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Original Article

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Sleep duration, plasma metabolites, and obesity and diabetes: a metabolome-wide association study in US women

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Abstract

Short and long sleep duration are associated with adverse metabolic outcomes, such as obesity and diabetes. We evaluated cross-sectional differences in metabolite levels between women with self-reported habitual short (<7 h), medium (7–8 h), and long (≥9 h) sleep duration to delineate potential underlying biological mechanisms. In total, 210 metabolites were measured via liquid chromatography-mass spectrometry in 9207 women from the Nurses' Health Study (NHS; $N = 5027$), the NHSII ($N = 2368$), and the Women's Health Initiative (WHI; $N = 2287$). Twenty metabolites were consistently (i.e. $p_{raw} < .05$ in ≥2 cohorts) and/or strongly ($p_{FDR} < .05$ in at least one cohort) associated with short sleep duration after multi-variable adjustment. Specifically, levels of two lysophosphatidylethanolamines, four lysophosphatidylcholines, hydroxyproline and phenylacetylglutamine were higher compared to medium sleep duration, while levels of one diacylglycerol and eleven triacylglycerols (TAGs; all with ≥3 double bonds) were lower. Moreover, enrichment analysis assessing associations of metabolites with short sleep based on biological categories demonstrated significantly increased acylcarnitine levels for short sleep. A metabolite score for short sleep duration based on 12 LASSO-regression selected metabolites was not significantly associated with prevalent and incident obesity and diabetes. Associations of single metabolites with long sleep duration were less robust. However, enrichment analysis demonstrated significant enrichment scores for four lipid classes, all of which (most markedly TAGs) were of opposite sign than the scores for short sleep. Habitual short sleep exhibits a signature on the human plasma metabolome which is different from medium and long sleep. However, we could not detect a direct link of this signature with obesity and diabetes risk.

Key words: habitual sleep duration; short sleep; long sleep; metabolomics; metabolites; obesity; diabetes; women; cohort studies; epidemiology

Statement of Significance

Little is known about the biological pathways linking sleep duration with chronic disease risk, such as obesity and diabetes. Metabolomics analysis is a promising tool to trace potential metabolic pathways of this association. Using LC–MS metabolomics profiling, we identified 20 metabolites which were clearly associated with habitual short sleep duration in more than 9000 US women, and which represent a signature distinctly different from patterns observed in long sleepers. Our results corroborate prior

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laboratory findings of short sleep-associated lipids and further the understanding of metabolic processes associated with short sleep. Independent replication of findings and further research, including longitudinal studies, on biological processes that mediate the association of short and long sleep with chronic health conditions is warranted.

Introduction

Sleep duration is associated with multiple health outcomes, with both short and long habitual sleep duration predicting higher risk of obesity, diabetes, cardiovascular disease, and mental and neurodegenerative disorders [1–6]. The public health consequences of these sleep disturbances are potentially substantial, because about 30% of US adults do not get the 7–9 h of daily sleep recommended by National Sleep Foundation [7], and numbers of adults reporting short sleep duration are rising [8–10]. However, there are knowledge gaps regarding our understanding of the extent to which short and long habitual sleep durations are causally related to adverse health outcomes, as well as a need to understand the mechanisms underlying potentially causal associations.

Short sleep duration is hypothesized to contribute to weight gain, obesity, and diabetes by altering timing and amount of food intake, disrupting energy balance, increasing inflammation, and impairing glucose tolerance, and insulin sensitivity [11]. The underlying mechanisms are less clear regarding long sleep duration, although poor sleep quality, sedentary lifestyle, unhealthy dietary choices, and misalignment between circadian and behavioral cycles might play a role [12]. On the other hand, there are plausible bidirectional relationships between habitual long sleep duration and obesity, diabetes, and depression [12]. Patel el al. [13] argue that depression and low socioeconomic status are potentially strong confounders or causal intermediates between long sleep and chronic health outcomes. Therefore, although both short and long sleep duration are associated with long-term health consequences, the underlying mechanisms appear to be different. A better understanding of the biochemical signatures associated with both short and long sleep duration, and their potentially mediating roles regarding obesity and diabetes would help identify prevention strategies, including therapeutic targets and/or the possibility of identifying individuals who might benefit the most from prevention efforts targeting sleep health. This need was recognized in the executive summary of a 2016 workshop of the National Heart, Lung, and Blood Institute calling for use of omics approaches to identify markers of long-term sleep behaviors [14].

So far, omics studies addressing consequences of sleep behaviors have been mostly of small size, were conducted in highly-controlled settings and have predominantly focused on acute sleep deprivation and short sleep duration. The current state of research and a comprehensive listing of relevant studies is summarized in two recent reviews [15, 16]. In brief, in several laboratory-controlled studies investigating the consequences of acute sleep restriction in young, healthy individuals, alterations of various metabolites in the plasma, such as tryptophan, serotonin, plasma amino acids, oxalic acid [17–19], and in particular lipid metabolites [17, 19–22] were observed. Of note, in a recent study of 16 normal-weight participants a set of 65 compounds were identified discriminating insufficient versus adequate sleep with 74% overall accuracy [23]. We are aware of only three studies in free-living individuals investigating the relationship between sleep duration and metabolomics [24–26]. Most likely due to small sample size, findings from two of these studies failed to demonstrate associations between metabolites and sleep duration, while in 205 overweight/obese participants of the "Satiety Innovation" (SATIN) study, 12 metabolites discriminating long sleep from short sleep duration were detected.

It remains unclear whether the findings can be translated to the effects of habitual sleep duration on free-living middle-aged to elderly women. In the present study, we examined associations of self-reported habitual sleep duration with plasma metabolites among 9207 mainly post-menopausal women from the Nurse's Health Studies I and II, and the Women's Health Initiative (WHI), aiming to identify markers of self-reported habitual sleep duration and thus delineating potential underlying biological mechanisms. We hypothesized that the metabolomic signature associated with short sleep duration is different from the signature associated with long sleep duration. Furthermore, we investigated whether and to what extent the metabolomic signatures can explain the increased risk of obesity and diabetes associated with short and long sleep duration (as compared to a sleep duration of 7–8 h).

Methods

Only a brief overview of the methods is given here. Full details are given in Supplementary material.

Study population

From the WHI, we used data of the WHI metabolomics sub-study, a nested case-control design on women without cardiovascular disease at baseline, in which 1153 participants who developed coronary heart disease after the baseline examination were selected as cases and were matched with another 1153 controls. In the NHS and NHSII, metabolomics profiling was part of various nested endpoint studies, with a total of 7449 women in NHS, and 3771 women in NHSII with metabolomics data available. The NHS, NHSII, and WHI studies were approved by the respective institutional review boards and conducted according to the Declaration of Helsinki.

Metabolomics profiling

For all three cohorts (NHS, NHSII, and WHI), metabolomic profiling was conducted at the Broad Institute of the Massachusetts Institute of Technology and Harvard University (Cambridge, MA, USA), using liquid chromatography–tandem mass spectrometry (LC–MS) methods. In our analysis, we included named metabolites which were assayed in at least 50% of samples, were above the limit of detection (LOD) in at least 75% of the samples assayed, had coefficients of variation (CVs) ≤25% (as measured in blinded quality control samples), and demonstrated stability with delayed processing ≤24 h after blood draw (defined as an intra-class correlation ≥0.67 comparing samples processed immediately with those processed 24 h later), for each of the three cohorts (NHS, NHSII, and WHI). Thus, 210 known metabolites were included in the current analysis, of which 157 were classified as lipids or lipid intermediates (Supplementary Table S1), and are identified via their Human Metabolome Database (HMDB) ID and HMDB common name throughout this manuscript [27].

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Exclusions

After excluding participants who had not reported on their habitual sleep duration and who had fasted less than 8 h before blood collection, 5027 females in the NHS, 2368 in the NHSII, and 2287 in the WHI remained for our final analysis population. More details are displayed in the flowcharts in Supplementary Figures S1–3.

Sleep duration assessment

Information on habitual sleep duration was captured via questionnaire at the baseline examination visit in WHI, the 1986 questionnaire in NHS and the 2001 questionnaire in NHSII, where respondents could choose from categories ≤5, 6, 7, 8, 9, or ≥10 h.

Statistical analysis

To reduce right skewness, all metabolite values were log-transformed. Within each cohort and nested endpoint study, the log-transformed metabolite values were converted to z-scores with a mean of 0 and a *SD* of 1. Missing values below the limit of detection were assigned half the lowest observed value.

The associations of short (<7 h) and long (≥9 h) sleep duration with the reference category 7–8 h were calculated for each metabolite individually in multi-variable-adjusted linear regression models with the log- and z-transformed metabolite values as the outcome, both in the NHS, NHSII, and WHI cohorts separately, and pooled. Variables adjusted for included age at blood draw, ethnicity, body mass index, habitual snoring, physical activity, diet quality, alcohol consumption, smoking status, menopausal status, lipid-lowering treatment intake, hormone therapy, presence of diabetes, history of depressive symptoms, case–control status, and arm/endpoint within study. *p*-Values were false-discovery rate-adjusted using the approach as recommended by Storey [28].

We applied LASSO (least absolute shrinkage and selection operator) regression [29, 30] on the pooled NHS, NHSII, and WHI data, and calculated a metabolite score for the propensity of short sleep duration for each participant using $\text{metabolite score} = \sum_{k=1}^{n} \beta_k \times \text{metabolite}_k$, where β_k is the corresponding regression coefficient from the LASSO regression model for metabolite k, metabolite_k is the z-score for metabolite k and n is the total number of LASSO-selected metabolites.

Furthermore, we used metabolite set enrichment analysis (MESA) [31] to identify groups of molecularly or biologically similar metabolites that were enriched among the metabolites associated with short and/or long sleep duration, based on the regression coefficients from the analyses described above.

Finally, we examined the associations of single metabolites as well as the metabolite score with prevalent (at the time of blood draw) and incident (after the time of blood draw) obesity and diabetes, using logistic regression and Cox proportional hazards models.

All statistical tests were two-sided at a significance level of 0.05, if not stated otherwise. The MESA analysis and generation of graphs was done in R (R Foundation for Statistical Computing, Vienna, Austria). The rest of the analyses was conducted using SAS, version 9.4 (SAS Institute, Inc., Cary, NC, USA).

Results

Study population

The flow charts defining the study's analytic samples are shown in Supplementary Figures S1–3 (for NHS, NHSII, and WHI respectively). After exclusions, the NHS population included 5027 participants, the NHSII 2368, and the WHI 2287 participants. There were marked differences in cohort profiles, most notably regarding the timing of sleep duration assessments and blood draws [NHS: sleep duration queried in 1986, blood drawn on average 3.5 years (*SD*: 0.4) later; NHSII: sleep duration queried in 2001, blood drawn on average 3.4 years (*SD*: 0.8) prior; WHI: both sleep duration assessment and blood draw occurred concurrently between 1994 and 1998]; the age distribution [mean (*SD*) age at blood draw 57.1 (6.9) in NHS, 44.9 (4.5) in NHSII, and 67.0 (6.9) in WHI]; ethnicity (proportion of whites, 93.2% in NHS, 96.3% in NHSII, and 79.6% in WHI); menopausal status (proportion of post-menopausal women, 72.1% in NHS, 15.3% in NHSII, and 100% in WHI); and prevalence of concomitant diseases, such as diabetes (NHS: 3.5%, NHSII: 1.7%, and WHI: 13.2%) and hypertension (NHS: 35.8%, NHSII: 16.9%, and WHI: 44.9%). Prevalence of ever night shift work was high in NHS (58.4%) and NHSII (69.6%).

The prevalence of self-reported habitual long sleep (≥9 h) was low and similar across cohorts (NHS: 4.5%, NHSII: 5.7%, WHI: 4.6%). Habitual short sleep (<7 h) was observed frequently, especially in WHI (NHS: 27.7%, NHSII: 25.5%, WHI: 40.6%). Further characteristics of study participants are shown in Table 1 and Supplementary Table S2 (stratified by sleep duration). While cohort characteristic heterogeneity may contribute to differences across cohorts in findings, the ability to see robust findings across these large and relatively diverse samples would enhance evidence for generalizability.

Associations of metabolites with short sleep duration

Sixty-four of the 210 metabolites were significantly associated with short sleep duration in at least one of the three cohorts on a nominal significance level of 0.05 in multi-variable, fully adjusted models, in which women with long sleep duration were excluded. We focus our discussion on metabolites which were either statistically significantly (nominal $p < .05$) associated with short sleep duration in at least two of the three cohort and the directionality of association was the same ("consistent" association), or for which the association was statistically significant after false-discovery rate adjustment in at least one cohort ("strong" association), as such findings are more likely to represent clinically relevant and generalizable associations.

In total, 20 metabolites (18 lipids) fulfilled any of these criteria (three both consistent and strong, 16 consistent, one strong). Eleven of the 18 lipids were triacylglycerols (TAGs; C50:5, C52:4, C52:6, C52:7, C54:3, C54:7, C54:8, C56:5, C56:7, C56:8, C56:9), one was a diacylglycerol (DAG; C34:3), two were lysophosphatidylethanolamines (LPEs; C18:1, C18:2), and four were lysophosphatidylcholines (LPCs; C18:1, C18:2, C20:4, C20:5). Of note, identified TAGs presented with higher double-bond content; out of the 24 analyzed TAGs with a lower (≤3) number of double bonds only one was identified to be associated with short sleep duration, while for TAGs with ≥4 double bonds 10 out of 21 were identified. All TAGs and the DAG were negatively associated with short sleep duration, meaning that levels were lower in women with short sleep duration (<7 h) compared to women who reported 7–8 h of sleep. Conversely, the LPEs and LPCs were positively associated with short sleep duration. Furthermore, two amino acids—hydroxyproline and phenylacetylglutamine—were identified, the first showing a positive association, and the latter a negative association with short sleep duration (Figure 1A; numbers and *p*-values in Supplementary Table S3). The three metabolites with both a consistent and strong association were LPE C18:1, LPC C18:2,

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Table 1. Study participants' characteristics of the Nurses' Health Study (NHS; 1989–1990), the Nurses' Health Study II (NHSII; 1996–1999), and the Women's Health Initiative (WHI; 1994–1998)

	NHS (*N* = 5027)	NHSII (*N* = 2368)	WHI (*N* = 2287)
Sleep duration, % (*N*)			
≤5 h	3.5 (178)	4.3 (101)	10.0 (228)
6 h	24.1 (1212)	21.3 (504)	30.7 (701)
7 h	43.0 (2160)	43.2 (1024)	34.6 (791)
8 h	24.8 (1249)	25.5 (603)	20.2 (461)
9 h	3.9 (194)	5.1 (120)	4.3 (98)
≥10 h	0.7 (34)	0.7 (16)	0.3 (8)
Age at blood draw [years]	57.1 (6.9)	44.9 (4.5)	67.0 (6.9)
Body mass index [kg/m^2]	25.8 (4.8)	26.0 (6.0)	28.7 (6.1)
White, % (*N*)	93.2 (4687)	96.3 (2280)	79.6 (1821)
Smoking status, % (*N*)			
Never smoker	46.9 (2358)	68.5 (1621)	47.5 (1086)
Past smoker	39.9 (2004)	23.9 (567)	39.8 (911)
Current smoker	12.9 (649)	7.5 (178)	11.5 (262)
Missing	0.3 (16)	0.1 (2)	1.2 (28)
Physical activity [MET-h/wk]	9.9 (3.5, 21.1)	11.9 (4.9, 24.0)	7.0 (1.5, 16.0)
Diet quality[a]	46.8 (40.0, 54.3)	44.8 (38.1, 52.1)	68.3 (59.4, 75.9)
Alcohol intake [g/day]	1.8 (0.0, 7.6)	0.9 (0.0, 4.0)	0.2 (0.0, 3.2)
Caffeine intake [mg/day]	206 (86, 386)	161 (45, 366)	177 (76, 185)
Menopausal status, % (*N*)			
Pre-menopausal	18.5 (928)	74.5 (1763)	0.0 (0)
Post-menopausal/no hormone treatment	40.0 (2009)	2.0 (47)	99.1 (2266)
Post-menopausal/hormone treatment	32.1 (1614)	13.3 (315)	0.9 (21)
Dubious/missing	9.5 (476)	10.3 (243)	0.0 (0)
Diabetes, % (*N*)	3.5 (176)	1.7 (41)	13.2 (303)
Hypertension, incl. anti-hypertensive medication use, % (*N*)	35.8 (1802)	16.9 (401)	44.9 (1028)
Lipid-lowering medication use, % (*N*)	3.0 (150)	4.8 (114)	13.2 (303)
Sleep medication use, % (*N*)	N/A	N/A	26.5 (607)
History of depressive symptoms[b]	5.7 (285)	21.4 (506)	10.8 (248)
Habitual snoring, % (*N*)	10.5 (527)	22.2 (526)	17.8 (408)
Self-reported diagnosis of sleep apnea, % (*N*)	5.0 (249)	7.8 (184)	N/A
Adequate sleep duration, % (*N*)	N/A	52.5 (1243)	N/A
Insomnia (WHIIRS ≥ 9[c]), % (*N*)	N/A	N/A	33.5 (766)
Self-reported ever night shift work, % (*N*)	58.4 (2935)	69.6 (1647)	N/A

Values are presented as means (*SD*) or medians (Q25, Q75) for continuous variables; and percentages (absolute numbers) for categorical variables.
N/A, information not available; WHIIRS, Women's Health Initiative Insomnia Rating Scale.
[a]Healthy Eating Index (HEI) 2005 for WHI, Alternative Healthy Eating Index (AHEI) without the alcohol and multivitamin components (Chiuve et al. 2012) for NHS and NHSII.
[b]Derived from the Burnam 8-item depression screening instrument using the cut point ≥0.06 for depressive symptoms for WHI, and from the MHI-5 scale using the cut point ≤52 for depressive symptoms for NHS and NHSII.
[c]Women's Health Initiative Insomnia Rating Scale (WHIIRS) (Levine et al. 2003).

and hydroxyproline. Of note, results of the NHS and NHSII cohorts were very similar (all point estimates of similar magnitude, 18 of the 20 metabolites statistically significant both in the NHS and the NHSII), while patterns were different and less clear in WHI with only two of the 20 metabolites (TAGs C52:4, C54:3) reaching statistical significance.

The 18 identified lipids were all strongly correlated with one another [partial Pearson correlation coefficients (pPCC) > |0.4| for most], while their correlations with hydroxyproline and phenylacetylglutamine were low (Supplementary Figure S4). The LASSO (least absolute shrinkage and selection operator) algorithm based on pooled NHS, NHSII, and WHI data included 12 of the 20 metabolites in its final model, indicating the redundancy of some metabolite associations with short sleep duration, which is in line with the strong correlations among the 18 lipids (Supplementary Table S4). The area under the receiver operating characteristic curve (AUC_{ROC}) of the metabolite score in predicting short sleep duration was 0.56 (95% CI = 0.55–0.58).

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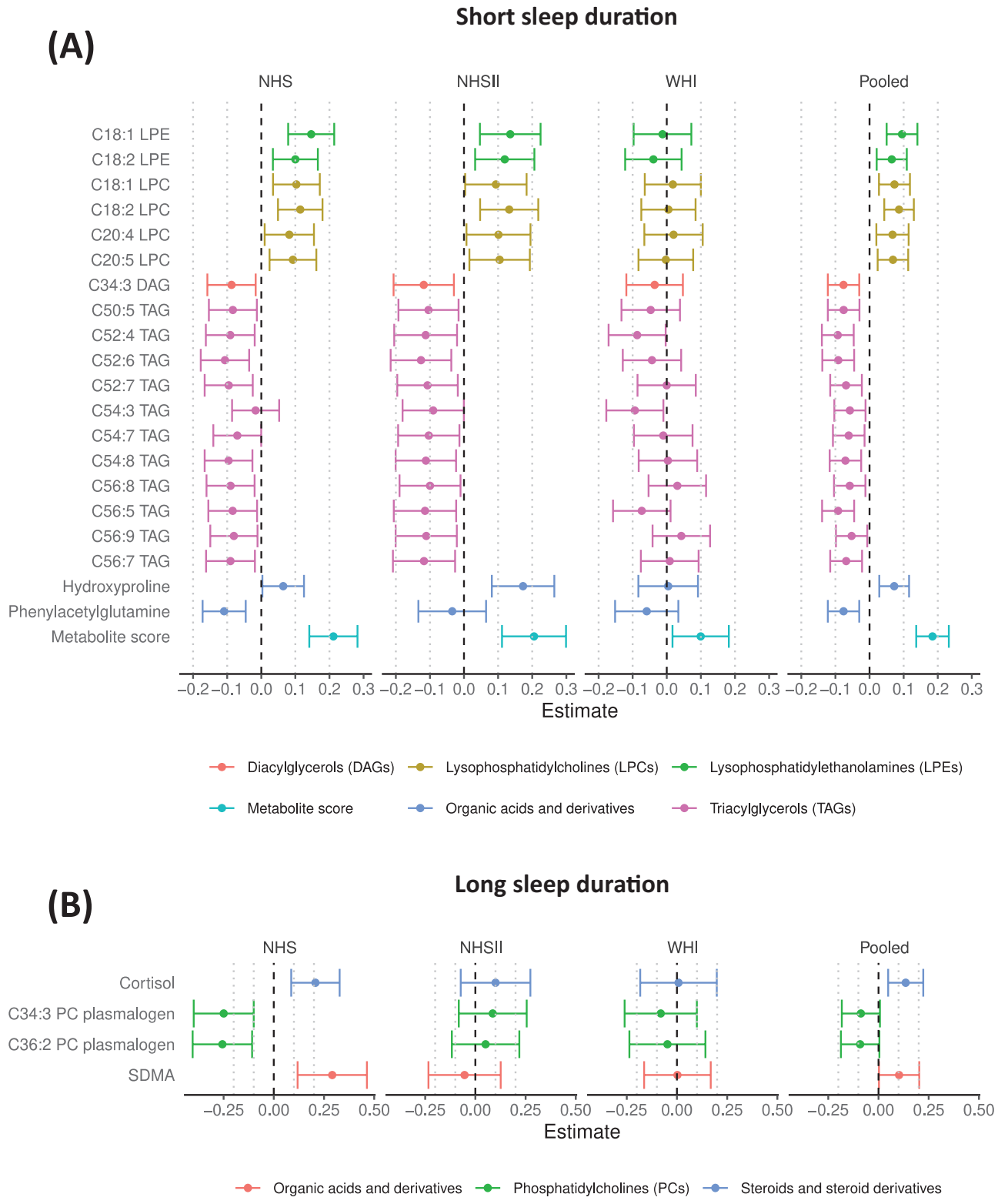

Figure 1. Associations between metabolites and short (A) as well as long (B) sleep duration from fully adjusted regression models, separately for NHS, NHSII, WHI, and pooled across all three cohorts. Regression coefficients for differences in the metabolite z-scores (dots), together with 95% CIs (whiskers), are given for short sleep duration (<7 h) vs. medium sleep duration (7–8 h) (A), and long sleep duration (≥9 h) vs. medium sleep duration (7–8 h) (B), obtained from linear regression models adjusted for age at blood draw (continuous), ethnicity (white vs. non-white), body mass index (continuous), habitual snoring (yes vs. no), physical activity (continuous), diet quality (continuous), alcohol consumption (0 g/day vs. >0–5 g/day vs. >5–10 g/day vs. >10–20 g/day vs. >20 g/day), smoking status (never- vs. ex- vs. current-smoker), menopausal status (pre-menopausal vs. post-menopausal vs. dubious/missing), lipid-lowering treatment intake (yes vs. no), hormone therapy (yes vs. no), presence of diabetes (yes vs. no), history of depressive symptoms (yes vs. no), case-control status, and arm/endpoint within study. Only metabolites which are consistently associated with short sleep duration (i.e. nominal $p < .05$ in at least two cohorts) or which are strongly associated with short sleep duration in any cohort (i.e. false-discovery rate-adjusted $p < .05$) are presented. The metabolite score is calculated as the propensity of short sleep duration for each participant. The association with short sleep duration therefore has to be positive by definition, and does not necessarily have to be the same as for the single metabolites which can contribute with a negative weight to the score. SDMA, symmetric dimethylarginine.

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There is growing evidence of bidirectional associations of some of the lifestyle- and health-related factors we considered as covariates in the fully adjusted main model with short sleep duration. Therefore, we compared results of the main model with results of minimally-adjusted models (i.e. adjusted for age, ethnicity, menopausal status, and arm/endpoint within study). Point estimates from minimally-adjusted models were in general close to the estimates from the fully adjusted main model. Applying the same criteria of consistency ($p_{raw} < .05$ in at least two cohorts) or strength ($p_{FDR} < .05$ in at least one cohort) of associations outlined above, we detected another nine metabolites associated with short sleep in the minimally-adjusted models: four acylcarnitines (C4–OH, C7, C9, C14; positive associations with short sleep duration), one phosphatylcholine (PC; C40:10; negative association), 2 TAGs (C58:7, C58:9; negative association), as well as biliverdin (negative association) and citrulline (positive association) (Supplementary Figure S5A; numbers and *p*-values in Supplementary Table S3).

Secondary analyses showed that the association of these 29 metabolites with short sleep duration were robust. Analyses restricted to (1) the extreme phenotype of <6 h sleep, (2) controls (data originated from nested case–control studies within NHS, NHSII, and WHI investigating various endpoints; cases developed major chronic diseases during follow-up), (3) women without history of depressive symptoms, (4) women not taking lipid-lowering medication, (5) women with a BMI < 25, and (6) women with a BMI ≥ 25 resulted in similar effect estimates for most associations. Treating sleep duration as a linear term did not substantially alter the results as well (Supplementary Tables S5 and S6). Of note, analyses restricted to the <6 h phenotype of short sleep duration, showed effect sizes larger than in the main model, as expected under the assumption of a dose–response relationship; however, CIs were wide due to the low prevalence (~5.0%) of <6 h of habitual sleep duration. Finally, analyses of (1) only women who never worked night shifts and (2) only women without a diagnosis of sleep apnea in NHS/NHSII did not indicate any differential associations between short sleep duration and metabolites in these subgroups (Supplementary Table S7).

When we analyzed classes of molecularly or biologically similar metabolites in metabolite set enrichment analyses, lipids were the main group of metabolites enriched in the short sleep duration group compared to the 7–8 h reference. Lipid groups acylcarnitines, LPCs and PEs had statistically significant positive enrichment scores (meaning that metabolites associated with higher levels of metabolites in short sleepers compared to 7–8 h sleepers were enriched), while DAGs and TAGs had statistically significant negative enrichment scores (Figure 2A). This is in line with the findings for single metabolites as reported in Figure 1A and Supplementary Table S3. This pattern is also seen in Figure 2B, showing multi-variably-adjusted beta-estimates for the association with short sleep duration for all analyzed lipids ordered by the total acyl chain carbon number. Lipids of lower total acyl chain carbon numbers (e.g. acylcarnitines, LPCs) were more likely to exhibit positive associations with short sleep duration, while lipids of higher carbon chain numbers (in particular TAGs) showed mostly negative associations, irrespective of the number of fatty acid chains.

Results of all regression analyses conducted for short sleep duration for all 210 metabolites are provided in Supplementary Dataset S1.

Associations of short sleep duration-related metabolites with obesity and diabetes

The majority of the 20 metabolites consistently and/or strongly associated with short sleep duration in fully adjusted models were significantly associated with obesity prevalent at the time of blood draw (17 statistical significant metabolites), prevalent and incident diabetes (15 and 16 significant metabolites, respectively), but not so much incident obesity (four significant metabolites; Figure 3; numbers and *p*-values in Supplementary Table S8). However, the direction of the metabolites' associations with obesity and diabetes were often in the opposite direction than the metabolites' associations with short sleep duration. For example, while the eleven identified TAGs were all decreased in short sleepers, decreases in these TAGs were also often associated with decreased risk of prevalent obesity and incident diabetes. Alternatively, LPEs and LPCs increased amongst short sleepers, yet higher levels of these metabolites were associated with decreased risk of prevalent obesity and incident diabetes. These are unexpected findings because they suggest that short sleep duration is associated with metabolite levels associated with lower obesity and diabetes risk, compared to the reference sleep duration of 7–8 h. The LASSO-derived metabolite score as a metric combining all of the short sleep relevant information carried by the individual metabolites was not associated with neither prevalent nor incident obesity or diabetes ($OR_{cross\text{-}sectional\ obesity}$ = 1.00, 95% CI = 0.95–1.06; $OR_{cross\text{-}sectional\ diabetes}$ = 1.02, 95% CI = 0.92–1.13; $OR_{incident\ obesity}$ = 1.09, 95% CI = 0.96–1.24; $HR_{incident\ diabetes}$ = 0.98, 95% CI = 0.92–1.05) (Figure 3 and Supplementary Table S8). Restricting analyses to only controls did not substantially affect the results (Supplementary Table S8).

These associations all being close to and not statistically significantly different from null associations indicate that the metabolite score does not contribute to obesity or diabetes risk, and thus cannot mediate potential associations between short sleep duration and obesity and diabetes. Specifically, in the pooled cohorts short sleep duration increased the risk of incident obesity substantially ($OR_{incident\ obesity,\ w/o\ metabolite\ score}$ = 1.50, 95% CI = 1.14–1.96), and when adjusting for the metabolite score, the risk only marginally changed ($OR_{incident\ obesity,\ with\ metabolite\ score}$ = 1.47, 95% CI = 1.11–1.93). Regarding incident diabetes, we only saw a weak, non-significant relationship with sleep duration ($HR_{incident\ diabetes,\ w/o\ metabolite\ score}$ = 1.05, 95% CI = 0.92–1.20), again with only a marginal change when adjusting for the metabolite score ($HR_{incident\ diabetes,\ with\ metabolite\ score}$ = 1.06, 95% CI = 0.92–1.21) (Supplementary Table S9). If mediation was plausible, one would expect a reduction in risk ratios after adjustment for the metabolite score.

Associations of metabolites with long sleep duration

We also observed associations of metabolites with long sleep duration. Thirty-nine of the 210 metabolites were significantly associated with long sleep duration in at least one of the three cohorts on a nominal significance level of 0.05 in multi-variably, fully adjusted models. However, only four of them (cortisol; C34:3 PC plasmalogen; C36:2 PC plasmalogen; symmetric dimethylarginine [SDMA]) had an FDR-adjusted $p < .05$ in the NHS cohort, and results were less clear with non-significant associations in NHSII and WHI (Figure 1B and Supplementary Table S10). Of note, omitting lifestyle- and health-related factors as covariates in the regression models changed results to a higher extent than for the analysis of short sleep duration associated metabolites. In minimally-adjusted models, ten metabolites were identified for which the associations were no longer significant after multi-variable adjustment (Supplementary Figure S5B and Table S10). Sensitivity analyses did not reveal major differences for various subgroups; however, analyses were limited by the small number of long

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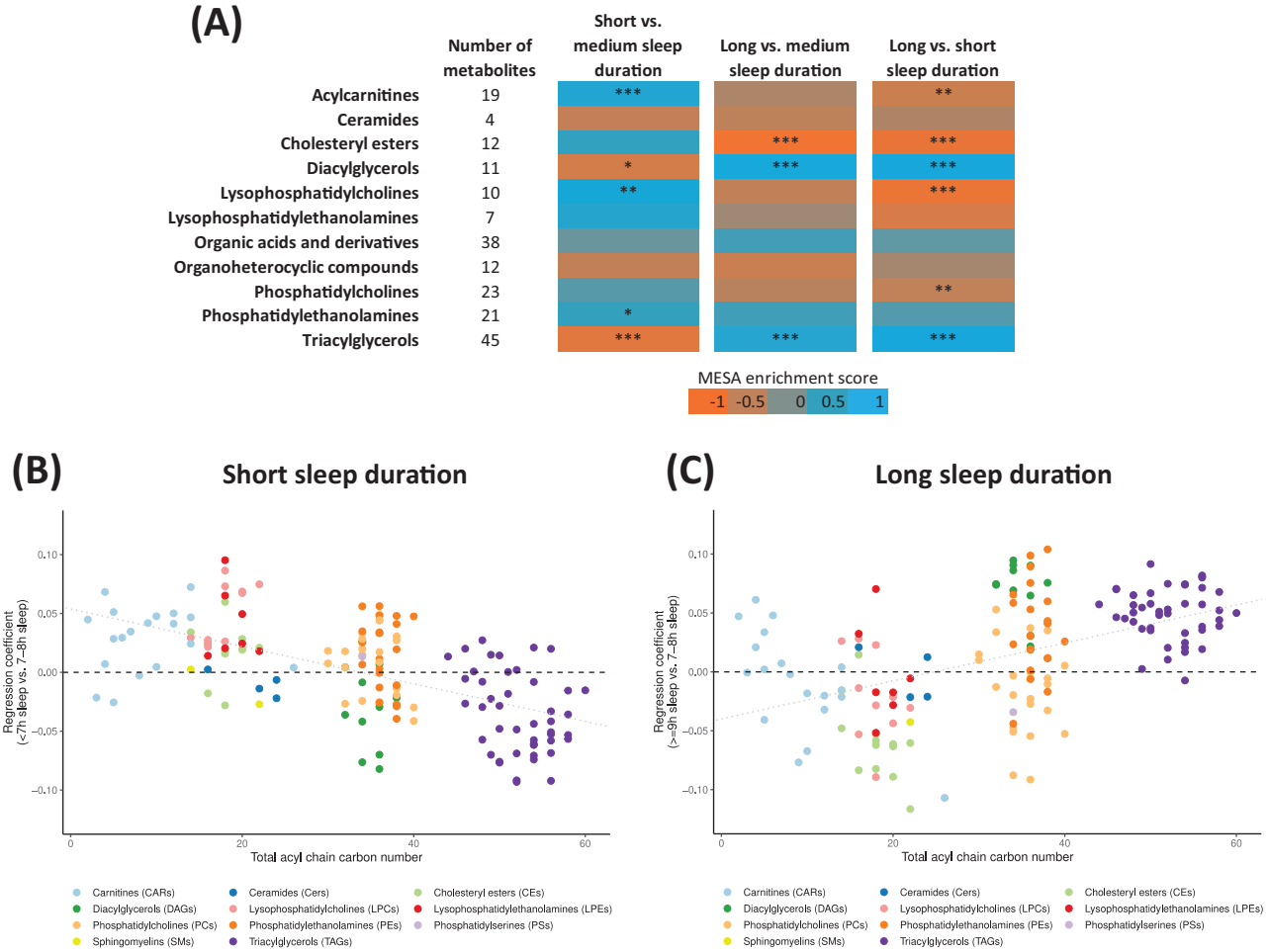

Figure 2. Metabolomics analyses by groups of metabolites. (A) Shows results of metabolite set enrichment analyses based on regression parameters from multi-variably adjusted models, comparing eleven groups of molecularly or biologically similar metabolites (chemical classes within lipids, HMDB superclasses for non-lipids) for short vs. medium sleep duration, long vs. medium sleep duration, and long vs. short sleep duration. Direction and magnitude of enrichment scores are indicated via color. ***Denotes an enrichment p-value < .001, **a *p*-value < .01, and *a *p*-value < .05, after Bonferroni-Hochberg adjustment for multiple testing of eleven groups. (B) Displays regression coefficients for the association of short sleep duration (<7 h), and (C) displays regression coefficients for the association of long sleep duration (≥9 h) [reference in both (B) and (C): medium sleep duration (7– 8h)] with metabolites of the HMDB class "Lipids and lipid-like molecules", plotted by the total acyl chain carbon number of the respective metabolite, irrespective of the number of fatty acid chains. Groups of different HMDB direct parents (i.e. chemical classes) are displayed in different colors. Regression coefficients used in (A) and displayed in (B) and (C) are from fully adjusted linear models applied on the pooled NHS, NHSII, and WHI data, adjusted for age at blood draw (continuous), ethnicity (white vs. non-white), body mass index (continuous), habitual snoring (yes vs. no), physical activity (continuous), diet quality (continuous), alcohol consumption (0 g/day vs. >0–5 g/day vs. >5–10 g/day vs. >10–20 g/day vs. >20 g/day), smoking status (never- vs. ex- vs. current-smoker), menopausal status (pre-menopausal vs. post-menopausal vs. dubious/missing), lipid-lowering treatment intake (yes vs. no), hormone therapy (yes vs. no), presence of diabetes (yes vs. no), history of depressive symptoms (yes vs. no), case-control status, and arm/endpoint within study. The grey, dotted lines in (B) and (C) show the regression lines through the depicted data points.

sleepers in the respective subgroups (Supplementary Tables S11 and S12).

In line with the inconsistent findings across cohorts, the LASSO algorithm based on pooled NHS, NHSII, and WHI data did not select any of the four metabolites as significant predictors of long sleep. Because of the lack of robust associations, we did not examine associations between long sleep-duration-related metabolites and obesity/diabetes.

In enrichment analyses, similar to the case of short sleep duration, only groups of lipids were enriched in the long sleep duration group compared to the 7–8 h reference. In particular, negative enrichment scores were observed for cholesteryl esters (CEs), and positive enrichment scores for DAGs and TAGs (Figure 2A). For long sleep duration, lipids of lower total acyl chain carbon numbers were more likely to exhibit negative associations with long sleep duration, and lipids of higher carbon chain numbers (in particular TAGs) were more likely to exhibit positive associations, a trend contrary to the one observed for short sleep duration (Figure 2C).

We then performed an enrichment analysis comparing long vs. short sleep duration, observing statistically significant differences in enrichment scores for six of the nine lipid groups, but not for the non-lipid metabolite groups (Figure 2A). When comparing long vs. short sleepers, 35 metabolites were significantly different after multi-variable adjustment in the pooled cohorts. Most of these metabolites were lipids, with twelve TAGs presenting higher levels in long sleepers as compared to short sleepers. However, after FDR adjustment only three metabolites [cortisol (increased in long sleepers), LPC C18:2 (decreased), and C56:5 TAG (increased)] were retained (Supplementary Table S13).

Results of all regression analyses conducted for long sleep duration for all 210 metabolites are provided in Supplementary Dataset S2.

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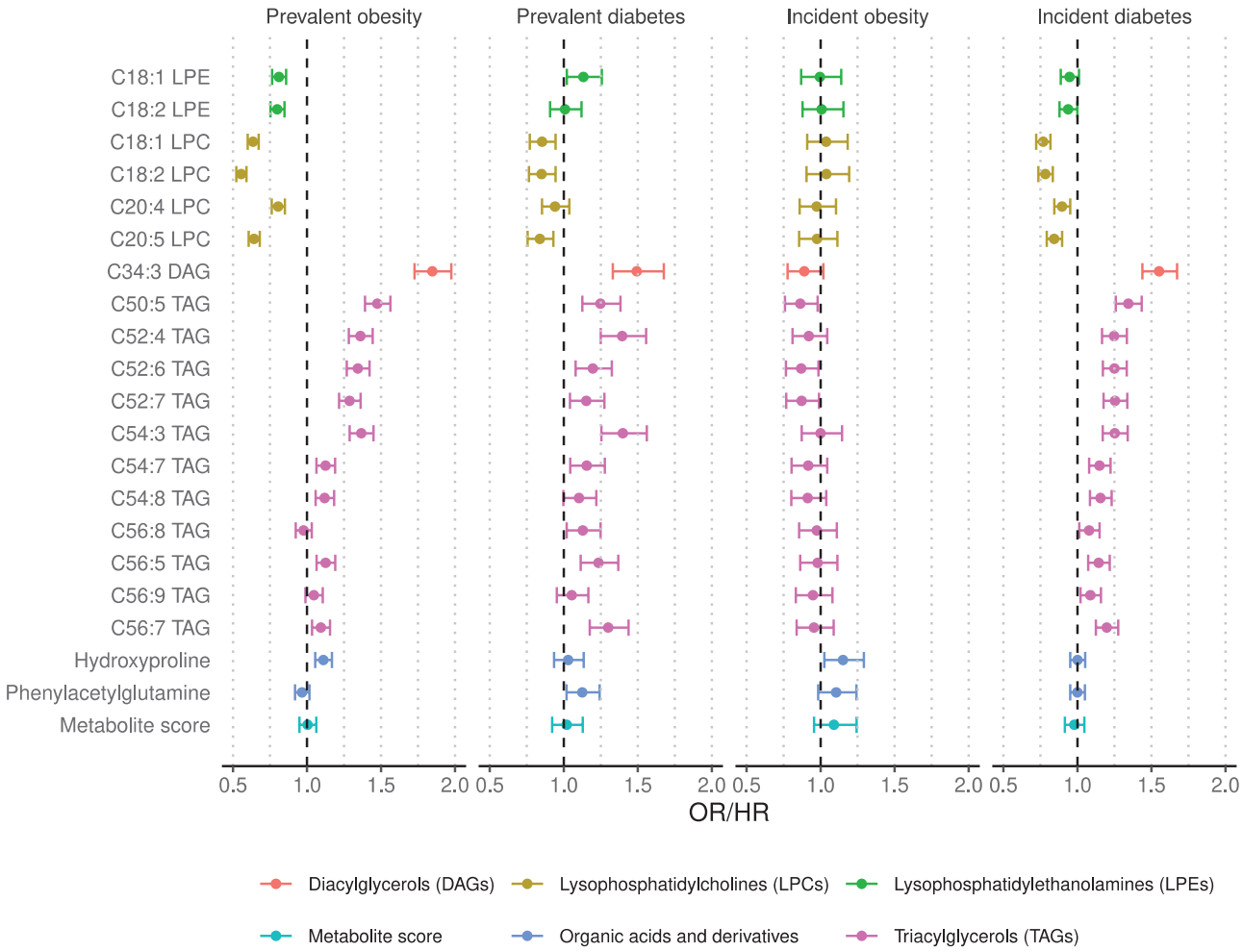

Figure 3. Cross-sectional and prospective associations of the 20 short-sleep associated metabolites (as identified in Figure 1) and the metabolite score with obesity and diabetes prevalence (at the time of blood draw) and incidence (after the time of blood draw) in the pooled NHS, NHSII, and WHI data. Women with long sleep duration (≥9 h) were excluded. Associations of the metabolites and the metabolite score were assessed using odds ratios (ORs) from logistic regression models for diabetes prevalence, obesity prevalence, and obesity incidence, and using hazard ratios (HRs) from Cox proportional hazards models for diabetes incidence. ORs and HRs are given as increase per standard deviation in the respective exposure variable. Separate models were built for each exposure variable. Exposure variables were included as linear terms. In the prospective analyses only women initially non-obese/free of diabetes were included. Incident obesity was assessed 6 years after the blood draw. All models were adjusted for age (continuous), case-control status (yes vs. no), ethnicity (white vs. non-white), cohort (NHS vs. NHSII vs. WHI), and BMI (continuous, except for the analysis of prevalent obesity).

Discussion

Our study of more than 9000 mostly post-menopausal women is the first to demonstrate systematic cross-sectional differences in plasma metabolite profiles between women with habitual short (<7 h) vs. medium (7–8 h) sleep duration in a non-experimental setting. Habitual short sleep was associated with consistent changes in several plasma lipids in the NHS and NHSII, but much less in the WHI. In particular, levels of two lysophosphatidylethanolamines and four lysophosphatidylcholines were increased, while levels of one DAG and eleven TAGs (all with a ≥3 number of double bonds) were decreased in short sleepers when compared to the 7–8 h group. Moreover, hydroxyproline and phenylacetylglutamine were increased. Still, a metabolite score derived from this set of metabolites was not significantly associated with obesity and diabetes, neither cross-sectionally nor prospectively. Furthermore, enrichment analysis assessing associations of metabolites with short sleep based on biological categories demonstrated significantly increased levels of acylcarnitines for short sleep. Associations of single metabolites with long sleep duration were less robust, but differences in some lipid classes, in particular increased levels of DAGs and TAGs, were observed in long sleepers compared to those with short and 7–8 h sleep.

To our knowledge, only three studies have investigated the association between short sleep duration and metabolite levels in non-experimental settings to date [24–26]. Two of them did not report cross-sectional associations [24, 25]. In both these studies, the participants were markedly younger than the women included in our analysis, and the exposure was derived from sleep durations reported during specific nights, rather than an assessment of habitual sleep duration. In the third study of 205 overweight/obese participants of the "Satiety Innovation" (SATIN) study [26], sleep duration was assessed via accelerometer, and 12 metabolites discriminating long sleep from short sleep duration were detected. However, the results are hard to compare with our findings, because of the use of different metabolomics platforms, and different categorizations for sleep duration. A limitation in all three studies is the relatively small sample size ($N < 300$ for all). As effect sizes of all our detected metabolites were modest (below

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0.2), sample sizes <300 are not sufficiently powered to detect effects, in particular when also accounting for multiple testing.

Furthermore, there are several laboratory-controlled studies of short-term sleep deprivation or restriction. For example, Weljie et al. [19]. assessed the effects of chronic sleep restriction by subjecting rats and young, healthy humans to a 5-day sleep restriction protocol. In humans, the authors observed decreases in DAGs/TAGs of higher carbon number and higher double-bond content and increases in seven LPC species. This is in line with our findings, where we see a very similar pattern with increased LPC levels, and decreased DAGs/TAGs levels of higher carbon number and higher double-bond content in short sleepers compared to 7–8 h sleepers. In particular, LPC C18:2, LPC C20:4, DAG C36:3, and TAG C54:3 are identified as relevant short sleep associated metabolites in both Weljie et al. [19]. and our study. Of note, DAG C36:3 was one of two metabolites reduced following sleep restriction not only in humans, but also in rats [19]. Depner et al. [23]. replicated this finding using an experimental insufficient sleep protocol. Thus, our finding corroborates the hypothesis that DAG 36:3 is a potential biomarker of short or insufficient sleep. Oxalic acid, the second metabolite identified in Welije et al. [19], was not annotated in Depner et al.'s [23] nor in our metabolomics data, thus warranting further examination.

A direct comparison of study findings can be complicated because of the use of different analytical metabolomics platforms. For example, many platforms used in prior studies detected limited numbers of PEs, PCs, LPCs, DAGs, and TAGs [17, 18, 20, 22]. Subjecting twelve healthy young males to a 24 h wake/sleep cycle, followed by 24 h of wakefulness, Davies et al. [17] reported increased levels in 27 metabolites (tryptophan, serotonin, taurine, 8 acylcarnitines, 13 glycerophospholipids, and 3 sphingolipids) when participants were sleep deprived compared with during sleep. Information on serotonin and taurine was not available in our study. Regarding tryptophan and sphingolipids (only 6 of them were measured on our metabolomics platform), we did not observe any changes in short sleepers in our data. However, regarding acylcarnitines and glycerophospholipids (PCs and LPCs), our results support the findings of Davies et al. [17]. We also identified four LPCs whose levels were increased in short sleepers, and although no individual acylcarnitines remained statistically significant after FDR correction in our main analysis, enrichment analysis showed statistically significant positive enrichment scores for the class of acylcarnitines. Increased acylcarnitine levels in participants exposed to acute sleep curtailment were also reported in a study by van den Berg et al. [20], although in this study no increase in glycerophospholipids was observed. Unfortunately, in both the studies of Davies et al. [17] and van den Berg et al. [20]. TAGs and DAGs were not measured. A study with a considerable overlap in measured DAGs and TAGs with our study is the work of Chua et al. [21]. However, in this study, subjects were acutely kept awake for 40 consecutive hours, which might explain why Chua et al. observed increases in various TAGs, in contrast to Weljie's [19] and our findings. As pointed out previously, metabolic effects of sleep restriction likely depend on the duration of curtailment [15], and this might, in addition to differences in participants' characteristics, be a reason for the observed differences in results.

Short sleep duration has been repeatedly associated with dyslipidemia in observational studies [26, 32, 33] and sleep restriction with changes in the lipidome in experimental studies [17–22]. This is reflected in the plentitude of lipids amongst our identified metabolites. However, we also identified two aqueous metabolites associated with short sleep duration, phenylacetylglutamine and hydroxyproline. Phenylacetylglutamine is the primary metabolite of the degradation of phenylacetate in the presence of glutamine in the liver, and prior reports point to a negative association of short sleep duration with phenylacetylglutamine in urine [34]. Phenylacetylglutamine has also been shown to be positively correlated with vegetable intake [35], and decreased levels in short sleepers would fit the hypothesized unhealthier eating behavior when sleep deprived [36–40]. Hydroxyproline is associated with dietary intake as well, but it is, in contrast to phenylacetylglutamine, abundant in red-meat, while levels are low in vegetables [41, 42], again consistent with increased levels in short sleepers in our study.

The sleep assessment in our study is also important to discuss. Habitual sleep duration does not differentiate between different short sleep phenotypes and does not provide information about sleep deprivation. Also, sleep duration assessment and blood draw were ~3.5 years apart in NHS/NHSII, which might introduce additional heterogeneity as sleep duration might change over time, probably diluting observed associations towards the null. Together, this might explain why in a study based on a subset of WHI and NHSII data, the metabolomic signature of habitual sleep quality was distinct from our findings; specifically, Huang et al. [43] reported positive associations of poor sleep quality with TAGs, while our study shows negative associations with short sleep. More work is needed to advance our understanding regarding short sleep phenotypes heterogeneity and the plasma metabolome.

In contrast to sleep quality, where a sleep-quality related metabolite score was strongly related to coronary heart disease risk [43], a metabolite score derived from our 20 identified short sleep duration metabolites was not significantly associated with obesity or diabetes, neither cross-sectionally nor prospectively, and irrespective of the set of covariates adjusted for. A reason for this might be the relatively poor ability not only of single metabolites, but also of the metabolite score (AUC_{ROC} = 0.56) to predict short sleep duration. In such a case, the metabolite score is not expected to be a good predictor of other sleep-related outcomes, either. The interrelationship between plasma metabolites and obesity and diabetes risk is complex with a lot of ongoing research (an overview is given in recent reviews [44–46]). It might be that focusing on a few single metabolites predictive of short sleep as potential mediators between short sleep and obesity and diabetes does not account sufficiently for these complex inter-relationships, even when considering them in a combined metabolite score. It is possible that examining the entirety of the metabolome from a wider perspective is necessary to better understand the biological mechanisms underlying the link between sleep and health; novel methodologies for such an approach were developed recently [47, 48].

The few significant associations of metabolites with long sleep duration were not robust across our three study cohorts. This is probably due to the low percentage (~5%) of women with long sleep duration in our cohorts, and thus limited statistical power. Statistical power is probably further reduced due the timely distance between sleep duration assessment and blood draw in NHS/NHSII. However, in enrichment analyses, where power is increased by aggregating metabolites into broader categories, long sleep showed a distinctly different metabolic signature compared to shorter sleep durations, even after multi-variable adjustments. This indicates that the associations between habitual long sleep and health outcomes go beyond associations produced by

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bidirectional relationships with e.g. depression and low socioeconomic status, as sometimes hypothesized [12, 13].

Another finding worth mentioning is that nearly all identified metabolites showed strong and consistent associations in the NHS and the NHSII, while the associations were in general much weaker and not statistically significant in the WHI cohort. Importantly, metabolites were all analyzed at the Harvard-MIT Broad Institute using identical metabolomics platforms, therefore the reasons for the observed differences cannot be attributed to platform differences. Notably, plasma samples were stored up to >30 years before metabolomics analysis, with longest storage times in NHS, and shortest storage times in NHSII. The potential impact of long-term storage on metabolomics has not been systematically examined [49], however, any potential degradation from long-term storage would likely lead to non-differential measurement errors and attenuate the associations. NHS and NHSII comprise only nurses and thus represent homogeneous and comparable populations, while the WHI population is more heterogeneous. Furthermore, with a baseline age of 67 years, WHI participants were older than the nurses in NHS/NHSII, and mostly retired. Women were more likely to have comorbidities or be short sleepers in WHI than in NHS/NHSII (~41% in WHI compared to ~27% in NHS/NHSII), and more than 25% of WHI participants used sleep medication. Finally, the prevalence of ever night shift work was high in NHS (58.4%) and NHSII (69.6%); however, subgroup analyses of only women who never worked night shifts confirmed the overall findings of NHS/NHSII, thus indicating that results were not substantially influenced by the high prevalence of women with a history of night shift work. Still, future studies in independent cohorts are needed to understand whether associations between sleep duration and metabolites are in general weaker for elderly populations, and, if yes, what the driving factors are.

Our study has several limitations. Data are based on different nested case–control studies within NHS, NHSII, and WHI for various endpoints, meaning about half of our study participants developed a major chronic disease (such as heart disease, cancer, diabetes, rheumatoid arthritis, etc.) during follow-up. Thus, data is not a random subset of the respective cohorts, which could undermine our ability to examine associations with secondary outcomes, such as obesity or diabetes. However, analyzing data of controls only did not substantially change our findings. Timing between sleep duration assessment and blood draw varied across studies as well. However, given the consistency of our findings with previous experimental studies and in view of the potential causal framework (Supplementary Figure S6), it appears plausible that the observed associations reflect physiological effects of short sleep duration on metabolism. Likewise, emerging evidence suggests that the relationships between sleep duration, body composition, and diabetes go beyond unidirectional associations [50–52]. Second, information about habitual sleep duration and other potential risk factors was self-reported in 1-h bins at a single time-point, and no objective measurements or sleep duration changes over time were available. However, self-reported sleep duration in the NHS was validated among 260 participants in 2002 with 6-day diaries ($r_{correlation}$ = 0.79) [53]. Furthermore, despite the crudeness of 1-h bins, unequivocal associations of extreme sleep phenotypes (both short and long) with various health outcomes and mortality have been previously demonstrated, both in the NHS [53–56], and the WHI [57, 58], indicating the validity of this questionnaire item. However, we were underpowered to detect meaningful associations with very short sleep (<5 h). Third, only 210 metabolites, and thus a small subset of all metabolites known to be physiologically relevant, were available for analysis, the majority (N = 157) being lipids. Thus, our findings are representative of lipid-related metabolites, but not the whole metabolome. Fourth, in our analysis we could not assess the circadian variation and rhythmicity known to be exhibited by a substantial proportion of metabolites [59, 60]. Blood samples were taken in the morning in a fasting status; the evaluation of diurnal patterns of metabolites, especially as related to sleep timing, might be relevant for future studies. Fifth, our study population consisted of only middle-aged and older women, most of them nurses. Additional data is needed to examine generalizability of patterns to other populations, such as men or younger women.

In summary, we demonstrated that habitual short sleep exhibits a metabolome signature different from medium and long sleep durations. Identified lipid species and directions of associations for short sleep resembled effects seen in experimental chronic sleep restriction studies, rather than acute sleep deprivation studies, indicating the generalizability of our results and adding evidence towards a potential causal relationship between short sleep duration and the identified metabolites. However, the signature we identified did not mediate the association between short sleep and obesity and diabetes risk, highlighting the need of further studies in this field of research.

Supplementary material

Supplementary material is available at *SLEEP* online.

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Data Availability

Data described in the manuscript may be made available upon application to and approval by the Channing Division of Network Medicine at Brigham and Women's Hospital, and Harvard T.H. Chan School of Public Health (email: nhsaccess@channing.harvard.edu) for NHS and NHSII data, and the Women's Health Initiative Publications and Presentations Committee (email: p&p@WHI.org.) for WHI data. Further information, including the procedures to obtain and access data is described at https://www.nurseshealthstudy.org/researchers, and https://www.whi.org/md/working-with-whi-data.

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