity against human umbilical vein endothelial cells that was reversed by adding anti-TNFα antibodies in the

cell culture [205]. These results are on casual observation opposite to our findings since we detected a decreased number of Pfr^+ positive $CD3^+\gamma\delta-T$ cells in OSA patients. However, one must consider, that the described cytotoxicity by Dyugovskaya, was TNF-α dependent which is not related to the Pfr/GrB pathway. Secondly, there are some issues to be discussed regarding the methodology. Although for the time when article was published the applied laboratory methods were impressing the authors do not discuss why of the initially 34 OSA patients and 19 controls in some tests less than half the participants remained and thus results were frequently for less than 10 participants in each group . Of course, the risk of technical failures by using sophisticated laboratory methods is always present. This can cause an understandable reduction in the number of analysed participants, but at least some explanation by the authors would be expected. Beside the fact that a statistical analysis in this small groups is questionable another important issue is the chosen protocol for the cytotoxicity test with Cr^{51} release test. Incubation time of the target/effector cell mixture is depending on the assay used, but most protocols recommend only a few hours since the test is supposed to investigate the rapid acting natural killer cell activity [295]. The two days incubation period used by the authors does not necessary investigate the Pfr/GrB induced NK cell activity but will also include the slower Fas/Fas ligand mechanism [296,297]. Therefore, the results from Dyugovskaya does not necessarily contradict our investigation. The second study publicated by the same group described in OSA patients an increased cytotoxicity of $CD16^+/CD56^+$ positive $CD8^+$ lymphocytes. Indirectly they showed that the cytotoxicity was related to perforin. Contrary to this, patients already on CPAP therapy demonstrated equal results in the cytotoxicity assay as controls. In this study the methods and protocol applied may also raise some concern regarding the results. The one-night CPAP treated group investigated by Dyugovskaya were distinct patients and not the same study participants measured before and after. Despite the fact, that the authors argue that the OSA severity of this one-night CPAP group was equal to the untreated patients, it remains questionable if this approach permits an analysis of the effect of CPAP on the cytotoxic immune system. Also, similar to the previously discussed study from the same group the number of participants included for the cytotoxic analysis was rather low (n=5 for controls, n=7 for OSA patients and n=6 for OSA patients following a single night CPAP) [206].

Our results did not demonstrate an increased cytotoxic potential in the investigated γδ-T cells. Neither did a follow-up sub-study over three months of positive pressure therapy reveal a significant change in the percentage of perforin positive cells within total lymphocytes or the respective subpopulations. Our sub-study included a total number of twenty patients realizing all three measurements which was substantially lower than the original OSA (noOSA and oOSA) group. This was related to the fact that either patients failed to appear at the scheduled appointments or due to problems within the cellular analysis. When investigating the effect size of the CPAP and APAP therapy via Cohen´s d we were able to demonstrate some interesting results. The total amount of Pfr positive lymphocytes increased after 3 months of APAP therapy reaching a small but relevant effect size. This indicates an augmentation of the cytotoxic potential of the lymphocyte population. The increase in NK cells reached a large effect size implicating that the result might become relevant for more than 79 % of the study group. This rise was detected in all investigated subpopulations with exception of the $CD3^-CD8^+$ lymphocytes. In this subset the percentage of perforin lymphocytes decreased after the start of the therapy reaching a mild effect size and remained lower after 3 months of therapy when compared to the initial value. The $CD3^-CD8^+$ lymphocytes group demonstrates some distinctive features when compared to normal $CD3^+CD8^+$ lymphocytes and NK cells. They have like NK cells a high cytotoxic potential and are mostly expressing the CD56 membrane molecule [298,299]. The described decrease in $CD3^-CD8^+$ lymphocytes is therefore supporting the results from Dyugovskaya, although not the same lymphocyte membrane markers were investigated. This observed bidirectional development of distinctive cytotoxic lymphocyte subpopulations may contribute to the understanding, why two -in immunological terms- opposite health problems like arteriosclerosis and cancer are encountered more frequently in OSA patients.

1) OSA is a stimulator of the inflammatory cascades leading to an increased risk of cardiovascular events. Especially coronary artery disease and plaque disruption have been associated with cytotoxic proteins like GrB in general [190] and with $CD8^+$ lymphocytes expressing the CD56 membrane molecule in special [148,185]. A decrease in this distinctive lymphocyte population might help to explain why PAP therapy in OSA patients reduces the risk of an acute coronary events [66].

2) Recent epidemiological studies found in OSA patients an elevated risk for cancer [64,213,215,300]. Due to the complexity of the interaction between sleep and the tumour defence it is difficult to prove a real cause-effect situation, but a decrease in the tumour surveillance of the immune is possibly involved [223]. Previously Gaoatswe and colleagues detected a reduced percentage of the invariant NK T cells in OSA patients [301]. This lymphocyte population increased following OSA therapy likewise to our observation regarding the perforin positive cytotoxic T lymphocytes (CTL) and NK cells. Additionally, we detected a reduced cytotoxic potential in γδ-cells of patients suffering of obstructive respiratory events. This might be of importance, since this lymphocyte population is highly linked to the tumour defence and is used in new therapeutic strategies of cancer therapy [302,303]. Nevertheless, our study was not designed to investigate this association and further evidence is needed to clarify if OSA and the OSA therapy influence tumorigenesis or not [304].

5.4 The importance of sleep fragmentation on the hemodynamic dipping in obstructive sleep apnea

Over the last decades clinical research accumulated a huge body of evidence regarding an intense relationship of OSA and arterial hypertension especially blood pressure non-dippers [83,305,306]. However, in a recent publication this concept has been questioned since the therapy of OSA with continuous positive airway pressure (CPAP) did not result in a decrease in cardio-vascular risk [281]. This study lines up with two other epidemiological studies that found only little or no longitudinal effect of OSA on blood pressure values [307]. On the other hand, there exist numerous studies confirming the risk of OSA on AHT especially in non-dipping blood pressure patients leading to specific recommendations in cardio-vascular guidelines [82-84,308,309]. There exist the need for some general considerations towards this point. Some of the apparently contradicting study results may be related to methodological issues of the study design [310,311]. Another, often forgotten factor is the fact that sleep is a function of the central nervous system. Previous works demonstrated some association between the sleepiness and the impact of OSA therapy on AHT [312,313]. Although Barbe et all found in the follow up study of the Spanish cohort a small decrease in ABP in non-sleepy OSA patients, the most important predictor for a positive therapy effect remained the change in the Epworth sleepiness scale [314]. Therefore, studies not investigating sleep and sleepiness, but only respiratory parameters might be insufficient. In this manuscript, we can demonstrate with a detailed beat-to-beat analysis of systolic blood pressure (SBP) and stroke volume (SV) that OSA influences the physiological decrease of blood pressure and stroke volume at sleep onset. The ability of the finger cuff-based blood pressure analysis to detect short term and sometimes small changes of the hemodynamic values makes it more adequate for this purpose than the 24 hours ambulatory blood pressure measurement (ABPM). We could confirm with this method, that in OSA patients the physiological decrease of the investigated hemodynamic parameters is reduced or absent at sleep onset. Furthermore, we were able to demonstrate that the short-term variability of the blood pressure was the more distinguishing parameter between OSA and controls than the SBP or the SV themselves. Both the non-dipping and increased short-term blood pressure variability are considered important risk factors for cardiovascular events

[309,315,316]. Our results did not only confirm a statistically significant difference between OSA and non-OSA patients, but we found additionally an either moderate or large effect size regarding the hemodynamic variations in OSA patients. Therefore, this study is of importance as it does not only confirm previous data, but also increases the credibility due to the controlled and detailed evaluation of systolic blood pressure (SBP) and stroke volume (SV).

To our knowledge, this is the first time that the evolution of hemodynamic parameters at sleep onset were investigated by a regression analysis. The standardized coefficient β (SCB) can be used to identify decrease (negative values) or increase (positive values) of the hemodynamic parameters. Further statistical analysis permitted the correction for known confounders like age or body mass index (BMI). As a proof of concept, we found in the first analysis a significant difference in the SCB of both SBP and SV when OSA patients were compared with controls. In OSA patients, the negative values in controls (thus decreasing SBP and SV) was either attenuated (SBP) or even reversed (SV). Although the prediction for the blood pressure evolution was improved by both the apnea/hypopnea index (AHI) and the arousal-index, however only the arousal-index remained significant in the final model.

Within the sleep medicine community there exists some controversy if the cardiovascular outcome in OSA patients is exclusively dependent on the respiratory parameters or not. There is evidence that intermittent hypoxia measured by the oxygen desaturation index (ODI) increases the risk of arteriosclerosis due to intravascular stress and subsequent hypertension [317]. On the other hand, sleep fragmentation without hypoxic respiratory events can also increase the activity of the sympathetic autonomous system and thus might also explain the high prevalence of arterial hypertension (AHT) in OSA patients [318,319]. In our study, AHI and ODI were interchangeable in the hierarchical block regression analysis and therefore not in favour of a more important impact of the oxygen desaturations. In obstructive sleep apnea the oxygen desaturations and arousals are usually associated. The relevance of the arousal index in our study may contribute to untangle this association and confirm that sleep fragmentation is relevant for the increased risk of cardiovascular diseases in OSA patients.

Nevertheless. there are two caveats to be observed in our study.

1. The study population is small, and results cannot easily be generalized

2. The arousal index and the AHI are practically interchangeable for patients with a severe OSA. International guidelines recommend a complete polysomnography to warrant the correct therapy decision in mild to moderate OSA patients. Our results underline this need since a non-dipping blood pressure profile may be related to sleep fragmentation and not necessarily to respiratory events.

While sleep fragmentation clearly increases the short-term variability of systolic blood pressure and decreases its physiological reduction at sleep onset it is not necessarily related to long-term vascular changes like arteriosclerosis. As discussed above OSA may lead to atherosclerotic plaque instability due to cytotoxic $CD8^+$ lymphocytes However, there is increasing evidence, that OSA might influence not only arterial hypertension but also other components of the metabolic syndrome [320].

5.5 Obstructive sleep apnoea independently predicts lipid levels: Data from the European Sleep Apnea Database

and

5.6 Hyperlipidaemia prevalence and cholesterol control in obstructive sleep apnoea: Data from the European sleep apnea database (ESADA)

The study protocol for the investigation of the cytotoxic proteins Pfr and GrB in OSA patients included routine blood analysis. The rationale behind this was to exclude relevant alterations in the inflammatory or metabolic system that might influence the immune system. As a not primary result we detected an increased level of triglycerides (TG) in both lean and obese OSA patients. Also, total cholesterol (cholesterol tot) and low-density lipoprotein (LDL) were higher in patients with obstructive sleep disordered breathing, although the results did not reach statistical significance. Interestingly, we detected significantly increased values of the gamma glutamyl transferase (gGT) in the obstructive sleep disordered breathing groups. Although speculative, this finding could indicate a possible relationship of OSA and the presence of non-alcoholic fatty liver disease without the modulation of obesity and intermittent hypoxia.

In the last decades, the relationship between OSA and components of the metabolic syndrome was subject of intense investigation. The definition of the metabolic syndrome has changed over the years. At present the National Heart Lung and Blood Institute defines as risk factors for the metabolic syndrome: 1) a large waist-line, 2) a high triglyceride level or being on triglyceride reducing medicine, 3) a low HDL Cholesterol level, 4) high blood pressure and 5) high fasting blood sugar (https://www.nhlbi.nih.gov/health-topics/metabolic-syndrome). Other components e.g. decreased insulin sensitivity, increased LDL cholesterol levels or non-alcoholic fatty liver disease are important regarding the risk for cardiovascular diseases but not included in this definition.

Already in 1994 Davies and colleagues investigated the plasma insulin and lipid levels in untreated OSA patients [8]. The study did not reveal any significant difference between OSA patients and controls. However, this study was clearly underpowered with 15 patients in the OSA group and 18 controls. In the following years several groups found mainly in animal experiments a positive relationship between OSA associated respiratory disturbances like intermittent hypoxia and the lipid metabolism confirming the importance of sleep related breathing disorders for the metabolic homeostasis [95,111,321].

Cholesterol metabolism is highly dependent on the liver pathway were an accumulation of triglycerides and cholesterol might induce non-alcoholic fatty liver disease (NAFLD) [119,322]. The possible relevance of OSA on the development of non-alcoholic fatty liver disease (NAFLD) has recently caught the interested the sleep research community [321,323]. The altered liver lipid metabolism caused by the activation of the hypoxia inducible factor-1 (HIF-1) might be the crucial to explain increased dyslipidaemia risk of OSA patients. Nevertheless, it remains under discussion if this OSA-NAFLD is independent of increased obesity tissue [17,321,323]. There exists a variety of animal models to confirm the link between hypoxia and the new synthesis of free fatty acids that are necessary for an increased hepatic secretion of triglycerides and very low-density lipoprotein (VLDL). Jun et al. showed that there was an increase in lipolysis within the adipose tissue of rodents when exposed to intermittent hypoxia [324]. Also, in a mouse model the presence of intermittent hypoxia increased the plasma free fatty acids and the lypolitic rate [325]. In humans the evidence is less clear. In a controlled study Chopra and colleagues could show, that OSA increases the nocturnal plasma free fatty acids, but also this study was underpowered since it included only 11 patients [326]. An indirect sign of altered lipoprotein metabolism was detected in a small sample of OSA patients. The fractional clearance rate for triglycerides was decreased and this effect was partially reserved by continuous positive pressure therapy [114].

From the Sleep Heart Health Study (SHHS) originated the probably first epidemiological study reporting a relationship between OSA and alterations within the metabolic system. Newman and colleagues described 2001 in a cohort of 4991 participants a significant positive relationship between pro-atherogenic lipid values and the

respiratory disturbance index (RDI) although after control for confounding factors like age and obesity the model reached significance only for the total cholesterol level [327].

In two studies from the ESADA cohort [328,329] we were questioning the impact of OSA on either the existing lipid levels, or as different perspective, on the prevalence of hyperlipidaemia. It is of interest, that both studies ended with the same conclusion: there exist a relationship between the respiratory parameters of OSA and the existence of hyperlipidaemia. In the first study when patients were divided in quartiles according to the AHI or ODI value we detected an elevated odds ratio for hypercholesterinaemia within the quartiles of the more severe OSA group. The result remained significant even after adjustment for several existing confounders like BMI, age, diabetes, or the presence of cardiovascular diseases. The same applied to LDL cholesterol and triglycerides while HDL cholesterol was significantly lower, especially in the highest quartile. Some limitations of this study must be mentioned. First: it is difficult to generalize results from cross sectional analysis on the general population since there is no clear time-line analysis to demonstrate a cause-effect relationship. Also, most patients were referred to the sleep centres due to suspected sleep disordered breathing. Therefore, participants within the cohort were pre-selected, and results might be only limited to patients with sleep related breathing disorders.

When comparing our results with the existing literature we found divergent results. In one review only 3 of 13 studies demonstrated a positive relationship between OSA total cholesterol, 6 of 13 regarding the triglycerides status and 4 from 13 described a negative relationship with total cholesterol [330]. Also, it remains controversial if positive airway pressure therapy has any impact on dyslipidaemias. Hu and colleagues could show in a metanalysis that CPAP influences the triglyceride level but not on other components of the lipid metabolism [15]. Data from Iceland obtained from a well-controlled study using home sleep apnea testing devices did not confirm a positive effect of CPAP therapy on the metabolic status [331]. Hence, there exists some discrepancy between the results from animal studies with a positive OSA-hypoxemia and dyslipidaemia relationship and the conflicting results from human studies. As one explanation might serve, that most of the existing evidence from human studies is based on cross sectional analysis without dyslipidaemia as a primary outcome of the study. Therefore, the study protocol was

possibly not designed to control for influencers of the lipid metabolism. Also, only large study groups like the ESADA cohort display enough power to extrapolate the small differences. Furthermore, in most studies the effect of OSA on the lipid metabolism is reduced to the presence and severity of hypoxic events. This applied even to those researchers that used polysomnographic sleep recordings for the diagnosis of OSA [114]. This is up to a point understandable, since as stated above, there exists an elaborated concept from animal models regarding the hypoxia induced stimulating of HIF-1 that results in an increased activity of several enzyme of the lipogenesis pathway e.g. the sterol regulatory element-binding protein-1 and stearoyl-CoA desaturase-1. Physiologically, the increased generation of triglycerides and very low-density lipoproteins in addition to NAFLD associated altered glucose metabolism could explain most findings [332-334].

There exists an increasing body of evidence that lipid metabolism is also dysregulated in non-hypoxic sleep disturbances including sleep fragmentation or circadian rhythm disorders [335-342]. Thus, the limitation on hypoxia to explain dyslipidaemias in OSA patients has been questioned recently.

If sleep time and sleep quality influence the metabolic system, these factors might explain while some studies failed to demonstrate an impact of OSA and its treatment on dysproteinaemia. In the ESADA group we questioned the relevance of the diagnosis method applied. A possible scenario would be a stronger dyslipidaemia/OSA relationship in centres using polysomnography (PSG) compared to centres with mainly operating with cardio-respiratory polygraphy (CR-PG). The distribution of the applied diagnostic method was balanced within the study population (47 % PSG versus 53 % CR-PG). However, in the multiple regression analysis, the relationship between OSA parameters and total cholesterol levels remained significant. As an additional point is to mention, that several centres with CR-PG recordings demonstrated the highest total cholesterol values of the total group. On the other hand, the prevalence of a dyslipidaemia diagnosis was lowest in the northern countries of the ESADA cohort, which predominantly use CR-PG while eastern countries with mainly polysomnography were the third in terms of a positive dyslipidaemia diagnostic. As such, we could not find

a convincing evidence, that the impact of OSA on dyslipidaemia values is dependent on the apnea diagnosing method applied.

5.7 Insomnia symptoms combined with nocturnal hypoxia associate with cardiovascular comorbidity in the European Sleep Apnea cohort (ESADA)

During the investigation of the lipid status in the patients of the ESADA cohort we detected a relevant regional influence. There exists already some support for the assumption that the geographical location is of importance for the incidence, prevalence and outcome of cardiovascular diseases [343]; updated data can be found within the homepage of the European heart network http://www.ehnheart.org/cvd-statistics.html). The ESADA cohort is covering several countries and cultures. This distinguishes ESADA from the other large cohorts investigating sleep related breathing disorders like the Sleep Heart Health Study, the Wisconsin Sleep Cohort, São Paulo Epidemiologic Sleep Study or, most recent, the Hypnolaus study. When our study population was divided in the main cardinal directions with the addition of a central European region the levels of total cholesterol differed with the highest values in the North and the lowest in the Western region. This is of some importance, since there exists already evidence, that both ethnical, cultural, and possibly geographical aspects could influence both sleep and sleep disordered breathing [344]. It is too early to decide if there is a real clinically relevant relationship between sleep disordered breathing and lipid levels or hypercholesterinaemia, but our results suggest, that conclusions from studies of one region in Europe do not necessarily apply in the same way to other regions.

This alleged geo-regional impact on sleep disturbances was furthermore investigated in another ESADA study. We found a clear regional influence regarding the severity of OSA and the prevalence of insomnia and excessive daytime sleepiness within the cohort. In general, OSA patients showed a higher severity in excessive daytime sleepiness, while cardiovascular diseases were more prominent in patients with insomnia symptoms.

However, this observation was not equally applicable for the investigated regions. The northern centres revealed the lowest AHI but higher prevalence of excessive daytime sleepiness than the South although the AHI was highest in the later.

The prevalence of sleep disturbances within Europe do not demonstrate an equal distribution. In a cohort of participants older than 50 years van de Straat and colleagues detected an important impact of both country localization and gender. Portugal showed the second highest prevalence in sleep disturbances resulting in values of 20.8 % for males and 36.8 % for females [345]. Only Poland surpassed this result. On the other hand, the excessive daytime sleepiness demonstrates a different pattern in Europe. Ohayon and colleagues detected in a questionnaire-based investigation of narcolepsy prevalence in Europe a clear North-South difference of sleepiness with significantly higher value in the United Kingdom and Germany when compared to Italy, Spain and Portugal [346]. Even the naps, traditionally more accepted in southern countries were higher in the northern countries. In a recent study Marta Gonçalves and colleagues investigated the prevalence of drowsy driving in Europe. Their results demonstrated that within the nineteen included European countries exists a clear difference in the risk of following asleep at the wheel. In this investigation Portugal belonged with an odds ratio of 1.34 to the four countries with the highest risk of following asleep while driving. Our results from the ESADA cohort are difficult to compare since the sleep centres were grouped in the geographical regions and not by countries. We could demonstrate for the South a low prevalence of isolated EDS but the highest values for the combination of EDS and insomnia. This might be of relevance since not only sleep disorders known to be associated with hypersomnolence like obstructive sleep apnea or restless legs syndrome but also insomnia and sleep restriction may cause significant daytime sleepiness [347]. Between countries and cultures not only the prevalence of sleep disturbances like insomnia or sleep restriction differ but also the perception of their consequences. As example may serve a survey study published by Léger and colleagues [348]. They detected in a cohort of 10132 individuals from the United States of America (USA), western Europe (France, Germany, Italy, Spain and United Kingdom) and Japan that sleep induction disturbances was highest in the Japanese population while the participants of the USA suffered more of insufficient sleep maintenance or poor sleep

quality. Furthermore, in the USA cohort the authors found the greatest impact of sleep disturbances on the personal activities, family life and social relationship while the Japanese group indicated the negative impact on professional activity as the biggest problem of their sleep disorder. This illustrates how different the relevance of sleep and sleep disturbances can be perceived depending on cultural or ethnical groups. In the ESADA cohort the ethical aspect is irrelevant since there was only a very low percentage of Non-Caucasian participants (< 1%). Nevertheless, the real or perceptive presence of insomnia or hypersomnolence was significantly different between the geographical regions. For the time being we are not able to explain this result. Beside cultural differences there exists also the possibility that dissimilar temperature or light exposure may be of importance [349,350].

We also showed that the prevalence of cardiovascular diseases varied significantly between the five regions. Of course, we cannot exclude, that regional differences in the admission procedure e.g. if the referral letters to indicate coexisting morbidities, would be of relevance. Nevertheless, the fact that the grouping in geographical regions included several different countries let this possibility appear less probable. As stated above, it is already known that within Europe the risk for cardiovascular disease is influenced by the geographical location. However, this has not been investigated in the context of sleep related breathing disorders. In a recent metanalysis Cuspidi et al. analysed a total of 14 publications regarding the presence of non-dipping blood pressure in OSA patients. The authors found different prevalence of non-dipping blood pressure between the study sites. The authors interpreted this to different study settings as well as clinical and demographical characteristics [351]. Yamagishi and colleagues compared the prevalence of sleep disordered breathing between Japanese and American Caucasian and Hispanics. He found a lower prevalence within the Japanese population with an equal relevance of the BMI [352]. Nevertheless, the results need to be taken with some care since the author compared different cohorts and sleep related breathing was measured by a single channel air-flow monitor. Recently, Grandner and colleagues summarized in a review the association of both obstructive sleep apnea and insomnia on the cardiovascular risk and referred also the ethical impact [353]. However, although the referred studies covered different time zones they were conducted in a common

cultural context and as explained above there was no relevance of the ethnical origin in the ESADA study.

5.8 Impact of temperature on obstructive sleep apnea in three different climate zones of Europe

In this large multicentre and multinational observational study, we could demonstrate, that the seasonal increase of outside temperature results in a very mild but significant worsening of obstructive sleep apnea (OSA). Interestingly, when using the Koeppen-Geiger climate classification system, the magnitude of this effect was modulated by climate zones. The highest impact of temperature on OSA was found in sleep centres belonging to the temperate oceanic climate (Cfb), followed by the region with warm summer humid continental climate (Dfb). Surprisingly, despite presenting the highest outdoor temperatures, we did not find any significant effect of ambient temperature in the sleep laboratories located in the hot-summer Mediterranean climate zone (Csa).

The relationship between temperature and sleep and sleep related events is complex. Since humans are homeotherm, an almost constant temperature must be generated via sweating (hot environment) or metabolic heat production (cold environment) [260]. An increase in the bedroom environment leads to an increase of the number and duration of awakenings during sleep [262,354]. Recently Obradovich and colleagues estimated that the predicted climate change up to 2050 will result in an increase of 6 additional nights of insufficient sleep per 100 individuals [355]. While the importance of the adequate environment for sleep quality is known for decades, the relationship between temperature and sleep disordered breathing is more recent.

In 2010 Zanobetti and colleagues were probably the first to describe a relationship between temperature and sleep disordered breathing. In this cohort originating from the Sleep Health Heart Study a short-term temperature exposure of 25,5º F was associated with an 11.54 % increase of the respiratory disturbance index (RDI). The authors detected additionally a clear seasonal effect of air pollution. During the summer period an interquartile increase in the particulate air matter of less than 10μm (PM_{10}) resulted in an 12,9 % increase of the RDI [268]. We did not investigate the association of air pollution and respiratory events during sleep, but in respect of the temperature-OSA relationship our results were comparable since we also identified an increase of the respiratory events during sleep in association with higher temperatures. This applied to

either fixed intervals or when temperature was investigated as a continuous variable. Contrary to this, in a controlled environment Valham et al found a decreasing apnea/hypopnea index with increasing temperature although the morning alertness and sleep quality was better at low temperature [356]. This study was investigating the effect of indoor room temperature on the respiratory events during sleep while there was no information regarding the daytime temperature values. A variability of respiratory events depending on the season has been previously described in children. Here the AHI increased during the Winter and Spring period [357]. The authors interpreted this result with the surge of virus infections during the colder months of the year. In the United States of America, the terms snoring and apnea are more frequently searched in the internet during wintertime. This could indicate that during the winter season this problem becomes more evident for the spouse [358]. The authors concluded that the increase of weight and alcohol consumption during the holiday period is unlikely to explain this result. In fact, we cannot exclude a bias of changed behaviour including increased alcohol consumption during the warmer season since the database does not allow to distinguish the change of habits before the sleep study. However, several sleep centres investigate the patients in laboratory environments, thus reducing the risk of a raised AHI only by ethanol ingestion. In one study from the Netherlands alcohol consumption was highest during the spring season and not summer [359], but this habit might differ between the countries included in the ESADA cohort.

The number of publications exploring the impact of temperature on respiratory parameters during sleep is still limited. Cassol et al. detected in a cross-sectional study in Porto Alegre/Brazil a seasonal impact on sleep apnea [360]. Interestingly, although the AHI was depending on temperature, it peaked during wintertime, thus demonstrating an inverse relationship compared to the results from Zanobetti's or the ESADA cohort but in concordance with results from Valham et al [268,356]. In another study from Germany Weinreich et al. showed that a short-term increase in temperature and ozone was associated with an increase in the AHI within the general population [361]. There might be some question regarding the method of this study since the AHI was assessed by a portable single channel monitor, but the result reflects the observation from Zanobetti et al. In another recent single centre study Cheng and colleagues demonstrated in a

cohort from Taiwan a seasonal effect on the presence of OSA with an increase of 2,8 events/h from the summer to the winter period and a significant result for temperature and investigated air pollutants in the cosinor analysis [271]. Nevertheless, in the multivariable linear model ambient temperature lost its significance while relative humidity result remained significant with a negative standardized coefficient β regarding the variance of AHI and Ozone in females. It is notable that the influence of pollutants became more visible in the NREM sleep when compare to REM sleep.

This demonstrates a relevant inhomogeneity within the studies investigating the relationship OSA and temperature. Already in 2015 the contradicting results regarding the impact of temperature on AHI by comparing apparently well conducted studies let to some puzzlement [362]. However, when comparing the larger cohorts, a distinguishing factor can be found. While cohorts investigated in warmer environment like Porto Alegre, Brazil or Taiwan, an increase of the AHI during the winter period is described, while in the more moderate climate of Germany the opposite was detected. The fourth study of Zanobetti was also in favour of a worsening of the AHI in the warmer environment. This study is for the interpretation of our results particularly interesting since the participants were recruited in several urban sites with different climate conditions. The same applies to the ESADA cohort, although in the later due to the international concept cultural differences may be of importance as well. This possible confounder was excluded by grouping our study sites according to the Koeppen/Geiger climate classification. Our result demonstrated a clear modulation effect of the climate zones that was not related to a single country. In fact, the Cfb climate zone included different countries like Belgium, the Czech Republic, Germany, Norway, Poland, Sweden and Scotland. With all respect for the European integration process during the last decades, it is unlikely that cultural differences are absent within this climate zone. Hence, it is surprising, that the strongest relationship between temperature and respiratory events during sleep was detected within this inhomogeneous cohort. Distinctive cultural behaviours are therefore unlikely to explain our results.

If elevated temperatures are of any relevance for the respiratory events during sleep one would assume the clearest results in the regions with the highest temperature which in our cohort would be the Csa climate zone. Surprisingly, all results were

negative. Once again, the multicentre/multinational design of ESADA is against a systematic error by the study design like it could be possible in a single centre study. One possible explanation might be that the population in the South regularly experience heat summer heat waves which might lead to various adaption processes [363].

The possible relevance of climate adaptation has been described in few cohorts. Zhang and colleagues demonstrated in a recent study a distinguishing geographical effect on nonaccidental and cardiovascular events by extreme cold or hot temperatures. The population investigated in several cities form the South of China was more sensitive to coldness and more resistant to heat than the northern cohort, while the inverse applied to the later city group [364]. Within Europe, the *Assessment and Prevention of Acute Health Effects of Weather Conditions in Europe (PHEWE)* project found between the Mediterranean and North-Central cities a possible difference in the temperature induced mortality. However, in this study the mortality was driven by colder temperatures [365]. Nevertheless, the same cohort detected that the hospital admission due to respiratory symptoms was related to high temperatures and higher in the Mediterranean cities [366]. The authors considered a possible explanation in the socioeconomic differences between the North and the South of Europe. We did not investigate the presence of respiratory diseases, but in our study only the Southern countries demonstrated no effect of raised temperatures on the respiratory events during sleep.

There are several other environmental factors that may be important do explain this North/South divergence. We used the Koeppen climate classification to form more homogeneous climate groups within the ESADA population. Although this does not allow an exact estimation of general conditions like relative or absolute humidity, it helps to estimate climate influences and has been already used in public health analysis [367]. One possible confounding factor for the analysis of the climate-heath interaction is the change of behaviour as an adaption processes [368]. In sleep medicine specific bedroom conditions may have a major impact on the results. Lappharat et al found in a study in Bangkok, Thailand a weak association between the AHI and RDI and the patient´s bedroom level of PM_{10} but not with the temperature or humidity values. Nevertheless, the results must be seen with caution since the sleep data was recorded

in a sleep-laboratory and not in the patient´s bedroom [369]. It is at present not known if the possible relationship between temperature and sleep disordered breathing is depending on the 24 hours temperature exposition or only on the bedroom condition. In both cases the artificial modification of the environment by air conditioner (A/C) will be of interacting with the results. In fact, we found for the Cfb and Csa climate zone a small but significant F change when the presence of A/C was included in the hierarchical block analysis. This applied to both AHI and ODI. In the Csa climate zone the inclusion of A/C resulted in a more negative standardized coefficient β regarding the effect of maximum temperature on AHI and ODI, but the results remained not significant. The ESADA database does not register if a A/C is present and activated. Therefore, we cannot correctly assess if on the day of the sleep study the A/C was used or not. Considering the patients discomfort in hotter sleep environment and the existence of sweating artefacts on the neurophysiological parameters of the polysomnography it is highly likely that A/C is used during the warmer summer nights within the Csa zone. For several sleep centres within the Csb zone home sleep apnea testing is the preferred diagnostic tool [62]. Hence, we have in this zone even less information regarding the presence of A/C. However, the prevalence of A/C in Csb residences is very low and has mostly been classified absent in this study. Although it is at present speculative, but if A/C is ameliorating the effect of outdoor temperature on the respiratory parameters during sleep this would explain two important observations:

1: It explains the negative results for the impact of higher temperature on OSA in the Csa group

2: It might help to understand the diverting results from studies performed in Brazil and Taiwan versus Germany and the United States of America, although the later cohort also covered several climate zones [268,271,360,361].

Strengths and limitations of this study.

The high number of patients included allows a detailed analysis of the influence of temperature on OSA within three different climate zones. We used in various models the minimum, average and maximum temperature for each month over the analysed time period. Thus, specific temperature extremes within a month are not assessed, but

with the total the number of sleep studies performed the impact of extreme weather conditions will be rather small. In three sites the climate zone is borderline between the chosen and an alternative climate zone. However, after consultation World Maps of Köppen-Geiger Climate Classification we were informed that the duration of the study allows the classification applied by us since climate zones are shifting due to the recent temperature development and in the future several study sites might shift the actual climate zone. Another important point is the fact that the ESADA cohort is recruited by sleep centres. Results must be therefore carefully interpreted regarding their applicability for the general population. This study was not designed to contribute to the discussion regarding climate change. On the site of the European Environmental Agency results from the Met Office Hadley Centre demonstrate a temperature increase in Europe of 0.09º C during the decade of 2007 to 2017. Compared to preindustrial temperature assessment this means an increase form 1.64ºC (1.59ºC-1.72ºC) to 1.73ºC (1.65ºC-1.83ºC) (https://www.eea.europa.eu/data-and-maps/indicators/global-and-european-temperature-9). It is questionable if this temperature increase would influence our results.

6 Conclusion

1) We have shown that exhaustive stress by physical exercise decreases the perforin and granzyme B containing cells in most lymphocyte subsets. Our data indicates that like physical exercise the stress induced by OSA provokes a significant reduction of perforin positive γδ-T cells in obese obstructive sleep apnea patients. This result may be important in understanding the possible association between obstructive sleep apnea and cancer.
2) Patients with obstructive sleep apneas demonstrate a reduced physiological decline of hemodynamic parameters like the systolic blood pressure and stroke volume. Perhaps even more important, the short-term variation of the hemodynamic parameters during sleep is highly increased, which is considered to be a relevant risk factor for cardiovascular diseases. Due a novel analysis to assess the progress of the hemodynamic parameters at sleep onset, we were able to prove, that sleep fragmentation by arousals is an important if not even the most relevant parameter for the direction of the blood pressure and stroke volume evolution.
3) We were able to show in two studies that sleep fragmentation without hypoxia is relevant for both the hemodynamic evolution at sleep onset and for the perforin content in the blood γδ-T cells. As a personal observation the author of this manuscript favours to score the respiratory effort related arousal (RERA) and maintain the 4 % decrease in the oxygen saturation as hypopnea definition. Together with the clinical signs of excessive daytime sleepiness, mood disorders (respiratory disturbance index) or cardiovascular morbidity (apnea/hypopnea index or respiratory disturbance index) this approach might avoid unnecessary therapy with positive airway pressure devices causing distress for both the publicly funded healthcare and the patient. The former due to the economic burden the later related to the necessity to wear a mask at night that does not improve and might even decrease the quality of life.
4) The results from the European Sleep Apnea Database (ESADA) adds new information regarding the impact of OSA on the lipid status. Interestingly, in this cohort the percentage of clinically known hyperlipidaemia reached less than half

of the real value diagnosed by peripheral blood analysis. This underlines the importance of analysing the OSA population regarding altered lipid values and other disorders of the metabolic syndrome.

5) We found a clear regional influence on the lipid status. Within the 5 analysed regions of the ESADA group the Northern countries demonstrated the highest values of cholesterol although there was no statistical difference with the eastern study sites. It is noteworthy that prevalence of hyperlipidaemia diagnosed by laboratory values did not correspond to the prevalence values combining clinical and laboratory data within the regions. As regional differences in the lipid status between the European countries has been described previously, this result should encourage research to identify and confirm these observations. Once again, there is a clear selection bias as only OSA patients were included in these studies and the results cannot be applied to the general population.
6) The prevalence of insomnia and excessive sleepiness differed between the five geographical regions. There is very little data available regarding this observation but the little evidence existing confirms our data about a nonuniformity of either sleepiness, insomnia or cardiovascular diseases within Europe.
7) We could demonstrate that there exists a positive relationship between temperature and the severity of OSA. Also, we are the first to show that climate zones influence this seasonal change of OSA severity with a reduction of the response to temperature in the warmest climate zone. These results remain to be further confirmed, but it could explain some of the divergent results obtained from recent studies in different climate zones.

7 Future Perspectives

Of the seven studies included in this manuscript four revealed a possible influence of the geographical location on the respiratory and non-respiratory sleep disturbances and their secondary diseases like altered lipid metabolism. This is surprising since the simplified pathomechanism leading to obstructive sleep apneas is the mismatch of external and internal pressure within the pharynx. Due to this mainly physical component of the disease the impact of obstructive sleep apnea (OSA) and its consequences should be equal between the different countries or regions independently if the diagnosis was done e.g. in Lisbon or in Turku. Anatomic structure of the face and upper airways are undoubtfully important. In fact within the existing interethnic studies the cranial structures were the only distinguishing variable to explain why the Chinese/Asian population after correction for BMI demonstrates a more severe OSA when compared to any other ethnic groups [370,371]. The ESADA cohort reveals a predominant Caucasian population (>95 %). Therefore, other possible external factors must be identified to explain our results. The fact that the ESADA group is not homogenous in the methods for OSA diagnosis has been discussed in several previous publications of the group. Nevertheless, the inclusion of the method (either polygraphy or polysomnography) in the regression analysis did not significantly change the outcome. Also, the sleep centres grouped by either the cardinal directions or the Koeppen-Geiger climate zones were divergent in their sleep apnea testing making a systematic error less likely.

If neither the phenotype nor the diagnostic procedure can be hold responsible for the observed regional differences external factors should be considered. Ambient factors like temperature or pollution variables showed some influence in previous publications. However, the different research groups described contradictory directions regarding the relationship between OSA and the ambient factors, especially temperature. Our results revealed a positive correlation between the temperature and the apnea/hypopnea index for the Northern countries of Europe but failed to detect any relationship between the two values in the South. One possible explanation is as discussed above the higher

prevalence of air-conditioner in the South. Nevertheless, there are other possible factors involved.

Geomedicine has been termed as a medical field that analysis the relationship between the localization of a person and the personal health. For example, there exist already several studies that are investigating geographical concentrations of toxic materials (natural or by pollution) on the prevalence of cancer [372]. But geomedicine can be also used for a broader approach. The question is up to what point beside local environment also behaviour factors induced by the geographical location will influence health outcome. As a simple example may serve nutrition. The geographical environment defines access to basic nutrients like fish, rice, wheat and several more. This directly influences the food-health relationship by either healthy or non-healthy diet e.g. the considered healthy food of Japan [373]. On the other hand, the cultural or religious restriction to specific food e.g. Islamic or Hinduism diets, may be inducing positive or negative effects on the human homeostasis and health [374]. In this case geomedicine is already overlapping with geopolitics. As an actual problem of geomedicine and geopolitics might serve the evidence that the diet encountered in some areas of China might have caused the severe global health problem Covid-19 [375].

Although it beyond the scope of this manuscript to discuss all the possible regional influencers of health one further important factor should be mentioned because it is causally linked to sleep medicine. The term geomedicine might also be applicable for the human light exposure. Light is the most important factor to entrain the circadian rhythm. Thus light can influence directly via melatonin, alteration of the circadian rhythm, sleep quality either directly or via sleep disturbances a numerous disorders of metabolic or cardiovascular system [349,376-379].

With the continuous evolution of informatic systems and analysis systems like neural networks a future perspective could be the integration of regional characteristics (geographical, environmental, cultural) beside the already known factors like anthropometric data or history of diseases. As such geomedicine has already taken a long way from London Cholera Map by John Snow designed during the Cholera epidemy in 1854 to the modern available information by geographic information systems (GIS).

This might help to a new individual risk assessment for each patient and possibly also to a more personalized therapeutic strategy.

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9 Addendum

Not included publications related to the objective of the investigation

9.1 Regulation of brain-derived neutrophic factor (BDNF) during sleep apnea treatment

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SLEEP DISORDERED BREATHING

Regulation of brain-derived neurotrophic factor (BDNF) during sleep apnoea treatment

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Background: Patients with obstructive sleep apnoea syndrome (OSAS) often display persistent cognitive dysfunction despite effective treatment with continuous positive airway pressure (CPAP). Brain-derived neurotrophic factor (BDNF) is a key mediator of memory and cognition, but its regulation in OSAS and during CPAP treatment is unknown.
Methods: Serum and plasma BDNF concentrations, BDNF secretion by peripheral blood mononuclear cells, and overnight polysomnography were evaluated in 17 men with newly diagnosed OSAS (as defined by a respiratory disturbance index of >10/hour with >70% obstructive events and corresponding daytime symptoms) and 12 healthy control men. In the patients all the parameters were monitored after 1 night and 3 months of CPAP treatment.
Results: There was no significant difference in baseline serum BDNF, plasma BDNF, or spontaneous BDNF secretion by peripheral blood mononuclear cells between untreated patients and controls. After 1 night of CPAP treatment there was a steep fall in median serum BDNF (from 18.0 ng/ml to 4.1 ng/ml) and plasma BDNF (from 58.7 pg/ml to 22.0 pg/ml) concentrations. Following 3 months of treatment BDNF concentrations did not return to baseline. In contrast, BDNF secretion was not suppressed by CPAP treatment.
Conclusions: Patients with untreated OSAS have normal serum and plasma BDNF levels. CPAP treatment is associated with a rapid decrease in serum and plasma BDNF levels which may reflect enhanced neuronal demand for BDNF in this condition.

The obstructive sleep apnoea syndrome (OSAS) is a major public health burden, with a worldwide prevalence of 4–6% among middle aged men.[1 2] OSAS is characterised by repeated airflow interruptions during sleep resulting in oxyhaemoglobin desaturations, sleep fragmentation, and functional impairment such as daytime hypersomnolence and cognitive dysfunction.[3] Cognitive dysfunction in OSAS includes deficits in memory, problem solving, and behavioural functioning.[4] The precise mechanisms underlying these changes are, as yet, unclear. Although sleep parameters can rapidly be normalised with continuous positive airway pressure (CPAP) treatment, deficits in cognitive performance often persist.[5] In addition, the effectiveness of CPAP does not correlate with neuropsychological improvement.[6] Thus, the relationship between CPAP treatment and cerebral dysfunction is still poorly understood.[5]

Brain-derived neurotrophic factor (BDNF) is a key mediator of neuronal and synaptic plasticity in adults.[7] It induces long term changes in synaptic composition, ion channel expression, and neurotransmitter production in neuronal structures of the brain.[8 9] Neuronal plasticity mediated by BDNF has been shown to be essential for cognitive functions and the consolidation of memory.[10] Reduced BDNF levels in the human brain are associated with cognitive deficits, impaired memory performance, and depression.[11 12] Substantial amounts of BDNF are stored in circulating human platelets (as reflected by high serum levels of BDNF), whereas low amounts of BDNF are found in human plasma.[13 14] There is evidence that peripheral blood BDNF levels relate to BDNF concentrations in the central nervous system.[15 16] This is in line with the finding that BDNF readily crosses the blood-brain barrier.[17] Despite the fact that sleep is essential for memory consolidation and the facilitation of learning,[18] very few data are available on BDNF regulation during sleep. Animal data suggest that sleep related neuroplasticity is associated with enhanced BDNF production and demand in the brain.[19 20] In addition, it has been speculated that the interplay between sleep and cognition might involve BDNF.[21] However, the regulation of BDNF in OSAS and during CPAP therapy is unknown. A study was therefore undertaken to investigate this regulation in a clinical setting.

METHODS

Study design

Twenty one men with newly diagnosed OSAS (inclusion criteria: respiratory disturbance index (RDI) >10/hour, >70% obstructive events, and corresponding daytime symptoms) were recruited. Seventeen male volunteers were recruited as presumably healthy controls (inclusion criteria: no history of snoring, sleep related breathing disorders, or daytime sleepiness). For both patients and controls, exclusion criteria were as follows: (1) any history of a malignant disease; (2) any chronic disease or medication having a major impact on the central nervous system or immune system; (3) signs or symptoms of an intercurrent infection. Before enrollment, participating subjects gave their written informed consent and answered a questionnaire regarding daytime sleepiness (Epworth Sleepiness Scale, ESS) and their medical history. The study was approved by the local ethics committee.

Both patients and controls underwent diagnostic overnight polysomnography using the Alice 3 polysomnography system (Heinen and Löwenstein, Bad Ems, Germany). Sleep measurements during polysomnography included standard electroencephalography (EEG), electrooculography (EOG)

Abbreviations: BDNF, brain-derived neurotrophic factor; CPAP, continuous positive airway pressure; ESS, Epworth Sleepiness Scale; 5-HT, 5-hydroxytryptamine; OSAS, obstructive sleep apnoea syndrome; RDI, respiratory disturbance index; TGFβ_1, transforming growth factor β_1

Table 1 Sleep characteristics in patients and controls

	Controls	Diagnostic night	1 night CPAP	3 months CPAP
RDI	2.3 (1.7–3.9)	57.8 (37.9–76.4)	5.9 (4.0–11.7)	3.3 (1.3–7.4)
Basal oxygen	94.5 (94.1–96.1)	88.5 (87.3–95.0)	93.0 (92.0–96.0)	96.0 (94.0–96.5)
TST (min)	339.0 (306.0–387.0)	337.2 (271.9–358.8)	327.0 (275.5–375.0)	341.5 (276.5–359.8)
SWS (%TST)	15.6 (13.2–20.4)	2.6 (0.0–12.6)	13.9 (6.8–19.3)	8.8 (6.7–11.4)
REM (%TST)	19.0 (14.5–19.5)	10.7 (8.3–18.0)	22.0 (17.5–28.7)	23.8 (17.4–32.6)
ESS	4 (3–4)	12 (10–16)	–	5 (3–7)

Parameters are displayed as median values with interquartile ranges for each time point in patients and controls.
RDI, respiratory disturbance index (number of apnoeas/hypopnoeas per hour); basal oxygen, mean oxygen saturation during sleep; TST, total sleep time within recording time; SWS, slow wave sleep (% of TST); REM, rapid eye movement sleep (% of TST); ESS, Epworth Sleepiness Scale (subjective sleepiness in the last month).

and electromyography (EMG) via electrodes.[22] In addition, nasal airflow was measured using a thermistor, and oxygen saturation using pulse oxymetry. Respiratory effort was analysed with thoracic and abdominal gauges. Following polysomnography, sleep stages and respiratory events were visually analysed and edited page by page following standard criteria of the American Academy of Sleep Medicine.[22 23]

In patients, the diagnostic night was followed directly by the first night with CPAP (1 night CPAP) and further polysomnographic monitoring after 3 months of CPAP treatment (3 months CPAP). CPAP titration was performed manually in the first treatment night and, if necessary, also during the control night. CPAP was applied using commercially available devices (SOMNOcomfort, Weinmann, Hamburg, Germany or CPAP S6, ResMed, Mönchengladbach, Germany). Compliance was measured using integrated hour counters in CPAP devices and appropriate compliance software.

Blood parameters and cell culture

Blood was drawn from the cubital vein in the morning at 06.00 hours following each polysomnography (while the patients and controls were still lying in the bed) into heparinised (plasma), additive free (serum) and EDTA containing (for cell separation and blood cell counts) containers and placed on ice immediately. Heparinised (plasma) and additive free (serum) containers were placed on ice for 60 minutes. Afterwards, serum and plasma samples were obtained by centrifugation for 15 minutes (2000 *g*, 4°C) and stored at −80°C until measured.[14] Differential blood cell counts, serotonin (5-hydroxytryptamine, 5-HT), BDNF, and transforming growth factor β_1 ($TGF\beta_1$) were measured as described previously.[14] Monocyte enriched peripheral blood mononuclear cells were isolated for cell culture experiments as previously described,[24] and 2×10^6 cells/ml were cultured in RPMI 1640 with 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin for 48 hours. Supernatants were aliquoted and stored at −80°C until measured.

Statistical analysis

Data were analysed using SPSS (SPSS Inc, Chicago, IL, USA). Most parameters were non-normally distributed so the Mann-Whitney U test was chosen for the comparison of groups (patients versus controls). For comparisons within patients at different time points, repeated measures ANOVA for related samples was used. Correlations were calculated using Spearman's correlation coefficient. p values of <0.05 were regarded as significant.

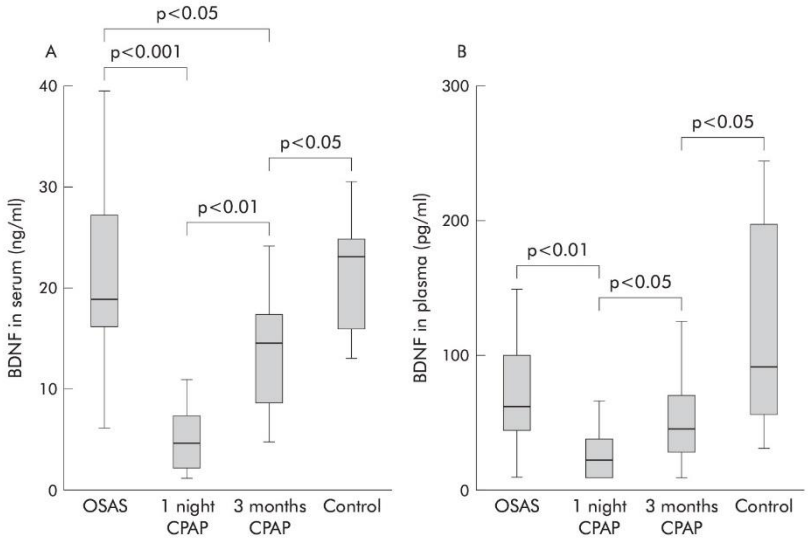

Figure 1 (A) Serum and (B) plasma concentrations of brain-derived neurotrophic factor (BDNF) in controls and patients with OSAS after the diagnostic night without CPAP (OSAS), after the following night with CPAP (1 night CPAP), and after 3 months of CPAP treatment (3 months CPAP). Box plot graphs display the median (line within the box), interquartile range (edges of the box), and the range of all values less distant than 1.5 interquartile ranges from the upper or lower quartile (vertical lines). Significant differences between groups or time points are marked with p values (levels of significance).

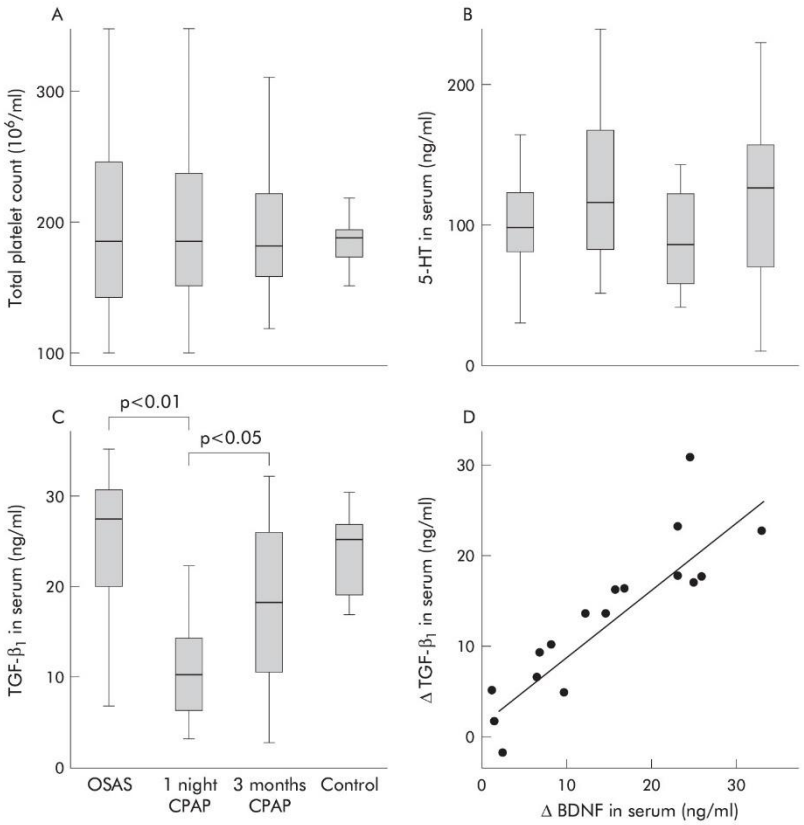

Figure 2 Comparison with platelet markers. (A) Platelet counts, (B) serum 5-hydroxytryptamine (5-HT) concentrations, and (C) serum transforming growth factor-β_1 (TGFβ_1) concentrations in the same groups as detailed in fig 1. Box plot details are as described in fig 1. Significant differences between groups or time points are marked with p values (levels of significance). (D) Serum levels of BDNF or TGFβ_1 after one night of CPAP treatment were subtracted from serum levels before CPAP treatment of each patient, giving the individual decrease in BDNF (ΔBDNF in serum) or TGFβ_1 (ΔTGFβ_1 in serum) after one night of CPAP treatment. Each point represents one patient; the line is the regression line calculated with SPSS.

RESULTS

Sleep characteristics and CPAP treatment

Four patients did not appear for the scheduled polysomnography after 3 months of CPAP treatment and were therefore excluded from the study. In the group of healthy controls, five volunteers had to be excluded due to sleep related respiratory events >10/hour during polysomnography. A final number of 17 men with sleep apnoea (mean (SD) body mass index 33.9 (4.1) kg/m^2, mean (SD) age 55.6 (10.5) years) and 12 healthy male volunteers with proven absence of sleep apnoea (mean (SD) body mass index 24.4 (3.1) kg/m^2, mean (SD) age 48.5 (8.2) years) were therefore evaluated. Two patients were included despite ESS values of less than 10. One patient was not holding a driving licence so the last question of the ESS was not applicable. The other patient suffered from drowsiness and sleepiness in situations demanding at least average attentiveness; his sleepiness was therefore considered clinically relevant. Patients were found to have medium (RDI >15/hour) to severe (RDI >30/hour) OSAS (table 1).[23] The median percentage of obstructive events was 99% (minimum 83%, maximum 100%). Following 1 night of CPAP treatment, almost all sleep parameters (except for slow wave sleep, SWS) were significantly improved and not significantly different from controls (p>0.05 for all parameters, table 1). In all of the 17 patients included in the analysis, CPAP devices were used >4 hours per night over the whole time period of 3 months. There were no significant changes in sleep parameters after 3 months of treatment with CPAP compared with the first night of treatment. There were no significant differences in sleep parameters between controls and patients after 3 months of CPAP treatment (table 1).

BDNF levels in serum and plasma

Before starting CPAP treatment there were no significant differences in serum and plasma BDNF levels between patients and controls (fig 1A, B). Serum and plasma BDNF levels in controls were in keeping with previous data obtained from a large group of healthy adults.[14] After the first night of CPAP treatment, median BDNF serum concentrations decreased from 18 ng/ml (interquartile range (IQR) 16.2–26.4) to 4.1 ng/ml (IQR 2.4–9.0) (fig 1A). Median BDNF plasma concentrations decreased from 58.7 pg/ml (IQR 35.4–129.3) to 22.0 pg/ml (IQR 8.0–37.6) (fig 1B). After 3 months of CPAP treatment, a significant increase in BDNF concentrations was found (compared with BDNF concentrations after the first night of CPAP treatment). However, serum and plasma BDNF levels were still lower than in controls. Serum (but not plasma) BDNF levels were still significantly lower than before CPAP treatment (fig 1). In patients with untreated OSAS there was no significant correlation between BDNF levels and the RDI (serum BDNF: $r = 0.32$, $p = 0.27$; plasma BDNF: $r = 0.39$, $p = 0.19$) or the ESS (serum BDNF: $r = 0.14$, $p = 0.62$; plasma BDNF: $r = 0.10$, $p = 0.73$). During

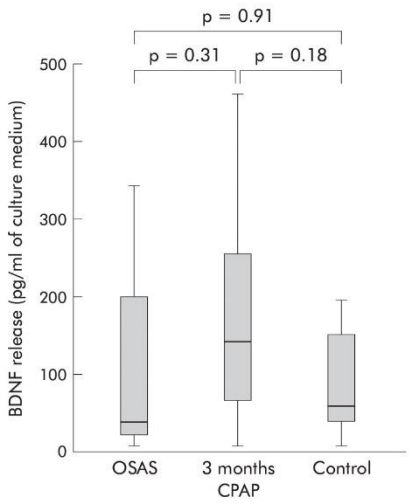

Figure 3 Spontaneous release of BDNF by mononuclear cells. Monocyte enriched peripheral blood mononuclear cells were cultured for 48 hours and BDNF concentrations measured in cell free supernatants. The figure shows BDNF concentrations (pg/ml) in supernatants of patients with OSAS before CPAP treatment (OSAS), after 3 months of CPAP treatment (3 months CPAP, n = 15 patients), and controls (n = 12). Box plot details are as described in fig 1. p values are given for each comparison between groups or time points.

CPAP treatment there were no significant correlations between sleep parameters or CPAP compliance and BDNF serum or plasma levels in patients with OSAS (data not shown).

Platelet counts and platelet markers

Despite the different range, median platelet counts were similar in controls and patients at all time points (fig 2A). To further elucidate the underlying mechanisms, the regulation of platelet markers was investigated. There was no significant difference in $TGF\beta_1$ and 5-HT levels in serum between controls and patients with untreated OSAS. In both controls and patients with untreated OSAS there was a correlation between serum BDNF and serum $TGF\beta_1$ levels (OSAS: $r = 0.72$, $p<0.01$; controls: $r = 0.73$, $p<0.01$), but no significant correlation between serum BDNF and 5-HT levels (OSAS: $r = 0.08$, $p = 0.77$; controls: $r = -0.06$, $p = 0.86$). One night of CPAP treatment led to a significant decrease in serum $TGF\beta_1$ levels but not in serum 5-HT levels (fig 2B and C). In addition, there was a strong correlation between the individual reduction in serum BDNF and $TGF\beta_1$ concentrations ($r = 0.90$, $p<0.001$) following the first night of treatment (fig 2D). In contrast, there was neither a change in $TGF\beta_1$ plasma levels following CPAP treatment nor a correlation between BDNF and $TGF\beta_1$ levels in plasma (data not shown).

Spontaneous BDNF release by mononuclear cells

In 15 of the 17 patients, cell culture experiments with monocyte enriched peripheral blood mononuclear cells were performed before and 3 months after initiation of CPAP treatment. Similar experiments were performed in control subjects (n = 12). There was no significant difference in the spontaneous release of BDNF between controls and untreated patients with OSAS ($p = 0.91$, fig 3). Despite a higher median level (138.9 pg BDNF/ml medium after treatment *v* 34.0 pg BDNF/ml medium before treatment), there was no significant difference in spontaneous BDNF release before and after 3 months of CPAP treatment ($p = 0.31$) in patients with OSAS (fig 3).

DISCUSSION

Despite normalisation of sleep parameters following successful treatment with CPAP, a large subpopulation of patients with OSAS continue to display cognitive deficits.[6] The aetiology of this phenomenon is as yet unknown, and there is no specific treatment for this clinical condition.[5] Our findings show that effective CPAP treatment impacts on circulating stores of BDNF, a key mediator of cognition in adults.

In patients with OSAS, 1 night of CPAP treatment resulted in a steep fall in previously normal serum BDNF concentrations. Due to relatively low BDNF levels in human plasma (<0.5 ng/ml), BDNF levels in human serum (10–30 ng/ml) serve as an estimate for the amount of BDNF stored in platelets.[14] Platelets, which acquire BDNF from external sources and release BDNF following agonist stimulation, appear to be a unique BDNF transportation system in the human body.[13] There is an abundant production of BDNF in peripheral tissues which is not completely attributable to a local function of BDNF in these tissues.[25] It has therefore been postulated that peripheral BDNF is taken up by neurones of the central and peripheral nervous system.[25] Circulating platelets may not only serve as a transportation system for BDNF, but also as a repository which can release large amounts of BDNF on acute demand in specific organs.[13] Since platelet levels of BDNF are not influenced by age, weight or height,[14] the amount of BDNF stored in platelets seems to be relatively stable. The median fall in serum BDNF concentrations to less than 25% of previous values after one night of CPAP treatment is therefore exceptional and has not been previously reported in other conditions.

Several lines of evidence point to a depletion of BDNF stores in platelets during the first night of CPAP treatment. Firstly, differences in BDNF serum concentrations were not attributable to a change in platelet numbers after starting CPAP treatment. Secondly, a suppression of BDNF production appears unlikely, since BDNF is neither synthesised by human platelets nor its precursors.[13] Analysis of platelet markers provided further evidence for a specific BDNF depletion of platelets. In a previous study with healthy adults (68 men and 72 women, 20–60 years old) we found a stronger correlation of BDNF with the platelet α-granule marker $TGF\beta_1$ ($r = 0.75$, $p<0.01$) than with the platelet dense core granule marker 5-HT ($r = 0.31$, $p<0.05$).[14] The analysis of men with untreated OSAS and control men in our present study supported these findings: there was a strong correlation of BDNF with $TGF\beta_1$ but not with 5-HT in serum. These data suggest that BDNF might be co-localised with $TGF\beta_1$ in platelet α-granules[14] rather than with 5-HT in dense core granules.[26] The parallel fall in $TGF\beta_1$ and BDNF serum concentrations following CPAP treatment is therefore compatible with a degranulation of platelet α-granules. This degranulation cannot be explained by an overall activation of platelets, since there is no evidence in the literature for an increase in platelet activity during the first night of CPAP treatment.[27] Instead, the concomitant decrease in serum and plasma BDNF concentrations suggests that BDNF is specifically removed from peripheral blood. The hypothesis that enhanced consumption rather than decreased production of BDNF accounts for low circulating BDNF levels during CPAP treatment is further supported by our cell culture experiments which found no decrease in spontaneous BDNF release by peripheral blood mononuclear cells after CPAP treatment.

In conclusion, this study has shown that CPAP treatment is associated with a decrease in circulating BDNF concentrations but not a decrease in BDNF secretion. We hypothesise

that this phenomenon reflects increased neuronal demand for BDNF during treatment, since neurones can acquire peripheral BDNF to change neuronal activity and synaptic transmission.[28 29] Low circulating BDNF levels in this critical time period could therefore interfere with cognitive improvement. It might be speculated that circulating BDNF does not reflect baseline cognitive performance, but the ability to react to unusual demand. This would explain the finding that circulating BDNF levels did not differ between controls and untreated patients with OSAS (in spite of presumably different cognitive performances). Notably, there was no correlation between the improvement in sleep parameters and the decrease in BDNF levels following CPAP treatment. In light of the reported discrepancy between the improvement in sleep parameters and cognitive functions during CPAP treatment,[6] these findings further support the hypothesis that BDNF might be specifically related to cognitive changes during treatment. Further studies are needed to elucidate whether low circulating BDNF levels or insufficient BDNF production relate to deficits in cognitive improvement during treatment with CPAP.

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The authors have no competing interests to declare.

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9.2 Increase in Perforin-positive Peripheral Blood Lymphocytes in Extrinsic and Intrinsic Asthma

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Increase in Perforin-positive Peripheral Blood Lymphocytes in Extrinsic and Intrinsic Asthma

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The cause of asthma, which has been linked to a chronic, T-cell-mediated bronchial inflammation, remains unclear. A number of other T-lymphocyte-mediated, chronic inflammatory disorders have been associated with autoimmunity and there are data indicating that autoimmune phenomena might also be present in asthma. Expression of perforin, a cytotoxic molecule produced by lymphocytes, has been implicated in the pathogenesis of autoimmune diseases. We therefore tested the hypothesis that allergic and intrinsic asthma might be associated with an increase in lymphocytes producing perforin by comparing the expression of intracellular perforin in peripheral blood lymphocytes of patients with extrinsic asthma (n = 13), intrinsic asthma (n = 7), and healthy control subjects (n = 18). Lymphocytes were identified using flow cytometry and subdivided into $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+$, and $CD56^+$ subpopulations after staining with appropriate monoclonal antibodies. The percentage of perforin-positive total lymphocytes was significantly elevated in patients with allergic as well as intrinsic asthma when compared with normal control subjects. Analysis of lymphocyte subpopulations also revealed a significant increase in the percentage of $CD3^+$, $CD4^+$, $CD8^+$, and $CD56^+$ cells expressing perforin in allergic asthma and a significant increase in the percentage of $CD4^+$ and $CD56^+$ cells in intrinsic asthma when compared with healthy control subjects. Perforin expression in $CD4^+$ cells in intrinsic asthma was also significantly elevated compared with allergic asthma. We conclude that allergic and intrinsic asthma is associated with increased expression of perforin in T-lymphocyte subsets. Arnold V, Balkow S, Staats R, Matthys H, Luttmann W, Virchow JC, Jr. Increase in perforin-positive peripheral blood lymphocytes in extrinsic and intrinsic asthma.

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Bronchial asthma is a chronic inflammatory disorder of unknown origin. Clinically, asthma can be divided into extrinsic/atopic asthma and an intrinsic/nonallergic variant. Whereas in extrinsic asthma allergens have been implicated in the development of airway inflammation and bronchospasm, the pathogenesis of intrinsic asthma remains unclear. Both allergic and intrinsic asthma have been linked to a chronic, T-cell- and eosinophil-mediated bronchial inflammation (1, 2) but the underlying etiologic mechanisms are still elusive. Based on clinical observations it has been hypothesized that asthma might have an autoimmune component (3). Several studies have reported organ- and nonorgan-specific autoantibodies in allergic and/or intrinsic asthma. Circulating autoantibodies directed against smooth muscle, thyroid, parietal cells, mitochondria, as well as antinuclear antibodies and IgG–anti-IgG antibodies have been described (4–7). In some of these studies autoantibody concentrations were more frequently detected in patients with intrinsic asthma (4, 6). In addition, the frequency of antinuclear antibodies was increased in patients with asthma and aspirin intolerance and these patients were more likely to suffer from clinical signs of autoimmunity (3). Other studies reported elevated concentrations of autoantibodies in both allergic and intrinsic asthma (4, 8). Yet, a pathogenetic role for these antibodies in asthmatic inflammation has never been conclusively demonstrated.

However, to our knowledge there are no studies that have investigated cell-mediated autoimmune phenomena in asthma. The chronic persistent nature of chronic allergic asthma and the chronic relentless course of intrinsic asthma have been associated with the accumulation of inflammatory cells in the airways, some of which have cytolytic potential. In addition to eosinophils (9), activated $CD8^+$ T lymphocytes (1) and natural killer (NK) cells (10) have been located in allergic and/or intrinsic asthma, and the CD4/CD8 ratio in peripheral blood of patients with intrinsic asthma was elevated compared with allergic asthmatics and normal control subjects (1). The mechanisms, however, by which these cells might contribute to the pathogenesis of asthma have not been studied further.

Perforin, a 60-kD pore-forming protein stored intracellulary which is produced by NK cells, gamma/delta (γ/δ) cells, cytotoxic $CD8^+$ T lymphocytes, and a small population of $CD4^+$ T lymphocytes (11–16) has been reported in elevated concentrations in several chronic inflammatory disorders with

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autoimmune features such as multiple sclerosis (17, 18), Takayasu's arteritis (19), or autoimmune thyroid disease (20). Perforin can induce apoptosis in a number of cells including T lymphocytes and thus might also play a role in the resolution of inflammatory immune responses by eliminating inflammatory (21–23) or virally infected cells (24, 25). Because several clinical (26) as well as immunological (1) features of intrinsic asthma are compatible with an autoimmune process, we hypothesized that there might be evidence for an increase in perforin expression in lymphocytes in patients with asthma.

METHODS

Subjects

Patients attending the local chest clinics and who were diagnosed as suffering from allergic or intrinsic asthma as previously described (1) and published (27) were randomly selected to participate in this study. There were 13 subjects with extrinsic asthma (7 male, 6 female) with a mean age of 43 yr (range, 22 to 74 yr) and a mean FEV_1 of 77.6% ± 5.9% of predicted (range, 44 to 105%). The seven patients with intrinsic asthma (1 male, 6 female) had a mean age of 55 yr (range, 41 to 64 yr) and a mean FEV_1 of 62.4% ± 8.2% of predicted (31 to 89%). Eighteen healthy volunteers (14 female, 4 male) with a mean age of 40 yr (range, 25 to 65 yr) and a normal FEV_1 served as control subjects. Further patient characteristics are listed in Table 1. All patients gave their informed consent and the study protocol was approved by the local ethics committee.

Cell Preparation of Mononuclear Cells

Mononuclear cells were isolated from 10 ml of venous blood containing 0.2% ethylenediaminetetraacetic acid (EDTA) obtained from patients with extrinsic and intrinsic bronchial asthma as well as from healthy volunteers on a gradient of Ficoll with a density of 1.077 g/L (Seromed, Berlin, Germany) as previously reported (28). Isolated peripheral blood mononuclear cells (PBMCs) were washed twice and resuspended in phosphate-buffered saline (PBS) supplemented with 2% heat-inactivated fetal calf serum (FCS; GIBCO, New York) at a concentration of 2×10^6 per milliliter.

Monoclonal Antibodies

Specific staining of the respective cell surface molecules was performed by anti-human CD3–phycoerythrin (PE) (clone UCHT1; Dako, Hamburg Germany), anti-human CD4-PE (clone, EDU-2; Cymbus Biotechnology, Hants, UK), anti-human CD8-PE (clone DK25; Dako), anti-human CD16-PE (clone 3G8; Immunotech, Hamburg, Germany), and anti-human CD56-PE (clone B-A19; Diaclone, Besancon, France). Anti-human perforin–fluorescein isothiocyanate (FITC) (clone δG9; Hölzel Diagnostika, Köln, Germany) was used to analyze intracellular perforin. IgG-FITC and IgG-PE served as isotype-specific controls (both from Dako).

Intracellular Perforin Staining

After incubation of mononuclear cells with either anti-CD3, anti-CD4, anti-CD8, anti-CD16, or anti-CD56 antibodies, cells were fixed in paraformaldehyde (4% in PBS) for 15 min on ice, washed twice, and permeabilized with saponin (0.1% in PBS). Subsequently cells were incubated with FITC-conjugated antiperforin antibodies for 30 min, washed twice again, and then analyzed by flow cytometry.

Statistical Analysis

Results are expressed as arithmetic means ± SEM. Differences between groups were analyzed using the Mann-Whitney sum rank test. Differences with p values < 0.05 were considered statistically significant.

TABLE 1

CHARACTERISTICS OF PATIENTS

Sex	Age (*yr*)	Duration of Asthma (*yr*)	FEV_1 Baseline (*L*)	FEV_1 (*% pred*)	IVC (*% pred*)	IgE (*kU/L*)	Medication
Characteristics of patients with allergic asthma							
Male	24	4	4.4	98	83	93.2	ICS, β
Female	22	2	3.4	105	90	ND	ICS, β
Male	29	20	2.7	61	86	ND	ICS, β
Male	47	1	4.0	95	84	ND	ICS, β
Male	45	6	2.9	70	86	98.2	ICS, β
Female	59	27	2.1	75	87	194	ICS, β
Male	55	2	4.1	103	90	57.4	ICS, β
Female	64	2	1.3	47	59	7.4	ICS, β, L
Female	46	13	1.3	44	74	137	ICS, β, T
Male	26	3	2.0	53	60	1,009	ICS, β, L, T
Female	49	—	2.4	82	92	ND	ICS, β, T
Male	74	54	3.1	89	88	170	ICS, β, T
Female	23	16	3.0	87	74	ND	ICS, β
Mean	43.3	12.5	2.8	77.6	81.0	220.8	
SEM	4.8	4.5	0.3	5.9	3.1	114.6	
Characteristics of patients with intrinsic asthma							
Female	47	5	1.4	49	62	ND	C, ICS, β
Female	63	10	2.2	82	85	35	ICS
Female	55	19	2.4	89	93	48	ICS
Female	63	40	2.1	81	92	ND	ICS, β, T
Female	41	31	0.8	31	43	29	C, ICS, β, T
Male	64	4	1.6	49	56	61.3	ICS, β, T
Female	53	1	1.4	56	67	16.0	ICS, β
Mean	55.1	15.7	1.7	62.4	71.1	66.7	
SEM	3.4	5.6	0.2	8.2	7.3	24.0	

Definition of abbreviations: β = β_2-agonists; C = oral corticosteroids; ICS = inhaled corticosteroids; L = leukotriene receptor antagonist; ND = not determined; T = theophylline.

RESULTS

Distribution of Lymphocyte Subpopulations in Peripheral Blood of Normal Control Subjects and Patients with Allergic and Intrinsic Asthma

The distribution of lymphocyte subpopulations was analyzed after incubation of cells with fluorescence-labeled antibodies against CD3, CD4, CD8, CD16, or CD56 and their subsequent differentiation by flow cytometry. As depicted in Figure 1 comparison of these lymphocyte subpopulations revealed no statistically significant differences in the percentage of $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+$, and $CD56^+$ subpopulations between patients with extrinsic or intrinsic asthma and healthy control subjects (Figure 1).

Perforin-Positive Lymphocytes

In contrast, when Ficoll separated mononuclear cells were fixed with paraformaldehyde and permeabilized by saponin, and then incubated with monoclonal antibodies against perforin there was a significantly higher percentage of cells expressing perforin in patients with extrinsic (35.3 ± 3.5%) and intrinsic asthma (37.7 ± 3.9%) compared with normal control subjects (21.7 ± 2.5%; $p < 0.05$ compared with allergic as well as intrinsic asthma) (Figure 2). However, there was no significant difference in the percentage of perforin-positive cells when cells from allergic and intrinsic asthma were compared.

Perforin Expression in Lymphocyte Subsets

To determine perforin expression in lymphocyte subsets, Ficoll-separated lymphocytes were incubated with monoclonal antibodies against CD3, CD4, CD8, CD16, or CD56 before fixation and permeabilization and then stained intracellularly with specific antibodies against perforin. Although the percentage of perforin-positive lymphocytes was highest in the $CD16^+$ and $CD56^+$ (NK cell) population, perforin$^+$/$CD3^+$, -$CD4^+$, and -$CD8^+$ cells were clearly detectable in all asthmatic patients. While there was no difference in the expression of perforin in the $CD16^+$ lymphocyte population, perforin expression in $CD3^+$, $CD4^+$, $CD8^+$, and $CD56^+$ lymphocytes in patients with allergic asthma was significantly elevated compared with normal control subjects (each $p < 0.05$). Similar results were obtained for $CD4^+$ and $CD56^+$ cells expressing perforin in patients with intrinsic asthma ($p < 0.01$). Although the percentage of $CD3^+$ and $CD8^+$ lymphocytes expressing perforin was elevated in patients with intrinsic asthma compared with normal control subjects, this difference failed to reach statistical significance. Interestingly, perforin expression in $CD4^+$ lymphocytes in intrinsic asthma was also significantly elevated compared with patients with allergic asthma ($p < 0.05$) (Figure 3).

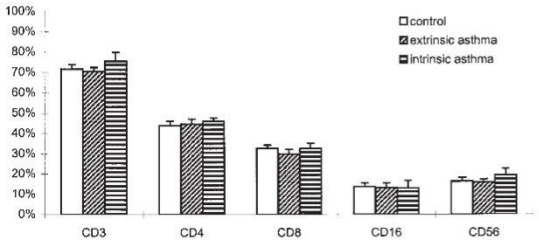

Figure 1. Composition of blood lymphocytes. Peripheral blood lymphocyte subpopulations from extrinsic (n = 13) and intrinsic asthma (n = 7) and healthy volunteers (n = 18) in percentage (%) of all lymphocytes. There were no significant differences in the distribution between the groups examined. Analyses were performed in whole blood. Values are mean ± SEM.

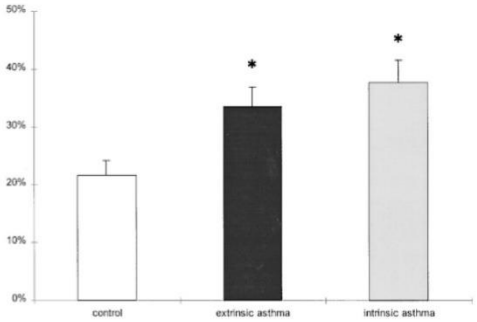

Figure 2. Percentage of perforin-containing lymphocytes. Comparison of the percentage of peripheral blood lymphocytes expressing intracellular perforin in healthy volunteers (n = 18), patients with extrinsic (n = 13) and intrinsic asthma (n = 7). As indicated by asterisks, extrinsic as well as intrinsic asthma had significantly increased percentages of perforin-expressing cells compared with normal control subjects. Values are mean ± SEM; *$p <$ 0.05.

Correlation with Clinical Parameters

There was no correlation between parameters of airflow limitation, duration of asthma, or concomitant medication and expression of perforin in peripheral blood lymphocytes and lymphocyte subpopulations in all the groups studied. Although the percentage of perforin$^+$/$CD3^+$ and perforin$^+$/$CD8^+$ lym-

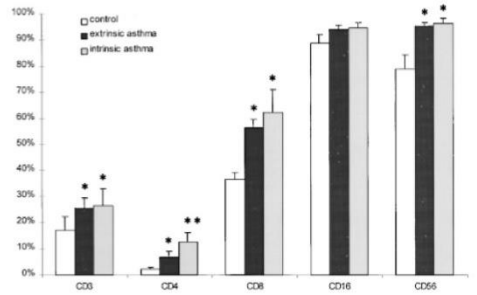

Figure 3. Percentage of perforin-containing subpopulations. Percentages of perforin-positive lymphocyte subpopulations from extrinsic and intrinsic asthma and normal control subjects. Both allergic and intrinsic asthma had significantly elevated percentages of perforin-positive cells in the total lymphocyte gate expressing perforin. All values are mean ± SEM. *$p < 0.05$. In contrast to the other lymphocyte subpopulations there was no significant difference in the percentage of $CD16^+$/perforin$^+$ lymphocytes between patients with asthma and normal control subjects. **$p < 0.05$ compared with allergic asthma.

phocytes were related to the age of the patients in the intrinsic asthmatic subgroup ($r = 0.8$ and $r = 0.77$), this failed to reach statistical significance when corrected for multiple correlations.

DISCUSSION

The etiology of bronchial asthma is still incompletely understood. Despite increasing evidence that allergen-dependent augmentation of bronchial inflammation plays an important role in maintaining the cellular infiltrate in bronchi of allergic asthma, a number of features in the chronic course of this disease remains unexplained. Current hypotheses fail to explain why asthma often persists in the absence of allergen, and at present the mechanisms underlying the pathogenesis of intrinsic asthma are unclear. The clinical observation that asthma often takes a chronic, protracted course, its association with activated T lymphocytes in allergic as well as intrinsic asthma (1, 2), its association with cell-mediated damage to the bronchial mucosa and processes of tissue remodeling (29), its responsiveness to corticosteroids, the demonstration of an increased frequency of autoantibodies (3–7) together with the increased incidence of intrinsic asthma in female patients (30), and its unpredictable course with exacerbations and remissions but sometimes purely progressive forms lend support to the hypothesis that bronchial asthma has an autoimmune background to its pathogenesis.

However, cellular events associated with autoimmunity have not been assessed in asthma. In this study we provide evidence that the percentage of perforin-positive lymphocytes in peripheral blood is increased in both allergic and intrinsic asthma as compared with normal control subjects. The observed difference in perforin expression between asthmatics and normal control subjects persisted when $CD3^+$, $CD4^+$, $CD8^+$, and $CD56^+$ lymphocyte subpopulations were investigated separately, suggesting that perforin expression in these lymphocyte subpopulations is upregulated in bronchial asthma. The observed difference in perforin expression by lymphocytes between patients with asthma and normal control subjects is unlikely the result of a selective redistribution of lymphocyte subpopulations because these were basically identical between the three groups (Figure 3). Thus, our data suggest that in the presence of a similar distribution of $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+$, and $CD56^+$ cells, the elevated, perforin-positive fraction of each of these subpopulations reflects a true increase in the peripheral blood of patients with asthma.

It has recently been reported that the percentages of $CD3^+$/perforin$^+$, $CD4^+$/perforin$^+$, and $CD8^+$/perforin$^+$ cells decline with increasing age (31). This observation is contrasted by our findings of markedly elevated perforin-positive lymphocytes and lymphocyte subpopulations, especially in intrinsic asthma, despite the fact that this patient population was somewhat older than the normal control subjects. Thus, in view of the findings of Rukavina and coworkers (31) the differences in perforin expression observed in our study might have been even more pronounced in age-matched populations. On the other hand, our observation of different percentages of perforin-expressing lymphocyte subpopulations in allergic asthmatics and normal control subjects despite a similar age distribution in these groups suggests that the observed differences in perforin expression cannot be attributed to the age of our study subjects alone.

Although this is the first study to report an increase in the percentage of perforin-expressing lymphocytes in patients with asthma, our study cannot provide conclusive evidence for the functional or clinical significance of the increased percentage of perforin-expressing cells in asthma. At present their relative cytotoxic potential must remain unclear since to date there are no studies that have satisfactorily addressed this question in human diseases.

In normal volunteers perforin expression has been detected intracellularly in peripheral blood NK and $CD8^+$ T cells but only in a small percentage of $CD4^+$ T lymphocytes (32). Using immunocytochemistry Nakata and coworkers even failed to detect any perforin expression in unstimulated $CD4^+$ T lymphocytes of healthy control subjects (16) which is in contrast to our findings where a small percentage of perforin-positive cells was detectable in healthy control subjects. An increase in the percentage of perforin$^+$/$CD4^+$ cells has been reported in infectious mononucleosis (16) and after treatment for Hodgkin's disease (33). Elevated percentages of $CD4^+$/perforin$^+$ T cells were also present in peripheral blood lymphocytes of patients with Wegener's granulomatosis (M. Schlesier, personal communication). Interestingly, the highest percentage of $CD4^+$/perforin$^+$ T lymphocytes was observed in patients with intrinsic asthma. Because little is known about the physiologic role and the putative cytotoxic functions of $CD4^+$ major histocompatibility complex (MHC) class II-restricted T cells (34), we can only speculate about the role of perforin$^+$/$CD4^+$ T cells in bronchial asthma. Increased perforin expression has been reported in several other chronic inflammatory disorders with autoimmune phenomena such as multiple sclerosis (17, 18), Takayasu's arteritis (19), or autoimmune thyroid disease (20) and Crohn's disease (35) in which it was localized to mononuclear cells, $CD4^+$, $CD8^+$, $CD16^+$, γ/δ T cells, or NK cells. Our findings of elevated numbers of potentially cytolytic $CD4^+$ and $CD8^+$ T lymphocytes in peripheral blood of these patients are therefore in line with our hypothesis of an autoimmune component to the pathology of asthma.

There are several possibilities by which the increased percentage of perforin-positive T lymphocytes might be associated with inflammation in asthma. Systemic interleukin-2 (IL-2) immunotherapy has been associated with an increased percentage of perforin$^+$/$CD4^+$ T cells, suggesting that the effects of IL-2 immunotherapy are also mediated by cytolytic lymphocytes (36). Similarly, perforin-mediated cytotoxicity has been associated with the vascular leak syndrome induced by IL-2 suggesting that IL-2 upregulates perforin expression (37). Elevated concentrations of IL-2 have been detected in bronchoalveolar lavage fluid of patients with intrinsic asthma (1) as well as following segmental allergen provocation in allergic asthma (2). Therefore, the elevated concentrations of IL-2 measured in patients with asthma might contribute to the increase in perforin-positive lymphocytes in our study population.

On the other hand, in an animal model of lupus erythematosus, perforin-deficient animals had more severe disease, suggesting that cytolytic lymphoid regulation plays a critical role in the immune homeostasis of these animals (38). Whether the increased percentage of perforin-positive lymphocytes observed in our patients reflects an increase in cytolytic activity inherent to the pathogenesis of asthma or instead a reaction of the immune system to remove abundant inflammatory cells remains at present unclear.

In conclusion, we provide evidence that bronchial asthma is associated with an increase in the percentage of cytolytic, perforin-positive lymphocytes. Whether these cells have a functional relevance in the pathogenesis of asthma requires further studies, and although there is circumstantial evidence linking perforin expression to the pathology of asthma, any causal relationship between asthma and perforin expression remains to be established.

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9.3 Diagnosis of Sleep Apnea by Automatic Analysis of Nasal Pressure and Forced Oscillation Impedance

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Diagnosis of Sleep Apnea by Automatic Analysis of Nasal Pressure and Forced Oscillation Impedance

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Detecting and differentiating central and obstructive respiratory events is an important aspect of the diagnosis of sleep-related breathing disorders with respect to the choice of an appropriate treatment. The purpose of this study was to evaluate the performance of a new algorithm for automated detection and classification of apneas and hypopneas, compared with visual analysis of standard polysomnographic signals. The algorithm is based on time series analysis of nasal mask pressure and a forced oscillation signal related to mechanical respiratory input impedance, measured at a frequency of 20 Hz throughout the night. The method was applied to all-night measurements on 19 subjects. Two experts in sleep medicine independently scored the corresponding simultaneously recorded polysomnographic signals. Evaluating the agreement between two scorers by a weighted kappa statistic on a second-by-second basis, we found that inter-expert variability and the discrepancy between automatic analysis and visual analysis performed by an expert were not significantly different. Implementation of this algorithm in a device for home monitoring of breathing during sleep might aid in the differential diagnosis of sleep-related breathing disorders and/or as a means for follow-up and treatment control.

Keywords: sleep-disordered breathing; diagnosis; classification; inter-observer variability

In recent years, the high prevalence of sleep-related breathing disorders has been increasingly recognized by epidemiologic studies (1, 2). One important aspect of the diagnosis of sleep-disordered breathing (SDB) with respect to the choice of an appropriate treatment is the detection and classification of different respiratory events, in particular, separating obstructive from central apneas. Diagnosis of SDB is usually performed by polysomnography (PSG) in a sleep laboratory, consisting of the measurement and recording of numerous signals used to analyze sleep and breathing. Whereas PSG currently represents the standard for the diagnosis of SDB, it is expensive, and access is limited. Moreover, the unfamiliar environment encountered in a sleep laboratory often impairs the patient's sleep. Therefore, efforts have been made to develop diagnostic approaches that rely on noninvasive, unsupervised measurements in the home of the patient (3).

One widely used approach consists of nocturnal outpatient measurements, including estimation of respiratory flow using thermistors or nasal prongs and monitoring of breathing effort by thoracic and abdominal strain gauges or respiratory inductive plethysmography. Using such devices, it is possible to distinguish central and obstructive apneas. However, signal quality may be reduced by dislocation of belts or due to patients' obesity (4).

Two other methods that have been extensively investigated in this context are the recording of pressure at the airway opening, mostly via nasal cannula (5–8), and the measurement of signals related to mechanical respiratory input impedance by the forced oscillation technique (FOT) (9–18). Both methods are highly sensitive with respect to the detection of disturbed breathing during sleep (5, 12, 13, 16). Moreover, both signals are simultaneously accessible via a nasal mask.

FOT signals in particular have been proposed as promising tools for classifying respiratory events as central or obstructive (10, 12). Argod and associates (19) have also suggested that central and obstructive hypopneas could be distinguished by analyzing nasal pressure recordings. Whereas episodes with decreased amplitude and a rounded contour are supposed to indicate a central origin, obstructive hypopneas should be associated with a flattened contour. However, this approach has not yet been investigated in a quantitative manner. Following a different approach, cardiogenic oscillations in nasal pressure signals during apneas could be used as indicators of their central origin. Ayappa and colleagues (20) have found cardiogenic oscillations in the continuous positive airway pressure (CPAP) flow signal during 60% of central apneas but never during obstructive apneas.

A further step toward a time-saving procedure for ambulatory diagnosis of SDB consists of developing an algorithm for automatic analysis of signals measured by a simple and robust device that can be easily applied by the patient. By providing time of onset, duration, and class of respiratory events, such an algorithm could quickly yield essential information about severity and type of possible sleep-related breathing disorders from data obtained during nocturnal home monitoring. Examinations using home recording equipment could thereby close a gap between screening, e.g., by oximetry, and full PSG or could sometimes even serve as substitutes for PSG in the sleep laboratory.

We have developed diagnostic software for off-line detection and classification of respiratory events on the basis of time series analysis of nasal mask pressure and of a FOT signal measured at a frequency of 20 Hz throughout the night. The goal of this study was to assess the quality of the underlying algorithm. For that purpose, we evaluated the agreement between the results of that algorithm and those of visual analysis of polysomnographic recordings performed by experts in sleep medicine. This agreement between automatic and visual analysis is compared with inter-expert agreement.

METHODS

Subjects

Nineteen male subjects were studied during an all-night PSG in the sleep laboratory of the Department of Pneumology at Freiburg Uni-

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formed by the other scorer. Onset, duration, and class of all detected events were stored for further analysis.

We used the weighted kappa statistic computed on a second-by-second basis to evaluate the agreement between scorers and automatic analysis instead of the more common procedure consisting of comparing the respective numbers of events detected in a recording. Weighted kappa computed on a second-by-second basis is more sensitive to subtle differences between two analyses that are due to varying interpretations concerning onset and duration of a particular event. On the other hand, the fact that κ_w implicitly reflects the degree of concordance with respect to detection, classification, and length of apneas and hypopneas in one single number is an important aspect for the optimization of the algorithm used for automatic analysis. Furthermore, the second-by-second approach guarantees that κ_w is a relatively smooth function of the parameters of the algorithm, which is a prerequisite for reliable optimization.

To evaluate the agreement between visual and automatic analysis, each of the 19 PSG recordings served as a test set for cross-validation once. Therefore, the 19 recordings were divided 19 times in rotation into a training set consisting of 18 recordings and a test set formed by the remaining one. For each of the 19 divisions, the following two steps were taken:

- FOT and mask pressure time series of the training set recordings were subjected to automatic analysis. The parameters of the algorithm were successively optimized to yield the maximum κ_w(*auto*, *scorer1*), i.e., the best possible agreement between automatic analysis and the first scorer when analyzing the training set.
- Using these parameters, the algorithm was applied to the remaining test set, yielding the results of automatic analysis to be used for cross-validation.

These results were used to compute κ_w(*auto*, *scorer1*) for each test set recording to quantify the agreement between the first scorer and automatic analysis. Furthermore, the agreement between the second scorer and automatic analysis was evaluated by calculating κ_w(*auto*, *scorer2*) in a similar manner, using the same parameters.

Moreover, the AHIs (AHI[*scorer1*], AHI[*scorer2*], and AHI[*auto*]) were computed, reflecting the respective numbers of events detected per hour of a recording.

RESULTS

The agreement of visual analysis of 19 PSG recordings performed by experts in sleep medicine with automatic analysis of the corresponding mask pressure and FOT signals is depicted in Figure 1, together with inter-expert agreement. Automatic analysis yielded results that are comparable to those of visual analysis of polysomnograms; the different values of the weighted kappa statistic are within their respective standard deviations (κ_w[*auto*, *scorer1*] = 0.45 ± 0.15, κ_w[*scorer1*, *scorer2*] = 0.50 ± 0.21, κ_w[*auto*, *scorer2*] = 0.40 ± 0.19, mean ± SD).

The same result is obtained when AHIs are compared. Here, automatic analysis is on average in between the two scorers (AHI[*scorer1*] = 34.2 ± 17.4, AHI[*scorer2*] = 25.4 ± 19.6, AHI[*auto*] = 30.5 ± 18.5, mean ± SD). Taking into account the predominant nature of detected events, three identical tentative diagnoses (mostly central, predominantly obstructive, or only hypopneas) would have been obtained by the different analyses for all recordings, except for one data set in which *scorer2* exclusively detected hypopneas, whereas *scorer1* and *auto* found obstructive apneas.

The weighted kappa statistic is most sensitive with respect to disagreements on single events if the total number of respiratory events in a recording is low. For example, the most prominent discrepancies between κ_w(*auto*, *scorer1*) and κ_w(*scorer1*, *scorer2*) are observed in a recording with an AHI of 5.1 per hour according to *scorer1* and 5.3 per hour according to *auto*, although similar severities of SDB were estimated by both automatic and visual analyses.

Examples of respiratory events and the respective classifications attributed by automatic analysis and scorers are presented in Figure 2. It shows obstructive apneas with paradoxical excursions of thorax and abdomen. The FOT signal is almost constant during these obstructive apneas at a high level compared with the periods of normal breathing preceding and following the event; no cardiogenic oscillations can be observed in any respiratory signal. Two examples displaying central apneas are given in the online data supplement. Event-by-event agreement between scorers and automatic analysis after optimization is presented in Table 2. The different numbers of events as detected and classified by one scorer (*scorer1*, *scorer2*, or *auto*) in all 19 recordings and the respective classifications as assigned by another scorer are displayed as: (a) *auto* and *scorer1*, (b) *scorer1* and *scorer2*, (c) *auto* and *scorer2*. For example, the number printed in bold in Table 2a indicates that a total of 97 events has been attributed to the category of hypopneas by automatic analysis, but classified as obstructive apneas by the first scorer.

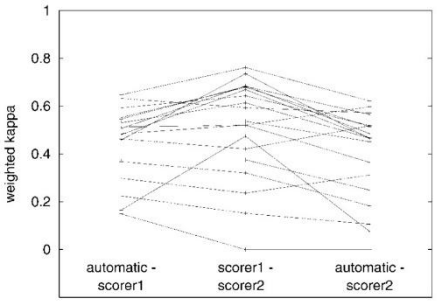

Figure 1. Agreement between automatic analysis and two scorers (automatic–scorer1, scorer1–scorer2, and automatic–scorer2) for recordings of 19 patients with SDB; weighted kappa was computed on a second-by-second basis using the weights displayed in Table E1 in the online data supplement.

The second scorer detected fewer events than both the first scorer and the algorithm. On the other hand, he judged a higher percentage of the detected events to be apneas rather than hypopneas. This is caused by different interpretations of the definitions for respiratory events, in particular of the terms "clear amplitude reduction" and "cessation of respiratory airflow".

Some events were classified as central apneas by scorers but as hypopneas by automatic analysis. This is due to the rather elevated variability of mask pressure during these events, caused by cardiogenic oscillations, but misinterpreted as breathing by the algorithm. Most of these events (140 of 199 in Table 2a, 175 of 223 in Table 2c) were observed in the recording of one subject. Nevertheless, automatic analysis revealed the central nature of these events in low levels of the FOT signal reflecting open airways.

DISCUSSION

As a main result of the present study, we found automatic detection and classification of sleep-related respiratory events on the basis of nasal mask pressure and FOT to be feasible and reliable. The algorithm provides results comparable to those of visual analysis of polysomnographic recordings performed by experts in sleep medicine. Discrepancies between two scorers (*see* Table 2) are mainly due to differing opinions concerning the duration of particular events and the extent of respiratory airflow.

Most events classified as mixed apneas by experts were assigned to obstructive apneas by automatic analysis, mainly due

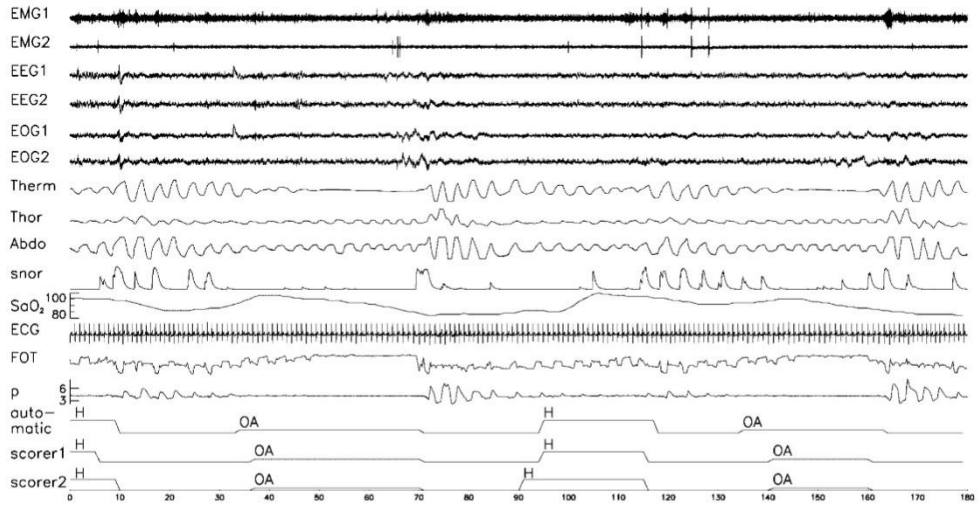

Figure 2. Examples of obstructive apneas. Three minutes extracted from a polysomnographic recording with submental (EMG1) and tibial (EMG2) EMG, two EEG channels, left and right electrooculograms (EOG1, EOG2), oronasal thermistor (Therm), thoracic (Thor) and abdominal (Abdo) inductance plethysmography, snoring sounds (snor), oxyhemoglobin saturation (Sa_{O_2}) as a percentage, electrocardiogram (ECG), estimated modulus of respiratory input impedance measured by FOT, and nasal mask pressure (p) in cm H_2O. The three bottom tracings show onset, end, and class of respiratory events as detected by automatic analysis of FOT and mask pressure as well as by visual analysis of full PSG except FOT signal performed by two scorers. H denotes hypopnea, and OA, obstructive apnea.

to the shortness of the central period at the onset of such events in our data sets. Occasionally, visually detected central apneas were classified as hypopneas by automatic analysis. This situation mainly occurred in the recording of one subject, where very pronounced cardiogenic oscillations in the mask pressure signal were observed during these events. However, as the algorithm correctly revealed the central origin of these events, the basic concern with respect to the choice of an appropriate treatment was fulfilled.

TABLE 2. OCCURRENCES FOR RESPIRATORY EVENTS AS DETECTED AND CLASSIFIED BY (*a*) AUTOMATIC ANALYSIS AND FIRST SCORER, (*b*) FIRST AND SECOND SCORER, (*c*) AUTOMATIC ANALYSIS AND SECOND SCORER

a.		*scorer 1*				
auto		N	H	OA	MA	CA
	N		959	15	0	11
	H	626	1,829	**97**	8	199
	OA	4	163	286	45	22
	MA	0	17	9	1	6
	CA	12	92	47	9	142
b.		*scorer 2*				
scorer1		N	H	OA	MA	CA
	N		186	9	0	5
	H	1,125	1,558	245	4	97
	OA	11	41	367	27	9
	MA	1	0	46	12	2
	CA	32	10	22	14	302
c.		*scorer 2*				
auto		N	H	OA	MA	CA
	N		400	21	3	6
	H	1,006	1,300	206	9	223
	OA	20	87	353	42	17
	MA	3	5	18	0	6
	CA	31	23	79	5	163

Esophageal pressure represents the gold standard for distinction of obstructive from central apneas and hypopneas; however, many patients refuse to be diagnosed by esophageal manometry (30). Because of its invasive nature, esophageal pressure monitoring is suspected to have negative side effects on sleep quality and upper airway dynamics (30, 31), the latter also influencing mechanical respiratory input impedance. Moreover, one goal of this study was to evaluate the agreement between our algorithm and experts in sleep medicine, and to compare this agreement with inter-expert variability, as encountered in a routine clinical setting. Routine examinations, however, do not include esophageal pressure monitoring in most sleep laboratories. As a consequence of all these aspects, we did not incorporate esophageal pressure into the set of signals monitored within the scope of PSG for this study.

A crucial issue is how the automatic analysis can deal with artifacts due to mask leaks, mouth breathing, swallowing, or yawning. Because we mainly addressed the fundamental possibility of automatically detecting and distinguishing obstructive and central apneas and hypopneas, our algorithm currently does not contain any features to reject such artifacts. Some of these artifacts, potentially misinterpreted as respiratory events by automatic analysis, can be indirectly detected on the basis of EEG, EMG, and Sa_{O_2} when they occur during wakefulness or are followed neither by an arousal nor by oxygen desaturation. In our data, however, this situation never occurred to an extent seriously deteriorating the agreement between automatic and visual analyses, possibly because the required duration of apneas and hypopneas is limited to a range of 10 to 240 seconds. Some other situations may also be identifiable using additional routines that could be included into the diagnostic software. For example, nasal inspiration and oral expiration re-

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2000;161:A332.

9.4 Sleep-Disordered Breathing and Cardio- and Cerebrovascular Diseases_ 2003 Update of Clinical Significance and future Perspectives

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Sleep-Disordered Breathing and Cardio- and Cerebrovascular Diseases: 2003 Update of Clinical Significance and Future Perspectives

Schlafbezogene Atmungsstörungen und kardio- und zerebrovaskuläre Erkrankungen: Update 2003 der klinischen Bedeutung und zukünftiger Entwicklungen

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Summary The purpose of this review is to summarize current knowledge about the link between sleep-disordered breathing (SDB) and cardiovascular and cerebrovascular diseases. Obstructive sleep apnoea (OSA) is a well-established risk factor for systemic arterial hypertension, and its treatment with continuous positive airway pressure leads to a decrease in daytime and night-time blood pressure profiles. Pulmonary arterial hypertension occurs in 20–30% of OSA patients and is usually mild. It is not yet clear if OSA *per se* leads to pulmonary hypertension or if the coexistence of chronic obstructive pulmonary disease with daytime and/or sleep-related hypoxaemia is required to provoke a persistent rise in pulmonary artery pressure. Furthermore, OSA is associated with nocturnal cardiac arrhythmias, especially cyclical fluctuations of the heart rate in response to recurrent apnoeas. Atrioventricular conduction blocks and ventricular premature beats are less often observed and seem to be confined to patients with severe OSA and those with accompanying ischaemic heart disease. The association between OSA and vaso-occlusive disease (i.e. atherosclerosis) is less clear. However, accumulating experimental and epidemiological data support such a link. Thus, OSA may lead to coronary artery disease (CAD) and stroke by promoting atherosclerosis. Correspondingly, patients with CAD or acute stroke show a high prevalence of SDB. Cheyne–Stokes respiration (CSR) is a specific pattern of central sleep apnoea occurring in

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patients with advanced congestive heart failure (CHF). If present, CSR clearly has a negative impact on the clinical course of CHF. Although the optimal treatment strategy for CSR is less well defined than that for OSA, the successful reversal of CSR might increase overall survival in affected patients.

Keywords Sleep-disordered breathing – obstructive sleep apnoea – central sleep apnoea – Cheyne – Stokes breathing – pulmonary disease – cardiovascular disease – hypertension – stroke.

Zusammenfassung Ziel dieser Übersichtsarbeit ist die Darstellung aktueller Zusammenhänge zwischen schlafbezogenen Atmungsstörungen und kardio- bzw. zerebrovaskulären Erkrankungen: Das obstruktive Schlafapnoe-Syndrom (OSAS) ist als unabhängiger Risikofaktor für die Entstehung einer arteriellen Hypertonie anzusehen. Die nasale Überdruck-Therapie (CPAP) führt zu einer Senkung des Tages- und Nachtblutdruckes. Ca. 20–30% der Patienten mit OSAS weisen eine pulmonal-arterielle Hypertonie auf; unklar ist jedoch, ob eine zusätzliche Lungenerkrankung mit Tages- und/oder Nachthypoxämie eine notwendige Voraussetzung zur Entstehung der pulmonal-arteriellen Hypertonie bei diesen Patienten ist. Herzrhythmusstörungen, insbesondere apnoebedingte Schwankungen der Herzfrequenz, treten bei OSAS gehäuft auf. Atrioventrikuläre Blockbilder oder ventrikuläre Extrasystolen sind seltener anzutreffen und scheinen auf schwere Formen des OSAS und solche mit zusätzlicher koronarer Herzkrankheit beschränkt zu sein. Die Zusammenhänge zwischen OSAS und der Entwicklung einer Arteriosklerose sind nicht vollständig geklärt, jedoch belegen experimentelle und epidemiologische Studien diesbezüglich eine enge Verbindung. Durch Induktion einer Arteriosklerose kann das OSAS zur Entstehung einer koronaren Herzkrankheit oder eines beitragen. Patienten mit einer koronaren Herzkrankheit oder einem apoplektischen Insult weisen dementsprechend eine hohe Prävalenz schlafbezogener Atmungsstörungen auf. Eine spezielle Form zentraler Apnoen stellt die Cheyne–Stokes Atmung dar, die bei Patienten mit fortgeschrittener Herzinsuffizienz gehäuft vorliegt. Gesichert ist der negative Einfluß der Cheyne–Stokes Atmung auf den klinischen Verlauf der Herzinsuffizienz. Obwohl die Behandlungsstrategie der Cheyne–Stokes Atmung weniger etabliert ist als beim OSAS, führt deren erfolgreiche Behandlung zu einer Steigerung der Lebenserwartung bei den Betroffenen.

Schlüsselwörter Schlafbezogene Atmungsstörungen – obstruktives Schlafapnoe-Syndrom – zentrale Schlafapnoe – Cheyne – Stokes Atmung – COPD – kardiovaskuläre Erkrankungen – Bluthochdruck – apoplektischer Insult.

Introduction

In 2000, the working group 'Kreislauf und Schlaf' of the German Sleep Society (DGSM) was founded. In this group, scientists combine their efforts to promote research in the field of sleep medicine and cardio- and cerebrovascular disease (CVD). CVDs are the most common life-threatening and debilitating diseases in the industrialized world. Recent studies have elucidated the importance of sleep-disordered breathing (SDB) with new epidemiological data and modern pathophysiological concepts supporting the hypothesis of a complex association and pathophysiological interaction of SDB and CVD. Thus, a better understanding of both disease entities is gained, which may result in a reduction of morbidity and mortality. The interaction of SDB and CVD can be regarded from various points of view: for example, in obstructive sleep apnoea (OSA), SDB plays an important role as a risk factor leading to the development of CVD. On the other hand, CVD (especially chronic heart failure) can cause SDB (i.e. Cheyne–Stokes respiration). The first aim of the working group was to summarize current knowledge about the interaction between SDB and CVD. The results of this collaborative work are reported in the present review paper. To avoid being too theoretical, the authors focussed on the interaction of SDB and relevant CVD, i.e. atherosclerosis, cardiac arrhythmias, systemic and pulmonary hypertension, coronary artery disease, heart failure, and cerebrovascular disease. All of these different subjects are similarly subdivided into introduction, epidemiology, physiology/pathophysiology, impact on clinical practice, therapeutic intervention, diagnostic and therapeutic recommendations, and conclusions and future perspectives in order to make life easier for the reader.

Atherosclerosis

H.-W. Duchna, R. Schulz

Introduction

Atherosclerosis forms the basis for many cardiovascular disorders, and patients with OSA present with a high comorbidity of CVD. However, it has been difficult to establish a cause–effect relationship between OSA and CVD because these patients typically present with traditional risk

factors for the development of CVD such as obesity and metabolic disease, i.e. hyperlipidaemia and insulin resistance. Keeping in mind these confounding factors, the interaction of OSA, vascular risk factors, and CVD has been called 'syndrome Z' [251].

Epidemiology

There is a low grade of evidence of epidemiological data dealing with systemic atherosclerosis in patients with OSA. As atherosclerosis is a causal factor in most CVDs, its prevalence in patients with OSA might be estimated from the prevalence rates of coronary artery disease, myocardial infarction, and stroke. Preliminary studies investigating the prevalence of carotid atheromas and stenoses indicate a significant correlation with the presence of OSA [1, 64], but the number of patients investigated is low.

Physiology/pathophysiology

Atherosclerosis is the end point of a vascular disease, which begins as a functional disorder of the complex interaction between blood with its cellular components, vascular endothelial cells, and vascular smooth muscle cells. The vascular endothelial cells are in control of vascular tone, immunomodulatory functions, growth, vascular permeability, cell adhesion, and vascular architecture via a multitude of enzymes and kinases [46, 131, 192]. Vascular endothelial cells, however, are integrated in systemic vascular reflex mechanisms, mainly influenced by sympathetic and parasympathetic tone, hormones such as catecholamines, atrial natriuretic peptide, angiotensin II, vasopressin, and others [85]. An endothelial dysfunction appears to play a key role in the development of atherosclerosis [91, 192, 193].

An endothelial dysfunction has been demonstrated in almost all known risk factors for CVD, as for example diabetes mellitus, smoking, hypercholesterolaemia, and arterial hypertension [27, 28, 35, 130, 180, 229]. Recent studies support the hypothesis that OSA also leads to vascular endothelial dysfunction. According to the results of three different studies, endothelium-dependent vascular relaxation is blunted in awake patients with OSA, in the absence of any other disease state or potential cause of endothelial dysfunction [26, 48, 107, 117]. These data from *in vivo* studies in humans are supported by the results of an investigation of vascular reactivity in rats, in which recurrent episodic hypoxia served as a model for OSA [232].

Another current pathophysiological concept is that the OSA-related stimuli of hypoxaemia and shear stress enhance vasoconstrictive and prothrombotic forces within the vascular milieu and thereby lead to accelerated atherosclerosis [39, 56, 67, 115, 121, 126, 138, 155, 191]. This is strongly suggested by abnormalities of biochemical markers of CVD, which have been described in patients with OSA. First of all, there is increased sympathetic tone as evidenced by elevated catecholamine levels in plasma and urine and by an increase of muscle sympathetic nerve activity [25]. Furthermore, endothelial nitric oxide (NO) generation is suppressed, thus giving an explanation for the above-mentioned reduction of endothelium-dependent vascular relaxation in OSA. In this context, decreased serum levels of NO-derived nitrite and nitrate have been found in OSA [100, 213]. In addition to its decreased release, NO is probably scavenged by excessively generated free oxygen radicals. This assumption is supported by the finding of an enhanced superoxide release from circulating neutrophils and monocytes in OSA patients [51, 211]. The role of the vasoconstrictor endothelin in the development of OSA-associated CVD is less well established. Some studies reported increased serum concentrations of endothelin in OSA [176, 195], whereas other investigations could not reproduce these findings [73]. Apart from these changes in vasoactive mediator systems, inflammatory markers of vascular injury are also upregulated in OSA. This concerns vascular adhesion molecules as evidenced by measurements of soluble VCAM, ICAM, and E-selectin [33, 166], levels of highly sensitive C-reactive protein [218] and cytokines such as IL-6 and TNF-α [242]. Finally, activation and aggregation of platelets and increased fibrinogen levels have been reported in OSA and are presumed to underlie the development of CVD in these patients [21, 34, 248]. The two major hypotheses regarding the development of CVD and atherosclerosis in OSA are summarized in figure 1.

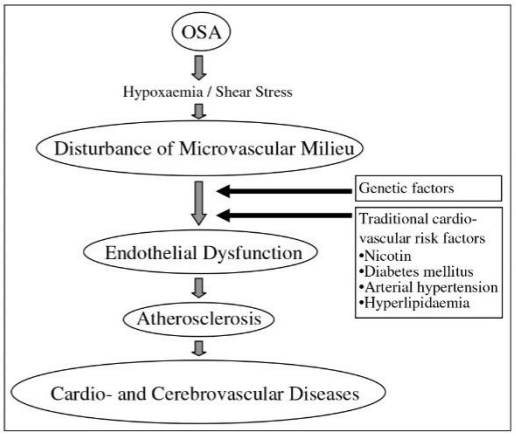

Figure 1. Hypothesis for the development of atherosclerosis and CVD in OSA.

The first direct evidence that OSA indeed leads to vascular remodelling and atherosclerosis is provided by ultrasonographic measurements of the intima media thickness of the common carotid artery (IMT-CCA). This parameter reflects the actual atherosclerotic burden of the organism and predicts the risk of future cardiovascular and cerebrovascular events in patients with or without pre-existing CVD. In this context, two independent studies have found an increase in IMT-CCA in untreated OSA patients as compared to matched control groups [210, 219].

Impact on clinical practice

Experimental data strongly suggest a negative effect of OSA-induced repetitive pressor surges and hypoxaemia on vascular endothelial function, although the level of clinical evidence is low. Endothelial dysfunction is a precursor of atherosclerosis. Abnormalities of microvessel function, structure and microvascular network are an important cause of hypertension, also likely to be central to many forms of atherosclerotic or hypertensive end-organ damage [125].

Therapeutic intervention

At present, there are no data available showing an improvement in atherosclerotic lesions by treatment of OSA *In vivo*

studies have shown that treatment of OSA with continuous positive airway pressure (CPAP) improves endothelial function [49, 99]. In a selected group of 11 patients with OSA, in the absence of any other disease state or potential cause for endothelial dysfunction, a 6-month therapeutic CPAP trial led to complete normalization of endothelium-dependent vascular relaxation, showing a restoration of endothelial cell function [49]. These data were confirmed by similar results in seven OSA patients treated with CPAP for 2 weeks, although a different technique investigating endothelial cell function had been used [99]. Furthermore, CPAP therapy is able to reverse the majority of the described biochemical alterations of the vascular system in OSA. Thus, there is good reason to speculate that by restoring normal vascular homeostasis, treatment of OSA may improve vascular function and thereby reduce cardiovascular morbidity and mortality in OSA.

Diagnostic recommendations

At present, a recommendation for screening patients with OSA for atherosclerotic lesions cannot be made. However, it is prudent to check for traditional risk factors (i.e. arterial hypertension, smoking, hyperlipidaemia, and diabetes). Investigation of endothelial function in order to estimate the 'atherosclerotic load' of a patient [57] would be of great value but is not practicable today.

Therapeutic recommendations

The above-mentioned studies suggest an improvement in important vascular functions in OSA patients treated with CPAP therapy. This led to the discussion of whether even non-sleepy patients with OSA should be treated with CPAP [88, 222]. Thus, future studies will have to prove the beneficial effects of CPAP therapy on cardiovascular outcome parameters in OSA.

Conclusions and future perspectives

OSA patients without overt CVD present with subtle vascular abnormalities such as impaired endothelial function and alterations of biochemical vascular markers, which can be reversed by CPAP treatment. There is now ample evidence that these subtle disturbances are the beginning of a cascade that may lead to the development of atherosclerosis and end in overt CVD (figure 1). Among other aspects, future studies should investigate the time course of the emergence of CVD in OSA, the possible role of vasoprotective mechanisms, and the impact of effective therapy on cardiovascular morbidity and mortality in OSA.

Cardiac Arrhythmias and Sleep Disordered Breathing

H.F. Becker, H. Hein, U. Koehler

Introduction

Research in SDB began in patients suffering from the pickwickian syndrome. As these patients have a high mortality – hospital mortality was 70% in pickwickian syndrome patients admitted to hospital due to decompensated ventilatory failure – and often die from sudden cardiac death [141, 142], broad interest in the occurrence and significance of cardiac arrhythmias as a possible clue to the increased mortality in these patients has evolved. It has become clear that not only pickwickian patients but also most patients with OSA exhibit characteristic arrhythmias. The current information concerning epidemiology, pathophysiology and clinical significance of cardiac arrhythmias in patients with OSA will be reviewed here.

Epidemiology

Concerning the epidemiology of cardiac arrhythmias, four different heart-rhythm disorders with different prevalence rates should be distinguished: (i) sinus arrhythmia/cyclical variation of heart rate, (ii) heart block (AV conduction block, sinuatrial block or sinus arrest), (iii) ventricular premature beats (VPBs) and (iv) atrial fibrillation.

Sinus arrhythmia/cyclical variation of heart rate (CVHR)

Sinus arrhythmia/CVHR is a typical finding in all patients with OSA except in cases with reduced heart rate variability due to diabetes or severe heart failure. CVHR is characterized by an abrupt increase in heart rate (HR) due to an arousal that terminates apnoeas, hypopnoeas, or obstructive snoring, followed by an HR decrease during respiratory events as a consequence of hypoxia and lack of thoracic expansion. This heart rate pattern is so typical that it may even be of diagnostic value: by the use of computerized analysis of Holter ECG alone, four algorithms of analysis correctly classified all patients with OSA out of a total of 35 patients. The best algorithm was able to detect 92% of 1-min epochs with or without breathing disorders correctly [173].

Heart block

In the early days of sleep research, heart block (second and third degree atrioventricular block [II° and III° AV block]), sinus arrest or sinuatrial block were thought to be highly prevalent in patients with OSA, and the reported incidence ranged between 18% and 50% [167, 235]. More recent data of unselected patients treated in a sleep laboratory revealed prevalence rates between 7% and 13% [15, 22, 142]. One publication has challenged previous results stating that the prevalence of bradyarrhythmias was not increased in patients with OSA compared to those without OSA [59].

Ventricular premature beats

Systematic evaluation of the incidence and severity of ventricular premature contractions (VPCs) in patients with OSA is still missing *Flemons* et al. [59] compared the incidence of frequent or complex VPCs in 173 patients studied because of suspected OSA, 76 of whom were diagnosed with OSA defined by more than 10 apnoeas and hypopnoeas per hour of sleep. The prevalence of VPCs was higher in patients without OSA, but the differences were not statistically significant.

In 29 heart failure patients with more than 15 apnoeas per hour of sleep – predominantly central apnoeas in 21 and obstructive apnoeas in eight patients – the effect of CPAP on VPCs was studied prospectively [102]. SDB was almost completely prevented by CPAP in 16 patients. In these patients, the number of hourly episodes of nocturnal VPCs was significantly reduced from 66 ± 117 to 18 ± 20 and the

number of couplets decreased from 3.2 ± 6 to 0.2 ± 0.21. In those 13 patients who did not respond to CPAP, VPCs remained unchanged. Based on the very limited data available, the influence of OSA on VPCs in patients without overt heart disease remains unclear, whereas SDB might worsen the occurrence of VPCs in heart failure patients.

Atrial fibrillation

Systematic evaluations of the incidence of atrial fibrillation in patients with OSA are missing. There is one report demonstrating that OSA is an independent risk factor for the development of atrial fibrillation following coronary bypass surgery [147]. The odds ratio for postoperative atrial fibrillation was 2.8 in patients with an oxygen desaturation index of ≥5/h compared to patients without SDB. Atrial fibrillation has been identified as an important risk factor for Cheyne–Stokes respiration [221].

Physiology/pathophysiology

CVHR is caused by an alternation between increased sympathetic and parasympathetic tone. Hypoxaemia-induced peripheral chemoreceptor stimulation leads to an activation of the sympathetic nervous system. As a consequence, ventilation increases and the resulting lung inflation has a vagolytic influence via the Hering–Breuer reflex. Therefore, hypoxaemia causes tachycardia in the presence of lung inflation; however, in the absence of lung inflation it causes bradycardia. Arousal at the termination of breathing disorders causes sympathetic activation and thus tachycardia. The repetitive occurrence of both mechanisms leads to CVHR, which is markedly attenuated by oxygen. The physiological meaning of bradycardia during apnoea-induced hypoxia might be a reduction in oxygen consumption by the heart, as this can be regularly demonstrated in humans before or during birth if the foetus is hypoxic, and also in diving mammals.

Heart block mainly occurs during REM sleep, most likely because parasympathetic activation and hypoxaemia are most pronounced in this sleep stage [17, 93, 111]. Hypoxia, tachycardia, sympathetic activation and increased blood pressure are possible mechanisms that might lead to ventricular premature beats in patients with SDB; however, the importance of these mechanisms remains uncertain.

Impact on clinical practice

CVHR seems to be a symptom that does not cause cardiovascular sequelae *per se*. It has been suggested that VPBs might play a role in the increased mortality rate of patients with OSA. However, there is no proof for this hypothesis. Heart block may lead to asystole of up to 15 s or longer. If this occurs exclusively during sleep, patients are often asymptomatic. In fact, OSA is the main cause of asymptomatic heart block occurring mainly during the night [151]. In 29 OSA patients with heart block, bradyarrhythmias could be completely prevented with CPAP treatment. Furthermore, none of these patients experienced syncope or died after an average of 54 months of CPAP treatment without implantation of a cardiac pacemaker [72].

Diagnostic recommendations

All patients with predominant nocturnal arrhythmias should be investigated with polysomnography in order to identify or rule out SDB.

Therapeutic intervention – therapeutic recommendations

CVHR is prevented with effective treatment for OSA. In most patients, effective treatment for OSA using CPAP or tracheotomy prevents patients from getting heart block [15, 108, 112, 235]. In only two out of 45 OSA patients, relevant VPCs occurred, and in one of these patients VPCs were no longer present with CPAP therapy [84]. There are no data available concerning the effect of CPAP treatment on atrial fibrillation in patients with OSA. It has recently been shown that SDB can be improved by atrial overpacing at a heart rate of approximately 15 beats per minute above the patient's spontaneous heart rate [68], but further research is necessary to support the clinical value of these findings.

Conclusions and future perspectives

In patients presenting with asymptomatic intermittent bradyarrhythmias, OSA should be considered as a possible cause. Episodes of heart block can be expected in approximately 20% of patients with severe OSA (apnoea–hypopnoea index [AHI] >60/h) and in approximately 7.5% of an unselected group of OSA patients. There is no threshold of SDB degree above which bradyarrhythmia exclusively occurs, although the risk increases with the amount of oxygen desaturation. REM sleep is an independent factor leading to heart block, irrespective of apnoea duration and oxygen desaturation. Effective treatment with CPAP leads to complete prevention of heart block in 80–90% of OSA patients. Sinus arrhythmia/CVHR is a typical finding in most patients with OSA and is also removed with effective CPAP treatment. Data concerning VPCs are scarce. There does not seem to be a clear link between OSA and VPCs in patients without overt heart disease, but SDB may be a factor that worsens VPCs in heart failure patients.

Sleep Disordered Breathing and Systemic Hypertension

B. Sanner, L. Grote

Introduction

The link between systemic hypertension and cardiovascular disease is well documented in the medical literature. It has been hypothesized that systemic hypertension is a short-term complication of OSA that mediates the association between OSA and CVD and increases cardiovascular morbidity in the long term. In fact, there is increasing evidence that OSA may cause systemic hypertension and that effective treatment of sleep-disordered breathing lowers high blood pressure [75].

Epidemiology

Epidemiological studies in the general population and in large clinical cohorts have demonstrated an independent association between OSA and systemic hypertension after controlling for confounders such as age and body weight [76, 122, 257]. A recent prospective study showed that patients with SDB have an increased incidence of systemic hypertension as compared with a non-SDB control group [174]. In summary, there is strong evidence based on high-quality epidemiological data that even a small degree of SDB causes elevation of blood pressure and systemic hypertension.

Physiology/pathophysiology

A number of pathophysiological mechanisms have been identified by case-control studies. They all suggest that SDB may cause sustained elevation of blood pressure [87]. Briefly, hypoxia and repetitive arousal from sleep are well-documented factors that cause an overall increase of sympathetic activity in patients with SDB [60]. Furthermore, changes in the renin–angiotensin system as well as in blood volume regulation are shown in OSA patients. Recent work has pointed out that vascular function is altered in OSA. It has been shown that the pressor response to hypoxia and to a given amount of angiotensin II is increased in OSA patients as compared with controls. In contrast, dilatory vascular response to the application of nitric oxide or to intra-arterial vascular β-2 receptor stimulation is attenuated in patients with SDB. However, it remains unclear whether altered vascular function is a cause or consequence of elevated blood pressure in SDB patients. In summary, there is pathophysiological plausibility and strong experimental evidence that SDB may cause systemic hypertension.

Impact on clinical practice

SDB is common in hypertensive patients (30–80%). In particular, OSA has been observed in up to 80% of patients with therapy-resistant hypertension [38, 124].

Therapeutic intervention

Treatment of SDB with CPAP results in a significant decrease in daytime and night-time blood pressure values in hypertensive patients [16, 43, 86, 137, 143, 146, 162, 175, 189, 230], indicating a causal relationship between the two conditions. High blood pressures or elevated heart rates at baseline – indicators of an increased sympathetic activity – may be predictors of a beneficial effect of CPAP therapy on blood pressure [204, 258]. Under CPAP therapy, also normotensive patients experience a decrease in blood pressure. On the other hand, not all hypertensive SDB patients show a blood pressure reduction in response to CPAP.

Diagnostic recommendations

Acute rises in blood pressure caused by SDB can be documented during sleep by invasive or noninvasive techniques. When daytime hypertension is suspected, blood pressure should be measured at least three times during two separate examinations. Blood pressure values above or equal to 140/90 mm Hg are considered hypertensive. Ideally, the diagnosis of hypertension should be verified by noninvasive ambulatory 24-h blood pressure monitoring or self-measurement. Given the epidemiological data, SDB proved to be the most common secondary cause of hypertension. Thus polysomnography should be performed in all patients with hypertension of unknown origin (so-called 'idiopathic hypertension'), especially in the absence of a nocturnal blood pressure dip [54].

Therapeutic recommendations

Usually, there is no association between the degree of blood pressure decrease with CPAP and polysomnographic parameters indicative of the degree of SDB improvement. This could be explained by the fact that hypertension – even if induced by SDB – might subsequently be perpetuated by vascular remodelling or some secondary renal response to chronic preglomerular vasoconstriction and altered perfusion. Furthermore, SDB is probably not the only cause of hypertension in these patients. For that reason, CPAP treatment can rarely replace medical treatment of systemic hypertension completely [118].

Conclusions and future perspectives

Hypertension and SDB are frequently associated: Approximately one-third of hypertensive patients have OSA, whereas about 50% of OSA patients are hypertensive. Epidemiological studies of the last years have documented that there is a causal relationship between these two conditions – independent of other known risk factors. Consequently, CPAP therapy has a significant blood pressure-lowering effect in a subgroup of hypertensive patients with OSA.

These data emphasize the need to consider OSA as a potential cause or aggravating factor in hypertensive patients, especially if hypertension is difficult to control. Furthermore, blood pressure monitoring should be performed in patients suspicious of OSA, and patients with 'idiopathic' arterial hypertension should be evaluated by full polysomnography, especially in the absence of a nocturnal blood pressure dip.

Coronary Artery Disease – Acute Myocardial Infarction

S. Andreas, U. Koehler, R. Staats

Introduction

Coronary artery disease (CAD), also named ischaemic heart disease, is defined as the manifestation of atherosclerosis in the coronary arteries. CAD may lead to coronary stenosis with flow limitation and consequently to an imbalance of myocardial oxygen supply and demand. Severity and duration of ischaemia determine the clinical manifestation as asymptomatic, stable or instable angina pectoris, myocardial infarction, arrhythmias, and sudden cardiac death.

Epidemiology

Today, CAD is the most common, chronic, life-threatening disease. According to data from the MONICA study, cardiovascular mortality in Germany affects 428 men and 272 women per 100 000 persons per year, with a slight decrease from 1993 to 1996 [252]. More than 60% of the overall cardiovascular mortality is attributed to CAD and >30% to cerebrovascular disease. There were 380 acute myocardial infarctions per 100 000 persons in the age group of 35–64 years. Similarly, the Physicians' Health Study revealed 440 cases of acute myocardial infarction (AMI) per 100 000 physicians per year [227]. Risk factors, life style, and socioeconomic circumstances are probably the most important explanations for a large regional variation [252].

Obstructive sleep apnoea and coronary artery disease

In a large study on the prevalence of OSA in CAD [149], 142 men with CAD verified by angiography were investigated by polysomnography using a pressure-sensitive bed. Thirty-seven per cent of patients had an AHI of 10 or more, which was significantly higher than that of age-matched controls [149]. A number of studies on patients with CAD who where

slightly overweight with a mean body mass index of 27 kg/m^2 yielded similar results, with an incidence of OSA between 35% and 50% [6, 41, 113, 148, 171, 214]. In one study, 101 unselected males aged less than 66 years were investigated by polysomnography 24 days (mean value) after they survived an acute myocardial infarction (AMI) [97]. About 30% of these patients had an apnoea index (AI) >5/h. Mean AHI in the patients was 12.7/h, while 53 male subjects of similar age but without evidence of ischaemic heart disease had an AHI of 3.7/h [97]. In an Italian study, the prevalence of apnoeas, chiefly of the central type, soon after clinical stabilization of unstable angina and following AMI, was similar and higher than that in stable CAD [152]. In 440 patients with OSA proven by polysomnography, CAD was demonstrated by angiography in 24.6% [203]. This high prevalence might be explained by a referral bias because the institution has a local reputation for CAD. The key question of public health importance is: Is OSA a risk factor for CAD? Although most studies found an association of OSA with CAD, they were of limited value because they were either hospital based with a selection bias, small in number, or lacked a control group. Cross-sectional associations from the baseline examination of the Sleep Heart Health Study [217] in 6424 individuals are compatible with modest to moderate effects of OSA on various manifestations of CVD within a range of AHI values that is considered normal or only mildly elevated. SDB was associated more strongly with self-reported heart failure and stroke than with CAD: The relative odds ratio for CAD was 1.27 (0.99–1.62 upper vs. lower AHI quartile) [217].

Physiology/pathophysiology – impact on clinical practice

In order to analyse the association of SDB, especially OSA, and ischaemic heart disease with its different manifestations such as chronic stable CAD and acute coronary syndrome (e.g. unstable angina and myocardial infarction), it is noteworthy to differentiate between acute effects and long-term effects of SDB with possible causal relationships. In general, myocardial ischaemia occurs as a result of diminished oxygen supply or increased oxygen demand in the case of inappropriate coronary reserve. Heart rate is an important determinant of myocardial oxygen consumption, especially during sleep without physical activity *Quyyumi* et al. [181] found episodes of ST-segment depressions in Holter ECG recordings preceded by an increase in heart rate as a result of arousal, lightening of sleep, body movements, and REM sleep in patients with CAD and nocturnal angina without evidence of OSA. Experimental data from animal studies exhibited an increase in coronary blood flow in REM sleep due to an increased sympathetic drive to the heart [42]. In the case of experimental coronary artery stenosis, the coronary blood flow was diminished in phasic REM sleep as a result of a mismatch between the increase in heart rate and diastolic perfusion [42]. In these circumstances, myocardial ischaemia is promoted by REM sleep, which was described first by *Nowlin* and coworkers in 1965 [165]. In OSA, myocardial oxygen supply is diminished by apnoea-associated hypoxaemia. Besides the effects of oxygen desaturation on myocardial ischaemia, OSA may reduce myocardial blood flow supply and/or increase oxygen demand by acute changes in heart rate and elevations of blood pressure-induced left ventricular after-load at the resumption of breathing at each apnoea termination. In addition, interventricular septum shift leads to an impediment of the diastolic function of the left ventricle. The frequency and clinical impact of nocturnal myocardial ischaemia in patients with OSA is unclear because of a lack of systematic studies. Asymptomatic ST-segment depressions during sleep were observed in seven of 23 patients with OSA without evidence of CAD by Holter ECG [79] *Franklin* et al. [63] found OSA in nine of 10 patients with nocturnal angina pectoris. During treatment of OSA with CPAP, nocturnal angina diminished and the number of nocturnal myocardial ischaemic events was reduced. In a study of 21 patients with OSA, *Schäfer* et al. [209] found asymptomatic nocturnal ST-segment depressions reflecting myocardial ischaemia only in those patients with angiographically proven CAD and in one patient with diffuse coronary vessel defects. The vast majority of these episodes was associated with apnoea-related oxygen desaturations and occurred predominantly in REM sleep. Microstructure of sleep was disturbed to a greater extent in ischaemic episodes than in control episodes. Ischaemic episodes led to more and severer arousals than control episodes, correlating with the extent of oxygen desaturation. In a more recent study, *Peled* et al. [172] investigated 51 patients with OSA and CAD and a control group of 17 patients with OSA without CAD (only 15 of the total had coronary angiography). Nocturnal ST-segment depression occurred in 10 patients with CAD, and no events were seen in the control group. The exacerbation of ischaemic events during sleep in OSA and CAD may be explained by the combination of increased myocardial oxygen consumption and decreased oxygen supply due to oxygen desaturation, with peak haemodynamic changes during the rebreathing phase of obstructive apnoea. Treatment with CPAP significantly ameliorated nocturnal ischaemia [172]. With regard to the different manifestations of ischaemic heart disease in selected subjects without risk factors for OSA, the frequency and extent of sleep apnoea was higher after an AMI or unstable angina than in stable CAD [152]. Moreover, in stable CAD, apnoeas were of obstructive type, whereas in unstable CAD the central type of apnoea was predominant without any correlation to left ventricular function. The increased sympathetic drive in unstable CAD [132] may have an inhibitory effect on respiratory drive as a possible cause of apnoea in these patients [152]. The sympathetic activation and coagulation disorders associated with OSA make it reasonable to believe that acute coronary syndromes with or without ST-segment elevation can be triggered by OSA. Treatment of AMI is focussed on the rapid reopening of the infarct-related artery, as detailed in national and international guidelines. To reduce angina, anxiety and oxygen consumption, morphine is used in the acute setting. This may lead to sleep and apnoeas when angina ceases. One study reports a high incidence of SDB in patients recovering from AMI [97]. In another study of AMI, patients' OSA was related to premature ventricular contraction but not to major complications of AMI [133]. Despite the greater incidence of cardiac arrhythmias during AMI in OSA patients, these patients have the same clinical course in hospital and mortality rate as non-OSA patients [133]. Whether patients with OSA and CAD are at increased risk of 'dying in their sleep' is not clear, although in general the frequency of AMI is highest in the early morning [153] due to increased sympathetic drive and changes in rheological factors. In patients with AMI occurring at night, the respiratory disturbance index (RDI) was significantly higher than in patients with AMI during wakefulness [114]. Besides the

acute effects of OSA on myocardial and cardiovascular physiology, a number of mechanisms link OSA and vasculopathy, leading to atherosclerosis and CAD in the long-term (see chapter on atherosclerosis).

Therapeutic intervention

Shahar et al. revealed that even an RDI considered normal or mildly elevated might enhance the risk of developing CVD including CAD, thus commencing the discussion of when to start OSA treatment [88, 217]. Though it is appealing that screening for OSA with the intention to treat patients with known CAD will reduce cardiovascular mortality, no study has specifically addressed this question. Some problems should therefore be mentioned: Since patients with CAD do not present to the medical system with complaints directly related to OSA, compliance with CPAP treatment is likely to be lower than that in a typical population of OSA patients [147, 170]. This might improve when OSA is considered to be a modifiable risk factor for CVD by all physicians involved in the treatment of patients with CAD. Related to this question is the problem of whether the treatment effect on blood pressure, sympathetic activity, endothelial function, etc., of OSA patients with an AHI >10/h and without significant daytime sleepiness will be as good as that in the published studies on 'classic' OSA patients with daytime sleepiness [49, 175]. However, since the underlying pathophysiology of the OSA-related cardiovascular complications and positive CPAP effects are clearly related to nocturnal apnoeas and oxygen desaturation [48, 175], the positive effects of CPAP on the cardiovascular system are unlikely to be influenced by daytime symptoms. There are several studies investigating the effects of CPAP therapy on CAD symptoms and risk factors of CVD in OSA patients. In OSA patients who also suffered from CAD, ECG recordings revealed ST-segment depression and therefore significant ischaemic events, often accompanied by nocturnal angina. CPAP therapy ameliorated the nocturnal ST-segment depression time and nocturnal angina [63, 79]. Venous vascular reactivity to bradykinin was found to be blunted in OSA patients as compared to controls. This effect was reversed with CPAP therapy [48]. In a recent study, *Imadojemu* and coworkers analysed the reactive hyperaemic blood flow and described an impaired arterial vasodilator response in OSA patients. CPAP therapy improved vascular function and decreased muscle sympathetic nerve activity [99]. Two studies detected decreased circulating NO levels in patients suffering from OSA. Serum level of NO increased significantly after overnight CPAP therapy [100, 213]. The relationship between OSA and hypertension is discussed in another part of this paper.

CPAP therapy positively influenced platelet aggregability, fibrinogen level, superoxide release, and cell adhesion molecule expression [21, 24, 33, 34, 206, 211]. Hence, future studies will disclose whether public awareness of a possible association between OSA and CAD will improve therapy compliance in non-sleepy patients with OSA. In patients unable to tolerate CPAP, however, alternative therapy strategies are required. Although less effective than conventional CPAP therapy, oral appliance (OA) devices proved to be beneficial with rare serious side effects in patients unable to maintain CPAP therapy and, when correctly indicated, as first choice therapy in selected OSA patients with low RDI [20, 71, 89, 183, 190]. Patients with AMI are usually monitored on an intensive care unit (ICU). If OSA in this setting leads to severe surges in blood pressure and/or myocardial ischaemia, treatment of OSA should be initiated even on the ICU, but there are no studies available supporting this hypothesis.

Diagnostic recommendations

Clinical examination in patients with CAD is directed by the underlying heart disease itself as well as cardiovascular risk factors and significant comorbidity. As detailed above, OSA is common in patients with CAD and is a significant and modifiable risk factor for CVD [129, 147, 217]. Therefore, OSA should be included in the diagnostic work-up of patients with CAD. If the CAD patient's medical history is positive regarding excessive daytime sleepiness, nocturnal angina pectoris, witnessed snoring, or apnoeas, polysomnography is recommended.

Therapeutic recommendations

A consensus statement published in 1999 recommended CPAP therapy in any OSA patients with an RDI exceeding 30/h or at a minimal threshold of 5/h if the patient is suffering from either excessive daytime somnolence, impaired cognition, mood disorders, insomnia, or documented cardiovascular disease [129]. The recommendation to treat non-sleepy patients with low RDI and CAD is further supported by cross-sectional results of the Sleep Heart Health Study [217]. In patients with AMI, CPAP treatment should be initiated on the ICU if OSA in this setting leads to severe surges in blood pressure or myocardial ischaemia, but there are no data supporting this hypothesis.

Conclusions and future perspectives

There is growing evidence suggesting that OSA is an independent risk factor for CAD. While studies with randomized therapeutic intervention are unlikely to be performed in the near future, it seems prudent to advocate CPAP therapy in patients with CAD and moderate to severe OSA even if they do not suffer from excessive daytime sleepiness.

Heart Failure

S. Andreas, I. Fietze, V. Töpfer

Introduction

Heart failure is defined by symptoms *and* objective evidence of cardiac dysfunction [186]. Symptoms may be breathlessness, ankle swelling, signs of venous distension, and fatigue. The severity of heart failure is classified by the New York Heart Association (NYHA), but there is a poor relationship between symptoms and cardiac dysfunction as well as prognosis. The underlying causes of heart failure are CAD and arterial hypertension, valvular disease, and idiopathic dilated cardiomyopathy. Clearly evidenced guidelines for the treatment of heart failure exist [186]. The prevalence of symptomatic heart failure in the general European population is 0.4–2% and increases rapidly with age [186]. The prognosis of heart failure is poor, albeit significant improvements in treatment have been gained. Still, about half of the patients diagnosed with chronic heart failure (CHF) will die within 4 years [186]. Recently, diastolic heart failure has been noticed to be common especially in the elderly population and

carries a prognosis nearly as grim as heart failure with systolic dysfunction [55].

Epidemiology

Javaheri et al. reported on 81 ambulatory male CHF patients with an left ventricular ejection fraction (LVEF) <45% [105]. The authors noted that 51% of their patients had an AHI >15/h. Most of the patients had Cheyne–Stokes respiration (CSR), but some more obese patients had obstructive apnoeas. Similar findings were made in a comparable CHF group [225] and in patients on a waiting list for heart transplantation [127]. In patients with an LVEF <45% investigated 1 month after an episode of pulmonary oedema, an AHI >15/h was reported in about 80% of 34 consecutive patients. Again, OSA was less common (25%) than CSR (75%) and was chiefly observed in the more overweight patients [239]. CSR seems to be more common in men [220], which might be explained by the higher ventilatory drive in men. It is our impression that presently the prevalence of SDB in appropriately treated CHF patients is less than 30%. This is likely the result of the increased prescription of ß-blockers and probably their direct influence on central controller gain (see Pathophysiology). There is insufficient knowledge about SDB in patients with diastolic heart failure. In one study, 11 out of 20 patients with diastolic heart failure had an AHI >10/h with mainly obstructive apnoeas [29]. Furthermore, there was an independent association between abnormal diastolic ventricular relaxation pattern and nocturnal oxygen desaturations in 68 patients with OSA [66].

Physiology/pathophysiology

In patients with impaired LVEF, a fundamental characteristic of SDB is the central origin of the disorder. Periodic breathing (CSR) with or without apnoea, central sleep apnoea, as well as other respiratory disorders such as hypopnoea and hypoventilation, all characterize the syndrome of SDB in CHF patients. The main factor leading to SDB in CHF patients, especially during sleep onset, is a fall in carbon dioxide ($PaCO_2$) tension below the apnoea threshold, with a consecutive decrease of central nervous outflow to respiratory muscles [104]. Engaged in this phenomenon are: carbon dioxide receptors in the medulla, the carotid body and the aortic arc; oxygen receptors located in the medulla and carotid body; ergoreceptors of the respiratory muscles; and central mechanisms regulating the sleep–wake rhythm. In CHF patients with CSR, hypocapnia is more pronounced than in CHF patients without CSR [223]. $PaCO_2$ levels are consequently only 1–3 mm Hg above the apnoea threshold during sleep in CSR patients, in comparison with healthy subjects, among whom this difference is 3–5 mm Hg. An increase of CO_2 in inhaled air to the level of 4% during sleep increases $PaCO_2$ and prevents occurrence of apnoea in CHF patients [128].

$PaCO_2$ in CHF patients with CSR is inversely correlated with pulmonary capillary wedge pressure (PCWP). An increase in PCWP leads to activation of pulmonary vagal afferent pathways, followed by hyperventilation and a fall in $PaCO_2$ [223]. Another mechanism possibly involved in generation of apnoea is enhanced peripheral and central chemoreceptor sensitivity. This corresponds to enhanced respiratory response, which is in turn correlated with the amount of CSR [7] and daytime hyperventilation [159]. Although the role of hypoxaemia in the genesis of CSR is not well known, it is possible that hypoxaemia elicits arousals that provoke hyperventilation.

During sleep, the extent of CSR is also evidently a function of sleep stage. Sleep-stage differences are based on the degree of impaired arousability in REM sleep, during which CSR is less common than in NREM sleep. One effect of an arousal-related stage shift is that the sleeper suddenly detects $PaCO_2$ as excessively high, which can in turn lead to hyperventilation. Further mechanisms responsible for CSR in CHF patients are increased blood circulatory time, enhanced sympathetic nerve activity, decreased body oxygen and CO_2 stores, upper airway instability, impaired LVEF, and respiration pattern preceding CSR [4, 77, 90, 109, 116, 124, 128, 156]. A correlation has been established, for example, between the cyclic length of periodic breathing and the degree of LVEF [77]. Changes in blood gas tension may provoke instability (underdamping) of the respiratory system, accompanied by exaggerated gas changes during CSR [116]. CSR is accordingly an expression of oscillations in feedback regulation of respiration by the above-stated disturbance variables, which prevent damping or physiological counter-regulation.

The question arises: Is there a relationship between central and obstructive apnoeas in CHF patients? Obstructive apnoea may provoke acute nocturnal decompensation with interstitial lung oedema, which in turn decreases functional residual capacity (FRC) [161] and leads to the above-stated changes in blood gas stores *Tkacova* et al. described a possible shift from OSA to CSR under conditions of progressively rapidly falling $PaCO_2$ and rising blood circulatory time, owing to deterioration in cardiac function [237]. Conversely, it is feasible that periodic breathing leads to instability in the upper airway due to pharyngeal oedema. It is also possible that obstructive breathing is followed by reduced respiratory drive during the waning phase of periodic breathing, with greater reduction of drive to the pharyngeal dilatator muscle than to the diaphragm. CSR has also been described for CHF patients during the day, as a symptom of disturbed autonomic regulation and poor survival outcome. Augmented chemoreceptor sensitivity [178], impaired autonomic control, and baroreflex inhibition [179] are possible mechanisms involved in the genesis of daytime CSR.

Impact on clinical practice

Clinical markers that indicate CSR among CHF patients are as follows: episodic hypoxaemia, numerous arousals during sleep, sleep fragmentation, daytime sleepiness [80], and heart rhythm disorders correlating with falls in $PaCO_2$ [98]. Other phenomena include nocturnal heart rate and blood pressure changes due to arousals, changes in sympathetic nerve activity [90, 160, 241], increased chemoreceptor sensitivity, and altered heart rate variability [164, 256].

Therapeutic intervention

Treatment options can be broadly divided into four groups: intensive heart failure treatment, pharmacological therapy, oxygen, and various forms of positive airway pressure such as CPAP, bilevel pressure ventilation, and adaptive pressure support servo-ventilation.

Intensive heart failure treatment

The first consideration is to optimize the heart failure therapy. Cardiovascular drugs improve left ventricular function,

decrease PCWP and favourably influence neuroendocrine activation. Recent studies have reported a decrease in central sleep apnoea (i.e. CSR) caused by heart failure treatment [37, 243]. Thus, before any specific therapy for CSR is undertaken, appropriate utilization (including dose adjustments) of cardiovascular drugs to optimize cardiovascular function should be undertaken [103].

Pharmacological therapy

Theoretically, respiratory-drive stimulants such as theophylline and acetazolamide can alleviate CSR beyond optimizing CHF by cardiovascular drugs [40, 53, 106]. In a study by *Javaheri* et al. [106], use of theophylline was associated with a significant reduction in AHI, but a reduction in the frequency of arousals or improvements in sleep structure were not documented. Theophylline did not lead to any improvement of cardiac function. Theophylline is problematic because it could increase minute ventilation in CHF patients with CSR whose minute ventilation is already elevated, and because of its potentially dangerous effects on cardiac output by causing cardiac arrhythmias. The effect of acetazolamide [18, 238, 250] on CSR has not been systematically evaluated in patients with CHF *Sakamoto* et al. [200] found that acetazolamide did not consistently reduce the frequency of respiratory events in patients with central sleep apnoea. In summary, theophylline or acetazolamide are not recommended for treatment of CSR in patients with CHF.

Oxygen

The rationale for using oxygen is that it increases oxygen and carbon dioxide stores and suppresses peripheral chemoreceptor drive, thereby dampening the respiratory control system and making it more stable *Hanly* et al. [78] investigated the effect of oxygen administration and demonstrated a significant decrease in AHI, arousal index, and degree of oxyhaemoglobin desaturation. In a subsequent randomized placebo-controlled study of intranasal oxygen given for 1 week, *Andreas* et al. [5] also documented a modest decrease of central apnoeas and hypopnoeas in patients with CSR. In addition, these patients experienced a significant increase in peak oxygen consumption during exercise without a change in the duration of exercise, peak heart rate, or quality of life. Furthermore, the hypercapnic ventilatory response (HCVR) was reduced by nocturnal oxygen [8]. More recently, *Staniforth* et al. [226] documented significant reductions in AHI and in overnight urinary norepinephrine excretion in patients with stable CHF and CSR while they were treated with nocturnal oxygen over a 4-week period. Similar acute effects were noticed in patients with chronic hypoxaemia due to chronic obstructive pulmonary disease [90]. More effective suppression of CSR may be achieved by adding carbon dioxide to oxygen therapy. Therefore, *Andreas* et al. [9] performed a study that evaluated the effects of nocturnal oxygen plus carbon dioxide on CSR, sleep, and sympathetic activation. Nocturnal combination of oxygen plus carbon dioxide reduced the duration of CSR and increased arterial oxygen saturation as well as mean transcutaneous carbon dioxide tension but markedly increased sympathetic activation.

Forms of positive airway pressure

Continuous positive airway pressure

CPAP is the most extensively studied therapy for CSR in patients with CHF and has been shown to alleviate this breathing disorder in association with substantial beneficial effects on cardiovascular function [253] *Takasaki* et al. [233] were the first to study the effects of CPAP in patients with CHF and CSR in a controlled trial in 1989. Application of CPAP was associated with a highly significant reduction in AHI, an increase in nocturnal SaO_2 and improvements in sleep structure [158]. These initial observations of beneficial effects of CPAP on CSR were confirmed by *Naughton* et al. [160] in a controlled trial of CPAP in patients with stable CHF and CSR. The group treated with CPAP experienced a decrease in the frequency of central events, associated with a reduction in minute ventilation and an increase in transcutaneous PCO_2. A randomized trial of CPAP was undertaken by *Naughton* et al. [160], with LVEF as the primary outcome measure. There was a greater improvement of LVEF in the CPAP group than in the control group. In another study, *Naughton* et al. [157] demonstrated that CHF patients with CSR had higher overnight urinary and daytime plasma norepinephrine concentrations than CHF patients without CSR. By using CPAP, there was a 40% reduction in overnight urinary norepinephrine and a 24% reduction in daytime plasma with a significant decrease in heart rate. The largest and longest randomized clinical trial of CPAP therapy for CHF involved 29 patients with and 37 without CSR [221]. Over a follow-up period of up to 5 years, patients in the CSR group who complied with CPAP therapy experienced a reduction in the combined rate of mortality and cardiac transplantation rate. In contrast, CHF patients without CSR but randomized to CPAP therapy did not experience any significant decrease in the mortality or cardiac transplantation rate.

Bilevel pressure ventilation

Willson et al. [254] recently reported preliminary data showing that CSR was abolished and sleep improved with noninvasive nasal ventilation using a time-cycled volume preset ventilator. In these studies, the prolonged use of noninvasive ventilation was also associated with a reduction in the AHI, a decrease in arousal index, and an improvement in cardiac function.

Adaptive pressure support servo-ventilation

Adaptive pressure support servo-ventilation (ASV) is a new approach to the treatment of CSR, in which a small but varying amount of ventilatory support is provided. The intention is to provide the hydrostatic benefits of low levels of CPAP while directly suppressing CSR and attendant sleep disturbance without causing overventilation. In a recent study by *Teschler* et al. [234], the acute effect of ASV on quality of sleep and breathing was compared with nasal oxygen, nasal CPAP, and bilevel spontaneous/time (ST) mode nasal ventilation. The authors described a better improvement in sleep and breathing with ASV than either nasal CPAP/bilevel ventilation or 2 L/min nasal oxygen. The authors concluded that sleep and breathing were better during 1 night of ASV therapy than during 1 night of oxygen or CPAP/bilevel pressure ventilation. Long-term studies of the effect of ASV on quality of life and cardiovascular function are presently under way.

Diagnostic recommendations

As detailed above, CSR, and to a lesser degree OSA, is common in CHF and is independently related to impaired left ventricular performance and increased mortality [81, 120, 217, 221]. Therefore, CSR and OSA have to be included in the diagnostic work-up of patients with CHF. Although OSA

often has a characteristic history, this seems to be much less the case for CSR in the setting of CHF [3, 225]. Full polysomnography is recommended in patients with CHF in order to analyse breathing patterns, arousals, and sleep structure, especially if nocturnal angina, excessive daytime sleepiness, witnessed snoring, or apnoeas are present.

Therapeutic recommendations

Of paramount importance is maximal conservative CHF treatment. It seems necessary to find the right method of treatment of CSR on an individual basis. However, we recommend trying oxygen therapy initially because it is effective and simple to use. In case of an insufficient therapeutic effect of oxygen, the next option is to use CPAP. CPAP is the most extensively studied therapy for CSR and is shown to alleviate this breathing disorder in association with substantial beneficial effects on cardiovascular function. The most effective suppression of CSR in patients with CHF is achievable with ASV. In severe cases of CSR with a high AHI or in cases where other options were ineffective, treatment with ASV is recommended. Using ASV, however, is not as simple as CPAP or oxygen, because special equipment and software is needed.

Conclusions and future perspectives

In conclusion, there is good evidence suggesting that CSR is common in CHF and is the cause of impaired sleep and sympathetic activation with concomitant unfavourable effects on left ventricular function and survival. However, large controlled studies are needed to test the hypothesis that successful treatment of CSR will reduce the high mortality of CHF.

Pulmonary Hypertension in Obstructive Sleep Apnoea Syndrome

W. Randerath, K.-H. Rühle, B. Sanner, H. Schäfer

Introduction

Pulmonary hypertension (PH) can be defined by a sustained elevation of the mean pulmonary artery pressure (PAP) to ≥20 mm Hg or of the systolic pressure to ≥30 mm Hg [74]. PH results from increases in resistance of blood flow in pulmonary venous drainage (e.g. elevated left ventricular diastolic pressure), in the pulmonary vascular bed (e.g. obstructive or restrictive pulmonary diseases) or from resistance of flow itself (e.g. thromboembolism). Syndromes associated with hypoventilation, namely the obesity–hypoventilation syndromes, OSA or neuromuscular disorders, are thought to lead to pulmonary hypertension. However, PH might be secondary due to hypoxic pulmonary vasoconstriction [74]. Other potential mechanisms are hypoxia-induced vascular endothelial dysfunction [56], pulmonary vascular remodelling [199], intrathoracic pressure changes, and autonomic reflexes.

Epidemiology

The prevalence of PH in OSA without underlying pulmonary disease is still controversial. Early studies reported prevalence rates between 20% and 80%. However, these investigations included patients with lung disorders, especially chronic obstructive pulmonary disease. According to these studies, impairment of lung function and hypoxaemia seemed to correlate best with PH. However, recent studies in patients without pulmonary disease also showed prevalence rates of about 30% of PH in OSA [11]. Studies in which pulmonary pressure was measured invasively found a prevalence of about 20%, whereas studies based on the noninvasive Doppler technique showed figures of 40% [119, 197]. One may conclude that PH can be found in OSA patients without pulmonary disease, which however can aggravate PH.

Physiology/pathophysiology

Increases in PAP have been described both acutely during a single apnoeic event and chronically in the course of the OSA. In NREM sleep, PAP reaches its maximum during the postapnoeic hyperventilation period, whereas in REM sleep, even long apnoeas are not necessarily associated with pressure increases [177].

While intravascular PAP decreases during apnoea and increases at the resumption of breathing, transmural pulmonary artery pressure (PAPtm, i.e. the correction for intrathoracic pressure swings) tends to increase progressively throughout an apnoea, with a maximum during the final occluded efforts and sustained during the early phase of hyperventilation [134]. Analysis of apnoea episodes in NREM sleep revealed a progressive increase in systolic mean PAPtm of 10 mm Hg towards the end of apnoea [208]. Among the underlying mechanisms of the acute changes, alveolar hypoxia was suggested to play an important role [134]. However, oxygen administration affected neither mean PAPtm nor the amplitude of pressure swings in most patients [135]. Other factors contributing to PAPtm changes are mechanical events caused by intrathoracic pressure swings with increased right ventricular preload and output or due to increased left ventricular afterload.

Beat-by-beat analysis of the underlying factors showed that hypoxia was a major determinant of the slow changes of PAPtm over the whole course of an apnoea and rapid changes in PAPtm were synchronous with intrathoracic pressure changes [136]. Analysing the contributing factors, *Schäfer* et al. [208] found hypoxaemia and intrathoracic pressure swings both independently associated with an increase of PAPtm. The authors did not find any association of arterial blood pressure as a rough estimate of left ventricular afterload with the changes in PAPtm in this study. According to the time course of pulmonary haemodynamics during the night, *Schäfer* et al. [208] did not find a progressive increase in PAP, in contrast to another study, which showed a trend towards a small progressive increase in PAP throughout the night [216]. The authors concluded that this increase reflects the cumulative effects of repetitive apnoeas and hypoxaemia. However, apnoea duration increased throughout the night in this study.

In 40% of OSA patients without overt CVD, *Sajkov* et al. described a slightly elevated PAP at rest, which rose significantly when pulmonary blood flow was increased [199]. Patients with or without sustained PH did not show any differences in the severity of SDB, lung function or body mass index. However, in patients with PH, the authors found more pronounced ventilation–perfusion mismatch and resting hypoxia. PH in these patients was thought to be the result of structural narrowing of the pulmonary vessels. The authors speculated that this remodelling may be caused by an increased pulmonary vascular pressure response to hypoxia or an increased small airways closure with regional lung hypoxaemia. In a more recent study, the same authors found

a decreased hypoxic pulmonary constrictor response, which was measured as the difference in PAP under hypoxic and hyperoxic conditions [198]. This might result from an impaired pulmonary vascular endothelial function, which is responsible for the vascular tone [10, 19, 56]. Moreover, *Sajkov* et al. described a reduction in the hypoxic pulmonary vascular reactivity under treatment with CPAP. They concluded that intermittent nocturnal hypoxia might cause pulmonary vascular endothelial dysfunction [198]. Recent studies suggest that genetic factors determine the link between hypoxia and manifestation of PH [47, 52].

Impact on clinical practice

In general, PH can lead to dyspnoea and right heart failure. It is often difficult or impossible to differentiate whether OSA or other pulmonary diseases are responsible for these symptoms. If present, the degree of daytime PH is mild in most patients with OSA alone. Hence, specific clinical symptoms of PH are rarely described in these patients. Although an association of OSA with PH has been shown conclusively, there is no correlation between the severity of OSA as measured by AHI and the severity of PH. Right ventricular failure and PH define, in part, one subtype of SDB, the pickwickian syndrome.

Diagnostic recommendations

The sensitivity of ECG or radiographic findings in the diagnosis of PH is unsatisfactory *Sanner* et al. demonstrated pulmonary wedge pressure and time spent below 90% SaO_2 during the night as independent predictors for PH when coexisting pulmonary disease was excluded. Other parameters of lung function or PaO_2 were not predictive for PH [205]. There are controversial results concerning the predictive value of resting PaO_2, AHI, or lung function. PAP can be evaluated invasively using right heart catheterization and noninvasively by using Doppler echocardiography [150, 196]. Invasive pressure measurement is the diagnostic gold standard, although *Sajkov* et al. described a good correlation ($P = 0.96$) between catheter and Doppler techniques in the investigation of PAP [196].

Therapeutic intervention – therapeutic recommendations

An early investigation in OSA patients treated with tracheostomy reported an improvement in PAP and right ventricular function [62]. In several studies, CPAP did result in a long-term improvement of PAP in patients with OSA [31, 215] *Chaouat* et al., for example, did not exclude patients with chronic obstructive pulmonary diseases, which might influence the level of PAP [31]. In contrast, *Sajkov* et al. studied the effects of CPAP in 20 patients without lung or cardiovascular disorders. Five of these subjects showed an elevation in mean PAP at baseline. The authors found that CPAP improved daytime PAP and total pulmonary vascular resistance, and the greatest improvement was shown in patients with sustained daytime PH [198] *Alchanatis* et al. described a sample of 21% of patients with PH out of 29 patients with OSA but without further CVD. In both groups, pulmonary hypertensive and normotensive, PAP fell significantly under treatment with CPAP for 6 months [2]. Though indicative of positive effects of CPAP therapy on PH in OSA, the therapeutic studies mentioned above have to be regarded with caution, as there was no control group.

Conclusions and recommendations

Mild PH is present in 20–40% of patients with OSA, but there is no correlation between the severity of OSA and the occurrence of PH. Although the pathophysiological background is still unclear, vascular endothelial dysfunction associated with increased vascular reactivity might be one important aspect. There is some evidence for the positive effect of long-term treatment with CPAP on PH in patients with OSA.

Pulmonary Hypertension in Chronic Obstructive Pulmonary Disease

W. Randerath, K. Rasche, K.-H. Rühle

Introduction

Chronic obstructive pulmonary disease (COPD) is often associated with nocturnal (i.e. sleep-related) hypoventilation, ventilation–perfusion mismatching, and consecutive O_2 desaturations [58]. As a consequence of the Euler–Liljestrand reflex and other mechanisms, pulmonary arterioles in the pulmonary circulation constrict and vascular resistance increases [36]. In the following, the pathophysiological mechanisms leading to an increase in pulmonary artery pressure will be discussed and the consequences for therapy elucidated.

Epidemiology

COPD is often diagnosed in patients with chronic cigarette abuse. The disease can be defined by clinical symptoms such as exertional dyspnoea, chronic cough and sputum production, and by lung function tests with reduced Tiffeneau index. COPD can be diagnosed in about 50% of all smokers older than 60 years [188]. In the daytime-hypoxic blue-bloater type of COPD, we often observe SDB and oxygen desaturations. In 30% of patients with COPD and daytime PO_2 values between 60 and 70 mm Hg, oxygen desaturations during sleep are diagnosed [185]. OSA and COPD are independent diseases, both possibly leading to PH [187, 244] (see chapter on pulmonary hypertension in obstructive sleep apnoea syndrome).

Physiology/pathophysiology

Most patients with COPD present with moderate or severe daytime hypoxaemia. During sleep onset, alveolar ventilation decreases slightly due to changes of the set point for CO_2. Especially during REM sleep, there is a further fall in ventilation with marked O_2 desaturations [45]. The two major causes of hypoventilation are a reduction in ventilatory effort, caused by an altered central nervous stimulation and a relaxation of thoracic muscles during REM sleep, whereas diaphragmatic ventilatory drive is blunted for anatomical reasons in COPD, especially in emphysema. The reduced central drive can be documented by reduced swings in the oesophageal pressure being observed mainly during periods of REM sleep with frequent eye movements. This decrease in ventilation is not counterbalanced by a hypoxic and hypercapnic ventilatory response because REM sleep mechanisms are blunting chemosensitivity. There is also a mild increase of upper airway resistance caused by the relaxation of the oropharyngeal airway. As a consequence of these mechanisms, O_2 saturation decreases dependent on

the resulting ventilation–perfusion mismatch. The duration of these episodes last as long as 10–20 min and can easily be discriminated from the apnoea-induced short desaturations with durations of 10–100 s. As a consequence of the sleep-induced O_2 desaturations, pulmonary artery pressure increases during these periods. In one study, integrated pulmonary artery mean pressure increased from 29.6 +/– 10 mm Hg during wakefulness to 41.2 +/– 14.4 mm Hg [194]. Besides the alveolar vascular reflex, the increase of cardiac output plays a role in the increase of pulmonary artery pressure [61]. It is controversially discussed whether the extent of nocturnal hypoxaemia is an additional factor contributing to the degree of PH. In patients with isolated sleep-associated hypoxaemia without severe daytime hypoxaemia, it was shown that despite nocturnal oxygen therapy, no significant change in daytime PAP could be observed compared with a control group breathing room air [30]. If COPD is combined with repetitive upper airway obstructions, i.e. OSA, the resulting hypoxaemia and pulmonary hypertension are more severe and the patients are more likely to develop right heart failure [23, 92, 95, 187, 228, 244].

Impact on clinical practice

COPD promotes development of PH by different pathophysiological mechanisms. Concerning sleep, both COPD-associated nocturnal hypoxaemia and additional OSA lead to significant rises in PAP. The severity of COPD-associated hypoxaemia during sleep can be predicted with sufficient precision by blood gas measurements in the evening before sleep onset, because according to one study, PO_2 values higher than 55 mm Hg during daytime combined with O_2 desaturations during sleep do not contain clinically important prognostic information concerning the development of PH [32].

Therapeutic intervention

In most patients with COPD and nocturnal hypoxaemia, nocturnal O_2 saturation (SaO_2) is increased to values above 90% by insufflation of O_2 through a nasal cannula at a flow rate of 2 L/min. Thus, total sleep time can be prolonged and sleep quality improved. In COPD patients, mean PAP fell significantly from 29.5 +/– 12.7 to 24.9 +/– 9.7 mm Hg in the first night of O_2 therapy [194]. Long-term oxygen therapy applied during 15–18 h/day led to a significant reduction in pulmonary artery pressure from 28.0 +/– 7.4 to 23.9 +/– 6.6 mm Hg after 31 months of therapy [245]. In a more recent study, *Raeside* et al. diagnosed a mean nocturnal PAP comparable to their PAP at exercise in 10 patients with COPD. This elevated nocturnal PAP could be reversed with oxygen [182]. The decrease in PAP could mainly be attributed to a decrease in pulmonary vascular resistance. The expectation that patients with resting hypoxaemia and hypercapnia treated with supplemental oxygen might develop progressive nocturnal hypercapnia as a consequence of reduced ventilatory drive caused by hypoxaemia could not be confirmed. In patients with stable COPD without OSA, transcutaneously measured PCO_2 did not increase more than 6 mm Hg [69]. In COPD patients with predominantly ventilatory failure, i.e. elevated levels of $PaCO_2$, (non-) invasive ventilator therapy may be necessary [139]. For COPD patients with additional OSA, see the chapter on pulmonary hypertension in obstructive sleep apnoea syndrome.

Diagnostic recommendations

Measurements of oxygen saturation during the night do not yield any additional value in the decision-making of whether oxygen therapy is indicated or not. Daytime arterial blood gas measurements are of sufficient prognostic value. Additional polysomnographic measurements are usually not indicated in COPD. However, polysomnography should be performed in COPD patients with a suspicion of coexisting OSA or in patients with unclear symptoms and findings such as daytime sleepiness, polycytaemia, cor pulmonale, or morning headaches.

Therapeutic recommendations

Daytime hypoxaemia in COPD is nearly always associated with hypoventilation and ventilation–perfusion mismatching during sleep and should be treated by supplemental oxygen therapy during the night. It has been shown that 14–16 h per day of oxygen therapy is superior to only nightly treatment with oxygen. In COPD patients with predominantly ventilatory failure, i.e. elevated levels of $PaCO_2$, (non-) invasive ventilator therapy may be necessary [139]. In COPD patients with additional OSA, both diseases should be treated consequently because these patients are likely to develop PH.

Conclusions and future perspectives

There is only a moderate correlation between nocturnal O_2 desaturation, hypercapnia severity and PH. Additional factors responsible for the development of PH, such as OSA, should be identified [184]. O_2 therapy reduces right-heart strain and improves life expectancy, but the work of breathing is only slightly improved. With intermittent positive pressure ventilation, the diaphragm can be unloaded in patients with ventilatory failure. Thus physical performance during the day can be ameliorated. However, especially in patients with COPD, noninvasive ventilation is not well tolerated and compliance after 6 months is only about 50%. We therefore need more intelligent ventilator devices with servo-ventilation to avoid sleep disturbances induced by mask and machine. Pharmacological therapy of PH with nitric oxide donors or endothelin receptor antagonists have to be studied in COPD patients first, before they can be considered as a further treatment option in future [92, 94, 228, 236, 244].

Sleep-Disordered Breathing and Cerebrovascular Disease

P. Clarenbach, A. Nachtmann, T.E. Wessendorf

Introduction – epidemiology

The high prevalence of SDB among patients with stroke has been confirmed in numerous studies, although methodological differences regarding patient age, time after stroke, or diagnostic methods somehow reduce the power of a general conclusion [14, 50, 83, 144, 145, 168, 207, 240, 247]. Most of the authors come to the following conclusions:

1 The overall prevalence rate of SDB in acute stroke patients is in the range of 40–60%.
2 OSA is the leading type of SDB; true central sleep apnoea is comparatively rare.

3 There is no correlation between stroke location and the diagnosis of coexisting SDB apart from a tendency of Cheyne–Stokes respiration to be more common in infratentorial strokes.

The Sleep Heart Health Study, a large population-based epidemiological study, confirmed an increased prevalence of stroke in SDB; the odds ratio for the highest AHI quartile (AHI >11/h) was 1.6 times (confidence interval [CI] 1.02–2.46) higher than that of the lowest quartile (AHI <1.4/h) [217]. In earlier studies using subjective questionnaires for evaluating snoring history, an even stronger association between snoring and stroke had been found [169, 224]. The fact of a similar prevalence of SDB in patients with transient ischaemic attacks (TIA) [13] and of similar anthropometric data in SDB [240, 247] with stroke as in SDB without stroke suggests that OSA preceded the event in most cases. This is further underlined by the observation that obstructive events tend to persist after stroke, whereas central apnoeas improve [168]. It should be noted that the vast majority of epidemiological studies investigating the relationship between SDB and cerebrovascular disease have been performed in patients suffering from TIA/stroke. In contrast, there is a paucity of data concerning the prevalence of TIA/stroke in patients with OSA. So far, only one retrospective survey addressed this question and found a prevalence rate of 8% [212].

Physiology/pathophysiology

Hypertension is regarded as the most important risk factor for stroke: The link between SDB and hypertension is now accepted as independent of other confounding factors (see above). However, there is evidence of other possible links apart from hypertension: Cerebral blood flow is impaired by SDB: During obstructive, but not central, events there is a significant decline in cerebral blood flow followed by an increase of up to 216% [12, 163]. Flow reduction correlates with severity of oxygen desaturation, which would be of particular relevance during REM sleep when cerebral blood flow and oxygen demands are normally highest, but when apnoeas are accompanied by the greatest degrees of hypoxia [110]. In patients with OSA, cerebrovasodilator reserve seems to be diminished, which can be restored with CPAP [44]. Patients with lesions in the intra- and extracranial circulation could therefore be at higher risk of stroke during respiratory events [1].

The link between atherosclerosis and OSA has been discussed above. An increased intima-media thickness as well as a higher prevalence of stenosis of the extracranial arteries has been confirmed in stroke patients [154, 219, 255]. Patients with ischaemic stroke and coexisting OSA have increased fibrinogen plasma levels [248], and the level of fibrinogen correlates with the severity of SDB. The consequences for increased blood viscosity and coagulability may further add to the increased risk of thrombotic events *Chin* et al. observed a reduction in overnight fibrinogen levels in OSA patients [34]. An effective treatment of OSA, e.g. with CPAP, can in fact improve other vascular risk factors beyond blood pressure, so that the risk/benefit ratio calculated from blood pressure changes may underestimate the true benefit [175].

Impact on clinical practice

There are only few data about the course of SDB after stroke: However, in most patients, SDB tends to persist at least for a 3-month period [123, 168] but shows a tendency to improve during the first 6–9 weeks [83]. Using a screening device without discrimination between obstructive and central events, *Szucs* et al. found persistent events in ischaemic but not in hemorrhagic stroke after 3 months [231]. Using pulse oximetry, *Good* et al. showed a worse functional outcome after 3 and 12 months in patients with higher desaturation indices [70]. This could not be confirmed in recent studies [101, 123], although *Iranzo* et al. found early neurological worsening associated with OSA. It has been speculated that some of the neuropsychological sequelae observed after stroke – and regarded as a consequence of the event – could in fact be partially due to coexisting SDB. OSA in elderly stroke patients is associated with delirium, depressed mood, latency in reaction and in response to verbal stimuli, and impaired ADL (activities of daily living) ability [202]. The evidence of an effect of SDB on morbidity and mortality is weak in patients with stroke, as no study has addressed this point in particular, and the original strong association between a positive history of snoring and short-term survival in acute stroke reported by *Spriggs* et al. [224] has not been confirmed by others *Good* et al. reports a correlation between mortality and oxygen saturation [70] and *Dyken* et al. found a mortality of 21% within 4 years in their stroke patients with OSA, but 0% in patients without OSA [50]. Mortality data in patients with CAD and SDB indicate a higher risk of stroke within the following 5 years [147].

The importance of central sleep apnoea (CSA) in stroke patients is an open question: Whereas CSA in heart failure has been associated with increased mortality, its relevance in stroke patients is not known. As these patients often have cardiac disease, too, the question remains whether CSA is a sign of underlying cardiac dysfunction. No study has addressed this point so far.

Therapeutic intervention

The role of treatment of SDB in stroke has yet to be determined: In a consecutive series of patients, *Wessendorf* et al. showed that CPAP is an option with an acceptance rate of up to 66% but with the need for intensive coaching during rehabilitation after stroke. CPAP was effective without increasing concomitant central apnoeas, but aphasia and functional disability predicted negative compliance. In case of acceptance, better subjective fitness and improved blood pressure control were observed [249]. This primary compliance rate could not be achieved in every setting [82], but *Milanova* et al. reported an acceptance rate of 50% in acute stroke [140]. One could speculate that treatment may be particularly important in the acute phase, when the survival of the penumbra is critical.

Among elderly stroke patients, in whom CPAP could not be initiated, oxygen treatment (3 vs. 0.5 L/min) for 8 days improved some cognitive symptoms in up to 53% of patients [65]. In a randomized treatment study, *Sandberg* et al. investigated the effects of CPAP in stroke rehabilitation and found positive effects on depression but not on functional outcome after 4 weeks of treatment. Compliance was a particular problem in patients with delirium and cognitive impairment [201] *Hui* et al. reached primary CPAP acceptance in 16 of 34 stroke patients with OSA, but only four proceeded to home treatment, with an overall low compliance after 3 months [96].

Diagnostic/therapeutic recommendations – conclusions and future perspectives

The high prevalence of SDB observed after stroke justifies a screening for SDB in stroke patients [144]. As milder forms of SDB, e.g. the upper airway resistance syndrome, are not of concern in this clinical setting of mostly elderly patients, simple forms of screening with a portable device or simple pulse oximetry may be sufficient [246]. Polysomnography, however, is the only diagnostic method to safely diagnose SDB and initiate adequate therapy. But one may argue that as long as the clinical consequences of treatment are not clear, diagnosis is useless. Therefore, randomized studies are needed to answer the important question about treatment relevance. Such studies are currently under way.

Conclusion

There is rapidly accumulating evidence for OSA being an important cardio- and cerebrovascular risk factor independent of confounding factors such as diabetes mellitus, hyperlipidaemia, and smoking. In particular, OSA is associated with a dose-dependent increase in systemic arterial blood pressure. Effective treatment of OSA with CPAP therapy lowers blood pressure values not only while asleep but also during daytime. Although somewhat less clear, OSA probably enhances atherosclerosis and thereby contributes to the emergence of vaso-occlusive disease such as CAD and TIA/stroke. Pulmonary hypertension and nocturnal cardiac arrhythmias are further features of OSA-related cardiovascular morbidity; however, they are usually less clinically important. CSR is frequently observed in the setting of advanced CHF (in earlier series in up to 50% of patients with an LVEF below 40%). It mainly occurs in elderly males and constitutes an adverse prognostic sign. Treatment options for CSR include medical stabilization of CHF, administration of nasal oxygen, and various forms of noninvasive ventilatory support. Another possible consequence of SDB (especially OSA, pulmonary diseases, or both) is PH, which leads to dyspnoea and right-heart failure. Depending on the causal type of SDB, administration of nasal oxygen or various forms of ventilatory support is recommended in these patients.

Based on the complexity of the interaction of sleep, SDB, and CVD, an exact diagnosis is important in order to initiate adequate therapy. Concerning diagnosis of SDB, full polysomnography is the only tool, besides the medical history, with which to simultaneously detect and analyse sleep structure, nocturnal arousals, disordered breathing, ECG and oxygen saturation in patients with CVD. These are the relevant facts physicians need to precisely define the underlying type of SDB. Nonlaboratory monitoring systems (NLMS) may help in risk-stratifying patients with suspected SDB, but often cannot precisely analyse the underlying sleep disorder, especially in the complex setting of a patient with CVD. As detailed above, adequate therapy of SDB can improve the outcome of CVD and is thus of great medical and socioeconomic importance.

Based on this review paper, some proposals for future research on the relationship between SDB and CVD can be made. First, the pathophysiological basis for the emergence of CVD in OSA needs to be further clarified. Second, the role of OSA in the development of atherosclerotic disease has to be studied further. Third, the long-term effects of CPAP therapy on cardio- and cerebrovascular end points have to be investigated. Fourth, the prevalence of Cheyne–Stokes respiration in chronic heart failure has to be evaluated. Finally, significant work needs to be done to stratify the value of different treatment options available for CSR/CHF. In the near future, at least some of these questions will be addressed by the members of the working group 'Kreislauf und Schlaf' of the German Sleep Society.

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