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Lower Plasma Choline Levels are Associated with Sleepiness Symptoms

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Abstract

Sleepiness and cardiovascular disease share common molecular pathways; thus, metabolic risk factors for sleepiness may also predict cardiovascular disease risk. Daytime sleepiness predicts mortality and cardiovascular disease, although the mechanism is unidentified. This study explored the associations between subjective sleepiness and metabolite concentrations in human blood plasma within the oxidative and inflammatory pathways, in order to identify mechanisms that may contribute to sleepiness and cardiovascular disease risk.

METHODS—An exploratory case-control sample of 36 subjects, categorized based on the Epworth Sleepiness Scale (ESS) questionnaire as sleepy (ESS ≥ 10) or non-sleepy (ESS < 10), was recruited among subjects undergoing an overnight sleep study for suspected sleep apnea at the University of Pennsylvania Sleep Center. The average age was 42.4±10.5 years, the mean body mass index (BMI) was 40.0±9.36 kg/m², median Apnea Hypopnea Index (AHI) was 8.2 (IQR: 2.5–26.5), and 52% were male. Fasting morning blood plasma samples were collected after an overnight sleep study. Biomarkers were explored in subjects with sleepiness versus those without using the multiple linear regression adjusting for age, BMI, smoking, Apnea Hypopnea Index (sleep apnea severity), study cohort, and hypertension.

RESULTS—The level of choline is significantly lower ($P=0.003$) in sleepy subjects ($N=18$; mean plasma choline concentration of 8.19 ± 2.62 $\mu\text{mol/L}$) compared with non-sleepy subjects ($N=18$; mean plasma choline concentration of 9.14 ± 2.25 $\mu\text{mol/L}$). Other markers with suggestive differences ($P<0.1$) include Isovalerylcarnitine, Alpha-Amino adipic acid, Spingosine 1 Phosphate, Aspartic Acid, Propionylcarnitine, and Ceramides (fatty acids; C14-C16 and C-18).

CONCLUSION—This pilot study is the first to show that lower levels of plasma choline metabolites are associated with sleepiness. Further exploration of choline and other noted metabolites and their associations with sleepiness will guide targeted symptom management.

Keywords

sleepiness; metabolites; obstructive sleep apnea; humans

INTRODUCTION

Sleepiness and cardiovascular disease share common mechanistic pathways; thus, metabolic factors that place one at risk for sleepiness may also predict cardiovascular disease risk. Both daytime sleepiness and excessive daytime sleepiness symptoms are associated with a higher risk of adverse cardiac events, such as stroke and coronary heart disease (CHD), as well as total and cardiovascular-specific mortality (1–5). Excessive daytime sleepiness is also a frequently reported symptom in obstructive sleep apnea (OSA), a common sleep disorder with an increased risk of cardiovascular disease (6). This increased risk in OSA is likely due to increased oxidative stress and inflammation caused by frequent and cyclic reductions in oxygen and rapid reoxygenation during sleep (i.e., cyclical intermittent hypoxia) (7). No prior study has explored the differential metabolome of subjects with OSA who complain of sleepiness versus those without. We explored important metabolites within the inflammatory, oxidative stress, and neuronal pathways within key panels: Acylcarnitines, Ceramides (Cer), trimethylamine N oxide (TMAO), Neurotransmitters and Amino Acids (AA).

Panels explored

Acylcarnitines were chosen given their link to proinflammatory signaling pathways (8) and their potential role in cognition and memory (9). The Ceramides panel was explored given their roles in both inflammation (10), and oxidative stress (11), specifically, involving Sphingosine-1-phosphate (S1P) (12–15), C14-cer (16), and C16-cer (17) and C18:1 (10). We explored the panel trimethylamine N oxide (TMAO) as circulating TMAO has been found to be associated with atherosclerosis (18). TMAO is derived from dietary choline through the action of gut flora (18). Sleep deprivation has been found to be associated with diminished choline plasmalogen levels (19). Choline is a precursor to acetylcholine, and acetylcholine is known to play a complex and significant role in memory formation and coordination, and lowered activity within the cholinergic pathway has been associated with memory impairment, as in Alzheimer's disease (20, 21). As amino acids are known for their role in obesity-related diseases and have independent associations with cardiovascular disease (22–26), we also explored this panel in our study. In addition, aspartic acid intake has been positively correlated with subjective napping (through a daily sleep diary), which may be used as a proxy for subjective sleepiness (27). N-methyl-aspartic acid has been shown to cause a dose-dependent decrease in slow-wave sleep and paradoxical sleep, and an increase in wakefulness (28).

The objectives of this study are to gain an initial understanding of the biological basis for sleepiness symptoms. We focused on metabolites within inflammatory/oxidative stress, and

neuronal pathways and provide evidence that levels of choline are associated with sleepiness.

METHODS

Sample collection

Two different patient cohorts, the *Mechanisms of Sleepiness Symptoms Study* (MOSS; N=16) and *Biomarkers of Obstructive Sleep Apnea* study (BOSA2; N=20) were included to evaluate biomarker differences in subjects with suspected sleep apnea. All subjects underwent an overnight sleep study for suspected sleep apnea and were recruited from the University of Pennsylvania Sleep Center (Figure 1). Both studies were approved by the University of Pennsylvania Institutional Review Board.

Fasting morning blood plasma samples were collected after an overnight sleep study. A schematic illustration of the panels analyzed and metabolites suggestively linked to sleepiness ($P<0.10$) are shown in Figure 2.

Overnight polysomnography

All study participants had in-laboratory sleep recordings, which included electroencephalogram, electrooculogram, electrocardiogram, chin and limb electromyogram, chest and abdominal piezo belts, finger oximeter and oral and nasal thermistors. The American Academy of Sleep Medicine alternative scoring method was used to score the studies (29). Sleep technicians scored polysomnograms and computed AHI as the number of apneas plus hypopneas divided by hours of sleep time. An apnea was 10 sec or more of airflow cessation, and a hypopnea was associated with a 3 % fall in oxyhemoglobin saturation or an arousal.

Sleepiness measurement

The Epworth Sleepiness Scale (ESS), a widely used standardized self-report instrument that assesses tendency to doze (30), was used to measure sleepiness. This questionnaire asks subjects to rate their chance of dozing during 8 common situations. The responses are based on a Likert-type scale ranging from 0 to 3, with 0 indicating no chance of dozing and 3 indicating a high chance of dozing. The sum of these responses determines the total ESS score, with higher scores indicating greater sleepiness (30). Subjects were categorized as having subjective daytime sleepiness if they had an ESS score ≥ 10 , based on prior studies (31, 32).

Panel of metabolites explored and mechanistic pathways

We used a targeted metabolomics strategy to profile subjects with suspected sleep apnea in a case-control study of subjects categorized by level of sleepiness. See Supplemental Table 1 for significant metabolites and their mechanistic pathways.

Acyl Carnitines UPLC-MS—Acyl carnitines (specifically C0-C18:1) were measured by Liquid chromatography-mass spectrometry (LC-MS). Briefly, 25uL of plasma was spiked with a purchased internal standard consisting of isotopically labeled acyl carnitines. The

samples were then extracted with cold MeOH:DCM (1:1) followed by centrifugation at 12,000 g for 10 minutes. The supernatant was transferred to another vial, dried down and reconstituted in running buffer. A calibration curve was made from a purchased acyl carnitine mix aliquoted at various concentrations and spiked with the same internal standard as the samples. The samples and calibration standards were analyzed on Thermo TSQ Quantiva mass spectrometer (West Palm Beach, FL) coupled with a Waters Acquity UPLC system (Milford, MA). Data acquisition was done using selective ion monitoring (SRM). Concentrations of each unknown were calculated against their respective standard curves (33).

Ceramides—Plasma ceramides, sphinganine, sphingosine, sphingosine-1-phosphate (S1P), were measured by previously described technique (34). Briefly a 25 μ l aliquot of plasma was spiked with an internal standards mixture prior to undergoing extraction. Data acquisition was done using a select ion monitor (SRM) after chromatographic separation and electron ionization on the Thermo TSQ Quantum Ultra mass spectrometer (West Palm Beach, FL), coupled with a Waters Acquity UPLC system (Milford, MA). Concentrations of each analyte were calculated against each respective calibration curve. Coefficient of variation of plasma analyzed with each batch of 40 samples over a one month period are 6.3%, 6.2%, 3.1%, 5.0%, 5.7%, 3.2%, 4.9% and 3.3% for sphingosine, sphingosine-1-phosphate, C16:0-ceramides, C18:0-ceramides, C20:0-ceramide, C22:0-ceramide, C24:1-ceramide and C24:0-ceramide respectively (34).

Trimethylamine N-oxide (TMAO)—Plasma betaine, choline, carnitine, TMA and TMAO concentrations were determined by LC-MS as described in Koeth et al. (35) and Kirsch et al with a few modifications (36). Briefly, a solution of D9-isotopes was spiked in plasma samples as internal standard. The mixture was deproteinized with cold methanol. Supernatant was dried down and resuspend in running buffer prior to injecting on a Sciex 6500 triple quadrupole mass spectrometer (Framingham, MA) coupled with a Cohesive TX2 LC system (Franklin, MA). Analytes were separated on a Grace Altima HP HILIC 150mm $\times$ 2.1mm, 5 μ m column prior to analyzing on the mass spectrometer via electrospray ionization mode. Data acquisition was done using selective ion monitoring (SRM). Concentration of each analyte was calculated against an 8-point standard curve for each respective analyte (35, 36).

Neurotransmitters and Amino Acids—Neurotransmitters and some amino acids such as Tryptophan and Taurine were measured by LCMS as previously described (37). Briefly, plasma samples were spiked with an internal standard mix consisting of isotopically labeled amino acids. They were then deproteinized with cold methanol followed by centrifugation at 12,000 g for 10 minutes. The supernatant was transferred to a different vial, dried down and derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate according to Waters' MassTrak kit. A 11-point calibration standard curve was spiked with the same internal standard mix and underwent the same derivatization procedure. Both derivatized standards and samples were analyzed on a Thermo TSQ Quantum Ultra mass spectrometer (West Palm Beach, FL) coupled with a Waters Acquity UPLC system (Milford, MA). Data

acquisition was done using selective ion monitor (SRM). Concentrations of the analytes of each unknown were calculated against their respective calibration curves (37).

Statistical analysis

Patient demographics, clinical characteristics, and biomarker values were summarized using mean (SD) or median (interquartile range) for continuous variables, and n (%) for categorical variables. To help visualize the relative magnitude of biomarker values, we generated a heatmap of normalized (set to same scale) biomarker values by sleepiness status using the heatmap function in R statistical software (www.r-project.org; R Core Team 2013).

Univariate analyses were first performed to compare differences between patients with and without sleepiness, with a two-sample t-test or Wilcoxon rank sum test used for continuous variables, and a Chi-square or Fisher's exact test used for categorical variables. The normality of biomarker values was checked using the Shapiro-Wilk test, and biomarkers whose values were not normally distributed were transformed using the Box-Cox transformation method. To examine the association between biomarkers and sleepiness (ESS ≥ 10 vs. ESS < 10), we applied multiple linear regression models in which each dependent variable (i.e., each biomarker separately as the outcome) was regressed against the binary sleepiness factor, adjusting for the following covariates: study cohort, age, BMI, smoking, Apnea Hypopnea Index (sleep apnea severity), and presence or absence of hypertension. All the statistical analyses were performed using the SAS version 9.4 (SAS Institute, Cary, NC). Given the exploratory nature of this study a two-sided p-value of less than 0.05 was considered to be statistically significant and a p-value < 0.10 suggestive of an association with sleepiness.

RESULTS

Summary statistics of patient demographics are presented in Table 1. The mean (SD) age was 43.3 (10.2) years in 18 sleepiness subjects and 41.4 (11.1) in 18 non-sleepiness subjects. No statistically significant difference was found between two groups ($p = 0.60$). As expected, the average ESS score in the non-sleepiness group was much lower than that in the sleepiness group [5.1 (2.2) vs. 14.1 (3.5); $p < 0.001$]. There were equal proportions of male and female in each group and in the overall sample. The hypertension rate in the sleepiness group was statistically higher than that in the non-sleepiness group (55.6% vs. 12.5%, $p = 0.009$). Other demographics including BMI, AHI, smoking, presence of cardiovascular disease and/or stroke, and use of exercise were comparable between the two groups (see Table 1).

Supplemental Figure 1 illustrates a heat map of normalized and color-coded expression values (Z scores) of all metabolites (X-axis) in 36 subjects (Y-axis), in which the green values indicate higher values with red color representing lower values. Clustering was done at the biomarker level (Y-axis). No significant patterns were spotted in the Figure.

Results for biomarkers showing suggestive differences ($p < 0.10$) between sleepy and non-sleepy individuals are shown in Table 2, after adjusting for relevant covariates. We also ran models with ESS treated continuously and the effect is reduced (data not shown), thus the

data are not linear and categorical ESS is a better predictor. Association results of sleepiness status with the complete list of biomarkers are presented in Supplemental Table 2. Choline showed the most significant association with sleepiness status; levels were found to be significantly lower in sleepy subjects compared to non-sleepy subjects [adjusted mean difference (SE) = -2.674 (0.804) μ M; $p=0.003$; Table 2, Figure 3]. A similar direction of effect (e.g., lower values in sleepy patients) was observed for all significant or suggestive biomarkers presented in Table 2, including the following which showed significant differences ($p<0.05$): Alpha-Aminoadipic-Acid ($p=0.022$), Sphingosine-1-Phosphate (S1P; $p=0.026$), Isovalercylcartine ($p=0.035$), Aspartic Acid ($p=0.039$), and Ceramides C14 ($p=0.040$), C16 ($p=0.040$) and C18 ($p=0.046$). In addition to these biomarkers, Sphinganine ($p=0.068$) and Propionylcarnitine ($p=0.074$) showed suggestive relationships with sleepiness.

DISCUSSION

The associations between choline and sleepiness may reflect previously established associations, as sleep restriction has been found to impact lipid concentrations in plasma (i.e. fatty acids) (38, 39), and a decrease in choline plasmalogen levels during sleep deprivation is consistent with prior work demonstrating lipids are susceptible to degradation by oxidative stress (19). This exploratory study is the first to show that lower levels of plasma choline metabolites are linked with sleepiness.

Choline is an important nutrient and precursor for the neurotransmitter acetylcholine and for phosphatidylcholine, a structural component of VLDL (40), and a key mechanism to export triacylglycerol from the liver. Choline may be obtained via the diet (41) and from endogenous biosynthesis predominantly in the liver through the action of phosphatidylethanolamine N-methyltransferase (PEMT) (42). Choline is present in the human diet as lecithin, which is a common name for phosphatidylcholine, with the main food sources as eggs, liver, soybeans, and pork (41, 43, 44).

Acetylcholine plays a significant role in memory formation and coordination, and lowered activity within the cholinergic pathway has been associated with memory impairment such as in Alzheimer's disease (21, 45, 46). In humans, a randomized, double blind, placebo-controlled study demonstrated that verbal memory (as measured by a logical memory passage from the Logical Memory subtest of the Wechsler Memory Scale-Revised) in older adults improves with 1000 mg/d dietary citicoline (CDP choline) supplementation (47). In another double-blind placebo controlled trial, patients with AD (average duration of illness being 4 years) given phosphatidylcholine (20–25 g/day of purified soya lecithin containing 90% phosphatidyl plus lysophosphatidyl choline) for six months demonstrated moderate improvements on multiple memory tests (48). It will be important to explore whether dietary supplementation will similarly improve sleepiness.

Our overall population had mild sleep apnea, which is important to recognize, as a prior study that exposed rats to severe intermittent hypoxia (IH) during sleep demonstrated reduced choline acetyltransferase (ChAT) immunoreactivity (49). IH treated animals demonstrated impaired working memory and significant reductions in CHAT-stained

neurons after 14 days of IH exposure (49). In a mouse model of Alzheimer's Disease (AD), Tg2576 genetically engineered AD mice with age-dependent β deposition in their brains displayed sleep abnormalities compared to control mice, and the authors of this study hypothesized that this may be due to cholinergic deficiencies in AD mice (50). Interestingly, when timed-pregnant Sprague-Dawley rats (Charles River Breeding, Raleigh, NC) were choline deficient, this significantly decreased the rate of mitosis in the neuroepithelium adjacent to the hippocampus in the fetal brain (51). Dietary choline availability alters the timing of migration, commitment to differentiation of progenitor neuronal-type cells in fetal brain hippocampal regions known to be associated with learning and memory (51). Choline appears to play a critical role in attentional processes (and by extension, learning processes), as well as memory (52). The exploration of choline levels and ChAT/AChE activity in humans with and without sleep apnea will be important to explore in order to clarify the link to sleepiness.

Overall, our findings are consistent with previous reports of choline playing a significant role in cognitive processes. The high-affinity choline uptake transporter (CHT) brings in choline from the extracellular space to presynaptic terminals, thereby enabling normal acetylcholine synthesis and cholinergic transmission (52). Thus, abnormalities in CHT capacity have been associated with decreased ability to perform tasks that require attentional processes (52), which suggest regulating CHT capacity may be a target for new pharmacological treatments for cognitive disorders (52). Cortical ACh release was higher in rats performing sustained attention tasks compared to rats conducting control tasks, highlighting the important role of choline in attentional processing (53). In another experiment, infusions of the cholinotoxin 192 IgG-saporin induced loss of cortical cholinergic inputs, which resulted in rats' impaired performance in a sustained attention test, while performance on non-attention related control activities remained unaffected (54).

Other metabolites that were related to sleepiness ($P < 0.1$) in this study were Isovalercylcartine, Alpha-Amino adipic acid, Spingosine 1 Phosphate, Aspartic Acid, Propionylcartinine and Ceramides and warrant further study. Of the acylcartinines explored, isovalercylcartinine and propionylcartinine were found to be related to sleepiness. Although isovalercylcartinine has not been studied in relation to sleepiness previously, isovaleric academia is a genetic condition that causes elevated levels of isovaleryl carnitine in plasma (55). One of the symptoms of this condition is lethargy, thus if isovaleryl carnitine levels in plasma are elevated, sleepiness may be increased (55), although this requires further exploration. Propionylcartinine plasma levels have also been found to be increased significantly in a small sleep restriction study of twelve healthy young male subjects in controlled laboratory conditions during sleep deprivation (56) which may explain the link in our study to sleepiness. Alpha-amino adipic acid was also found to be associated with sleepiness in our study, and it has been found to be linked to oxidative stress (57). Aspartic acid was also linked with sleepiness and has previously been found to be positively correlated with subjective napping (through a daily sleep diary), used as a proxy for subjective sleepiness, in a study of 459 post-menopausal women (27). Ceramides were also associated with sleepiness in this study, and as ceramides are linked to oxidative stress (17); it will be important to clarify this association further.

The strengths of this study are that this is the first study exploring the metabolic profiles of subjects among subjects with and without sleepiness. We were able to obtain robust clinical information such as apnea hypopnea index. One of the limitations of this study is that the sample size was small, thus the statistical power to detect small to moderate effects was limited and findings should be interpreted with caution. We did not adjust for multiple comparisons when interpreting the p-values, as this is a hypothesis generating study. As the measurement of sleepiness phenotype was subjective, the sleepiness scores are also subject to misclassification bias. Further, the study was cross-sectional in nature and had a targeted population of subjects with suspected sleep apnea, thus preventing broad generalization of results.

The mechanism through which inadequate sleep and sleep apnea may impair the cholinergic pathway and influence sleepiness warrants further study. Future studies should also consider the collective impact of dietary choline, genetics (i.e. SNPs in genes involved in choline metabolism), inflammation, and oxidative stress on attentional processes, sleepiness, and cardiovascular disease risk. The continued exploration of choline and other noted metabolites and their associations with sleepiness would guide targeted symptom management, which may include dietary/supplement recommendations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- As sleepiness and cardiovascular disease share common molecular pathways, metabolic risk factors for sleepiness may predict cardiovascular disease risk.
- This is the first study to explore the association of metabolites and sleepiness in subjects with suspected sleep apnea.
- Lower plasma choline is present in subjects with sleepiness (Epworth Sleepiness Scale score ≥ 10) with suspected sleep apnea, which highlights a potential target for treatment options.

Figure 1:
Study cohorts included from the University of Pennsylvania

Figure 2: Panels analyzed and metabolites linked to sleepiness ($P < 0.10$ in adjusted models)

Figure 3. Differences in plasma choline concentration between subjects with sleepiness versus those without sleepiness.

Table 1.

Summary statistics of patient demographics and clinical characteristics by Sleepiness status

	Sleepiness			
	No (N = 18)	Yes (N = 18)	Total (N = 36)	P-Value
Age	41.44 (11.05)	43.33 (10.15)	42.39 (10.50)	0.60
Epworth Sleepiness Scale (ESS)	5.08 (2.20)	14.10 (3.53)	9.81 (5.43)	<0.001
Apnea Hypopnea Index, events/hr	8.2 (3–28.10)	10.05 (1–23.80)	8.2 (2.5–26.5)	0.64
BMI, kg/m ²	36.29 (9.88)	35.63 (9.09)	35.96 (9.36)	0.84
Total minutes sleep time	356.0 (300.0 – 422.2)	384.5 (357.0 – 434.1)	358.8 (325.0 – 428.2)	0.36
Gender				
Female	9 (50.00%)	9 (50.00%)	18 (50.00%)	0.99
Male	9 (50.00%)	9 (50.00%)	18 (50.00%)	
Smoking				
No	14 (87.50%)	11 (61.11%)	25 (73.53%)	0.13
Yes	2 (12.50%)	7 (38.89%)	9 (26.47%)	
Hypertension				
No	14 (87.50%)	8 (44.44%)	22 (64.71%)	0.009
Yes	2 (12.50%)	10 (55.56%)	12 (35.29%)	
Cardiovascular disease				
No	16 (94.12%)	18 (100.00%)	34 (97.14%)	0.49
Yes	1 (5.88%)	0 (0.00%)	1 (2.86%)	
Stroke				
No	16 (94.12%)	18 (100.00%)	34 (97.14%)	0.49
Yes	1 (5.88%)	0 (0.00%)	1 (2.86%)	
Heart Failure				
No	16 (94.12%)	18 (100.00%)	34 (97.14%)	0.49
Yes	1 (5.88%)	0 (0.00%)	1 (2.86%)	
Exercise				
No	5 (55.56%)	1 (16.67%)	6 (40.00%)	0.29
Yes	4 (44.44%)	5 (83.33%)	9 (60.00%)	

Data are presented as mean (SD) or median (IQR: 25th - 75th percentile) or N (%). Significant values are in bold.

Table 2.

Results of the multiple regression analyses of biomarkers as a function of Sleepiness (Yes vs. No) and adjusted covariates.

Dependent Variables	Estimate	SE	P-value
choline (uM)	-2.674	0.804	0.003
alpha-Aminoadipic-acid [*] (uM) [*]	-0.234	0.097	0.022
Sphingosine 1 Phosphate [*] (uM) [*]	-0.235	0.100	0.026
Isovalerylcarnitine (uM) [*]	-0.151	0.067	0.035
Aspartic Acid(uM) [*]	-0.799	0.368	0.039
C16- Ceramides (uM) [*]	-0.008	0.004	0.040
C14-Ceramides (uM) [*]	-0.030	0.017	0.040
C18:1- Ceramides (uM) [*]	-0.008	0.004	0.046
Sphinganine [*] (uM) [*]	-0.060	0.032	0.068
Propionylcarnitine [*] (uM) [*]	-0.031	0.017	0.074

^{*}. Biomarker values are normalized before regression analysis.

Note: adjusted covariates include study center, age, BMI, smoking, Apnea Hypopnea Index (sleep apnea severity), and hypertension.