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Original Article

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Human serum proteomics reveals a molecular signature after one night of sleep deprivation

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Abstract

Study Objectives: Sleep deprivation is highly prevalent and caused by conditions such as night shift work or illnesses like obstructive sleep apnea. Compromised sleep affects cardiovascular-, immune-, and neuronal systems. Recently, we published human serum proteome changes after a simulated night shift. This pilot proteomic study aimed to further explore changes in human blood serum after 6 hours of sleep deprivation at night.

Methods: Human blood serum samples from eight self-declared healthy females were analyzed using Orbitrap Eclipse mass spectrometry (MS-MS) and high-pressure liquid chromatography. We used a within-participant design, in which the samples were taken after 6 hours of sleep at night and after 6 hours of sleep deprivation the following night. Systems biological databases and bioinformatic software were used to analyze the data and comparative analysis were done with other published sleep-related proteomic datasets.

Results: Out of 494 proteins, 66 were found to be differentially expressed proteins (DEPs) after 6 hours of sleep deprivation. Functional enrichment analysis revealed the associations of these DEPs with several biological functions related to the altered regulation of cellular processes such as platelet degranulation and blood coagulation, as well as associations with different curated gene sets.

Conclusions: This study presents serum proteomic changes after 6 hours of sleep deprivation, supports previous findings showing that short sleep deprivation affects several biological processes, and reveals a molecular signature of proteins related to pathological conditions such as altered coagulation and platelet function, impaired lipid and immune function, and cell proliferation. Data are available via ProteomeXchange with identifier PXD045729. This paper is part of the *Genetic and other molecular underpinnings of sleep, sleep disorders, and circadian rhythms including translational approaches* Collection.

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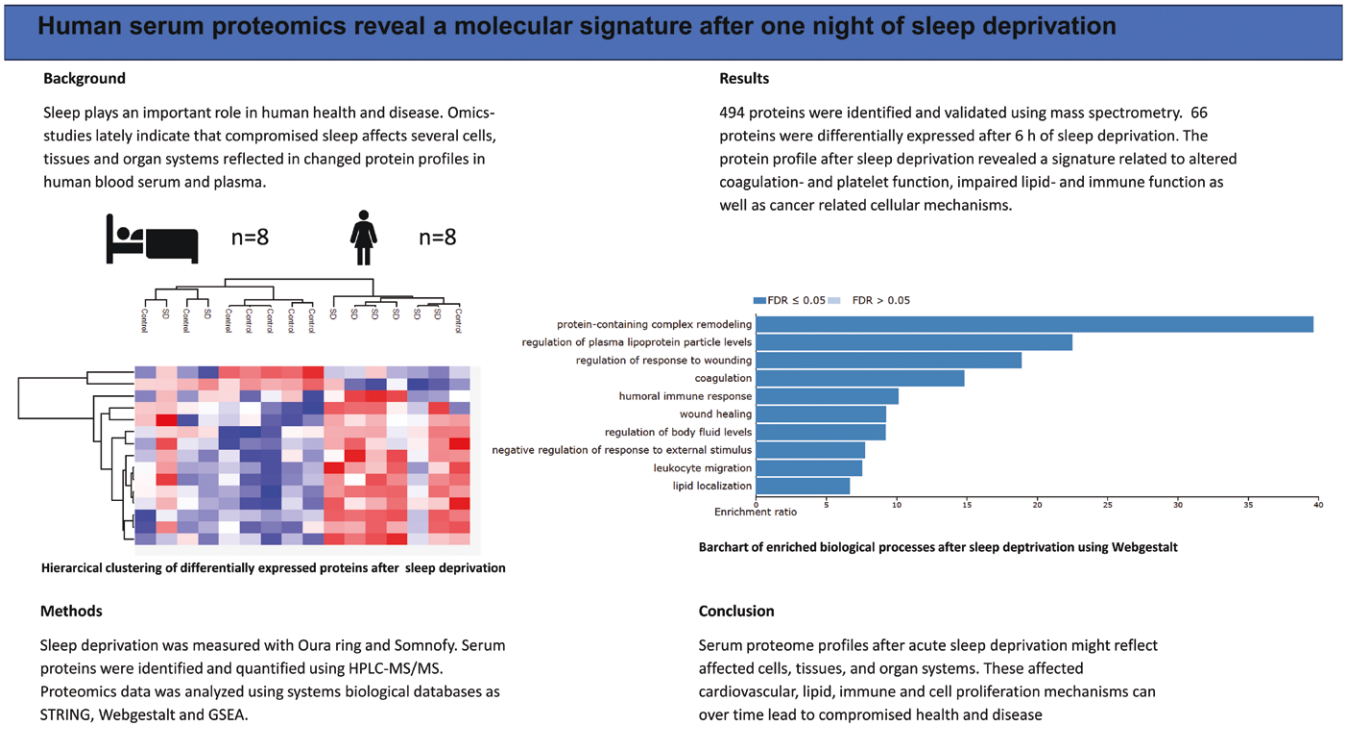
Graphical Abstract



Statement of Significance

The affected cellular mechanisms, biological processes and molecular functions after acute sleep deprivation are reflected in the differentially expressed proteins in human blood serum. Our study contributes to the protein profiling of sleep deprivation, which has profound implications for several physiological and metabolic processes. The identification of possible biomarkers in one of the most clinically used specimen, human blood serum, are needed. Furthermore, achieving more knowledge of the affected cellular and molecular mechanisms of compromised sleep is essential for the development of tools for managing lack of and mistimed sleep as in shift work. More knowledge on the cellular level is also needed for the management and treatment of sleep-related disorders improving sleep quality, sleep quantity, and overall quality of life.

Key words: human blood serum; proteomics; sleep deprivation; cellular stress; functional enrichment analysis

Sleep deprivation is becoming increasingly prevalent. Several conditions such as night or alternating shift work have been proven to lead to circadian rhythm disruption or sleep deprivation that affects cellular processes and molecular mechanisms. These effects result in altered gene and protein expression and are proposed to be involved in the development and progression of various mental, cardiovascular, metabolic, immune, and neurodegenerative disorders [1–8].

Loss of sleep is also a symptom of several diseases such as obstructive sleep apnea (OSA) and depression, thereby causing sleep deprivation to be bidirectionally linked to several of its pathologies by changes in cellular mechanisms. Insufficient sleep or altered circadian rhythms change the transcription and translation. This can be detected by measuring the changes in mRNA and protein levels [3, 4, 9, 10].

Cellular and molecular changes due to sleep deprivation measured in blood and/or urine can provide a molecular signature related to conditions caused by sleep deprivation such as OSA. Such biomarkers could potentially be used diagnostically. However they could also be used prognostically and as predictors of comorbidities [11, 12]. The molecular mechanisms affected by sleep deprivation are still not fully understood even though sleep–wake associated studies using the omics-methodology, as well as technologies used to characterize and quantify molecules and their interactions at the genome-wide level, are increasing [4]. The effects of partial sleep deprivation using a proteomic and systems biological approach are still sparse [5, 9, 10, 13, 14].

This research project is part of an ongoing study that examines the effects of sleep deprivation in human blood serum. We aim to expand the existing pool of molecular signatures for sleep deprivation by protein expression profiling in serum and saliva. A recently published paper from our research group has identified several differentially expressed proteins (DEPs) in human serum after 6 hours of sleep deprivation that could be linked to various biological processes and pathological conditions [15]. The goal of the present study was to conduct statistical and system biological analyses of human blood serum proteome changes after 6 hours of sleep deprivation at night using a different part of the human serum proteome. Different bioinformatic software and biological databases have been used with the aim of discovering biological processes and functions that are affected by one night of sleep loss.

Materials and Methods

Experimental setup, data acquisition, and data preanalysis

Human blood serum samples were acquired from eight self-reported healthy voluntary female participants, 22 to 57 years

old, non-medicated, and with no history of neurological or psychiatric disease and analyzed using mass spectrometry (Orbitrap Eclipse) in combination with high-pressure liquid chromatography (HPLC Dionex Ultimate 3000 Basic HPLC System). All of the participants gave their informed consent to take part in this study and approval for the study was given by the Regional Ethical Committee (REK, 2019/254).

Each participant was their own control, and two samples were taken from each of them. The first sample was obtained after 6 hours of sleep at night, after the participants went to bed at 22:00, and the second sample after 6 hours of sleep deprivation the following night. Both samples were taken at 4:00 a.m. The samples were depleted for the most abundant proteins for proteins with a lower abundance to be detected (Thermo Fishers High Select™ Top 14 Abundant Protein Depletion Resin mini spin column) and trypsinated. After fragmentation, mass spectrometry and liquid chromatography were used for the analysis. The number of peptides identified for a protein determined the confidence of the protein identification. Detailed descriptions of study participants, sleep monitoring (Oura-ring and Somnofy) and serum sampling can be found in Bjørkum et al. 2021 [15].

Digestion

The samples were depleted for the top 14 most abundant proteins using Thermo Fisher's High Select™ Top 14 Abundant Protein Depletion Resin mini spin columns, 10 µL of serum were added to each column. The protein concentration was measured using Pierce BCA protein Assay. In Eppendorf lo-bind tubes the samples were mixed with paramagnetic beads (Sera-Mag Speed beads, GE healthcare) in a 1:10 ratio. The peptide-bead mixture was then subjected to agitation before the addition of 100% ethanol to 70% ethanol solution. The solution was agitated at 1000 rpm for 7 minutes in RT. The sp3 reaction mixture was placed on a magnetic rack and the supernatant was removed. The procedure was repeated twice with 80% ethanol to remove the remaining lysis buffer.

Digestion buffer containing 100 mM AmBic/1mM $CaCl_2$ and trypsin concentration of 0.2 µg/µL. 50 µL digestion buffer were used for each sample. The samples were sonicated for 30 seconds and incubated for 16 hours at 37°C in a thermomixer at 1000 rpm. After digestion, the samples were centrifuged at 13 000 rpm at 24°C for 3 minutes. The tubes were placed on the magnetic rack until the beads had settled onto the tube wall before the supernatant was moved to a fresh tube. 50 µL of 0.5 M NaCl in H_2O was added to each sample.

Tubes were sonicated for 30 seconds in a water bath and centrifuged at 13 000 rpm at 24°C for 3 minutes. The tubes were placed on the magnetic rack and when the beads settled onto the tube wall, the supernatant of these tubes were moved to the tube with the previous supernatant. The amount should be 100 µL and combining these two supernatants would release all peptides from the beads. Next, 200 µL of 0.1% TFA was added to make the total solution amount 300 µL.

Desalting

Oasis 96 well cartridges, Waters, USA, was used for desalting. Cartridges were activated by adding 500 µL of 80% ACN, 0.1% formic acid (FA), **centrifuge 200 ×*g* for 1 minute, and discard the flow through. Wash cartridges by adding 500 µL of 0.1% TFA, the centrifuge 200 ×*g* for 1 minute, and allow the discard to flow through, and then repeat this step twice. Add samples and centrifuge 100 ×*g* for 3 minutes, discard flow through. Wash twice with 500 microliters of 0.1% TFA, centrifuge 200 × g for 1 minute. Elute the sample using 100 microliters of 80% ACN, and 0.1% FA. Centrifuge 100 × g for 3 minutes. This time, keep the flow through. Elute sample in 96 well elution plate. 100 microliters of 80% ACN, 0.1% FA. Centrifuge 100 × *g* for 1 minute, repeat once. The samples were freeze-dried prior to TMT labeling.

Mass spectrometry

About 0.5 µg protein as tryptic peptides dissolved in 2% acetonitrile (ACN), 0.5% FA, were injected into an Ultimate 3000 RSLC system (Thermo Scientific, Sunnyvale, CA, USA) connected online to Orbitrap Eclipse mass spectrometer (Thermo Scientific) equipped with EASY-spray nano-electrospray ion source (Thermo Scientific). For the trapping and desalting process, the sample was loaded and desalted on a pre-column (Acclaim PepMap 100, 2 cm × 75 µm ID nanoViper column, packed with 3 µm C18 beads) at a flow rate of 5 µL/minute for 5 minutes with 0.1% TFA (trifluoroacetic acid). Peptides were separated during a biphasic ACN gradient from two nanoflow UPLC pumps (flow rate of 250 nL/minutes) on a 25 cm analytical column (PepMap RSLC, 50 cm × 75 µm ID EASY-Spray column, packed with 2 µm C18 beads). Solvents A and B were 0.1% FA (vol/vol) in dH2O and 100% ACN, respectively. The gradient composition was 5% B during trapping (5 minutes) followed by 5%–7% B over 30 seconds, 8%–22% B for the next 145 minutes, 22%–28% B over 16 minutes, and 35%–80% B over 15 minutes. Elution of very hydrophobic peptides and conditioning of the column were performed during 15 minutes of isocratic elution with 90% B and 20 minutes of isocratic elution with 5% B, respectively. The eluting peptides from the LC column were ionized in the electrospray and analyzed by the Orbitrap Eclipse. The mass spectrometer was operated in the DDA mode (data-dependent acquisition) to automatically switch between full scan MS and MS/MS acquisition. Instrument control was through Tune 2.7.0 and Xcalibur 4.4.16.14.

Survey full scan MS spectra (from m/z 375 to 1500) were acquired in the Orbitrap with resolution R = 120 000 at m/z 200 after accumulation to a target value of 4e5 in the C-trap, and ion accumulation time was set as auto. FAIMS was enabled using two compensation voltages, –45V and –65V, respectively. During each CV, the mass spectrometer was operated in the DDA mode (data-dependent acquisition) to automatically switch between full scan MS and MS/MS acquisition. The cycle time was maintained at 0.9 s/CV. The most intense eluting peptides with charge states 2 to 6 were sequentially isolated to a target value (AGC) of 2e5 and maximum IT of 120 milliseconds in the C-trap, and isolation width maintained at 0.7 m/z, before fragmentation was performed with a normalized collision energy of 30%, and fragments were detected in the Orbitrap at a resolution of 30 000 at m/z 200, with first mass fixed at m/z 110. The spray and ion-source parameters were as follows: ion spray voltage = 1900 V, no sheath and auxiliary gas flow, and capillary temperature of 275°C.

The raw files were searched in Proteome Discoverer 2.5 and data were curated using the Perseus package.

The raw data were loaded into Protein Discoverer 2.5 (ThermoFisher) and searched against the human database uploaded from Uniprot.org [16]. The results were then preprocessed in Perseus [17]: The data were log2-transformed since proteomics studies normally work with log-transformed values, and verified to be normally distributed which was a prerequisite for subsequent statistics. To assess the strength of the correlation of the protein expression between the samples, Pearson correlation analysis was performed. Of the 793 proteins identified by mass spectrometry, only 494 proteins were considered for further analysis after manual filtering based on valid values. From 7904 values (494 proteins × 16 samples), 831 were missing and replaced using normal distribution.

Data analysis and statistics

A paired two-tailed *t*-test for the 494 proteins was performed using the t.test function in the statistical computing software R [18] version 4.2.2 in order to identify the proteins with significant differential expression (p-values ≤ .05 were considered statistically significant) after 6 hours of sleep deprivation. Multiple hypothesis testing (MHT) was performed using Bon-Ferroni, as well as the less strict Benjamini-Hochberg approach; however, with no significant results. Using a paired *t*-test ensured that the correlation between the two groups (control and sleep deprivation) was accounted for (each participant was their own control) and it reduced the variability within the data and increased the power of the test.

Of the 71 significantly changed proteins found, only those with no more than four values missing (of the 16 values obtained by mass spectrometry for each participant) were considered for further analysis. For the 66 proteins fulfilling these criteria, the fold-change was calculated from the log2-transformed data to compare the relative difference in the protein expression levels of the control and sleep deprivation conditions.

Gene ontology analysis

The SwissProtIDs of the 66 significantly changed proteins were put into two different databases WebGestalt and STRING to perform systems biological analyses.

In WebGestalt, an over-representation Analysis (ORA) was first carried out using our data and the human genome as a reference set as well as default advanced settings (correction for MHT via Benjamini-Hochberg) [19]. By gene ontology (GO) slim classification, the affected biological process, cellular compartment, and molecular function categories were identified. Furthermore, top enriched biological process categories based on their significance level could be pointed out by the ORA.

Secondly, the STRING database with the default settings was used to create a network that showed the interactions between the significantly changed proteins based on known information and statistical calculations [20]. All of the active interaction sources were considered. The network was exported into the open-source software Cytoscape [21] version 3.9.1. After importing the list of fold-changes into Cytoscape as well, the nodes in the network representing the proteins were grouped and colored according to the fold-change after sleep deprivation of the respective proteins. In Cytoscape, network-based functional enrichment analysis with default settings (correction for MHT via FDR) was performed regarding the biological process, cellular compartment, and molecular function categories to identify the biological themes that were overrepresented in the dataset of the significantly changed proteins. The results were compared with those from WebGestalt.

Further analyses were conducted with the ClueGO App [22] version 2.5.9 within Cytoscape that does not rely on protein–protein interactions and is a more traditional approach to identifying enriched functional categories. The functional analysis performed within ClueGO was carried out for the biological process, cellular compartment, molecular function, and immune system process gene ontology categories. The default settings were used (correction for MHT via Bonferroni).

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was performed in the GSEA software [23, 24] version 4.3.2 to find the gene signatures that were significantly enriched and associated with our sleep deprivation data. GSEA focuses on gene sets, pathways, and processes with common biological function or regulation, and tests how they are distributed within a dataset. For the analysis, the accession IDs and intensities of the proteins of the dataset were required. First, the 494 proteins in our dataset were ranked based on the correlation between their expression and the class distinction (in our case control and sleep deprivation). The metric for ranking genes was set to tTest. The number of permutations was set to 250 to match the default from Perseus where our data was preprocessed, and all of the other parameters were left on default (correction for MHT via FDR).

Statistical testing was then carried out on all the different gene sets collections downloaded from the MSig database [25] to determine whether these gene sets were distributed randomly throughout our ranked dataset or if hits were mainly found at the extremes regarding gene expression (lowest or highest degree of expression) which could indicate a correlation between sleep deprivation and the tested gene set. The enrichment score (ES) described the maximum deviation from zero and to which degree the tested gene set was overrepresented at the extremes of our ranked gene expression dataset. The leading-edge subset consisted of genes of the tested gene set that appeared in our ranked dataset at or shortly before the ES and made up the core of the gene set that accounted for the enrichment signal and core enrichment (CE).

By using gene sets instead of single genes or proteins (as in the GO analyses), the strengths of GSEA include higher reproducibility and easier interpretation. It considers all of the genes of an experiment and not only those above an arbitrary cutoff in terms of significance, thereby capturing the collective behavior of the gene expression levels related to health and disease.

Only gene sets created from human data were considered during the evaluation. For the significantly enriched gene signatures associated with our sleep deprivation group, we also analyzed the overlap of the genes of the CE and the gene symbols of our 66 significantly changed proteins after sleep deprivation and were identified with the classical *t*-test in R.

Comparative analysis with other sleep-related datasets

To further analyze our data, the significantly changed proteins after sleep deprivation were made into a separate gene signature by translating our significantly changed proteins back into their corresponding gene symbols and into a GMT file. It was then tested with GSEA how our proteins were distributed in other sleep-related datasets that have been published online (using the same settings as before) and if our gene signature of significantly changed proteins was significantly enriched in any of them. The datasets from different studies and their additional information were found and retrieved via the Proteomics Identifications Database Archive (identifier PXD) [26] and NCBI Gene Expression Omnibus (identifier GSE and GPL) [27]. The distribution of our gene signature of significantly changed proteins after sleep deprivation was tested within the data of the following studies (more details on the individual study protocols can be found in the original papers).

The data with the accession number PXD032734 modeled a study from Cheng et al., 2022 where DEPs in the serum of children with different severities of OSA were identified using a tandem mass tag (TMT) based proteomic analysis [14]. The participants were 2–13 years old, and the study randomly selected 12 serum samples, three samples from each group (mild, moderate, and severe OSA, and control), for proteomics analysis in which they identified 752 proteins. In the process of GSEA, the UniProt Knowledgebase was used throughout for protein searches and ID conversions via the ID mapping tool [16].

To match the methods of this study better with our own, it was also tested which proteins of PXD032734 from Cheng et al. 2022 were differentially expressed in the OSA groups when undergoing a t-test in R, and how these DEPs overlapped with the CE of the GSEA as well as our 66 significantly changed proteins after 6 hours of sleep deprivation at night. However, the difference is that the *t*-tests performed on this other dataset from Cheng et al. 2022 were unpaired since they had different participants for the control and intervention group, whereas the eight participants in our study were their own control.

After our gene signature of significantly changed proteins after sleep deprivation was significantly enriched in the moderate phenotype of OSA (which also leads to sleep deprivation) from the dataset PXD032734 in Cheng et al. 2022, it was tested in another pediatric OSA study from Becker et al. 2014 in which morning and bedtime urine samples were collected from 13 healthy children and 14 children with OSA, who were 2–12 years old [28]. The samples were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis that led to 674 urine proteins being identified across all of the patient samples.

The dataset GSE98582 from Uyhelji et al. 2018 consisted of a study where 17 healthy adults were assigned to two groups that both spent seven consecutive days in a sleep laboratory [29]. After a baseline phase of 2 days with normal sleep of 10 hours each, 11 participants underwent continuous wakefulness for 62 hours (sleep deprivation group) while six control participants continued to have 10 hours of sleep every night. Blood was collected at 12 timepoints (T) at regular intervals to determine the gene expression using Affymetrix GeneChip Human Gene 1.0 ST arrays. T1-4 and 9–12 were during the baseline phase and the recovery phase, respectively, and the participants went to bed at 10 pm (in line with our own sleep protocol). Blood was obtained at 8 am the next day. During the experimental phase (starting at 8 am on day three), T5-8 marked different sleep deprivation durations for the sleep deprivation group on day 4: 10, 14, 18, and 22 hours of sleep deprivation, respectively. In this study, 8623 peptides were found in total, and a gene symbol could be assigned for 8007 of them.

The Affymetrix IDs were converted to gene symbols using R and the respective file from platform GPL6244 (Affymetrix Human Gene 1.0 ST Array) so that the data could be used for GSEA. Our gene signature was tested in each of the timepoints 5–8 in order to see if it was enriched in any of these conditions.

Results

DEPs (66) after one night of sleep deprivation

In this study, 793 proteins were identified in human blood serum by Orbitrap Eclipse mass spectrometry analysis. The Pearson correlation analysis that was performed showed a relatively good correlation between the samples with correlation coefficients from 0.86 to 0.98 (Figure 1). After filtering criteria and adjustments, as described in the materials and methods section, out of 494 proteins identified and considered for further analysis, 66 proteins were found to be significantly differentiated by the paired t-test in R after 6 hours of sleep deprivation at night (uncorrected *p*-value ≤ .05; Table 1). Out of 66 proteins, 63 proteins were upregulated, and three proteins were downregulated.

The protein-protein interaction network that was created in STRING and Cytoscape for the 66 proteins significantly changed (Figure 2) showed that 61 of the 66 proteins were linked to one or more proteins based on databases (e.g. text mining), experiments, and predicted interactions such as protein homology, gene neighborhood, or co-expression.

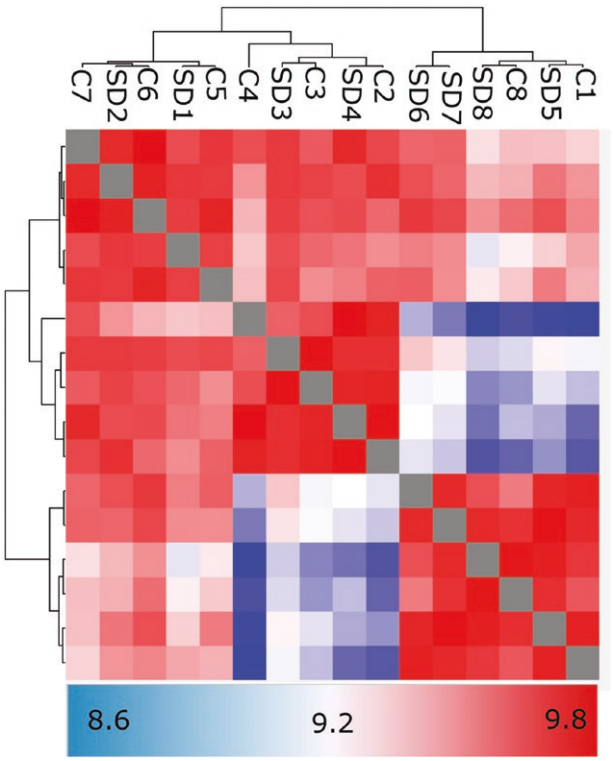


Figure 1. Column Pearson correlation analysis performed in Perseus, indicating a high correlation between the serum samples. C for the control sample and SD for the sleep deprivation sample. The scale represents the correlation coefficient were 10 is 100% correlation.

Enriched proteins after 6 hours of sleep deprivation are involved in wound healing and metabolism

Functional enrichment analyses performed in WebGestalt and Cytoscape using the ClueGO plugin regarding the biological process, cellular function, and molecular function categories revealed the most affected GO categories (Supplementary Figure S1a and S1b) based on the 66 DEPs after sleep deprivation. There was a large overlap between the results from WebGestalt and those from Cytoscape: more than 75% of the proteins were involved in the following biological process categories: biological regulation, regulation of the cellular process, response to stimulus and metabolic process, and in the cellular compartment categories extracellular space and (membrane-bound) organelle. Two of the categories of molecular function, protein binding, and ion binding, included more than half of the proteins.

In WebGestalt, GO analysis revealed the top 5 enriched biological processes (based on the significance level) of the DEPs after sleep deprivation that were related to the categories of protein activation cascade, platelet degranulation, (blood) coagulation, and hemostasis (Table 2). Three of the sixty-six proteins were involved in all the top 5 enriched biological processes: APOH, KNG1 and SERPING1.

Further functional analysis was carried out in the Cytoscape plug-in ClueGO and revealed enriched GO groups in the categories biological process, cellular compartment, molecular function, and immune system process, as well as the most represented terms within these groups having the greatest number of our DEPs involved (Table 3). In the biological process and cellular compartment categories, around two-thirds of the terms of the individual GO categories were associated with either wound

Table 1. Significantly Changed Proteins (66) in Human Serum After 6 Hours of Sleep Deprivation

SwissprotID	Gene symbol	Protein name	*P*-value	Fold-change (log2)	# of peptides
Upregulated proteins (63)					
P06727	APOA4	Apolipoprotein A-IV	.0010	1.016	45
P08514	ITGA2B	Integrin alpha-IIb	.0025	1.107	8
O43852	CALU	Calumenin	.0028	1.054	6
P05556	ITGB1	Integrin beta-1	.0035	1.053	5
Q6ZNG0	ZNF620	Zinc finger protein 620	.0042	1.037	1
Q14515	SPARCL1	SPARC-like protein 1	.0055	1.051	11
Q9NPY3	CD93	Complement component C1q receptor	.0073	1.099	6
P06733	ENO1	Alpha-enolase	.0075	1.070	4
P02549	SPTA1	Spectrin alpha chain, erythrocytic 1	.0081	1.028	1
A1L4H1	SSC5D	Soluble scavenger receptor cysteine-rich domain-containing protein SSC5D	.0122	1.044	1
P21926	CD9	CD9 antigen	.0123	1.095	1
Q13201	MMRN1	Multimerin-1	.0125	1.033	14
Q8WUA8	TSKU	Tsukushi	.0125	1.063	3
P04180	LCAT	Phosphatidylcholine-sterol acyltransferase	.0133	1.030	10
Q14766	LTBP1	Latent-transforming growth factor beta-binding protein 1	.0140	1.047	18
P24593	IGFBP5	Insulin-like growth factor-binding protein 5	.0143	1.047	6
P09172	DBH	Dopamine beta-hydroxylase	.0146	1.034	15
Q15113	PCOLCE	Procollagen C-endopeptidase enhancer 1	.0156	1.037	8
P10909	CLU	Clusterin	.0166	1.020	21
Q6UXB8	PI16	Peptidase inhibitor 16	.0168	1.040	8
P22891	PROZ	Vitamin K-dependent protein Z	.0178	1.022	10
Q15582	TGFBI	Transforming growth factor-beta-induced protein ig-h3	.0178	1.022	16
Q03591	CFHR1	Complement factor H-related protein 1	.0179	1.028	12
O95445	APOM	Apolipoprotein M	.0180	1.008	11
P35443	THBS4	Thrombospondin-4	.0213	1.070	10
Q12860	CNTN1	Contactin-1	.0214	1.056	7
P08253	MMP2	72 kDa type IV collagenase	.0226	1.029	11
O00533	CHL1	Neural cell adhesion molecule L1-like protein	.0246	1.048	22
P05109	S100A8	Protein S100-A8	.0248	1.064	2
P04114	APOB	Apolipoprotein B-100	.0251	1.004	309
P10720	PF4V1	Platelet factor 4 variant	.0254	1.021	5
Q5SYB0	FRMPD1	FERM and PDZ domain-containing protein 1	.0262	1.027	1
Q92496	CFHR4	Complement factor H-related protein 4	.0285	1.032	9
P35542	SAA4	Serum amyloid A-4 protein	.0290	1.017	5
Q06033	ITIH3	Inter-alpha-trypsin inhibitor heavy chain H3	.0292	1.019	24
P33151	CDH5	Cadherin-5 OS = Homo sapiens	.0298	1.030	14
P07359	GP1BA	Platelet glycoprotein Ib alpha chain	.0298	1.038	7
P00746	CFD	Complement factor D	.0309	1.028	11
P98160	HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein	.0311	1.059	24
P12111	COL6A3	Collagen alpha-3(VI) chain	.0313	1.043	27
Q07954	LRP1	Prolow-density lipoprotein receptor-related protein 1	.0330	1.054	9
P01042	KNG1	Kininogen-1	.0331	1.019	42
Q6UY14	ADAMTSL4	ADAMTS-like protein 4	.0344	1.154	4
P07996	THBS1	Thrombospondin-1	.0350	1.020	53
P04196	HRG	Histidine-rich glycoprotein	.0356	1.020	28
P23142	FBLN1	Fibulin-1	.0359	1.021	21

Table 1. Continued

SwissprotID	Gene symbol	Protein name	P-value	Fold-change (log2)	# of peptides
P02649	APOE	*Apolipoprotein E*	.0374	1.016	24
Q13093	PLA2G7	*Platelet-activating factor acetylhydrolase*	.0376	1.091	3
P02776	PF4	*Platelet factor 4*	.0390	1.016	4
P00748	F12	*Coagulation factor XII*	.0395	1.015	21
P13473	LAMP2	*Lysosome-associated membrane glycoprotein 2*	.0411	1.040	4
P02765	AHSG	*Alpha-2-HS-glycoprotein*	.0416	1.022	13
P00734	F2	*Prothrombin*	.0425	1.016	37
P51884	LUM	*Lumican*	.0430	1.020	13
P02753	RBP4	*Retinol-binding protein 4*	.0434	1.016	12
Q9NZP8	C1RL	*Complement C1r subcomponent-like protein*	.0441	1.024	11
Q92859	NEO1	*Neogenin*	.0444	1.070	3
P02749	APOH	*Beta-2-glycoprotein 1*	.0451	1.020	21
P05155	SERPING1	*Plasma protease C1 inhibitor*	.0457	1.020	21
Q12913	PTPRJ	*Receptor-type tyrosine-protein phosphatase eta*	.0469	1.125	5
P02766	TTR	*Transthyretin*	.0469	1.021	11
O00391	QSOX1	*Sulfhydryl oxidase 1*	.0482	1.025	20
Q8NBP7	PCSK9	*Proprotein convertase subtilisin/kexin type 9*	.0484	1.052	6
Downregulated proteins (3)					
Q9NWX6	THG1L	Probable tRNA(His) guanylyltransferase	.0332	0.962	1
O00187	MASP2	*Mannan-binding lectin serine protease 2*	.0380	0.969	12
P0DJI8	SAA1	*Serum amyloid A-1 protein*	.0420	0.927	4

All significantly changed proteins in human serum after 6 hours of sleep deprivation with no more than four missing values (out of 16), up- (63) and downregulated (3) with p-values ≤ .05. Each protein is listed with their SwissProtID, gene symbol and protein name. The fold-change (log2) shows the degree of change in the protein concentration in the sleep deprivation samples compared to the control samples. The number of peptides indicates how many peptides have been found for the respective protein. Proteins that have been identified by two or more peptides are shown in italics. The adjusted p-values after the Bonferroni correction, as well as after the less strict Benjamini-Hochberg correction, were all non-significant, which is why they are not listed here. This was to be expected due to the low sample size of this pilot study and the large number of proteins tested in the analysis.

healing or cholesterol transport as well as high-density lipoprotein particle or vesicle lumen, respectively. For the molecular function categories, half of the terms were associated with groups within which lipoprotein particle receptor binding was the most represented function. Granulocyte chemotaxis comprised more than two-thirds of the terms as the most represented process in the immune system process categories.

Enriched gene signatures identified with GSEA map changes in molecular pathways caused by 6 hours of sleep deprivation

There were 24 gene sets/signatures with common biological function or regulation that were significantly enriched (p-value ≤ .05) within the sleep deprivation condition of our ranked dataset of 494 proteins and overrepresented at the extremes (top or bottom) of it, indicating a correlation between the respective gene sets and our dataset (Supplementary Table S1). In half of these gene signatures, the CE compromised ≥ 30% of our significantly changed proteins after sleep deprivation.

From the GO collections, such notable enriched gene sets were the regulation of the protein catabolic process, transmembrane receptor protein serine-threonine kinase signaling pathway and muscle structure development in the GO biological process category, vesicle membrane, (plasma) membrane protein complex and endoplasmic reticulum lumen in the GO cellular compartment category, and growth factor binding in the GO molecular function category. In the cell type signature gene sets collection, the CE of a gene set associated with skeletal muscle endothelial cells consisted of almost half of our DEPs. They also compromised a third of the CE of a gene set related to the up-regulated genes in anaplastic thyroid carcinoma (compared to normal thyroid tissue).

With one of our main focuses being on the altered biological processes caused by sleep deprivation, we looked further into those gene sets: 6 of the 24 significantly enriched signatures were part of the GO biological process collection and the most abundant protein from our list of significantly changed proteins within the CE of these gene sets was LRP1, as part of the CE in four of the six gene sets. PCSK9, ITGB1, and LTBP1 were each involved in two of the six gene sets.

The 66 DEPs after 6 hours of sleep deprivation gave a signature related to the protein profile of moderate OSA

Testing the distribution of the gene signature of our 66 DEPs after sleep deprivation within PXD032734 from Cheng et al. 2022 revealed that it was significantly enriched in the phenotype moderate OSA (p-value < .01), but not in the other two phenotypes, mild and severe OSA. In the moderate condition, 15 of our 66 significantly changed proteins compromised the core of the gene signature that accounted for the enrichment signal (Figure 3). The shift

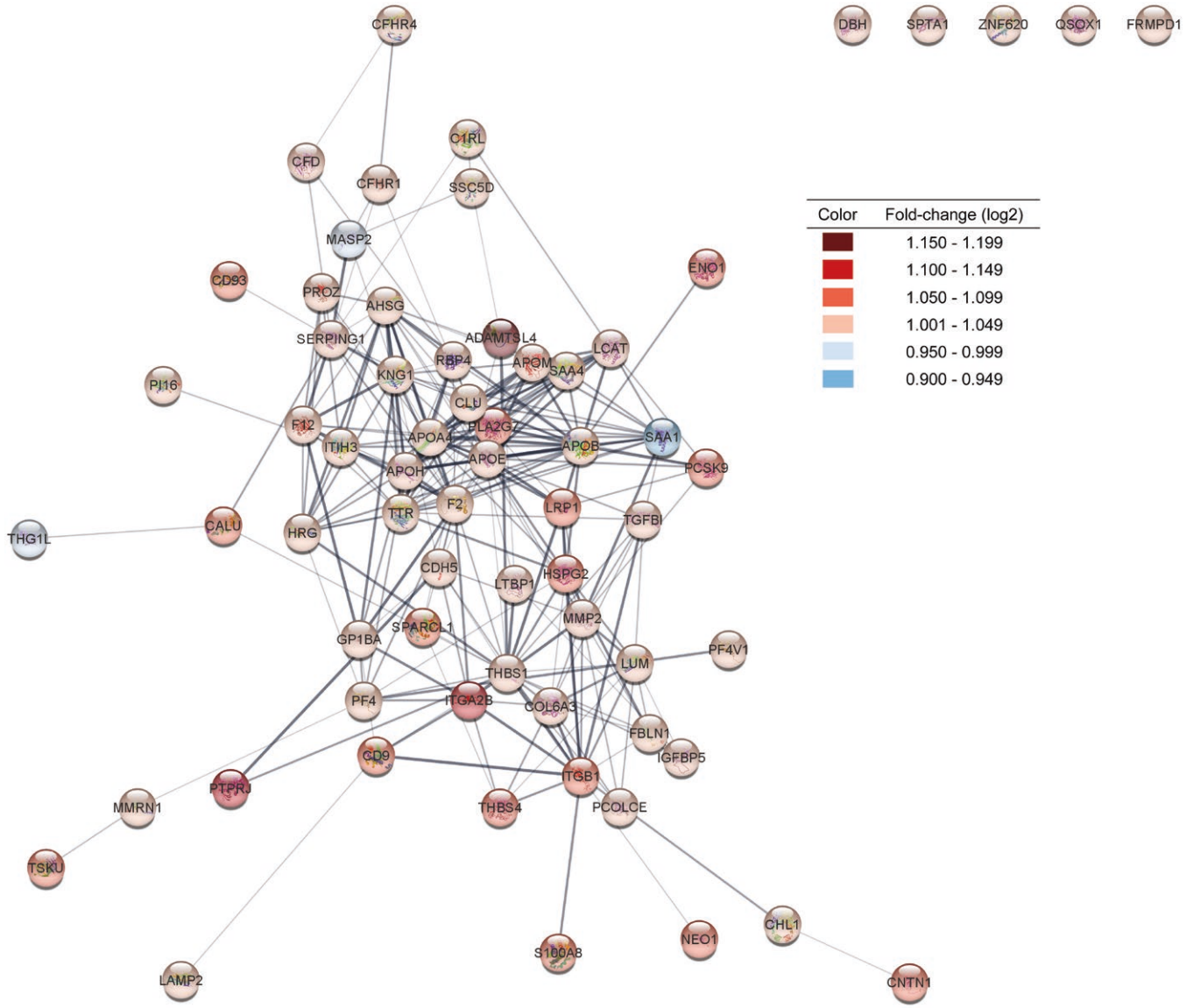


Figure 2. Protein-protein interaction (PPI) network of the significantly differentially expressed proteins after 6 hours of sleep deprivation. Each node in the figure represents a protein described with its gene symbol and the lines between the boxes represent physical or functional protein-protein interactions. The different shades of the nodes represent the respective degree of fold-change (log2) of the protein after 6 hours of sleep deprivation at night (higher saturation corresponds to a higher fold-change). PPI enrichment score *p*-value < 1.0e-16. The network was created using STRING database version 12 and modified in Cytoscape version 3.9.

of the hits toward the left shows the positive correlation between the upregulated proteins of the moderate OSA condition and our DEPs after sleep deprivation, which were also mostly upregulated. Two of the fifteen proteins of the CE, APOH, and TTR, were also significantly upregulated (p-value ≤ .05) in moderate OSA versus control when testing the PXD032734 data in an unpaired t-test in R.

When testing the distribution of our gene signature in the dataset from Becker et al. 2014, the enrichment was not significant. However, an overlap of five of the nine proteins of the core enriched genes from Becker et al. 2014, with the CE proteins from Cheng et al. 2022 could be observed: RBP4, AHSG, APOH, TTR, and IGFBP5.

Our gene signature was also not significantly enriched within the GSE98582 sleep deprivation data from Uyhelji et al. 2018. However, when testing the gene signature within the data of timepoint 6 (14 hours of sleep deprivation), the family-wise error rate (the probability of making at least one error among all the tests conducted) and, therefore, corrected for MHT, was the lowest (0.144) of all the timepoints and the enrichment plot of the GSEA showed a trend toward a positive correlation of the upregulated proteins of the sleep deprivation condition and our gene signature (Supplementary Figure S1).

Discussion

In this study of human blood serum, out of 494 proteins, 66 were found to be differentially expressed after 6 hours of sleep deprivation at night, 63 upregulated and three downregulated. We suggest that these proteins could serve as biomarkers for sleep deprivation and be considered as a part of a protein signature for OSA.

Sleep deprivation of 6 hours at night affects coagulation, hemostasis, platelet, and endothelial function

The use of systems biological databases enabled us to find possible clinically relevant trends in cellular and molecular changes reflected in serum level changes of DEPs in our dataset. Both

Table 2. Top Five Enriched Biological Processes of the 66 Significantly Changed Proteins

Biological process	Enrichment ratio (ER)	Proteins involved (gene symbol)
Protein activation cascade	36.8	APOH, C1RL, CFD, CFHR1, CFHR4, CLU, GP1BA, F2, F12, FBLN1, KNG1, MASP2, SERPING1
Platelet degranulation	30.8	AHSG, APOH, CD9, CFD, CLU, HRG, ITGA2B, ITIH3, KNG1, LAMP2, MMRN1, PF4, QSOX1, SERPING1, THBS1
Blood coagulation	13.7	APOE, APOH, CD9, F2, F12, FBLN1, GP1BA, HRG, ITGA2B, KNG1, MMRN1, PF4, PF4V1, PROZ, SAA1, SERPING1, THBS1
Coagulation	13.6	APOE, APOH, CD9, F2, F12, FBLN1, GP1BA, HRG, ITGA2B, KNG1, MMRN1, PF4, PF4V1, PROZ, SAA1, SERPING1, THBS1
Hemostasis	13.5	APOE, APOH, CD9, F2, F12, FBLN1, GP1BA, HRG, ITGA2B, KNG1, MMRN1, PF4, PF4V1, PROZ, SAA1, SERPING1, THBS1

The processes are sorted by the highest enrichment ratio (ER). The ER describes the proportion of proteins in our dataset of 66 proteins that belong to a particular biological process divided by the proportion of genes in the background set that belong to the same process. The respective associated significantly changed proteins after sleep deprivation are listed with their gene symbols. FDR ≤ 0.05 for all biological processes (values given in Cytoscape per default).

findings from Webgestalt and ClueGO (Cytoscape) indicate a change in the biological processes of wound healing and coagulation after sleep deprivation. In our study, APOH, KNG1, and SERPING1 were upregulated after sleep deprivation and were all involved in the wound healing ClueGO term as well as the top 5 enriched biological processes in Webgestalt ranked by the significance level and that relate to the categories of protein activation cascade, platelet degranulation, blood coagulation, and hemostasis. KNG1 was also found to be significantly upregulated in the serum of adults with OSA [30] and strongly expressed in the serum of rats in chronic sleep deprivation [31]. Kininogen 1 (KNG1) is a proinflammatory mediator that is essential for the intrinsic blood coagulation pathway and its higher expression has been associated with several cardiovascular diseases such as pulmonary artery hypertension and thrombosis [32–34] as well as neurodegenerative diseases such as Alzheimer's and Parkinson's disease [35–37]. Serpin Family G Member 1 (SERPING1), also increased in the present study, exhibits protease inhibitory activity and thereby negatively regulates coagulation, complement activation, and vascular endothelial permeability [38] and it was also found to be related to chronic sleep deprivation and cardiovascular function [31]. However, in our previous study, Bjørkum et al. 2021, SERPING1 was decreased. This was in line with two earlier reports by Thompson et al. 2010 after 6 hours of sleep deprivation [39] and by Becker et al. 2014, in urine from a pediatric OSA study. We have seen discrepancies in both our own and other studies regarding the possible biomarkers for sleep deprivation most likely due to different sleep deprivation protocols and sleep disorders resulting in a different amount of lack of sleep, comorbidities, preanalytical and methodological issues as well as the measurement of the gene transcripts or proteins in different tissues or body fluids. However, the only solution is to repeat and continue to try to nail down the real effects of sleep deprivation in these different contexts for being able to have more certainty over time to reveal the affected cellular and molecular changes after sleep deprivation in different cells and tissues as well as in body fluids such as serum, which is one of the most used clinical analytical specimens.

Two other proteins, Prothrombin (F2) and Platelet factor 4 (PF4), which were both involved in four of the top 5 enriched biological processes in Webgestalt and the wound healing term in ClueGO, were also found to be significantly upregulated in the proteomic profiles of patients with insomnia [40]. Our results match those of another insomnia study where PF4 was again significantly overexpressed in patients with insomnia compared to controls [41], and those from a study where sleep deprivation in rats led to higher release levels of PF4 and altered platelet activation [42]. Prothrombin (F2) plays a critical role in blood coagulation processes as well as platelet activation and is activated by thrombin in the final enzymatic step of the coagulation pathway. That is also important, in addition to the coagulation process, in biological processes such as inflammation and atherosclerosis [43]. The consequent massive thrombin generation holds a large risk of thromboembolic complications with high morbidity and mortality [44].

Sleep deprivation of 6 hours at night affects lipid- and cholesterol profile and transport

Analyses performed with ClueGO in the GO biological process category further showed altered cholesterol transport function after sleep deprivation. Altered lipid metabolism and compromised lipid transport were shown to affect neurons and glial cells and they are bidirectionally linked to sleep duration and circadian rhythms [45]. Two of our significantly changed proteins that were associated with this ClueGO term, low-density lipoprotein receptor-related protein 1 (LRP1) and proprotein convertase subtilisin-kexin 9 (PCSK9), were also two of the four most abundant proteins of our DEPs after sleep deprivation within the CE of the GO biological process gene sets that were significantly enriched when testing our data in GSEA. The proprotein convertase subtilisin-kexin nine is involved in systemic inflammation, endothelial dysfunction, and the development of cardiovascular diseases. Among other mechanisms, PCSK9 can bind to LRP1 to promote lipoprotein concentration [46]. Inhibitors of PCSK9 are used worldwide to treat hypercholesterolemia patients by lowering lipid concentrations as well as to reduce the incidence of atherosclerosis [47].

Our findings are also associated with changes in the immune responses/activation of inflammation associated with immune-associated thrombosis events, atherosclerosis, or cardiovascular diseases. In addition, low-density lipoprotein receptor-related protein 1 (LRP1) is also a major receptor for APOE, which was among our DEPs after sleep deprivation, and is involved in Aβ clearance and turnover in the brain as well as connected to Alzheimer's disease in which Aβ accumulation is one of the pathological hallmarks [48]. Apolipoprotein E (APOE) transports triglycerides and cholesterol in multiple tissues and, thereby, plays an essential role in lipoprotein metabolism. High plasma levels of APOE appear to be associated with a higher risk of

Table 3. Gene Ontology (GO) Biological Process, Cellular Component, Molecular Function and Immune System Process Groups of the 66 Significantly Changed Proteins

Group	# of terms	% of terms	Most represented term	P-value	# of proteins	Associated proteins (gene symbols)
GO biological process						
Group11	63	43.15	*Wound healing*	$1.75 \cdot 10^{-14}$	20	*APOE, APOH, CD9, CNTN1, F12, F2, FBLN1, GP1BA, HRG, ITGB1, KNG1, MMRN1, PF4, PF4V1, PROZ, S100A8, SAA1, SERPING1, THBS1, TSKU*
Group10	38	26.03	*Cholesterol transport*	$2.40 \cdot 10^{-8}$	9	*APOA4, APOB, APOE, APOM, CLU, LCAT, LRP1, PCSK9, TSKU*
Group9	10	6.85	Positive regulation of wound healing	$2.52 \cdot 10^{-7}$	7	APOH, CNTN1, F12, F2, HRG, ITGB1, THBS1
Group8	9	6.16	Cholesterol transport	$2.40 \cdot 10^{-8}$	9	APOA4, APOB, APOE, APOM, CLU, LCAT, LRP1, PCSK9, TSKU
Group7	8	5.48	Cholesterol transport	$2.40 \cdot 10^{-8}$	9	APOA4, APOB, APOE, APOM, CLU, LCAT, LRP1, PCSK9, TSKU
Group6	7	4.79	Negative regulation of endothelial cell migration	$1.73 \cdot 10^{-3}$	4	APOE, APOH, HRG, THBS1
Group5	4	2.74	Regulation of transforming growth factor beta1 production	$4.69 \cdot 10^{-4}$	4	LTBP1, LUM, THBS1, TSKU
Group4	2	1.37	Acute inflammatory response	$4.01 \cdot 10^{-5}$	7	AHSG, CNTN1, F12, F2, S100A8, SAA1, SAA4
Group3	2	1.37	Vascular associated smooth muscle cell proliferation	$1.04 \cdot 10^{-2}$	3	DBH, IGFBP5, MMP2
Group2	1	0.68	Complement activation	$8.47 \cdot 10^{-5}$	7	C1RL, CFD, CFHR1, CFHR4, CLU, MASP2, SERPING1
Group1	1	0.68	Cell adhesion mediated by integrin	$5.88 \cdot 10^{-3}$	4	HRG, ITGA2B, ITGB1, MMRN1
Group0	1	0.68	Cysteine-type endopeptidase inhibitor activity	$1.20 \cdot 10^{-2}$	3	AHSG, HRG, KNG1
GO cellular compartment						
Group6	8	40.00	*High-density lipoprotein particle*	$5.02 \cdot 10^{-17}$	10	*APOA4, APOB, APOE, APOH, APOM, CLU, LCAT, PLA2G7, SAA1, SAA4*
Group5	5	25.00	*Vesicle lumen*	$9.14 \cdot 10^{-13}$	16	*AHSG, APOB, APOH, CFD, CLU, DBH, HRG, ITIH3, KNG1, MMRN1, PF4, QSOX1, S100A8, SERPING1, THBS1, TTR*
Group4	3	15.00	Extracellular matrix	$1.42 \cdot 10^{-23}$	28	ADAMTSL4, AHSG, APOA4, APOE, APOH, CLU, CNTN1, COL6A3, F12, F2, FBLN1, GP1BA, HRG, HSPG2, KNG1, LTBP1, LUM, MMP2, MMRN1, PCOLCE, PF4, S100A8, SERPING1, SPARCL1, SSC5D, TGFBI, THBS1, THBS4
Group3	1	5.00	Ficolin-1-rich granule membrane	$1.54 \cdot 10^{-3}$	3	CD93, CLU, LAMP2
Group2	1	5.00	Blood microparticle	$5.04 \cdot 10^{-11}$	11	AHSG, APOA4, APOE, C1RL, CFHR1, CLU, F2, HRG, ITGA2B, KNG1, SERPING1
Group1	1	5.00	Platelet dense granule	$1.73 \cdot 10^{-4}$	3	APOH, ITIH3, LAMP2
Group0	1	5.00	Endoplasmic reticulum lumen	$6.51 \cdot 10^{-16}$	18	ADAMTSL4, AHSG, APOA4, APOB, APOE, CALU, CLU, COL6A3, F2, IGFBP5, KNG1, LTBP1, PCSK9, PROZ, QSOX1, SERPING1, SPARCL1, THBS1
GO molecular function						
Group11	11	33.33	Lipoprotein particle receptor binding	$4.17 \cdot 10^{-8}$	6	APOB, APOE, CLU, HSPG2, LRP1, PCSK9
Group10	7	21.21	*Lipoprotein particle receptor binding*	$4.17 \cdot 10^{-8}$	6	*APOB, APOE, CLU, HSPG2, LRP1, PCSK9*
Group9	2	6.06	Opsonin binding	$2.42 \cdot 10^{-5}$	4	CD93, CFHR1, CFHR4, MASP2
Group8	2	6.06	Fibronectin binding	$2.42 \cdot 10^{-8}$	6	FBLN1, IGFBP5, ITGB1, MMP2, SSC5D, THBS1
Group7	2	6.06	Apolipoprotein binding	$7.09 \cdot 10^{-4}$	3	LCAT, LRP1, PCSK9
Group6	2	6.06	Serine-type endopeptidase activity	$1.08 \cdot 10^{-9}$	13	C1RL, CFD, CNTN1, COL6A3, F12, F2, HRG, ITIH3, MASP2, MMP2, PCSK9, PROZ, SERPING1
Group5	2	6.06	Heparin binding	$6.03 \cdot 10^{-11}$	12	APOB, APOE, APOH, F2, HRG, KNG1, PCOLCE, PF4, PF4V1, SAA1, THBS1, THBS4

Table 3. Continued

Group	# of terms	% of terms	Most represented term	P-value	# of proteins	Associated proteins (gene symbols)
Group4	1	3.03	Cysteine-type endopeptidase inhibitor activity	$2.86 \cdot 10^{-3}$	3	AHSG, HRG, KNG1
Group3	1	3.03	Fibrinogen binding	$3.16 \cdot 10^{-6}$	3	CDH5, FBLN1, THBS1
Group2	1	3.03	Transforming growth factor beta binding	$1.21 \cdot 10^{-3}$	3	LTBP1, THBS1, TSKU
Group1	1	3.03	Collagen binding	$3.71 \cdot 10^{-6}$	6	ITGB1, LUM, PCOLCE, SPARCL1, TGFBI, THBS1
Group0	1	3.03	Integrin binding	$3.45 \cdot 10^{-5}$	7	CD9, FBLN1, ITGA2B, ITGB1, TGFBI, THBS1, THBS4
GO immune system process						
Group2	5	71.43	Granulocyte chemotaxis	$1.13 \cdot 10^{-2}$	6	PF4, PF4V1, S100A8, SAA1, THBS1, THBS4
Group1	1	14.29	Antimicrobial humoral immune response mediated by antimicrobial peptide	$2.47 \cdot 10^{-2}$	4	F2, HRG, PF4, PF4V1
Group0	1	14.29	Complement activation	$3.28 \cdot 10^{-3}$	7	C1RL, CFD, CFHR1, CFHR4, CLU, MASP2, SERPING1

Functional analysis performed with the Cytoscape plug-in ClueGO. All *p*-values listed were adjusted for multiple hypothesis testing via Bonferroni correction (default setting in ClueGO) and $p \leq .05$. The number of terms describes how many processes, components or functions are represented in each respective group of the GO category and the percentage of terms describes the relative representation of each group. Groups that have ≥ 20% of the terms assigned to them are marked in italics. The most represented term in every group of each GO category is the term with the greatest number of significantly changed proteins involved of all the terms in the group. The respective gene symbols of the proteins involved are listed in the associated proteins column. If there were multiple terms with the same number of proteins involved in a group, the term with the higher percentage of associated genes found in ClueGO was listed.

ischemic heart disease, possibly mediated by triglyceride-rich lipoproteins [49]. APOE is also closely related to the nervous system and the regulation of expression has important connections to several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis. However, many associations between APOE and the risk of pathogenesis in those diseases remain unclear [50].

Sleep deprivation of 6 hours at night affects immune- and cell-proliferating mechanisms

Of all the DEPs after 6 hours of sleep deprivation, the protein ADAMTSL4 showed the highest fold-change compared to the control condition. ADAMTS-like (ADAMTSL) proteins are secreted glycoproteins residing in the extracellular matrix that have crucial roles in major biological pathways reflected in evolutionary conservation [51]. ADAMTSL4 was significantly increased in our study and is strongly associated with immune-related biological processes in glioblastoma multiforme (GBM, WHO grade IV), making it a promising prognostic biomarker for primary GBM [52]. Evidence has been provided that ADAMTSL4 also has the potential to serve as a diagnostic and prognostic indicator for Burkitt lymphoma, one of the most aggressive forms of non-Hodgkins lymphomas [53]. Hong et al. 2023 showed that ADAMTSL4 was expressed at higher levels in thyroid cancer [54]. In alignment with the results from Hong et al. 2023, we found a gene signature of anaplastic thyroid carcinoma to be significantly enriched within our dataset of 494 proteins using GSEA. From our DEPs, two other interesting proteins were found in the CE of this gene set: ENO1 and ITGB1. Alpha-enolase (ENO1) is a multifunctional oncoprotein whose overexpression can be observed in a variety of cancer types and that makes it an important cancer biomarker [55]. ENO1 enhances processes such as cancer cell proliferation and invasiveness via different signaling pathways [56]. As a subfamily of integrins (cell surface receptors), integrin-beta 1 (ITGB1) also plays an important role in cancer. The ITGB1-induced focal adhesion kinase pathway causes the upregulation of anti-apoptotic proteins and is associated with critical processes such as migration and angiogenesis in several cancer types [57].

Therefore, an association of the overexpression of proteins such as ADAMTSL4, ENO1, and ITGB1 with various tumor phenotypes indicates how sleep deprivation might affect tumor development and progression.

Sleep deprivation of 6 hours at night can affect neuronal and muscular functions

Several gene sets (24) from the Molecular Signatures Database (MSigDB, https://www.gsea-msigdb.org/gsea/msigdb) were found to be significantly enriched in the sleep deprivation condition of our dataset of 494 proteins when testing them in GSEA. One enriched gene set from the GO biological process category was the transmembrane receptor protein serine-threonine kinase signaling pathway. Cyclin-dependent kinase-like 5 (CDKL5), which is a gene encoding a serine-threonine kinase, regulates neuronal migration, axonal growth, and synaptic development [58]. The characteristics of CDKL5 deficiency are severe sleep disruption [59] and sleep apnea [60]. The gene sets "protein catabolic process" and "muscle structure development" from the GO biological process category, the gene set "growth factor binding" from the GO molecular function category and a gene set associated with skeletal muscle endothelial cells were also found to be enriched within our dataset.

Sleep deprivation of 6 hours at night versus OSA—comparison of proteomic datasets and single proteins

Moreover, our signature of DEPs after 6 hours of sleep deprivation was found to be significantly enriched within the moderate pediatric OSA condition of the proteomic dataset from Cheng et al. 2022, indicating an overlap and correlation between their OSA dataset and our sleep deprivation dataset. In the present study,

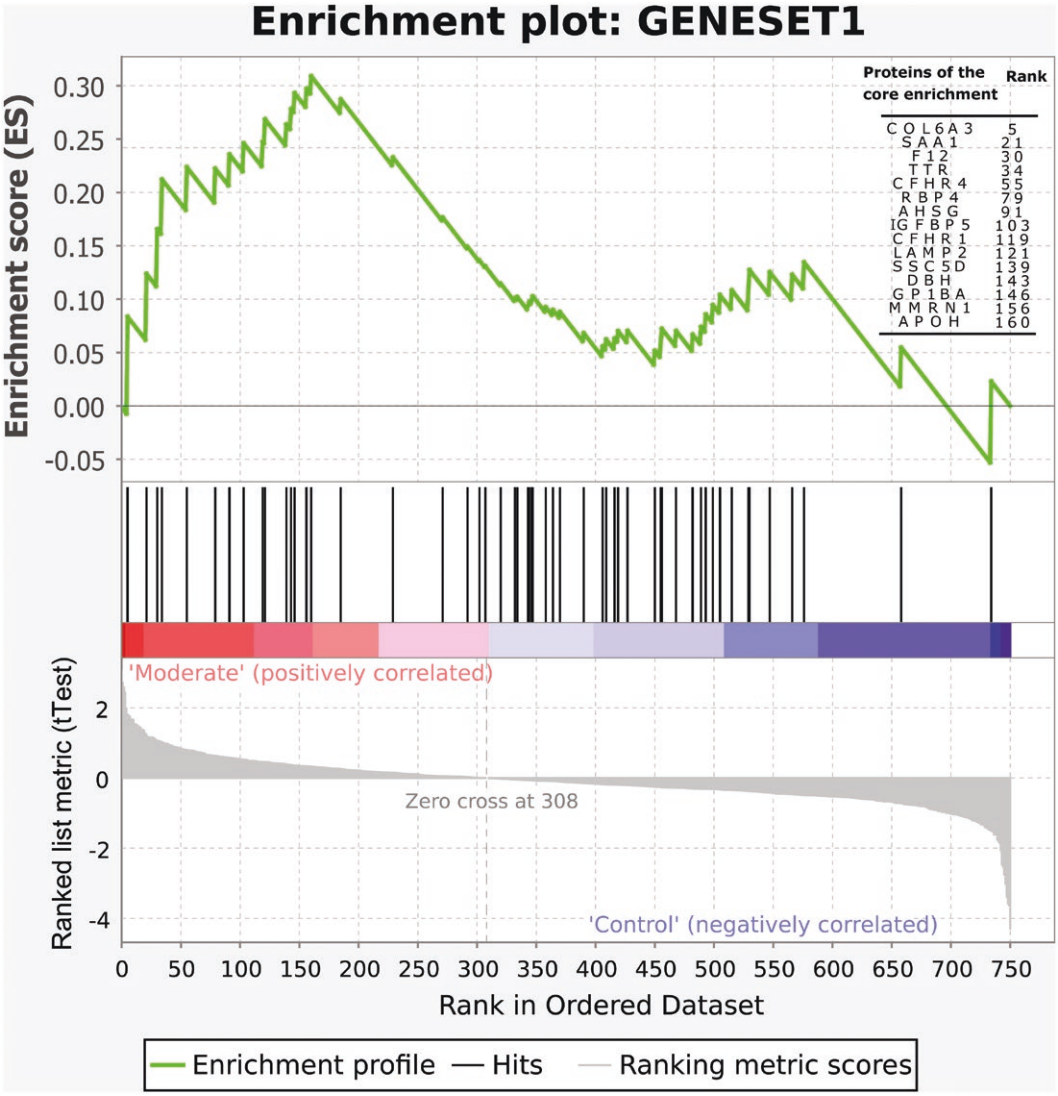


Figure 3. Enrichment plot of our gene signature of significantly changed proteins within moderate OSA phenotype of the ranked dataset PXD032734 from Cheng et al. 2022. The metric for ranking the proteins of the data were set to tTest within GSEA. The ranking causes the upregulated proteins of the moderate OSA condition to be on the left, the unchanged proteins in the middle, and the downregulated on the right. The vertical lines represent hits with proteins from our gene signature. The proteins of the core enrichment are listed with their gene symbols along with their rank.

when testing our DEPs towards the OSA dataset, the CE was made up of 15 of our 66 DEPs of which some will be discussed in the following. Furthermore, Cederberg et al. 2022 used plasma to identify and quantify proteins assayed using the SomaScan aptamer-based multiplexed platform (SomaLogic Inc., Boulder, CO, USA), which utilizes aptamers and hybridization to quantify proteins from small amounts of human plasma, and found some of the respective assay proteins changed in OSA also related to pathways involving endothelial function, blood coagulation, and inflammatory response which is in line with our findings in this pilot study.

From the 15 proteins, APOH and transthyretin (TTR) were also upregulated when testing the data from Cheng et al. 2022 in an unpaired *t*-test in R. In addition, TTR was overexpressed in a study where they tested serum proteomic changes in adults with OSA [30], in which the APOH serum levels were significantly higher in Chinese males with OSA and hypopnea [61]. Transthyretin (TTR) is a highly conserved protein that can bind to thyroid hormones and retinol-binding proteins, and plasma TTR is mainly secreted by the liver. Although TTR is negatively correlated with inflammation, cholesterol can bind to TTR and promote TTR aggregation/amyloid formation that contributes to oxidative stress and inflammation [62]. Furthermore, an independent association between OSA severity and higher total cholesterol were reported [63]. Apart from its carrier functions, several functions of TTR in the nervous system have also been proposed and TTR levels were found to be elevated in the plasma and cerebrospinal fluid of neuronal pathologies such as frontotemporal dementia [64] and Parkinson's disease [65]. However, the role of TTR in Alzheimer's disease (AD) is controversial as some studies report a protective role of TTR in AD [66].

COL6A3 was the protein with the highest rank of the 15 proteins in the CE when ranking them with a tTest in GSEA, thereby being one of the most upregulated proteins of the moderate OSA condition of the Cheng et al. 2022 dataset. The extracellular matrix protein collagen VI isoform alpha 3 (COL6A3) has been reported to be involved in fibrosis and inflammation in adipose tissue and the upregulated expression of COL6A3 in adipocytes

has been associated with insulin resistance [67]. Furthermore, COL6A3 was increased in osteosarcoma tissues and thought to create beneficial conditions for the development of tumors by contributing to the remodeling of the extracellular matrix as well as activating the PI3K/AKT pathway, which is one of the most important cancer-related pathways [68].

SAA1 was found in the CE of the OSA dataset from Becker et al. 2014 and downregulated in our study. We also found SAA4 to be one of the significantly upregulated proteins after sleep deprivation in our dataset. Sleep restriction was shown to increase Serum amyloid A (SAA) serum levels in healthy mice and humans, linking sleep loss to some of its associated comorbidities such as obesity and insulin resistance [69]. Study results vary regarding the connection between SAA and different neuronal diseases: SAA1 was significantly downregulated in both the serum and skin of Parkinson's disease patients [70], but there is also evidence that the continuous elevation of SAA is related to neuronal inflammation and the development of amyloidosis which is a symptom of Alzheimer's disease [71]. However, in Bjørkum et al. 2021 we found SAA4 to be downregulated, we reported in our Tab. 8 that Maret et al. 2007 [72] and Thompson et al. 2010 too found SAA4 upregulated after 6 hours of sleep deprivation. Therefore, we concluded that most likely our previous measurement might have been more unreliable than the measurement in this study. Furthermore, Fibulin-1 (FBLN1) here in this dataset was downregulated but was increased in Bjørkum et al. 2021. Here, two other reports also went in the opposite direction regarding this protein, Thomson et al. 2010 found FBLN1 upregulated after 6 hours of sleep deprivation and Møller-Levet et al. 2013 found FBLN1 downregulated after 1 week of 5.7 hours of sleep per the 24 hours sleep deprivation protocol [73]. Therefore, as mentioned earlier, methodological issues could possibly sometimes explain the discrepancies in the changed level of mRNAs and proteins after sleep deprivation.

Complement factor H-related protein (CFHR4), which promotes complement pathway activation via binding to the C-reactive protein (CRP) [74], was also found to be significantly upregulated in the serum of patients with insomnia compared to the controls [41] and Multimerin 1 (MMRN1), which supports the adhesion of platelets and many other cell types, was highly related to chronic sleep deprivation in rats [31]. We also found both these proteins upregulated in this pilot study.

Retinol-binding protein 4 (RBP4) is a major transport protein of retinol (vitamin A) and is known to associate with TTR to form the retinol/RBP4/TTR complex that is released into the bloodstream [75]. Plasma levels of RBP4, which was also reported to be associated with APOH and insulin resistance [76], were higher in patients with OSA and positively correlated with visceral fat areas and triglyceride levels in those patients [77]. RBP4 was also significantly increased in the hippocampal transcriptional profiles of sleep-deprived rats compared to the control group [78]. With RBP4 being so closely linked to TTR and APOH, respectively, we observed all of them to be upregulated after sleep deprivation.

The Serum Soluble Scavenger with 5 Domains (SSC5D) upregulated in our dataset is a member of the scavenger receptor cysteine-rich superfamily (SRCR-SF) that has essential roles in inflammation and immunity and is reported to be related to several cardiovascular diseases, and elevated serum levels of SSC5D were found to be significantly increased in patients with heart failure, making it a possible biomarker and therapeutic target [79].

In a recent study from 2023, 4 of the 15 proteins, APOH, F12, COL6A3 AND CFHR1, as well as F2, which was also among our DEPs after 6 hours of sleep deprivation, were downregulated in the serum of narcolepsy patients [80]. Although this is speculative, it could be argued that it makes sense for these proteins to be downregulated in narcolepsy as it increases the total sleep duration during the day when we found them to be upregulated in a sleep-deprived setting.

Our results from some of the 15 proteins of the CE when testing our DEPs after sleep deprivation in the moderate OSA condition of the dataset from Cheng et al. 2022 suggest that improvements in sleep quality may reduce the levels of some of the previously discussed proteins that could be beneficial for the prevention of affected cellular mechanisms which, if repeated over time and maybe in the long run, lead to health complications such as insulin resistance, neuronal pathologies, hypertension, and inflammation.

Limitations

To validate our own data within related datasets has methodological limitations. In both OSA studies the participants were children [14, 28] and in the study from Becker et al. 2014, they used urine instead of serum samples. In the insomnia study by Uyhelji et al. 2018 gene transcripts were analyzed instead of proteins in our study. In our study, the participants were exclusively female adults and there is evidence that suggests that females might have a higher degree of responsivity to sleep deprivation compared to males [81].

It is also important to acknowledge that our pilot study had only eight participants. However, we tried to validate our results in the context of larger datasets that have been published [14, 28, 29]. Additionally, the participants underwent a short period, 6 hours of sleep deprivation, during one single night. The proteome changes in serum therefore might be limited. Our findings, in this pilot study only, provide indications of the possible cellular and physiological effects long-term or recurrent sleep deprivation might have over time.

In conclusion, our study was able to reveal another set of human serum proteins that were altered by sleep deprivation and could connect similar biological processes to sleep deprivation that have been identified before with slightly different methods applied in Bjørkum et al. 2021. Our results support our previous findings, in addition to results from other sleep-related studies, that sleep deprivation affects several biological functions based on DEPs classified into groups of proteins associated with regulation, binding, or transport, and, thereby, also associates with protein changes that can be found in clinically relevant pathological conditions like altered platelet function and coagulation, oxidative stress, impaired immune function, affected cardiovascular and neurodegenerative cellular mechanisms, and cell proliferation.

Supplementary material

Supplementary material is available at *SLEEP Advances* online.

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Data Availability

The data underlying this article are available at PRIDE with the unique identifier PDX045729.

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