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DNA methylation pattern for insufficient sleep and recovery

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ACADEMIC DISSERTATION

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Посвящается папе

All things are difficult before they are easy
Thomas Fuller

Abstract

Chronic insufficient sleep affects basic physiological processes and increases risk for various mental and somatic disorders. Despite the growing number of omics-studies, sleep laboratory studies conducted in human samples, as well as various experiments in animals, the biological mechanisms underlying the health consequences of curtailed sleep are not fully understood. This thesis was inspired by the hypothesis that the consequences of sleep loss may be reflected as changes in the epigenetic processes, with DNA methylation (DNAm) selected as the most feasible to study. The aim of this thesis was to elucidate biological pathways associated with chronic insufficient sleep, as well as to explore how transient and reversible DNAm changes triggered by sleep loss are.

In the first study, a cross sectional genome-wide DNAm analysis (Epigenome-wide Association Analysis, EWAS) was performed in relation to self-reported insufficient sleep in individuals from a population-based sample and in relation to insufficient sleep (shift work disorder) among shift-workers from an occupational cohort. No genome-wide significant differences in DNAm were observed in cases versus controls. The study revealed that insufficient sleep was accompanied by the loss of methylation and DNAm alterations in genes enriched in nervous system development pathway. The karyoplot evidenced for several clusters of CpGs on various chromosomes, including a cluster of 12 CpGs on chromosome 17. The genes corresponding to these CpGs were previously associated with a rare genetic condition accompanied by disturbed sleep and inverted circadian rhythm.

The second study examined dynamic DNAm changes in relation to recovery from a shift work disorder in the occupational cohort of shift workers across the genome. The results indicated that recovery during vacation leads to the restoration of DNAm and specifically affects genes involved in the activity of N-methyl-d-aspartate (NMDA) glutamate receptors. These findings provide evidence for the dynamic nature of human methylome and suggest CpG sites in genes Glutamate Ionotropic Receptor NMDA Type Subunit 2C (*GRIN2C*), cAMP Responsive Element Binding Protein 1 (*CREB1*), and Calcium/calmodulin Dependent Protein Kinase II Beta (*CAMK2B*) as putative indicators of recovery in a shift worker with shift work disorder.

In the third study, we studied the effect of depressed sleep on DNAm in a sample of adolescents with comorbid depression and insomnia as compared to healthy controls. No genome-wide significant differences in DNAm appeared in cases versus controls. However, the top findings of DNAm analyses were enriched in the synaptic long-term depression (LTD) pathway, emphasizing the role of sleep in synaptic plasticity and the widespread physiological consequences of disturbed sleep.

Based on these findings, it can be concluded that chronic insufficient sleep is associated with a specific DNAm pattern in blood leukocytes, evidencing for the systemic physiological wide-spread consequences of curtailed sleep. Some of these specific DNAm alterations appeared to be reversible, once individuals restored sleep during two weeks of vacation. Altogether, this thesis contributes to an understanding of the changes triggered by sleep loss in a highly complex and dynamic regulatory mechanism, human DNA methylome.

Contents

List of original publications

This thesis is based on the following publications:

Publication I	Alexandra Lahtinen, Sampsa Puttonen, Päivi Vanttola, Katriina Viitasalo, Sonja Sulkava, Natalia Pervjakova, Anni Joensuu, Perttu Salo, Auli Toivola, Mikko Härmä, Lili Milani, Markus Perola, Tiina Paunio. A distinctive DNA methylation pattern in insufficient sleep. *Scientific Reports*, 2019, 9(1):1193.
Publication II	Alexandra Lahtinen, Antti Häkkinen, Sampsa Puttonen, Päivi Vanttola, Katriina Viitasalo, Tarja Porkka-Heiskanen, Mikko Härmä, Tiina Paunio. Differential DNA methylation in recovery from shift work disorder. *Submitted.*
Publication III	Antti-Jussi Ämmälä*, Anna-Sofia Urrila*, Aleksandra Lahtinen, Olena Santangeli, Antti Hakkarainen, Katri Kantojärvi, Anu E. Castaneda, Nina Lundbom, Mauri Marttunen, Tiina Paunio. Epigenetic dysregulation of genes related to synaptic long-term depression among adolescents with depressive disorder and sleep symptoms. *Sleep Medicine,* 2019, 61:95-103. *co-first authors

Author's contributions

Publication I	Performed quality control and preprocessing of the DNA methylation data, interpreted the methylation data, performed all statistical and bioinformatics analyses, made figures, and wrote the paper.
Publication II	Performed quality control and preprocessing of the DNA methylation data, interpreted the methylation data, performed statistical analyses and bioinformatics analyses, made figures, and wrote the paper.
Publication III	Performed quality control and preprocessing of the DNA methylation data, contributed to the statistical analyses, performed and interpreted pathways analyses, wrote corresponding sections on the DNA methylation analyses and pathways.

The publications are referred to in the text by their roman numerals. All publications are reprinted at the end of this book with permissions (where applicable) from the publishers.

Abbreviations

27K	Illumina Infinium HumanMethylation27
450K	Illumina Infinium HumanMethylation450
AHI	Apnea hypopnea index
AIS	Athens Insomnia Scale
ASMN	All Sample Mean Normalization
BDI	Beck Depression Inventory
BH	Benjamini-Hochberg
BMI	Body-mass index
BMIQ	Beta MIxtrute Quantile dilation
C. elegans	*Caenorhabditis elegans*
CpG	DNA methylation site: cytosine nucleotide followed by guanine nucleotide
CRP	Cross-reactive probes
DILGOM	DIetary, Lifestyle, and Genetic determinants of Obesity and Metabolic syndrome study
DMP	Differentially methylated position
DMR	Differentially methylated region
DNAm	DNA methylation
DNMT	DNA methyltransferase
DSM	Diagnostic and Statistical Manual of Mental Disorders
DSWPD	Delayed sleep-wake phase disorder
EDS	Excessive daytime sleepiness
EEG	Electroencephalogram
EWAS	Epigenome-wide association study
FDR	False discovery rate
GO	Gene ontology
GSEA	Gen Set Enrichment Analysis
GWAS	Genome-wide association study
ICSD	International Classification of Sleep Disorders
IPA	Ingenuity Pathway Analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
K-SADS-PL	Schedule for Affective Disorders and Schizophrenia for School-Age Children and Lifetime version
LINE-1	Long interspread nuclear element

LTD	Long-term depression
LTP	Long-term potentiation
MDD	Major depressive disorder
meQTL	Methylation quantitative trait loci
MGI	Mouse Genome Informatics
MRI	Magnetic resonance imaging
NMDA	N-methyl-d-aspartate
NREM	Non-rapid-eye-movement
NSD	Nervous system development
OMIM	Online Mendelian Inheritance in Man
ORA	Over-representation analysis
OSA	Obstructive sleep apnea
PDSS	Pediatric daytime sleepiness scale
PTSD	Post-traumatic stress disorder
QC	Quality control
REM	Rapid-eye-movement
RGD	Rat genome database
RLS	Restless legs syndrome
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SCD	Stearoyl-CoA desaturase
SCN	Suprachiasmatic nucleus
SD	Standard deviation
SHY	Synaptic homeostasis hypothesis
SMS	Smith-Magenis syndrome
SNP	Single nucleotide polymorphism
SQN	Sub-Quantile Normalization
SWAN	Subset-quantile within array normalization
SWD	Shift work disorder
SWA	Slow wave
SWS	Slow-wave sleep
TSD	Total sleep deprivation
TSS	Transcription site start
UTR	Untranslated region

1 Introduction

Sleep is essential for our health. According to the recommendations based on population-based studies, in order to promote optimal health adults should sleep 7-8 hours, and deviations from the recommended sleep duration are associated with an increased risk for various diseases [1]. Insufficient sleep is globally highly prevalent, often under-reported, spreads across all age groups, brings rather high economic costs to the society, and causes wide range of adverse medical and mental dysfunctions [2].

The ’24-hour society’ and globalization have brought both technological and structural changes in worktime arrangements, meaning the increase of irregular worktime arrangements and shift work. Approximately 20% of the European workforce and 15% of the U.S. workforce are engaged in some type of shift work [3], [4] and are potentially at risk to suffer from insufficient sleep. Shift work disrupts both circadian rhythm and sleep homeostasis leading to increased risk of various health issues, such as sleep disturbances and shift work disorder (SWD) [5]. Considering such a large proportion of shift workers in the global workforce, the societal implications of the adverse health effects associated with shift work are estimated to be substantial.

Technological advances have been made in ‘omics’ in the last decade, including genomics, epigenomics, transcriptomics, metabolomics, and proteomics. Genome-wide assessment of DNA methylation (DNAm) is now affordable and promising avenue to uncover molecular processes underlying complex disorders. Insufficient sleep and mistimed sleep have been shown to strongly affect cell transcriptome – both in rat models [6], [7] and in human cohorts [8], [9]. The effect of acute sleep deprivation on human methylome was recently investigated in the selected cohort of young healthy males at Uppsala University [10], [11], but the DNAm changes associated with chronic insufficient sleep in workers remain underexplored. The study of the epigenetic changes at molecular level could foster the assessment and enhance methods of prevention and cure of the long-term health risk associated with the chronic sleep curtailment.

Human methylome is responsive to the environment, but its dynamics is still poorly understood. In particular, we still have limited information on the short-term changes in DNAm patterns in humans. Longitudinal DNAm studies in human are scarce, and to the

best of our knowledge, there are no studies of DNAm on sleep with longitudinal data. Moreover, the reversible nature of DNAm makes it a potential tool to predict and estimate the effect of environmental change, as well as to pinpoint specific molecules that can serve as important indicators of the environment-disease interaction.

The major objective of this thesis was to identify deviating DNAm pattern in individuals suffering from chronic insufficient sleep. The first study (Publication I) included two cross-sectional epigenome-wide association studies (EWAS) conducted in two complementary samples of cases and controls from 1) sub-sample of the population-based FINRISK and 2) an occupational cohort of shift workers. In 1) cases were selected based on the self-reported evaluation of insufficient sleep, while in 2) cases constituted shift workers with SWD symptoms, such as excessive sleepiness and insomnia. Both samples overlap in terms of common component – insufficient sleep, as SWD symptoms include excessive sleepiness and insomnia.

In the second study (Publication II) we continued with the occupational cohort of shift workers and performed paired EWAS to investigate the effect of vacation on DNAm compared to a working period in shift workers with SWD and without. SWD, a common condition among the shift workers, results in reduction of sleep quality and quantity and has adverse consequences for health and work performance [12].

In the last study (Publication III) we assessed the effect of disturbed sleep and depression on DNAm and explored underlying biological processes in adolescents suffering from comorbid insomnia and depression. This cross-sectional EWAS was performed in the patient cohort from the Helsinki University Central Hospital and healthy controls. Insomnia and depression frequently co-occur and share many features and molecular mechanisms which are still poorly understood [13].

2 Review of the literature

2.1 Normal human sleep

Sleep stages. Normal human sleep occurs in a regulated order of two main states: rapid-eye-movement (REM) and non-rapid-eye-movement (NREM), the latter further divided into the light (N1 and N2) and deep (N3) stages. The NREM sleep stages are identified by the amplitude of the slow wave oscillations in the electroencephalogram (EEG). N1, the lightest stage, is characterized by a lowering EEG frequency, while N2 stage shows slow brain waves with occasional bursts of the rapid waves. The deepest stage N3 occurring mostly during the first half of the night is also referred as slow-wave sleep (SWS) [14]. During SWS EEG is characterized by the high-amplitude slow waves, as well as by sleep spindles and ripples [15]. Sleep normally begins with N1 and progresses through N2 to N3, followed by a REM sleep episode. Such cycle of alternating stages repeats several times during the night and lasts approximately 1.5 – 2 hours. A reversible, consolidated to one main phase, human sleep occurs during the dark period of the day [16].

Sleep duration. The duration of sleep per day varies considerably in the population. The core determinator of sleep duration is genetic, although its heritability established from a recent meta-analysis of the twin studies appeared to be quite low, numbering 38% [17]. Normally sleep lasts between 6 and 8 hours per day; for instance, a self-reported estimation from the Health2000 population-based study conducted in Finland gave a mean of 7.51 hours [18]. In addition to the genetic component, sleep duration is strongly affected by gender and age. Women reported to sleep 0.23 hours more in average than men. Among all participants, the group of short sleepers (sleep duration 6 hours or less) included 16.7% of men and 12.5% of women, while long sleepers (sleep duration 9 hours or more) included 10.5% of men and 16.1% of women. Among 7,262 respondents, short and long sleepers numbered 14.5% and 13.5%, respectively. In regards to age, sleep duration is shown to evolve in a complex pattern: according to a meta-analysis representing 3,577 subjects aged 5-102, objectively measured sleep duration significantly decreased with age [19]. However, the aforementioned population-based study showed a U-shaped relationship between age and self-reported sleep duration, possibly reflecting co-morbidities increasing with age or changes in sleep architecture over the lifetime.

Two-process model of sleep regulation. According to the widely accepted two-process model of sleep regulation, sleep is governed by two separate but interacting processes: a circadian component C and a homeostatic component S [20]. Process C determines the timing of sleep and is mainly directed by the genetically driven internal clock located in the suprachiasmatic nucleus (SCN) in the anterior hypothalamus (reviewed in Section 2.2.2 Circadian rhythm disruptions). Process S depends on the duration of the preceding wake period and accounts for a sufficient amount of sleep. During wakefulness homeostatic sleep pressures increases and is followed by a period of sleep. A prolonged wake period results in the prolonged recovery sleep that contains more SWS than a normal sleep [14]. The interplay of circadian component C and homeostatic component S defines the amount of sleep and its timing – a sleep-wake cycle. Any failure in this interaction results in insufficient sleep and in a feeling of tiredness in the morning, with these effects being much more severe, should sleep disturbances persist for a longer time.

Sleep and plasticity. Plasticity is broadly defined as the ability of nervous system to change its structure and function in response to the environmental changes. Regarding synaptic plasticity, such changes refer to the selective activity-driven strengthening (long-term potentiation, LTP) or weakening (long-term depression, LTD) of the individual synapses. A theory linking synaptic plasticity with sleep was proposed by Tononi and Cirelli to suggest that restoration of the synaptic homeostasis is the fundamental function of sleep [21]. Synaptic homeostasis hypothesis (SHY) proposes that during wake, due to ongoing learning, the synaptic strength in many brain circuits increases mediated by synaptic potentiation and results in LTP. Such net increase in synaptic strength occurs at high-energy costs, leads to saturation and creates a need for synaptic renormalization. During sleep, once brain is disconnected from the environment, neural circuits undergo renormalization, meaning downscaling of the synaptic strength or pruning. According to SHY, our sleep is ‘the price to pay’ for plasticity [22].

A large number of studies in rodents and humans indicate that sleep plays active role in memory consolidation, which is thought to be based on the changes in the strength of synaptic connections, LTP and LTD processes [23]. According to ‘active system consolidation’ concept introduced by Jan Born [15], the neuronal replay of memories occurring during sleep accounts for the formation of the long-term memory. Originated in the hippocampus, these repeated reactivations of neuronal representations occurring mostly

during SWS propagate across the memory networks and lead to the formation of the stable long-term memories in neocortical networks. Regarding synaptic plasticity, such reorganization of the newly encoded representation comprises strengthening of some synapses and weakening of others, as well as formation of new synaptic connections with neurons outside of this encoded representation. Thus, sleep promotes both synaptic upscaling and downscaling, which either mediate an increase in the contribution of the selected neurons to the memory storage or reduce this contribution. The process of active systems consolidation is consistent with SHY and is currently regarded as an integrative basis for sleep-dependent formation of long-term memory [15].

Molecular regulation of sleep and wakefulness. The cellular processes underlying sleep and wake states were extensively investigated in the studies of gene expression changes in animals. Early experiments in 1970-1980s evaluated global changes in brain RNA and protein synthesis in relation to sleep and waking or sleep deprivation and found that both RNA and protein synthesis was increased during sleep and decreased in sleep-deprived animals. In mid-1990s, the studies of associations between sleep/wake and gene expression led to the discovery of Fos, NGFI-A and P-CREB - transcription factors involved in synaptic plasticity. These findings evidenced for major changes in the patterns of expression across the genome that could be triggered by transition of the brain from one state to another [24]. Indeed, a study of brain gene expression in spontaneously asleep, sleep-deprived, and spontaneously awake rats conducted by Cirelli *et al*. revealed that sleep and wake states were associated with different sets of differentially expressed genes and, furthermore, sleep-related and wake-related transcripts related to different biological functions [7]. For example, wake-related transcripts included those involved in energy metabolism (*GLUT1*, mitochondrial genes), glutamatergic neurotransmission (*Narp, Homer*), memory acquisition and LTP (*Arc, NGFI-A, BDNF*), cellular stress (*HSP, Bip*), and transcription activation (*Per2, NGFI-A, NGFI-B, CHOP*). Sleep-related up-regulated transcripts comprised genes involved in LTD and memory consolidation (*calcineurin, CAMK4*), membrane trafficking and maintenance (*Rabs, Arfs, NSF*), transcription deactivation (*NF1, Id2*), GABAergic neurotransmission (*dlg3, gephyrin*), and positive regulation of translation *(eEF2, eIF4AII*). The presence of genes associated with translation during sleep suggested that sleep is not a quiescent state characterized by global inactivity. It may enhance the synthesis of specific proteins, and actively mediate certain aspects of neuronal plasticity.

Since acute sleep loss was found to affect broadly gene expression, could sleep pressure also drive genome-wide changes at the level of epigenetic regulation? Massart *et al.* compared DNA methylation and hydroxymethylation genomic brain profiles of sleep-deprived and non-sleep-deprived mice [25]. The statistically significant effect of sleep deprivation on DNA methylation was observed in genes related to neuritogenesis (*Rab11b*), synaptic plasticity (*Pcdh19*) and glutamatergic transmission (*Wnt5a, Dlg4*). Largest changes in hydroxymethylation were linked to genes involved in the processes of cell death *(Daxx, Tnf*), neurotransmission (*Nrxn1, Nlgn3*), and cell signaling (*Akt*). The study also explored transcriptomics of sleep-deprived mice and revealed strong changes in the expression of genes involved in synaptic transmission (NMDA receptors), circadian rhythm (*CLOCK*), and activity dependent signaling pathways (*CREB1, CREM*). These findings supported the hypothesis that in animals sleep, deprivation has widespread impact on both the brain transcriptome and methylome.

2.2 Insufficient sleep

According to self-reported U.S. data, it is estimated that the proportion of the short sleepers in the general population increased from 7.6% in 1975 to 35% in 2014 [26]-[29]. Unlike sleep duration, the prevalence of sleep disturbances at the population level is difficult to estimate. A self-reported assessment of 150,000 Americans found that the prevalence of the troubles falling or staying asleep ranged from 13.7% (ages 70-74) to 18.1% (ages 18-24) for men, and from 17.7% (ages 80 and older) to 25.1% (ages 18-24) for women [30]. Data from 2007 regarding the prevalence of the self-reported sleep symptoms (mild and moderate severe) revealed the following numbers: difficulty falling asleep 18.8%, sleep maintenance difficulties 20.9%, early morning awakenings 16.5%, daytime sleepiness 18.8%, non-restorative sleep 19.7%, and frequent snoring 31.5% of adults [31]. Epidemiological studies on sleep duration, sleep quality, and insomnia have shown that insufficient sleep is associated with various adverse health outcomes, including cardiovascular [32]-[35], inflammatory [36], [37], and metabolic diseases [38]-[40], cancer [41], [42], cognitive decline [43], and increased rates of mood disorders [44], [45]. Short sleep was also significantly associated with mortality, as found in the recent meta-analysis combining data from 5,172,710 participants [46].

The genetic factors underlying curtailed sleep have been investigated in twin and genome-wide association studies (GWAS). From the twin studies we know that heritability estimates of self-reported sleep length varies moderately between 31% and 44% [47], [48], with similar numbers for sleep quality (44%) [47] and insomnia symptoms (28% to 48%) [49], [50]. The recent findings from GWASes based on UK Biobank data have shown that self-reported sleep duration is a complex trait with a genome-wide single-nucleotide polymorphism (SNP)-based heritability estimated at modest 9.8%. The 78 genome-wide significant genomic loci explained 0.69% of the variance in sleep duration [51]. The GWAS on insomnia based on combined data from UK Biobank and 23andMe resulted in 202 genome-wide significant genomic loci (956 genes) explaining 2.6% of the variance and SNP-based heritability numbered 7% [52]. This study revealed a strong overlap between insomnia and psychiatric traits: for example, the strongest genetic correlations were found between insomnia and depressive symptoms (r = 0.64, *P* value = 1.21E-71), followed by anxiety disorder (r = 0.56, *P* value = 1.40E-7). The findings both from twin studies and GWASes suggest that the role of genetic factors in sleep duration and insomnia is relatively moderate and large proportion of variance is explained by the environmental factors and by the interaction of genes and environment.

The causes of insufficient sleep may include behavioral, social or work-related factors, circadian disruptions, sleep disorders, as well as poorly understood processes occurring in numerous somatic and psychiatric disorders.

2.2.1 Curtailed sleep

2.2.1.1 Voluntary sleep curtailment

The timing, environment and constraints of sleep are different across the human societies, making upstream social and environmental influences on individual sleep sufficiency extremely complex. According to the social-ecological model, voluntary sleep curtailment results from different complex and overlapping factors that evolve at the individual, social, and societal levels (Figure 1). At the individual level, factors influencing sleep include genetics, general health, lifestyle, beliefs, habits, and so forth. For instance, in the urban environment, such daily routines as exercise and diet, use of technology, and lifestyle habits have a huge impact on sleep duration and quality, as reviewed in [53]. In addition to above

mentioned gender and age-related differences, sleep practices, choices and beliefs vary greatly: bed-sharing with infants, engagement in social events, daily naps affect sleep and to a certain extend overlap with factors from the social level [54]. One example of a biological factor influencing lifestyle and sleep would be a chronotype, a trait with a strong genetic influence, where from the twin study heritability numbered 49.7% [55]. Thus, an evening-type chronotype increases risk for social jet lag, which may lead to curtailed sleep and symptoms of insomnia due to imbalance of the sleep-wake schedule and the intrinsic circadian rhythm [56].

Home and family environment play important role in sleep, for example larger household size was associated with greater sleep insufficiency, as well as were marital statuses 'married', 'living with a partner', and 'divorced' [57]. Both lower socioeconomic position and low education level were linked to a shorter sleep duration in the study of 6,928 adults from California [58], though the study on relationship between income and insufficient sleep has given contradictive results, depending on the adjustments for covariates [57]. Several studies reviewed in [54] have investigated the association between sleep quality and neighborhood, showing that areas with increased crime rate, environmental noise and light adversely impact sleep. The relationship between work and sleep is especially important: a survey of Basner *et al*. [59] indicated that work was the primary determinant of sleep duration. Holding multiple jobs increased risk for short sleep duration, while self-employed respondents were less likely to be short sleepers. Unemployment was linked to a longer sleep duration but also to a larger amount of sleep disturbances. Working in shifts is a well-known factor associated with the insufficient sleep and will be reviewed in the Section 2.2.2.

The societal level includes several factors known to impact sleep, among which use of technology and globalization are particularly relevant ones. When in 2011 the National Sleep Foundation conducted interviewed Americans regarding use of technology in the bedroom, the results were striking: firstly, 90% of Americans used some sort of a device in bed, and secondly, the use of such devices as smartphones, laptops, video game consoles used in the hour before bedtime was associated with reports of difficulties to fall asleep and non-restorative sleep [60]. More studies supported this finding indicating that light emitted by devices negatively affects sleep [61], as well as certain mental engagement like video gaming can result in sleep disruption and reduction of the self-reported sleep quality [62]. The use of technology goes hand in hand with the globalization: social interactions with

family members and friends, commercial activities, such as online shopping, as well as work responsibilities, including 24/7 emails and business in various time zones, can considerably impinge on sleep.

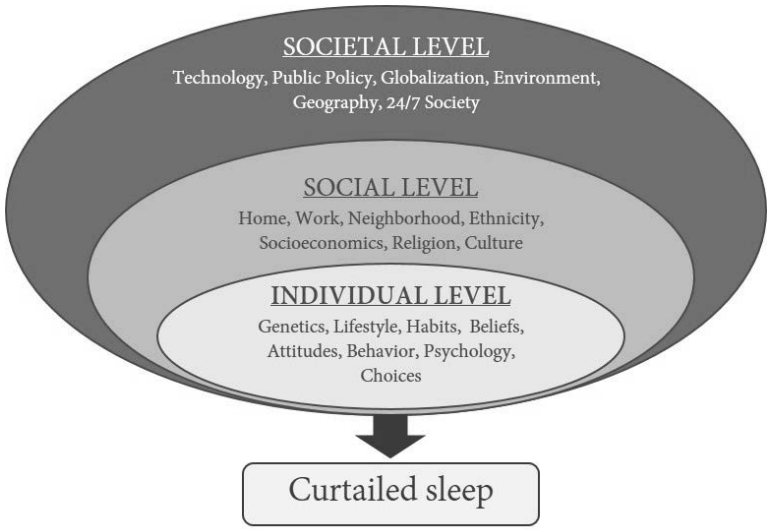


Figure 1: Factors contributing to voluntary sleep curtailment, organized in three levels, according to the social-ecological model of sleep. Edited from [54] with permission from Elsevier Inc.

2.2.1.2 Pathological sleep curtailment

It tends to be the rule and not the exception that insufficient sleep results from a combination of extrinsic (environment) and intrinsic (biological) factors, and the relative contribution of work-, social- or disease-related factors are impossible to identify. However, the statement of "can't sleep versus won't sleep" may help to distinguish voluntary sleep curtailment from a sleep disorder, whereby insufficient sleep occurs due to inability to obtain or maintain sleep or due to mistimed sleep. This chapter focuses on those common sleep disorders which include insufficient sleep as a prominent symptom, and which are most often studied in the general population.

One of the most common sleep disorders, the definition of **insomnia** (F51.0 in ICD-10) is comprises following criteria: a) disturbance of sleep onset or sleep maintenance, or poor

sleep quality; b) the sleep disturbances persist over a one-month period and occur at least three times a week; c) insufficient sleep length and poor quality impair daily activities, and d) the affected individuals show worries about insomnia and its consequences [63]. According to the epidemiological studies, the prevalence of insomnia varies depending on the study design, studied population and the definition used: the latest self-reported U.S. data from 34,712 adults gave an estimation of 27.3% [64], while in European countries the numbers varied from 5.7% (Germany) to 19% (France) [63]. Insomnia is found to be more frequently reported by women than men, and the prevalence is known to increase with age. In fact, insomnia represents an extremely common health complaint in persons aged 60 years and older and is expected to expand rapidly in this group from 205 million currently to 2 billion by 2050 [65]. According to the International Classification of Sleep Disorders-3 (ICSD-3), based on chronicity of symptoms insomnia subtypes include chronic insomnia disorder, short term insomnia disorder and other insomnia disorder [66]. By the definition, chronic insomnia lasts at least three months with symptoms occurring at least three times per week. The treatment of chronic insomnia includes pharmacologic agents, such as benzodiazepines and related drugs, as well as non-pharmacologic approach, for example, cognitive behavioral therapy [63]. Short term insomnia can be temporarily associated with a stressor and should generally resolve within three months.

Insomnia frequently co-occurs with mood disorders, such as major depressive disorder (MDD) or anxiety. A clear majority of individuals suffering from MDD also report insomnia symptoms [13]. According to longitudinal studies, chronic insomnia was associated with a likelihood of 2-3 times higher of developing a depression or anxiety [67]. Though the underlying pathophysiology of the link between depression and insomnia is poorly understood, it is known that two conditions share bidirectional relationship, with a stronger evidence for insomnia preceding depression. The longitudinal study conducted in a Finnish twin cohort of 18,631 individuals revealed that poor quality of sleep predicted life dissatisfaction (odd ratio 2-3), while life dissatisfaction did not consistently predict poor sleep [68]. In elderly persons, insomnia frequently co-occurs also with chronic pain, cancer, cardiovascular disease, and medication use [65].

As adolescence is characterized by prominent changes in sleep patterns and increased vulnerability to mental disorders, the incidence of both MDD and insomnia simultaneously increases. According to the epidemiological studies, the prevalence of depression in

adolescents varies between 5 to 8%, with a cumulative incidence reaching 15-20% by 18 years old. As many as 10-40% of adolescents report unspecific symptom of disturbed night sleep or significant daily sleepiness. These symptoms of poor sleep and depression are reported more frequently after the onset of puberty. The epidemiological studies also evidenced for insomnia preceding depression and provided similar numbers for an increased risk of subsequent onset of depression – two- to threefold [69].

Obstructive sleep apnea (OSA) is characterized by repeated cessations of breathing during sleep associated with arousals with or without oxygen desaturations. OSA is further characterized by reduced sleep duration, sleep fragmentation, and oxidative stress. The standard measure to define the severity of OSA takes into account the number of apneas and hypopneas that occur per hour of sleep, referred as apnea hypopnea index (AHI) [70]. OSA is a highly prevalent condition: a 2015 study in Switzerland estimated that 50% of men and 23% of women had moderate OSA [71]. In the United States the prevalence has increased and numbered 24-26% in men and 17-28% in women of 30-70 years old age group, with the proportion of affected individuals increasing with advancing age and increased body mass index [72]. Despite being such a common condition, OSA remains underdiagnosed, with approximately 82% of men and 93% of women remain unidentified [73]. The reported symptoms vary considerably, but snoring, daytime sleepiness, fatigue and witnessed apneas are the most common complaints. Patients with severe and moderate OSA are at higher risk to develop various comorbid conditions, such as stroke, myocardial infarction, hypertension, diabetes, arrhythmias, and depression [74].

EDS is reported by approximately half of the patients with **restless legs syndrome** (RLS), a common sensorimotor disorder characterized by an urge to move and uncomfortable sensations in the legs worsening during rest and at night. Other sleep disturbances of this group include difficulty falling asleep and frequent night awakenings and can affect quality of life severely enough to warrant treatment [75]. As estimated from the community-based samples, the prevalence of RLS ranges from 7 to 23%, with an average of 10%, making it a relatively common sleep disturbance [76].

2.2.2 Circadian rhythm disruptions

Circadian rhythm. The circadian rhythms are near-24-hour oscillations found in virtually every physiological process in the human body and brain. The cluster of ~50,000 neurons within SCN termed as master clock coordinates tissue-specific rhythms according to the light signal from the outside world [77]. The photic entrainment originates in the eye and involves a conveyance from a small fraction of retinal ganglion cells containing light sensitive photo pigment melanopsin via the retinohypothalamic tract to the SCN [78]. Furthermore, the light signal passes from the SCN to adrenergic fibers innervating the pineal gland which regulates melatonin synthesis [79]. The circadian rhythm is entrained mainly, but not solely, by light and in healthy humans this intrinsic circadian period lasts slightly longer than 24 hours [80]. Each day master clock resets the human daily cycle in accordance to the daily cycle of light and darkness, therefore circadian rhythm is established by both internal master clock cycle and earth's day/night cycle [81].

At the level of individual cells, molecular rhythms are generated by interactive positive and negative transcriptional-translational feedback loop involving transcription factors. The positive loop is promoted by the heterodimerization of CLOCK (NPAS2 in vasculature tissue) and BMAL1. The resulting heterodimers bind to enhancer boxes in gene promoters and activate transcription of the clock-controlled genes encoding CRY and PER proteins. The latter ones accumulate in the cytoplasm during circadian cycle, eventually dimerize, travel to nucleus, and repress own transcription, thus acting as negative feedback loop regulators [77]. Many other proteins – transcriptional cofactors, kinases, phosphatases – participate in the regulation of the core molecular clock. Unlike genes regulating sleep homeostasis, circadian genes are widely studied and well characterized.

Sources of circadian disruptions. Circadian rhythm is governed by both genetic and environmental factors. Chronotype or circadian preference defines individual's preferred timing of sleep and wake and is independent of the environmental factors. We commonly separate individuals into the morning people ("larks", prefer early sleep timing and early waking), evening people ("owls", prefer later bedtime and later waking), and intermediate types falling between the two extreme types. There is a great variability in circadian timing, with age, gender and environmental factors explaining a substantial proportion of it. Genetic

variation is known to contribute as much as 50% to a population variability of the chronotype, according to the twin and family studies [82].

While genetic factors may predispose to sleep disorders driven by circadian misalignments in a minority of individuals, the environmental factors affect a much larger proportion of human population. Certain changes in the environment can disorganize the circadian system, and since circadian system is intertwined with sleep/wake cycle, sleep may become a subject to disruption. The sources of circadian disruptions include those related to age (phase delay occurring at puberty) and ageing, particular disease states (comprising both common disorders, such as Alzheimer's disease, and rare disorders, such as Smith-Magenis syndrome, SMS, characterized by inverted circadian rhythmicity of melatonin), circadian rhythm sleep/wake disorders, changing photoperiods (seasonal disorders), jetlag, and work schedules (shift work, social jetlag, early starting times in schools) [79].

During adolescence, the sleep patterns undergo changes: typically, teenager sleep is shorter, meaning later bedtime and early school starting time on weekdays and delayed due to various social activities. Both intrinsic bioregulatory factors resulting from puberty-driven changes and such psychosocial factors as bedtime autonomy, social networking, screen time and academic pressure push for a delay of the timing of sleep [83]. Many adolescents shift towards an evening chronotype and experience misalignment between own biological rhythm and social schedule, which results in daily fatigue and sleepiness, decreased school performance and behavioral problems.

Social jetlag. Term 'social jetlag' refers to the chronic jetlag-like phenomenon caused by modern work schedules and reflects the misalignment between internal circadian clock and actual sleep times during the week. Hence, as many as 87% of Northern Europeans show at least 1 hour of discrepancy between sleep time on workdays versus weekends [79]. This difference is especially pronounced for adolescents (and even more for those with evening type of chronotype), as bedtime tends to progressively delay during teenage years, both on weekends and weekdays, while morning rising is earlier on weekdays versus weekends. Such 'living against the clock' results in chronic sleep loss, and therefore social jet leg has been extensively researched in relation to the risks for cardiometabolic disorders [84], [85], obesity [86], and behavioral ramifications, such as alcohol abuse and smoking [87].

Delayed sleep-wake phase disorder (DSWPD). According to ICSD-3 classification, DSWPD, a circadian rhythm sleep/wake disorder, can be recognized for the individuals with a complaint of insomnia and a chosen delayed timing for the major sleep episode. The prevalence of DSWPD depends on the population and numbers around 1% in adults without insomnia symptoms, as estimated from the population-based cohorts; however, among the adolescents and young people it ranges between 7 to 16% [88]. In the majority of individuals with DSWPD the circadian timing is normally aligned with the solar cycle, but the sleep episode is delayed resulting in chronic sleep loss. DSWPD commonly occurs among the teenagers due to such psychosocial factors as bright light during evening and night hours and late-night activities. The studies conducted in the young adults and adolescents also evidenced that the prevalence of moderate and extreme evening phenotypes is higher among the individuals with DSWPD [89].

Shift work and SWD. According to the Directive 2003/88/EC of the European Parliament and of the Council - Article 2, shift work is defined as "any method of organising work in shifts whereby workers succeed each other at the same work stations according to a certain pattern, including a rotating pattern, and which may be continuous or discontinuous, entailing the need for workers to work at different times over a given period of days or weeks". Accordingly, "shift worker" means any worker whose work schedule is part of shift work". As night is the rest phase for humans, night shift workers are at a particular risk of circadian rhythm and sleep disruptions. Rotating shift schedule typically includes rotations of 3-day periods of early/ late/night shifts and rest days. Since circadian system is inert, such schedule does not allow the master clock to fully adapt to the night shifts [90]. Irregular shift work schedules, prevalent in the health care and transportation sectors, are less studied and suggested to have similar negative health impact, as night shift work.

Both night (shift between 24:00-6:00 hours) and early morning shift work (starting between 4:00-7:00) commonly result in insufficient sleep: according to the studies performed in shift workers, the sleep at night following the night shift is truncated by 2-4 hours and the loss involves N2 stage and REM sleep [91]. Up to one-third of shift workers add a compensatory daily nap which frequently exceeds an hour. Night work is also accompanied by daily sleepiness which often worsens in the early morning [92], [93]. Similar consequences, such as sleep curtailment and daily naps were observed in relation to the morning shifts, in addition, shift workers complained feeling not refreshed after sleep and reported difficulties

awakening [94]. Rotating shift schedule differs in terms of the number of consequent shifts (speed) and the direction of rotation. Few intervention studies have shown that rapidly forward-rotating shift system (morning-afternoon-night) seem to decrease insomnia and daytime sleepiness, increase alertness, and improve work performance [95]-[98].

As a result of circadian misalignments, a subset of shift workers are at great risk to develop SWD, a circadian rhythm sleep/wake disorder characterized by complaints of insomnia and/or excessive sleepiness temporarily associated with shift work and not explainable by another medical condition or medication use [99]. After a longer recovery period, such as vacation, SWD primary symptoms likely ameliorate, and normal sleep/wake function should be restored. The prevalence of SWD varies and is estimated that one in five shift workers suffers from SWD. A relatively small population-based study reported that 14%-32% of night workers and 8%-26% of rotating shift workers experienced SWD [100]. A study in oil rig workers in the North Sea estimated the prevalence rate of similar 23% [101]. Notably, the prevalence rates vary depending on whether ICSD-2 or ICSD-3 criteria are applied: ICSD-2-based prevalence of SWD among the hospital shift workers was higher and numbered 7.1%-9.2% (shift workers without nights), 5.6%-33.5% (shift workers with nights), and 16.7% (permanent shift workers) [102]. The vulnerability to experience SWD symptoms differs remarkably between the individuals, with age and gender known to play an important role [103]. In one of our studies in shift workers [104], we showed that genetic factors also contribute to the vulnerability to circadian disruption in relation to shift work. Precisely, a variant rs12506228 located downstream of the melatonin receptor 1A gene (*MTNR1A*) was associated with job-related exhaustion in shift workers and with changes in DNAm in the TSS of *MTNR1A*.

In addition to significant costs to employers, related to decreased work performance and increased errors and accidents, SWD is linked to adverse health effects, including cardiovascular, gastrointestinal, mental, and metabolic disorders, as well as alcohol abuse, obesity, and psychosocial distress [100], [103].

Jet lag. Like shift work, jet lag causes circadian rhythm and sleep disruptions. Jet lag results from the fact that environmental cues and light-dark cycle are in conflict with change of timing of the internal circadian clock and its symptoms are usually temporary and rather mild [81]. Notably, jet lag is known to be more severe after flying east than after flying

west. However, flight attendants are at particular risk to suffer from chronic jet leg, due to travel through multiple time zones and irregular work schedules. Thus, a study of 4,011 U.S. flight attendants showed that jet leg was associated with increased rate of sleep disorders: men and women had 3.7 and 5.7 times the reported prevalence, as compared to general population, but, surprisingly, the risk for sleep disorders did not correlate with the job tenure [105]. Smaller study conducted in female flight attendants found that transmeridian flights affect sleep quantity and quality: in particular, during the three days following the flight participants reported an increase in the number of awakenings, feelings of non-restorative sleep and sleepiness in the mornings, sleep restlessness, and difficulties to fall asleep [106]. A relatively recent study in the female flight attendants using both diaries and actigraphy indicated that these workers suffer from sleep disturbances, both in terms of sleep quality and quantity, at significantly higher rate, than teachers [107].

2.3 DNA methylation

The term “epigenetics”, literally meaning “above genetic” or “in addition to changes in genetic sequence”, refers to the set of biochemical mechanisms which are not coded in the DNA itself and which result in heritable changes in the gene expression [108]-[110]. Though debates over this term continue, we usually categorize the epigenetic modifications into the three groups: DNA modifications, histone modifications, and non-coding RNA (Figure 2).

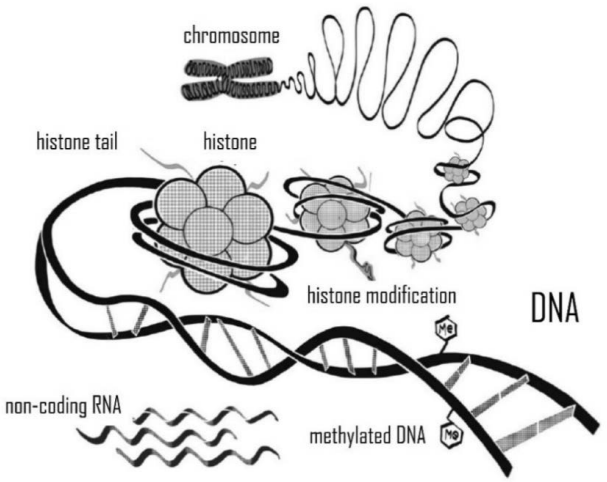


Figure 2: Epigenetic mechanisms of a human cell. Three levels of epigenetic mechanisms include the level of DNA covalent modifications, the RNA level represented by non-coding RNAs, and the protein level meaning chemical modifications to the N-terminal histone tails. Edited from [109] with permission from Springer Nature.

At the DNA level, two types of chemical DNA modifications received the most attention - methylation and hydroxyl-methylation of the cytosine-phosphate-guanine (CpG) dinucleotides [111]. Histone modification occurs at the histone amino terminus and can affect gene regulation by altering the accessibility of the DNA sequence by transcriptional machinery [112]. RNA level encompasses various non-coding RNAs which exert effect on gene regulation via silencing [113] or remodeling of chromatin [114].

Epigenetics mechanisms are crucial for various organism functions and any misbalance in them can lead to the adverse health effects. Today, a wide variety of disease-associated epigenetic dysregulations has been reported for many disorders and behaviors. This thesis focuses on the DNAm that, thanks to the technological advances of the last decade, is by far the most widely studied epigenetic mechanism.

2.3.1 Creating a pattern

In mammals, DNAm is achieved almost exclusively via addition of a methyl group to the 5th position of the cytosine residue followed by the guanine residue. The methyl group from co-factor S-adenosyl-methionine (SAM) is introduced by one of the three enzymes belonging to the family of DNA methyltransferases (DNMTs) (Figure 3A). The human genome encodes five DNMTs, of which three are canonical enzymes catalyzing the addition of the methyl group to the cytosine – DNMT1, DNMT3A, and DNMT3B. DNMT1 recognizes hemimethylated strand from the DNA replication and adds methyl groups to the cytosines on the newly synthesized strand providing mitotic heritability of DNA methylation patterns [115]. Both DNMT3A and DNMT3B come to the action after the global demethylation took place during early stages of embryonic development and perform *de novo* methylation across the genome [116]. Although such model suggests that DNMT1 is responsible for the maintenance of the DNAm pattern, while both DNMT3 enzymes establish it after the global erasure, it is an oversimplification. Few studies mentioned in a recent review of Frank Lyco [115] have given evidence that all three DNMTs tightly coordinate the activity and are involved in both *de novo* and maintenance of DNAm genomic pattern. Interestingly, both DNMT3A and DNMT3B, but not DNMT1, can also convert 5-methylcytosine back to cytosine, acting bidirectionally as DNA methyltransferases and as dehydroxymethylases [117].

The distribution of the CpGs varies greatly in mammalian genomes: in the bulk of genome, for instance, in the DNA repetitive loci, gene bodies or intergenic loci, as well as in tandem repeats of the centromeric, pericentromeric, and subtelomeric regions, the pattern exhibits low density and high level of methylation which is important for the genomic stability [118], [119] (Figure 3B). However, the most prominent feature of the vertebrate DNAm patterns is the presence of CpG islands or clusters of unmethylated CpGs that tend to locate at the 5’

end of the gene [120]. Approximately 1-2% of the total number of CpGs account for such CpG islands, which are typically DNA regions of 500 base or longer with at least 50% enrichment of GC content. Exceptionally, CpG islands located on inactive X-chromosomes in females are known to be hypermethylated [118]. In addition to the CpG islands situated next to the gene promoters, some CpG clusters of unknown function are found between the genes (intergenic CpGs) or within the gene bodies (intragenic CpGs). Thus, of the nearly 28 million CpGs present in the human genome, 20-40% are generally unmethylated, as compared to the remaining hypermethylated part [121].

Almost 60% of the CpG islands are associated with the gene promoters and are unmethylated in the human genome allowing the transcription of the corresponding genes [122]. Already in 1975 two papers [123], [124] independently proposed that methylated cytosine in CpG can serve as a heritable epigenetic mark interpreted by the DNA-binding proteins and, by some mechanism, can 'silence' the gene. Technological advances in the last couple of decades have shown that, indeed, actively transcribed genes are associated with the unmethylated promoter sequences, however, such model of silencing is grossly oversimplified (Figure 3C). The majority of these studies focused on the effect of the methylated CpG islands located next to the transcription start sites (TSS) of the genes [122]. However, DNAm is context-specific and, for example, gene expression and DNAm in the first intron demonstrated quasi-linear inverse association across different tissues in fish [125]. Another controversial example is the function of the methylated CpGs located in the gene body. A study of Maunakea *et al*. [126] demonstrated that the intragenic methylation affects the transcription of the corresponding gene indirectly via activation of the alternative promoters. As much as 40% of genomic CpG islands do not co-localize with the promoters in TSS (so-called 'orphan' CpG islands marked as intragenic and intergenic CpG in Figure 3B) but demonstrate similar promoter-like characteristics as promoter-localized CpG islands [127]. CpG clusters situated 2 kb upstream of the TSS (distal regulatory elements in Figure 3B), characterized by a much lower density of CpGs, and sometimes referred as CpG shores, are involved in the regulation of the gene expression in human cancers but this role is poorly understood [128]. Despite numerous correlative observations reviewed here, our expertise to assess the function of the methylated CpG at particular genic location remains surprisingly limited.

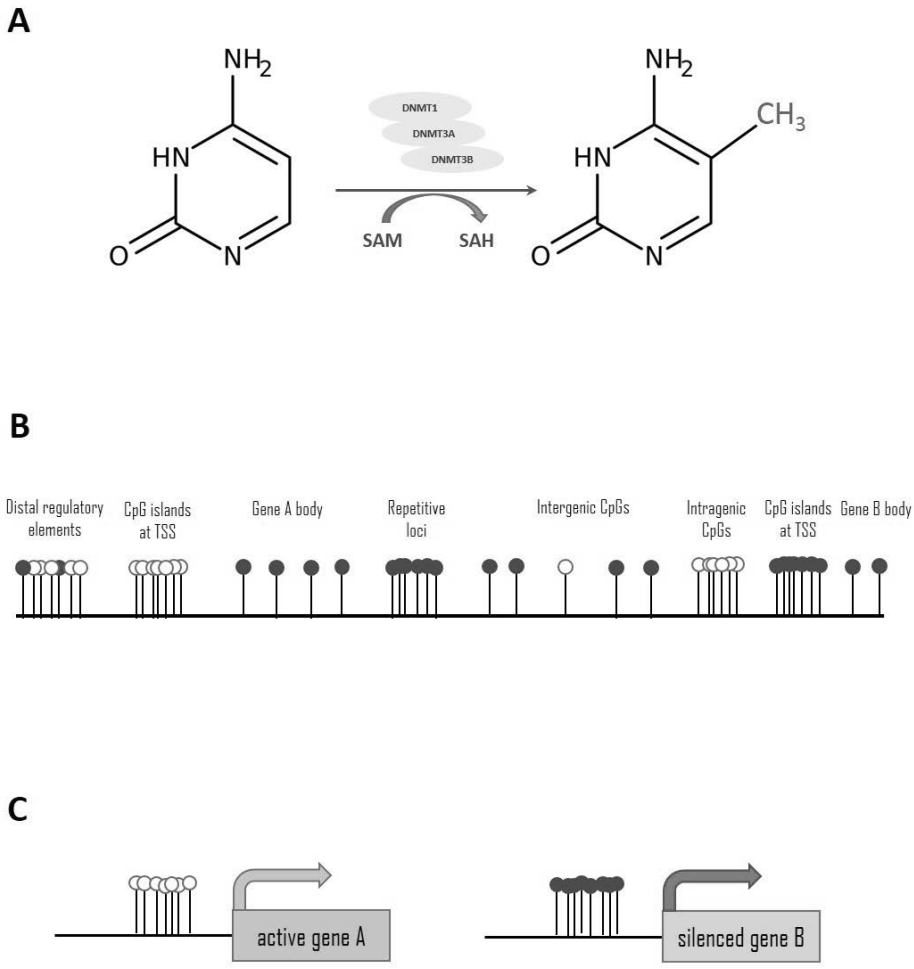


Figure 3: DNAm pattern in mammalian genomes. **A)** Conversion of cytosine to 5-methylcytosine by DNA methyltransferases DNMT1, DNMT3A and DNMT3B; SAM, S-adenosylmethionine, a donor of methyl group, and SAH, S-adenosylhomocysteine. Edited from [129] and [130] with permission from Elsevier Inc. and Wolters Kluwer Health Inc., respectively. **B)** Genomic CpG distribution. White circles, non-methylated CpGs, dark blue circles, methylated CpGs. TSS, transcription start site. Edited from [119] with permission from Ivyspring International Publisher. **C)** Effect of DNAm at promoter CpG islands on the gene transcription, a simplified model. Gene A with unmethylated CpG islands at TSS is active with transcriptional state indicated by a green arrow; gene B with methylated CpG islands at TSS is silenced and repressed transcription is indicated by the red arrow. Edited from [131] with permission from Elsevier Inc.

2.3.2 Modifying a pattern

Summarizing the previous chapter, DNAm is a heritable, reversible, tissue and cell-specific modification that affects gene expression in a complex manner. Genomic DNAm landscape is relatively stable and needs to be preserved across the cell divisions. However, unlike genomic sequence, DNAm profile changes through the life as a result of physiological changes, environmental influence, and disease-associated alterations (Figure 4).

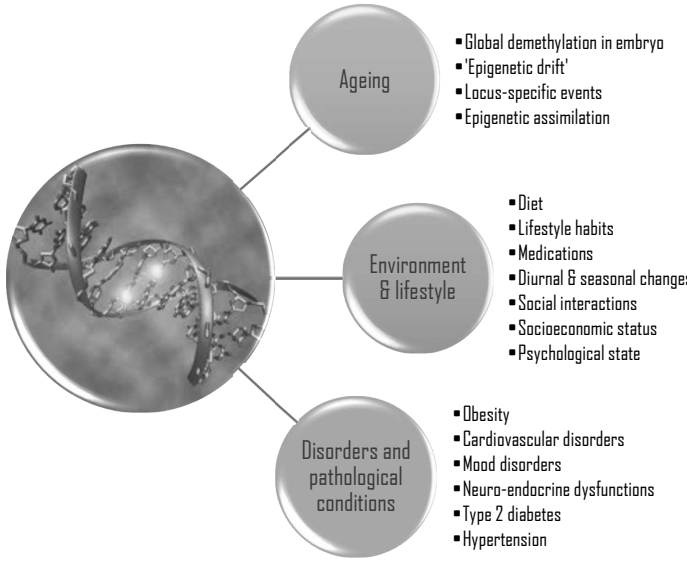


Figure 4: Major modifiers of human methylome – age, environment, and pathologies. The three factors are tightly related and inter-dependent resulting in an oversimplified schematic. For example, the widespread effect of genetic variation on DNAm characterized in recent studies of [132], [133] is missing here. Edited and reconstructed from [109], [134], [135].

Ageing. The major physiological change in genomic DNAm pattern arises during early stages of development when massive demethylation occurring in the genome of pre-implantation embryo serves as a starting point to establish cell-specific DNAm patterns [136]. Once established, such pattern is strictly maintained during cell division to sustain cell identity. Nevertheless, despite the same starting point, human methylomes diverge over the lifetime, with epigenetic similarities being lost even in the pair of monozygotic twins

[137]. The age-associated DNAm differences accumulate, firstly, due to the phenomenon defined as “epigenetic drift” involving stochastic changes in baseline methylation levels, and, secondly, due to the directional and probably programmed changes in the specific regions of our genome. As an example of epigenetic drift, generally hypermethylated CpGs located in the heterochromatic regions of the genome, such as transposons and repetitive elements undergo loss of methylation [138]. An elegant comparative study performed by Heyn *et al*. [139] on the methylomes of newborns versus centenarians demonstrated that more hypomethylated CpGs were observed in virtually all genomic compartments of centenarians, for example, in regulatory regions, promoters, intergenic, both exonic and intronic sequences.

Broad research of age-associated locus-specific DNAm changes gave rise to several mathematical models estimating biological age with astonishing accuracy. Thus, Horvath ‘epigenetic clock’, a prediction tool based on DNAm state of the 353 CpGs across the genome, estimates DNAm age with an error of 3.6 years robustly across various tissues and cell types [140]. The gap between individual DNAm age and true chronological age that Horvath defined as age acceleration has been associated with various age-related pathologies, such as neurodegenerative diseases [141]-[143], and cancer [144], [145].

Curiously, diverging from the early adulthood, human methylomes were observed to converge at the later stages of life. Recent studies of very old twin pairs [146], [147] gave evidence to a paradoxical phenomenon of ‘epigenetic assimilation’ meaning a reduction of epigenetic variability in very old individuals.

Environment. The possible influence of various environmental cues on DNAm has attracted considerable interest. Starting from the groundbreaking study on the association between environment and methylomes of monozygotic twin pairs conducted by Fraga *et al.* [137], methylome divergence occurring due to the differences in lifestyle was also confirmed in the longitudinal twin study of Martino *et al*. [148]. Table 1 lists examples of association studies on some well-studied environmental cues and DNAm conducted in adult human cohorts, along with the corresponding sources.

Factor	Tissue	Phenotypic change/major finding	Sources
Tobacco smoke	Lung, blood	Lung cancer, inflammation, heart disease	[149]-[151]
Air pollution	Blood	Decrease methylation in DNA repetitive elements with unknown effect	[152]
Asbestos	Pleural tissues	Ageing and susceptibility to different diseases	[153]
Arsenic	Tumor samples, blood	Increased risk for bladder cancer. Arsenicosis, skin cancer	[154], [155]
Silica	Blood	Silicosis, lung cancer	[156], [157]
Benzene	Blood	Increased risk of acute myeloid leukemia	[158]
Ultraviolet radiation	Skin samples	Genome-wide hypomethylation, skin ageing	[159]
Alcohol consumption	Blood	Robust DNAm signature of a heavy consumption	[160]
Coffee and tea consumption	Blood	DNAm changes in genes involved in estradiol metabolism and cancer (women only)	[161]
Antioxidant and vitamin rich diet	Blood	DNAm changes in mismatch repair enzymes	[162]
Folate depletion	Blood	Global hypomethylation	[157], [163]
Vitamins D, B supplements	Blood	No effect on long interspread nuclear element (LINE-1) methylation	[164]
Vegetarian diet	Blood	Disturbed methyl group metabolism	[165]
Caloric restriction	Adipose tissue blood	DNAm changes in genes involved in weight control and insulin secretion	[166]
Famine	Blood	Obesity, hypertension, cardiovascular diseases.	[167], [168]
High-intensity walking	Blood	Suppression of pro-inflammatory cytokines	[169]
Non-specified regular physical activity	Liver biopsies, muscle biopsies	Change of methylation status of *MT-ND6.* Changes in insulin sensitivity	[170], [171]
Endurance exercise	Muscle biopsies	DNAm changes in regulatory enhancers	[172]
Leisure-time activities	Blood	Moderate changes in methylation profile, cancer-specific pathways?	[173]
Early life stress	Post-mortem brains. Blood.	Suicide. Serotonin transporter *SLC6A4* affected. No robust findings.	[174]-[176]
Socioeconomic status	Blood	DNAm changes in inflammation-related genes	[177]
Educational attainment	Blood	Low-education-methylome resembled one from a smoker	[178]

Table 1: DNAm candidate-gene and epigenome-wide studies of the environmental influences in adult human cohorts, studied tissues, and health effects (if known) or major finding. The chemical and physical environmental cues are extensively studied, as well as various nutritional factors and exercise. The studies on childhood adversity and socioeconomic factors are limited due to the low availability of the longitudinal studies. DNAm studies on sleep and shift work will be discussed in Sections 2.4 and 2.5.

Pathological conditions. Due to rapid technological advances of the last decade, a growing body of studies has been published seeking out correlations between DNAm and disease traits. Figure 5 provides an overview of some common human disorders, excluding developmental syndromes and cancers, where modified patterns of DNAm have been revealed by the EWASes.

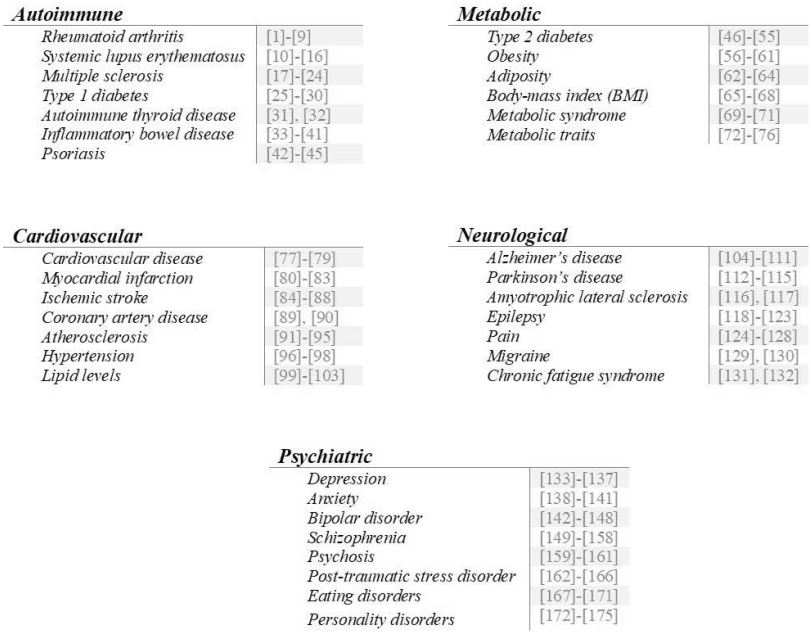


Figure 5: Some common human diseases with studied DNAm patterns. The results are from the systematic PubMed search carried out 15-17 of April, 2020, with the following criteria: a) study conducted in 2010-2020; b) epigenome-wide study; c) case-control/twin study; d) array-based technology applied in study. For the sources marked in green, see Appendix. DNAm studies on sleep disorders are reviewed in Section 2.4.

This systematic search presented in the Figure 5 was carried out due to two reasons. Firstly, I wanted to emphasize that exploration of the human methylome touched an impressive number of the most common disorders affecting humans. For couple of decades, DNAm analysis stayed far behind genome-wide association studies (GWAS), but once the platforms profiling epigenetic changes became available, more and more EWASes reached

publications in scientific journals. Though a typical EWAS cohort includes tens or maximum few hundreds of individuals, not hundreds of thousands as in GWAS, for such complex disorders, as schizophrenia or rheumatoid arthritis we already can find several studies performed on various cohorts of patients. EWAS might be still behind GWAS, but it is catching up rapidly. Secondly, just within the same disorder, we can observe a great variety of research questions, study designs and applied methods. For instance, if we look at the multiple sclerosis, we notice that DNAm studies include monozygotic twins discordant for multiple sclerosis and non-twin case-control study, the study performed in blood and brain samples, as well as the study where secondary-progressive and relapsing-remitting individuals were compared with healthy controls. The titles of quite many EWAS studies involve words “signature”, “pattern” or “biomarker”. Growing larger in terms of sample sizes due to cost reduction, exploration of DNA methylome attracts researches from different medical fields, and study by study we are approaching closer in the understanding of molecular processes behind complex human pathologies.

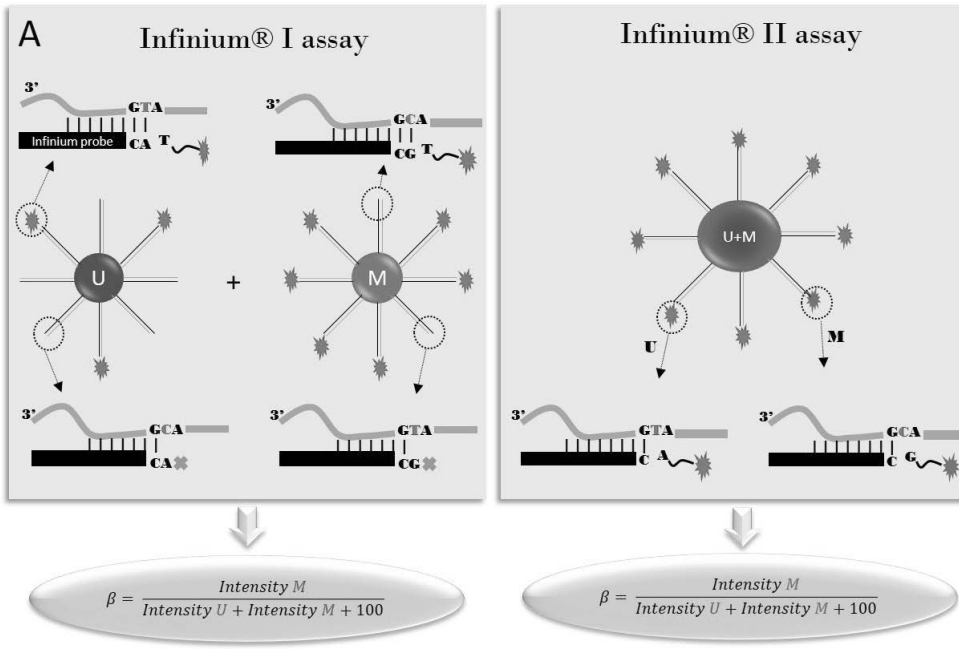
2.3.3 Exploring human methylome

Epigenome-wide association study aims to reveal statistically significant correlation, but whether DNAm is causal in the pathogenesis of certain disease remains unclear. We also know that quite many of the specific environmental factors, such as diet, lifestyle habits (Table 1) are linked to the somatic and mental disorders. As such, DNAm variation can be affected by genetic variations, environmental factors, and stochastic processes of ageing and /or by all the above. Methods for genome-wide DNAm analysis include three major principles used to distinguish between methylated and unmethylated cytosines that are adapted for array-based-based platforms: restriction endonuclease-based, immunoprecipitation-based, and bisulfite conversion. The vast majority of the EWASes listed in Figure 5 were carried out using Illumina Infinium® HumanMethylation microarrays, and since all studies in this thesis were performed on IlluminaHumanMethylation450 array, I will focus on this assay in the section devoted to the technological aspects of measuring DNAm.

2.3.3.1 Genome-wide DNAm profiling using Infinium® technology

Based on the Infinium® Technology, Illumina Infinium HumanMethylation450 BeadChip (450K) contains 482,421 CpG sites, 3,091 non-CpG sites and 65 random SNPs targeting 26,658 CpG Islands in 21,231 genes, with a global average of 17.2 probes per gene [179]. The validation studies, following the release of 450K assay in 2011, confirmed a strong correlation with an assay of previous generation, Illumina HumanMethylation27 BeadChip (27K), as well as high concordance between biological replicates [179], [180]. The correlation values between 450K and whole genome bisulfite sequencing data numbered 95%, indicating that data generated by the new assay are consistent with the results from the 'gold standard' of DNAm measurement, whole genome bisulfite sequencing. Considering that DNAm changes are typically mild, manufacturers emphasized that 450K array is capable to detect the differences of 20% in methylation level with 99% confidence. Though in 2015 Illumina 450K was followed by a larger version, IlluminaHumanMethylation850 ('EPIC'), it remains the most widely used Illumina array in epigenetic research as the best compromise in terms of costs, sample throughput and coverage.

The foundation of the Infinium technology is the assessment of cytosine methylation at the CpG site through quantitative genotyping of the C/T polymorphism generated by the bisulfite conversion [181]. Whereas 27K was based on a single type of assay (Infinium I), its extension 450K is a hybrid utilizing two different chemical assays Infinium I and Infinium II (Figure 6A). Such two-probe or, in fact, two-assay design results in divergence between methylation beta-values retrieved from Infinium I and II assays, leading to a specific type II bias during analysis [182]. To overcome this issue, several within-array normalization algorithms, along with the specialized steps for preprocessing, were developed as 450K analysis pipelines and further integrated into R software packages (Figure 6B).



B	*methylumi*	*minfi*	*wateRmelon*	*ChaMP*	*RnBeads*
Import raw data files *.idat*	✓	✓	✓	✓	✓
Background correction	✓	✓	✓	✗	✓
Adjustment for type II bias	✓	✓	✓	✓	✓
Cell composition correction	✗	✓	✗	✗	✓
Batch effect analysis	✗	✓	✗	✓	✓
Batch effect correction	✗	✓	✗	✓	✓
Probe filtering	✓	✓	✓	✓	✓

Figure 6: Infinium HumanMethylation450 BeadChip technology and data preprocessing pipeline. **A)** Infinium hybrid assay used in 450K array. Infinium I (27K) utilizes two beads per CpG probe, methylated and unmethylated, sharing the same color channel. To extend 27K array, a second assay type was added. Infinium II uses a single bead and two color channels. Due to the particular probe characteristics, i.e. CpG density within the probe, assays show different dynamic ranges, creating divergence of beta-values and type II bias during analysis. **B)** Illumina 450K preprocessing pipeline. Freely available R packages for 450K data preprocessing include *methylumi*, *minfi*, *wateRmelon*, *ChaMP*, and *RnBeads*. The R package *minfi* is used in this thesis. Figures modified and adapted from [182], [183] with permissions.

The typical pipeline for the processing of 450K data includes import of the raw data (IDAT files) and quality control procedures aiming to assess the success of the bisulfite conversion and array hybridization steps. Next, importantly for the hybrid design, within-array normalization is performed, comprising background correction and adjustment for type II. Over the past years few methods were developed and successfully validated in cancer cell lines, for instance, 'peak-based correction' [182], Beta MIxtrute Quantile dilation (BMIQ) [184], Subset-Quantile Within Array Normalization (SWAN) [185], Sub-Quantile Normalization (SQN) [186], All Sample Mean Normalization (ASMN) [187], and data-driven approach [188]. In all studies for this thesis, we selected SWAN, a method that was derived from normalization methods applied for the microarray expression platforms [185]. Validated in the kidney and rectum cancer cell lines, SWAN substantially reduced the differences in the distributions of beta-values derived from two assays, decreased technical variability between arrays, and, importantly, maintained sensitivity by showing a better detection of the differential methylation. Though SWAN was developed as a within-array normalization procedure, it is capable to conduct batch effect correction when comparing between arrays. Needless to say that other methods, such as ASMN, BMIQ and SQN demonstrated comparable performance in the evaluation of different pipelines and serve as valid alternatives for within-array normalization procedure [187], [189].

Batch effects are widespread in high-throughput experiments and are critical to address [190]. Few possibilities to minimize or avoid them include a) application of supervised or unsupervised correction methods (reviewed in [183]), b) careful plan of a study design (described in detail in the next section), and c) adjustments for technical variables of the study, e.g. use of methylation array and slide as covariates in the regression model.

The most common tissue for measurements of genome-wide DNAm is whole blood which is a heterogeneous mixture of different cell types. This presents an issue: in fact, DNAm profile observed from whole blood is a collection of the DNAm profiles from different types of the white cells. In order to explore how much the distribution of the different types of cell explain the observed variability in the DNAm, Jaffe and Irizarry [191] conducted a study on publicly available datasets from Illumina450K platform. The authors found a strong evidence that cell types displayed larger variability than age, as well as confirmed that cell type proportions change with age and strongly correlate with global DNAm profile. Currently the most common approach for the users of 450K array is to estimate cell counts

from DNAm data, as described in the study of Houseman *et al*.[192], check for the possible confounding and perform adjustments for the cell counts in the regression model, if considerable confounding was detected.

The last preprocessing step in the 450K analysis pipeline is probe filtering. Several types of CpG probes are typically removed from subsequent analyses:

- probes that failed the hybridization step and showed detection P value > 0.01;
- probes located on sex chromosomes (11,232 probes on X chromosome, 416 probes on Y chromosome);
- cross-reactive probes (CRP) which are found to co-hybridize to various sequences and, thus, generating spurious signals (4400 probes) [193];
- polymorphic CpGs overlapping known SNPs, as DNAm detected for such probe can be affected by underlying genetic polymorphisms (16,770 probes) [193].

Typically, 7-15% of the probes are removed in the preprocessing steps and the final number of remaining probes is usually reported in the section describing methods of the study.

The beta-value derived from the 450K preprocessing pipeline represents the measure of intensities of the umethylated and methylated status at the interrogated CpG site and ranges between 0 and 1 (Figure 6A). Another common way to measure the methylation level would be a logit transformation of bets-value, (log2 ratio of the intensities of methylated prove vs unmethylated probe), or M-value. Though beta-values are considered to be more intuitive for biological interpretation, they possess severe heteroscedasticity for highly methylated and unmethylated probes. In a comparison study of beta- and M-values [194], the M-value method demonstrated a better performance and statistical validity for conducting differential methylation analyses. Still, beta-values are widely used in EWASes and, for example, found a robust application in the clustering of methylation data [195].

Summarizing this chapter, I would like to emphasize that development of Infinium platform resulted in an exponential growth of 450K data and made it an ideal choice for measuring DNAm. However, the challenges associated with the array design are considerable, and thanks to the extensive development of the various pipelines and software packages we are currently able to conduct EWASes involving hundreds of samples and make discoveries that were earlier possible only with the sequencing-based methods.

2.3.3.2 Steps towards a successful EWAS

DNAm EWAS investigates the DNAm state at genome-wide scale in a sample of individuals and estimates whether differences at the CpG loci are associated with a trait. It would be natural to assume that conducting EWAS could follow similar principles as conducting GWAS, but several key concepts related to the DNAm phenomenon pose challenges and render design of EWAS conceptually different from GWAS. Firstly, though a given CpG site is either methylated or unmethylated, the DNAm in blood averages over millions of copies of DNA and what we measure is the percentage of cells that are methylated. Thus, DNAm measurement represents quantitative measure, unlike categorical character of genotype information in GWAS. Secondly, the dynamic nature of DNAm profile is an issue that we know very little about, in particular, our knowledge about time scale of these changes is still fairly limited. Thirdly, since DNAm is greatly affected by natural variation, we need larger sample sizes for EWAS than for GWAS, for any given trait. Below I will discuss these and other crucial concepts underlying recommendations for conducting a standardized EWAS (summarized on Figure 7).

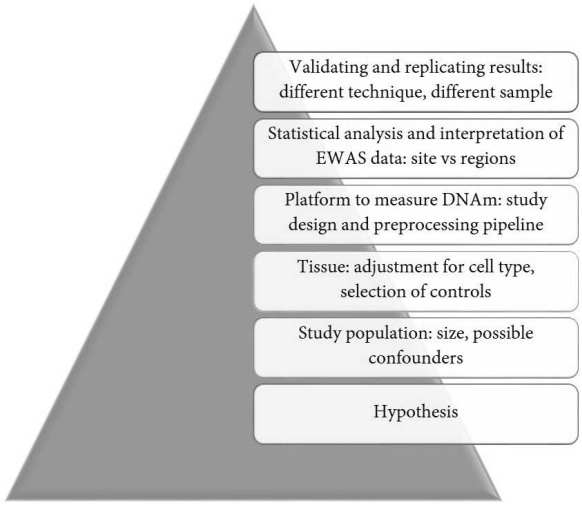


Figure 7: Steps towards a successful EWAS. Figure adapted from [196].

Hypothesis. Starting with a hypothesis essentially increases the odds to obtain biologically interpretable results from EWAS. As commonly the research question involves DNAm changes associated with a disease, it might be useful to consider possible genetic risk factors and environmental exposures that modulate both methylome and risk for the disease. In this case, clearly outlined hypothesis covering all variables will determine the choice of the study design and selection of cohorts and tissue for the study [197].

Study sample selection. As mentioned earlier, large biological variability in DNAm necessitates to conduct power calculations in order to select a sample size large enough to detect small effects of DNAm on phenotype. The presence of confounders in the population is another issue in EWAS. Among the biggest influencers of DNAm are age, gender, genetics, smoking, alcohol consumption, diet, and medication [198]. Chronological aging-associated DNAm changes have been intensively studied and for those I refer reader to a comprehensive review of Zampieri *et al*. addressing global reconfiguration of methylome during lifetime [118]. What is more surprising is that aging-associated DNAm changes can be identified in a population of the middle-age individuals with age range of only 9 years [199]. The research on sex-specific autosomal DNAm patterns has been so far inconsistent, though in a large meta-analysis McCarthy *et al*. identified sex-specific methylation profiles and particular autosomal CpG loci differentially methylated by sex [200]. Approximately 20% of the inter-individual variation in DNAm is known to be attributed to genetics [201]. Smoking is known to affect DNAm profile and some studies on smoking-related changes in DNAm are mentioned in Table 1 previously. Interestingly, a study of Tsaprouni *et al*. not only identified specific loci associated with a smoking status (current smokers, ex-smokers and never-smokers), but also demonstrated that cessation of smoking reverses the effect on DNAm only partially, with some loci not returning to never-smoking DNAm levels even after 10 years [202]. Similarly, alcohol alters DNAm even at small quantities: a rather small study on 165 females indicated that alcohol use resulted in widespread changes in DNAm, though further pathway analysis of the genes corresponding to the affected loci did not reveal any significant results [203]. Dietary factors are pointed out in Table 1 and remain important influencers of methylome, but we rarely have precise information about those for a studied cohort. In the same manner, for a population-based cohort, use of medication is rarely fully reported, while in the selected patient cohorts such information will be most likely available for stratification or statistical corrections. Therefore, two possibilities to

control for population structure and biological variability exist: a) to select a relatively homogenous population with respect to mentioned characteristics, for instance, to restrict age of participants and/or focus on one gender; and b) to adjust a regression model for known covariates, such as smoking status, alcohol consumption, genetic background, as well as age and gender if study includes a group of individuals of various ages and both genders.

Tissue selection. Several studies addressed the tissue specificity of the DNAm pattern [204]-[206]. For cancer EWASes which stayed out of scope of this thesis, the selection of the appropriate tissue may be easier, though the purity of the sample and choice of control material remain problematic. For other diseases, due to unavailability of the disease-relevant tissues, the tissue of choice is commonly blood, which requires cautious interpretation of findings and validation of results. As mentioned in the previous chapter, the common practice is to determine the distributions of the different cell types in blood by methods embedded in the preprocessing algorithms and adjust for the cell counts in the statistical analyses. In this case the study sample needs to be large enough to permit statistical corrections for the six types of cells, calculated by the widely used Houseman method [192].

Platform selection and batch effects. Choosing the right method for DNAm analysis was thoroughly reviewed in Kurdyukov & Bullock review [207] and extends far beyond this thesis. Illumina 450K is currently a good compromise of cost, sample throughput, and CpG coverage, but as any microarray technology it remains prone to batch effects and requires careful experimental design. Three issues should be addressed with particular care: a) balanced design, i.e. equal distribution of cases and controls between chips; b) random processing of samples, avoiding any possible groupings that may affect the measurement, and c) processing of all samples at the same time and under same strict conditions [196].

Statistical analysis. Currently, consensus regarding the optimal data analysis approach for identifying differential DNAm in human cohorts is missing. Two major strategies include identification of differentially methylated positions (DMPs) and differentially methylated regions (DMRs). The methods to identify DMPs involve linear regression methods with control for confounding variables, followed by multiple-testing adjustments, for example, using false discovery rate. Due to variability of the individual DNAm measurements, this approach may lead to underpowered analyses. Identifying DMRs means search for the

regions of contiguous CpGs associated with the disease. Thus, R packages *minfi* and *bumphunter* allow to detect DMRs, though the interpretation of the findings where region contains both hypo- and hypermethylated CpGs is problematic [198], [208]. Identifying methylation quantitative trait loci (meQTL) requires examining of DNAm, genetic variants, and expression data and enables to create a comprehensive map of relationship between epigenetic disease-related changes and regulation of gene expression [198].

Validation. Among two possibilities – to use a different sample or a different methylation technique – the replication in a comparable but independent study sample is more common. However, using a different technique in the validation would be probably essential, as any errors or inconsistencies in the statistical analysis or technical failures of the assay might introduce same bias in both original and validation studies [196].

2.3.3.3 From DMP to biological pathway

Similar to GWAS, next step after the regression studies and computation of *P* values for each CpG probe is the assessment of statistical significance and control of the multiple testing using Benjamin-Hochberg false discovery rate (FDR) or Bonferroni correction method. Most commonly, the choice of the multiple correction method is made in the favor of FDR, since Bonferroni method is regarded as too conservative for epigenome-wide studies and calculated q value (FDR-adjusted *P* value) represents a good balance between the number of true and false positives [209]. The widely used threshold of statistical significance defining CpG as DMP is q value < 0.05 but other thresholds, such as 0.1 and even 0.3 can be considered. Another parameter established from the association studies is hypomethylation or hypermethylation of the CpG which is estimated from the value of the beta coefficient in the regression model, with hypomethylation defined by a negative value. Next, DMPs are usually ranked according to *P* values or q values and mapped to genes using Infinium HumanMethylation450k BeadChip annotation data. From this step, two strategies to proceed with the interpretation of these results include study on individual genes (5-10 top ranked by statistical significance) and study on gene sets using pathway analyses applied to 10-500 top ranked genes. Both approaches are appropriate and frequently enrichment analyses are followed by the detailed research on individual genes from observed pathways.

Individual gene studies. The annotation data for 450K assay includes various types of information for each CpG that can be of interest for studies of gene transcription regulation. Few examples include chromosome and exact coordinate of the CpG, information about genomic location (Island, Shore, Shelf), location in relation to the gene and its transcription site (5' Untranslated region (5'UTR), Body, 3'UTR, TSS200, TSS1500), enhancer status (True, Not available), and relation to promoter (Promoter_associated). In addition to the annotation provided by Illumina, the Ensembl Regulatory Build developed within Ensembl project provides a detailed overview of the regulatory activity of genome, for instance, predicting transcription factor binding sites, promoter, and enhancer for a given gene [210]. Ensembl genome browser is an annotation platform integrating tools and data resources to facilitate genomic analyses [211]. The possibilities of Ensembl platform regarding the studies of gene functions or association with clinical disorders are enormous; for example, in this thesis we explored in detail disease and phenotype data integrated from multiple databases and catalogues [212], e.g. GWAS Catalog [213], ClinVar [214], Online Mendelian Inheritance in Man (OMIM®, McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University https://omim.org), Mouse Genome Informatics (MGI) [215], and Rat Genome Database (RGD) [216].

Gene set studies. Extending EWAS findings to pathway mechanisms underlying complex diseases has been and, perhaps, always will be a major challenge. Despite the absence of guidelines and common outlines and variability in results, the use of pathway-based analyses have grown exponentially in the last decade [217]. Furthermore, over 70 pathway analysis methods were proposed so far and, for example, once differentially expressed genes are identified, the selection of method includes both gene set analysis methods (also known as non-topology methods operating only with gene names) and topology-based methods. The latter require the input of other information, such as the direction of change (fold changes in gene expression analyses defining down or upregulated gene expression) or interactions between genes (activator/inhibitor) [218]. EWAS findings are limited to gene names, as our knowledge whether a hypermethylated CpG is associated with up- or downregulation of the gene expression is still limited. Thus, common way to proceed with the set of genes corresponding to DMPs from EWAS results is to conduct gene set analyses. Below Table 2 summarizes steps and concepts essential for the pathway analyses of EWAS data.

Step	*Concepts, examples, and sources*
Study design	Two approaches exist 1) hypothesis-driven candidate pathway analysis with targeted *a priori* known pathways to assess and 2) genome-wide pathway analysis utilizing available data on extensive range of pathways, widely used in studies of complex diseases [217].
Pathway annotation data	Pathway annotation databases are roughly divided into freely available databases and commercial databases. Freely available are widely used due to easy access and transparence of terms and features. Prominent examples include Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), PANTHER, Reactome, Biocarta, WikiPathways [219]-[224]. Commercial databases often offer user-friendly interface and pathway graphics but might require a significant investment. An example of a commercial pathway analysis tool is Ingenuity Pathway Analysis (IPA) [225] used in Publications I&III of this thesis. Users of pathway databases need to be aware of the criteria used for inclusion in pathways, as well as update intervals. Simultaneous use of several databases for the same input data typically results in divergent results, due to the differences in annotations. The general recommendation is to use multiple databases and conduct a systematic comparison of the results obtained from the different tools [226]. One approach is to use an integrative web-based platform containing various gene-set libraries and embedded visualization tools, for example, FUMA GWAS and Enrichr [227]-[229] used in this thesis. Both platforms provide also tissue specificity and chromosome/location enrichment analyses.
Input data	A set of gene names is used as an input for genome-wide pathway analyses. The most challenging aspect is the number of genes serving as input. The minimum threshold appeared to be ten genes, while the upper limit did not exceed 400. Common way is to submit all genes with statistical significance < 0.05 (adjusted/unadjusted *P* values) or below other chosen threshold. Alternatively, input list may include all genes replicated in another dataset. Some studies seem to conduct pathway analyses with various numbers of input genes (100 – 400), in order to assess robustness of findings (reviewed in [217]).
Statistical testing	In the context of genome-wide approach, pathway enrichment tool utilizes competitive enrichment method based on one of the three analytical frameworks: threshold-based, rank-based or z-score-based. The threshold-based, or over-representation analysis (ORA) uses hypergeometric, Fisher's exact test, or χ^2 test to identify the pathways that are overrepresented among the genes serving as input [218]. ORA is used in all tools mentioned earlier. The rank-based approach requires the genes to be ordered by the significance and then tests this ranked list for pathways having a lower ranking than overall distribution. Examples of statistical testing include Kolmogorov-Smirnov test and Wilcoxon rank sum test, approach is applied in GenGen and Gen Set Enrichment Analysis (GSEA) [217], [218]. Z-score-based approach is used in GSA-SNP and involves calculation of the deviation from a normal distribution accounting for the size of pathways [230].
Post-analysis considerations	Sources of bias to discuss include: a) gene set size – use of permutation and choice of threshold; b) correction for multiple testing – FDR or Bonferroni, if not too conservative; c) replication of findings in an independent data set [217].

Table 2: Conducting pathway analysis of EWAS data: summary of steps and concepts.

2.4 Sleep and DNAm

There is a substantial evidence to hypothesize that DNAm is affected by sleep. Before any of DNAm studies on sleep were conducted in humans, a pioneering genome-wide study investigating the effect of acute sleep deprivation on DNAm was performed by Massart *et al*. in mice [231]. The discovered DNAm differences between sleep-deprived and non-sleep-deprived male mice were enriched in pathways related to synaptic plasticity and neuritogenesis. Shortly after, **acute sleep deprivation** became the focus of several epigenome-wide [11], [232] and candidate gene-based [233], [234] studies. All four studies were carried out in the same sample of 16 healthy young men and DNAm was measured following one night of total sleep deprivation (TSD). Just a single night of wakefulness resulted in several outcomes:

- DNAm measured from blood is significantly affected by TSD across the genome in healthy humans. Precisely, 269 DMPs /119 genes with BH-corrected *P* values <0.05 were differentially methylated in sleep versus sleep deprivation [232];
- TSD leads to the tissue-specific alterations in methylome and transcriptome, with anabolic adipose tissue signature being different from a skeletal molecular signature, involving changes in clock genes *CRY1* and *PER1* (adipose tissue, hypermethylated promoter and enhancer region) [11], [234]. EWAS conducted in adipose tissue resulted in 148 DMRs with FDR-corrected *P* values <0.05, while no significant DMRs were observed in skeletal muscle after TSD;
- TSD has an impact on lipid metabolism and has the ability to contribute to the development of obesity via changes in gene expression of liver enzymes [233]. In particular, three CpGs located in TSS region of stearoyl-CoA desaturase 1 (SCD) were significantly differentially methylated after TSD and the changes in methylation correlated with the activity of SCD;
- TSD-affected biological pathways estimated from EWAS include metabolic fuel utilization, the Notch (genes *NOTCH4*, *RING1*, *HES1*, *CCND1*, *MAML1*), and Wnt signaling (*CCND1*, *FZD8*, *FRAT1*, *FZD6*, *WNT4*), with q-values <0.05 [11], [232];
- Correlation between the DNAm and gene expression was weak and required larger samples [11], [232], [234].

The focus of several DNAm studies carried out in larger population-based cohorts was **sleep duration**. Huang *et al.* [235] investigated genome-wide DNAm profiles in 173 young adults with short vs. long sleep duration and found 87 DMPs/52 genes with FDR-corrected P values <0.05. Furthermore, this study combined EWAS with assessment of gene function in *Caenorhabditis elegans (C. elegans)* and used a replication cohort for the DNAm investigation for genes whose perturbation affected *C.elegans* sleep. Though independent verification in a different cohort resulted in rather poor replication rate, the study revealed six genes with conserved roles in regulation of sleep duration in *C.elegans* and humans and proposed them as candidate genes for the future sleep studies (*B4GALT6, DOCK180, GNB2L1, PTPRN2, ZFYVE28, NPY*). A recent population-based study aimed to investigate the association of DNAm with sleep in 465 children using blood samples and 450K array [236]. Though this study assessed self-reported sleep problems, as well as sleep patterns estimated by actigraphy, the only significant association was found for DNAm level of a single module of nine genes and sleep duration. All but one gene from this module locate in the chromosome 17q21.31 region, with largest amount of sites annotated to *MAPT* - a key regulator of Tau proteins in the brain. Earlier in a cohort of 351 adolescents, Jansen *et al.* [237] found that associations between DNAm in LINE-1, marker of global methylation, and sleep duration are sex-specific: both long and short-sleeping boys have lower methylation, than boys in the reference category, while girls have higher LINE-1 methylation. Unfortunately, Jansen's study utilized candidate gene-based approach and lacked evidence whether sex-specific changes occur at other parts of the genome.

The only epigenome-wide DNAm study in monozygotic twins was performed for the twins discordant for **diurnal preferences** [238]. No global changes in the methylome were observed, as well as identified DMPs were only nominally significant, which might reflect the small sample size (15 discordant pairs). The GO enrichment analysis for 500 top DMPs resulted in over-represented terms related to cell adhesion and calcium ion binding – two categories essential for circadian clockwork.

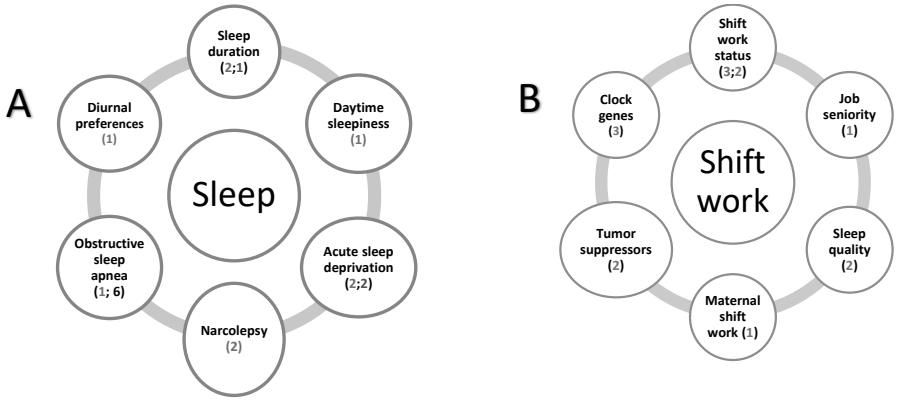
A relatively recent multi-ethnic EWAS on **daily sleepiness** revealed four genes with affected DNAm levels, however three of them were specific to African-Americans only [239]. This study utilized two main independent cohorts, a replication cohort of UK biobank, as well as SNP and gene expression data. The meta-analysis for two cohorts resulted in 14 associations, in 13 of which decreased methylation was correlated with an

increase in sleepiness. Interestingly, of several associations found in EWAS, the only gene with observed correlation between DNAm and gene expression or sleepiness was *RAI1*, a gene involved in SMS and OSA, located at chromosome 17.

Genome-wide DNAm in relation to **narcolepsy** was researched in two studies of Shimada *et al.* [240], [241]. A combined-rank approach for the top DMPs from blood samples [240] evidenced for 95% of hypomethylated probes and high number of sites located outside of CpG islands, with the top site corresponding to adenosine A1 receptor (*ADORA1*). The latest study on narcolepsy utilized DNA samples extracted from lateral hypothalamic and temporal cortical brain regions and revealed 77 DMRs, with the top association for myelin basic protein (*MBP*) in lateral hypothalamus [241]. In addition, authors reported that these DMRs significantly overlapped with DMRs found for the multiple sclerosis, indicating that two diseases might share partially their pathogenesis.

DNAm patterns of **OSA** stayed in focus of at least six candidate gene-based studies and one EWAS (See Figure 8A for the references). However, the results obtained from the methylation studies on OSA may reflect more hypoxia, rather than sleep loss. Although latter is an important factor in the pathophysiology of OSA, the metabolic disturbances due to intermittent hypoxia are equally important.

Overall, DNAm studies on sleep and sleep disorders number less than twenty (Figure 8A). In order to explore the associations between chronic sleep loss and methylome, we need to consider DNAm studies performed in the cohorts of shift workers (Figure 8B).



Sleep duration [235]-[237]
Daytime sleepiness [239]
Acute sleep deprivation [11], [232]-[234]
Diurnal preferences [238]
Narcolepsy [240], [241], [251]-[257]
Obstructive sleep apnea [251]-[257]

Shift work status [242]-[246]
Job seniority [247]
Sleep quality [248], [249]
Maternal shift work [250]
Tumor suppressors [249], [258]
Clock genes [248], [259], [260]

Figure 8: DNAm studies on A) sleep and sleep disorders and B) conducted in shift workers. Numbers in parentheses indicate: EWASes, blue; studies utilizing candidate gene approach, green.

2.5 DNAm studies in shift workers

There have been numerous studies to evaluate differences in DNAm profiles of night shift workers, as compared to day workers, in various occupational cohorts. To begin with, I summarize findings from four **epigenome-wide studies**, three of which have been performed in the cohorts of female nurses and one in the hospital-based cohort of mothers and infants. The pioneering study of Zhu *et al.* compared DNAm levels across 27K sites between ten female long-term night and day shift workers [242]. Despite a very small sample size, 4,752 genes, including *CRY2* and *CLOCK*, showed differential methylation at *P* value <0.05 after adjusting for multiple comparisons, with two-thirds being hypermethylated in the night shift workers. Using IPA network analysis and all 4,752 genes as input, a pathway related to DNA repair was found featuring several cancer-relevant genes, including *CDKN2A*, *BCAS3*, and *ESR1*. Considering a large number of genes submitted for pathway analysis, small sample size, as well as the smallest Illumina array used for DNAm profiling, these results should be interpreted with caution. A larger study of Bhatti *et al.* [245] compared DNAm profiles of 59 actively employed night shift workers

and 65 day workers and found that 65% of the 473,800 tested CpGs were hypomethylated, indicating a systemic loss of methylation among the shift workers. The CpGs in several core circadian genes, including *CLOCK, CSNK1D, CSNK1E, NPAS2, PER1, PER2, PER3*, and *RORA* were significantly less methylated (FDR-corrected *P* values <0.05) among the night shift workers. GO enrichment analysis indicated that terms related to host defense and immunity were significantly represented among the genes corresponding to DMPs. Though no cancer-related pathways were found from the enrichment analysis, such prominent loss of methylation genome-wide and, in particular, in circadian genes, may contribute to the increased risk of cancer. Several studies on transcriptomics indicated that alterations in the gene expression of circadian genes were associated with increased risks of various cancers [261]-[265]. Once Bhatti's sample was extended to 197 females (111 night shift vs. 86 day shift workers) [246], EWAS resulted in no significant findings in relation to shift work status. The top findings were hypomethylated and corresponded to the genes linked with various cancers (*BACH2, JRK, RPS6KA2*) and type-2 diabetes (*KCNQ1*). Similarly, no significant DNAm differences were found in relation to the chronotype of night shift workers. The latest EWAS in relation to shift work was performed in placental tissue from 237 participants who were engaged in night shifts work or not, and reported an overall trend towards hypomethylation among the top 57 DMPs [250]. Enriched GO-terms belonged to "cell-cell adhesion", and notably, among the top 298 DMPs submitted for the enrichment analysis, the only clock gene was *PER1*.

Candidate gene-based approach comprised two groups of genes that were extensively studied in shift workers: circadian genes and tumor suppressors. The findings of DNAm studies of **circadian genes** in shift workers have been consistent with the study of Bhatti: *PER1*, *PER2*, and *BMAL1* showed nominally significant decreased methylation in night shift workers [259]. Furthermore, for instance a higher number of night shifts was associated with a decrease of *PER2* methylation, while *PER1* and *BMAL1* methylation decreased with a longer lifetime duration of night shift work. However, once the sleep quality and sleep duration were included in the study of Bukowska-Damska *et al*. [248] as outcomes, adjusted for the shift work status and chronotype, the findings on DNAm levels of circadian genes became inconsistent. Thus, *PER2* methylation increased among the morning chronotype day workers who slept 6 hours and less, and same occurred to *PER1* methylation among the evening chronotype workers who slept 6 hours and less per day. Adjustments for chronotype

and shift work status resulted in lack of significant associations for other circadian genes, even though the sample comprised 710 women. It is plausible to assume that female shift workers represent a heterogeneous sample where job seniority, number of shifts per night, total night shift work duration and other factors affect DNAm in a complex manner and should be considered as potential confounders in DNAm studies.

Circadian disruption in shift workers was hypothesized as one of the molecular mechanisms increasing risk of cancer. Of five studied circadian genes *CLOCK, BMAL1, CRY1, PER1*, and *PER2,* increased DNAm levels in breast cancer cases among the night shift workers were found in *CLOCK, BMAL1*, and *CRY1* (all nominally significant associations), as compared to breast cancer-free controls [260]. None of these studies assessed gene expression, therefore results suggest that epigenetic regulation of circadian genes is rather complex and DNAm of clock genes may contribute to cancer development in shift workers.

Regarding the **tumor suppressors**, no significant findings were observed in the study of association between sleep quality and DNAm levels of the selected **tumor suppressor genes** in shift workers [249]. In a recent study of Carugno *et al.* [258], however, night shift workers had clear reduction of methylation in *ESR1, TP53, BRCA1*, and *BRCA2*.

2.6 Studies of DNAm dynamics

The majority of existing EWASes are cross-sectional and unable to evaluate DNAm changes over the time and establish the direction of causality. Given the dynamic nature of the human methylome, longitudinal studies are ideal for capturing such dynamic changes that accompany progression of the disease or those induced by environment. However, today, only a limited number of longitudinal DNAm studies have been performed. A systematic search in PubMed conducted 11.05.2020 using key words ‘DNA methylation’, ‘dynamic changes’, ‘dynamics’, ‘longitudinal analysis’ resulted in 351 studies. Of those, only studies conducted in human samples (adults or adolescents, $N \geq 6$), with epigenome-wide assessment of DNAm changes were taken for further investigation. After removing a) twin studies, b) prenatal, development, early childhood studies, c) studies on ageing, and d) studies on cancer samples, 23 longitudinal epigenome-wide association studies completed between 2015 and 2020 remained for the discussion in this chapter.

The vast majority of these studies used Illumina 450K array (18 studies), three studies profiled subjects with EPIC array [266]-[268], and two studies performed DNAm measurement on 27K array [269], [270]. The number of profiled participants varied greatly, with minimum of six [271] to the biggest cohort comprising 1,344 individuals [272]. Half of studies involved less than 100 individuals, five studies included few hundreds but less than a thousand [266], [268], [269], [273]-[275], and six studies performed DNAm measurements of more than a thousand individuals [272], [276]-[280]. Replication in an independent cohort was carried out in six studies [273]-[275], [277], [279], [281]. Concerning the significant findings, all but one study reported individual DMPs or DMRs (significant after correction for multiple testing comparisons), with a single study exploring differentially methylated signatures and differentially methylated pathways [282]. In virtually all studies, DNAm analyses were followed by some type of gene set enrichment analysis, network analysis or clustering.

Of 23, twenty-one study utilized paired design, one study measured DNAm at three time points [283], and one study profiled individuals across six sampling points [271]. According to the time interval between first and last measurement, I divided all studies into the four groups: 1) 'very long', 11-12 years; 2) 'long', 5 – 10 years; 3) 'short', 12 months – 24 months, and 4) 'very short', 1 day- 6 months.

'Very long'- term dynamics. Altogether four paired studies explored DNAm changes during prolonged time: two were concerned with chronic alcohol consumption [270], [279], one study was performed for the smoking status [278], and one - for BMI [280]. All studies in this group concluded that trait-associated DNAm changes occurred genome-wide, however, the amounts of modified CpGs are strikingly low. Both alcohol consumption [279] and smoking status [278] assessed in large cohorts were accompanied by changes in 300-400 DMPs (less than 0.1% of 450K probes). Both studies also estimated the reversibility of changes, for instance, authors of alcohol study mentioned 218 DMPs with 100% degree of reversibility observed in former drinkers. Despite a large sample, BMI study resulted in 310 DMPs associated with BMI, but only 34 of them showed changes longitudinally after 11 years [280]. This study also lacked any assessment of reversibility.

'Long'- term dynamics. This groups of five paired studies assessed DNAm changes occurring within 5-9 years. Two studies on the smoking status [269], [272] produced results

consistent with a 'very long'-term study [278]: a small amount of significant distinctive changes in 22-52 CpGs and slow reversion of methylation levels to never smoker's level during several decades after cessation of smoking [272]. The BMI study of Sun *et al.* [274] established causality in the relationship of obesity and DNAm, indicating that 'obesity is the cause, not a consequence, of changes in DNAm over time'. Interestingly, causality was evidenced from unidirectional changes of only 25 DMPs observed after 6.2 years in the main and replication cohorts of totally 1,120 individuals. A large study of Ward-Caviness *et al.* [277] on the incidence of myocardial infarction combined three cohorts and explored DNAm changes in the range of 7-9 years. Nine DMPs defined as 'epigenetic fingerprint' of myocardial infarction were consistently revealed in two cohorts and further tested for its prediction in the third independent cohort. Despite modest results from prediction analysis, this is the only longitudinal DNAm study of cardiovascular condition integrating gene expression and metabolomics data. Last in this group, a study on anxiety disorders conducted in the cohort of 47 adolescents over 5 year follow-up detected four distinctive trajectories of DNAm changes according to the differentially methylated signatures and diagnosis [282]. In the persistent group (individuals suffering from anxiety at both time points), 27 differentially methylated pathways were observed (adjusted *P* values <0.05), accompanied by global hypermethylation in the central nervous system development pathway. Notably, the study used saliva samples from non-medicated adolescents of both genders and aimed to uncover gene ontologies, rather than isolated genes.

'Short'- term dynamics. The group of 'short'-term dynamics included six paired studies and a study with six time points [271], all investigating changes occurring within 12-24 months. Three studies investigated DNAm changes in relation to psychiatric disorders after one year follow-up: bipolar disorder (N=95) [284], conversion to psychosis (N=39) [285], and depression (N=59, adolescents) [281]. Perhaps, due to small sample sizes, the amount of statistically significant changes ranged from one to nine DMPs, with large amount of nominally significant results. Surprisingly to be in this group, two studies on neurodegenerative disorders – pre-symptomatic dementia [286] and Parkinson's disease progression and medication [268] – revealed 8 and 138 DMPs, respectively, all corrected for age and assessed for overlap with Horvath clock [140]. A relatively small study on metal exposure and subclinical atherosclerosis in a cohort of 23 middle-aged men identified ~ 0.1% of DMPs with changes occurred after two years of exposure [287]. Finally, the 'six-

time point study' involved six individuals profiled during long-term isolated environment, Mars-500 mission [271]. Despite a small size, phenotype synchronization algorithm applied in the study resulted in 5,326 DMPs (1.1% of 450K probes) enriched in pathways related to mood, circadian rhythms, and glucose state. Needless to say, longitudinal personal DNAm dynamics was earlier explored across 28 data points, measured over a period of 36 months in a 54-year old human volunteer with elevated glucose condition [288]. This study provided evidence for relatively slow DNAm changes, occurring 80-90 days prior to glucose elevation. Overall, personal methylome seemed to be extremely stable longitudinally, with minor changes taking place in regions associated with gene regulation.

'Very short'- term dynamics. This group comprised seven short-term studies, all of which were concerned with some type of distinct exposure: medication [276], [283], bariatric surgery [289], meditation [290], combat experience [273], [275] or acute alcohol withdrawal [266]. Both metformin [283] and fenofibrate [276] therapies, as part of clinical trials aiming to explore drug-induced alteration of DNAm profiles during one or three weeks, respectively, resulted in few DMPs affected by drug. Similarly, a study on adiposity state of normal and obese women before and after bariatric surgery identified two differentially methylated genes [289]. A 'meditation' study was performed in a cohort of 17 experienced meditators, profiled before and after a day of intensive mindfulness-based practice [290]. Surprisingly, 61 DMPs enriched in genes associated with immune response and ageing were identified, evidencing for very rapid changes in human methylome over a short time window. The study of Snijders *et al.* [273] compared DNAm patterns in three military cohorts before and after ~ 6 months combat exposure, in relation to the development of post-traumatic stress disorder (PTSD). Though majority of DMPs failed replication, the most prominent findings involved DMRs corresponding to genes, previously associated with PTSD in the longitudinal military cohorts [275]. Last for this group, a study of Witt *et al.* [266] investigated DNAm changes in 99 male patients during acute alcohol withdrawal and after two weeks of recovery, as compared to age-matched controls. A relatively large number of observed DMPs (2,876) witnessed for prominent genome-wide changes associated with withdrawal, mainly involving genes related to immune system response. Similar to the abovementioned study of Dugué *et al*. [279], the differences in DNAm levels between cases and controls diminished after the recovery, indicating partial reversibility of alcohol-related methylation.

Common for all studies is the fact that outside of the early stages of development and within such a relatively short period of human life as a decade, human methylome is more stable than dynamic. Nevertheless, various environment exposures, lifestyle habits, as well as underlying pathological conditions modify DNAm inducing a limited amount of precise changes. According to some of these findings, certain modifications are reversible, for instance, upon cessation of smoking some of the DNAm changes are reversed to the baseline (non-smoker's status) relatively fast and some take decades [278].

***

The studies from the last two groups explored DNAm changes over short time intervals, similar to Publication II of this thesis. However, to the best of our knowledge, there is no longitudinal study which has examined epigenome-wide DNAm changes in relation to sleep, sleep disorders, circadian rhythms or shift work in human cohorts.

3 Aims

Our main hypothesis was that sleep loss induces systemic changes across the human DNA methylome. The goal of this thesis was to explore DNAm changes of blood leucocytes associated with curtailed sleep, as well as to investigate how transient and reversible such changes are.

The specific aims for each publication were:

1. to identify and characterize deviating DNAm pattern in relation to insufficient sleep. In this study, we used two independent datasets: the DILGOM cohort including individuals reporting insufficient sleep and occupational cohort of shift workers suffering from insufficient sleep as part of SWD diagnosis. (Publication I)
2. to observe DNAm changes in relation to a manifesting circadian sleep/wake disorder, SWD, and the recovery from it. In this study, we used paired data from an occupational cohort of shift workers, in combination with sleep diaries and questionnaires collected during work and after vacation. (Publication II)
3. to study the effect of sleep disturbances and depression on DNAm and explore underlying biological processes in adolescents suffering from depressed sleep. (Publication III)

4 Materials and Methods

We used DNAm data (Infinium HumanMethylation450k), questionnaires, and clinical data from Finnish Institute for Health and Welfare (DILGOM, general population-based sample), Finnish Institute of Occupational Health (AIRLINE I and II, occupational cohort of shift workers), and Helsinki University Central Hospital (ADSLEEP, selected cohort of patients and healthy staff). Table 3 lists the sample datasets, basic characteristics of the participants, and the study designs used in each publication.

Publication	Cohort	N (Mean age ± SD)	%, men	Study design
I	DILGOM	79 (39.3 ± 7.3)	100	Cross-sectional
	AIRLINE I	26 (44.9 ± 9.0)	100	Cross-sectional
II	AIRLINE II	32 (43.8 ± 8.8)	78	Paired measurements
III	ADSLEEP	17 (16.0 ± 0.8)	100	Cross-sectional

Table 3: Sample datasets and study designs used in Publications I-III. SD, standard deviation.

4.1 Study samples

4.1.1 Characteristics of the participants, sampling, and study approvals

Prior to the analyses performed in Publication I, we found that age and gender strongly affect the DNAm in that sample. From corresponding EWASes we obtained a) 25.6% (122,721) and b) 7.9% (37,780) of 479,954 CpGs with false discovery rate (FDR)-corrected *P* values for age and gender, respectively. Thus, two samples used in the Publication I (DILGOM and AIRLINE I) were matched in age and gender, leaving out all women and older men. In the Publication II, we included all subjects with paired data of both genders (AIRLINE II), as no replication sample was used for these analyses. Finally, ADSLEEP used in the Publication III included only boys with depression and sleep disturbances recruited from the Helsinki University Central Hospital Department of Adolescent Psychiatry. The healthy controls recruited from the hospital staff were all men, as well.

DILGOM (I). Dietary, Lifestyle, and Genetic determinants of Obesity and Metabolic syndrome (DILGOM) is a sub-study of the FINRISK 2007 established to assess risk factors

for cardiovascular and metabolic disorders in Finnish population [291]. The DILGOM population-based subsample comprised 517 individuals (54% women) aged 25-74 years, out of which 88 men aged 25-50 with available data on alcohol consumption and smoking status (FINRISK survey questions #115 and #87, respectively) were considered for the Publication I. After defining the exclusion criteria for the study of sleep insufficiency (see 4.1.2 Phenotypes), we selected 79 men (age = 39.3 ± 7.3) for the methylome-wide analyses. The blood samples were collected between 7 a.m. and 1 p.m., after a 10-hour fasting period.

AIRLINE (I). Forty-two shift workers from a Finnish airline company aged 27-60 (21% women) participated in the questionnaire-based study carried out in the Finnish Institute of Occupational Health [292]. All participants completed questionnaire on general health, working times, lifestyle habits including smoking status and alcohol consumption, and answered specific questions on insomnia and sleepiness. The shift work schedule included morning (shift starting before 6 a.m.) and/or night shifts (shift lasting at least three hours between 11 p.m. and 6 a.m.), in addition to the evening shifts. After the shift work disorder status was assessed (see 4.1.2. Phenotypes), we selected 26 men (age = 44.9 ± 9.0). The blood sample collection was performed between 7 a.m. and 11 a.m. from a healthy shift worker with no infection episode in the last 7 days. AIRLINE I included the blood samples taken in the lab during the working period and never from a participant after the night shift.

AIRLINE (II). Out of 42 shift workers, described in the section above, we selected 32 shift workers aged 27-60 (22% women) with questionnaire data and blood samples collected twice. The first blood sample collection referred to the working period was performed as described in the section AIRLINE I. The second blood sample was collected in the lab in the morning from each participant after at least two weeks of vacation, with the same conditions of being healthy and with no episode of infection in the last 7 days. For the Publication II study we obtained paired blood samples and complete responses in the questionnaires for 32 participants.

ADSLEEP. The ADSLEEP sample comprised twenty adolescent boys, with blood samples available for 17 individuals (age = 16.0 ± 0.8). The blood samples were obtained at varying time intervals from the clinical interviews used to assess depression symptoms and sleep disturbances (see 4.1.2. Phenotypes). All participants a) were free from psychotropic and

other medications, b) did not have somatic conditions, and b) did not show any structural pathologies in the brain anatomy, according to brain magnetic resonance imaging (MRI).

All studies were performed according to the principles of the Declaration of Helsinki. Sample collection and study designs were reviewed and approved by the appropriate ethics committees. All participants or their custodians provided written informed consent.

4.1.2 Phenotypes

Across all the studies, various sleep phenotypes were tested using case-control study design. Publication I was concerned with phenotypes "insufficient sleep" and "shift work disorder status". Publication II aimed to compare individuals SWD versus healthy shift workers. Finally, Publication III comprised cases with both depression and sleep symptoms and healthy controls. Secondary analyses performed in the Publications II and III involved several additional phenotypes tested by applying correlation tests or in the association studies.

Publication I. DILGOM sample was dichotomized based on the FINRISK survey question "Do you, in your opinion, sleep enough?" as described in Aho *et al*. [293]. Among 88 men aged 25-50, seventeen were deemed as cases suffering from insufficient sleep (answers "Seldom or almost never"), sixty-two formed a control group (answers "Yes, almost always" and "Yes, often"). We excluded nine men answering "I cannot say" and ended up with 79 men in total. At least one of the following symptoms were reported by all cases: insomnia during last month, short sleep defined as 6 hours or less or tiredness in the mornings. The majority of cases (94%) stated two or three of these sleep related symptoms, while among the controls, the majority (84%) reported none or one of them.

Twenty-six AIRLINE men were divided into the SWD (cases) and non-SWD (controls) groups based on: a) questions estimating the shift type and evaluation of the working day-specific symptoms of insomnia and sleepiness, and b) a three-week sleep diary and actigraphy monitoring to evaluate a reduction of total sleep time, as required by ICSD-3 criteria [99]. Seventeen shift workers constituted SWD group reporting symptoms of insomnia and/or sleepiness "often/continuously" in relation to shift work only and reduced

total sleep time. Non-SWD group included nine men lacking significant insomnia and sleepiness.

Publication II. For the paired methylome-wide analyses performed in the Publication II, SWD and non-SWD groups of AIRLINE cohort were defined in the same way as described earlier. Both genders were included in the study, totaling 32 shift workers (21 cases and 11 controls).

For the secondary analyses of recovery in SWD group, we used two questions from the questionnaire filled during work and vacation: (Q5) "How often do you not feel fresh after sleep?" and (Q6) "How often do you feel daytime sleepiness?" The following formula defined the change in recovery symptoms estimated for each of the 21 cases:

$$CHANGE\_SYMPTOMS = (Q5 + Q6)_{WORK} - (Q5 + Q6)_{VACATION}$$

Three groups were established depending on the calculated value of CHANGE_SYMPTOMS: a well-recovered (n=10, value 2 or 3); recovered (n=5, value 1); poorly recovered (n=6, value 0 or -1).

Similarly, we calculated the difference between the M-values measured during working period and vacation, as follows:

$$CHANGE\_METHYLATION = M\text{-}VALUE_{WORK} - M\text{-}VALUE_{VACATION}$$

Publication III. The psychiatric evaluation of ADSLEEP participants was performed by a clinician using a semi-structured diagnostic interview, the Schedule for Affective Disorders and Schizophrenia for School-Age Children and Lifetime version (K-SADS-PL) [294]. Nine cases were confirmed to suffer either from a depressive disorder (n=8) or a circadian rhythm sleep disorder (n=1). None of them manifested psychotic features or was diagnosed with a bipolar disorder. None of the eight controls was diagnosed with Diagnostic and Statistical Manual of Mental Disorders (DSM) -IV axis I disorders. The sleep symptoms were assessed as part of K-SADS-PL interview attachment for affective disorders. Six items on sleep included initial insomnia, middle insomnia, terminal insomnia, sleep/wake rhythm disturbance, non-restorative sleep, and hypersomnia. All cases manifested clinically significant sleep symptoms and none of the controls did, as rated in the previous study [295].

For the secondary analyses, the depressed mood was assessed using the shorter version of Beck Depression Inventory (BDI-21) [296], BDI-19, which excludes sleep and tiredness

items. The insomnia symptoms were evaluated using self-reported questionnaire Athens Insomnia Scale (AIS) [297], while the tiredness was measured using the self-related Pediatric Daytime Sleepiness Scale (PDSS) [298]. In addition, the study included objective measures on sleep and vigilance.

4.2 DNA methylation data

In all studies, CpG methylation was performed using Infinium HumanMethylation450k BeadChip (Illumina, Inc., San Diego, California, USA). All samples were derived from peripheral blood, DNA extracted using standard laboratory methods, bisulfite converted using EZ DNA Methylation Kit (Zymo Research, Irvine, California, USA), as described in the manufacturer's instructions, and assayed on the Illumina 450k array. We conducted post-array processing and normalization for all studies, as described in Karlsson Linnér *et al*. [299]

DNA methylation data processing flowchart including preprocessing, quality control (QC) report, SWAN [185], exclusion of the probes [193], and generation of the support files including cell counts [192] is shown in Figure 9.

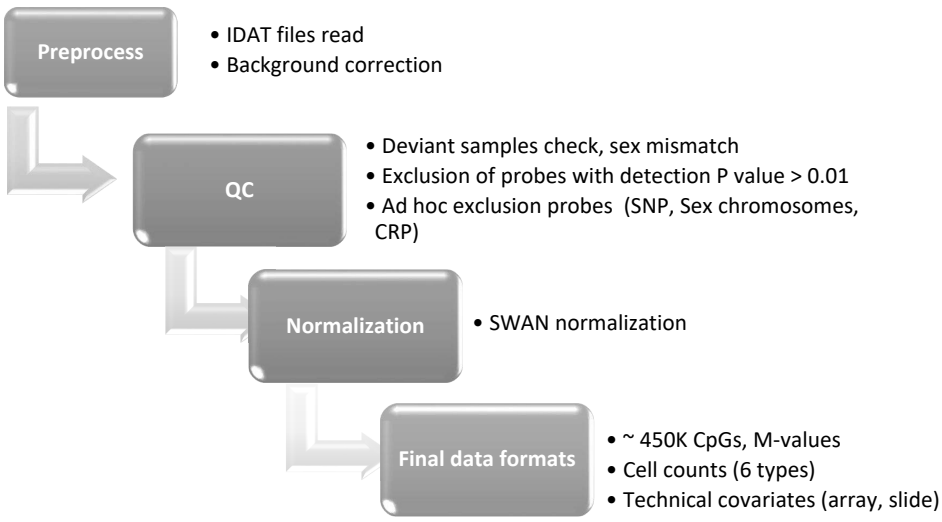


Figure 9: Data preprocessing steps, according to R package *minfi* [208].

After these steps, the final number of CpG probes numbered 479,953 (DILGOM), 433,479 (AIRLINE I and II), and 439,128 (ADSLEEP). The methylome-wide analyses in all studies were conducted using M-values (log 2 ratio of the intensities of methylated probe versus unmethylated probe), as described in Aryee *et al*. [208].

4.3 EWAS models

Publication I: we used multiple linear regression adjusted for covariates.

Publication II: to estimate an effect of being on vacation (versus working) on each methylation site, we fit a linear least-squares model involving i) a group effect for both the SWD and control groups; ii) a vacation-vs-work specific effect for each group; and iii) the covariates. The significance of a vacation-specific effect, in either, each, or both groups, was evaluated using a variance ratio (F) test, while controlling for the between-group and covariates.

Publication III: we used an empirical Bayes moderated t-test.

Table 4 lists EWAS models for each study and corresponding covariates, where applicable.

Publication	Phenotype	N, n cases/ n controls	Covariates
I	insufficient sleep	79, 17/62	age, gender, alcohol, smoke,
	SWD	26, 17/9	array, slide, 5 types of cells
II	SWD, time	32, 21/11	age, gender, alcohol, smoke, array
III	depression, sleep	17, 8/9	none

Table 4: EWAS models used in Publications I-III.

For each CpG probe, we acquired *P* value, FDR-corrected *P* value, hyper-or hypomethylation level defined from the value of the beta coefficient in the EWAS model, and a gene name where it was defined from the Infinium HumanMethylation450k BeadChip annotation data.

4.4 Bioinformatics analyses

For the gene set enrichment analyses and pathways (I-III), an unranked list of gene names served as the input with the default settings for bioinformatics analyses. For the study of associated phenotypes (I), we selected the following key words as input to the Phenotype tool of Ensembl, to RGD, and to the UniProt: a) sleep, b) syndrome, and post-hoc we also added c) visual/retinal abnormalities added *post-hoc*. For the study of tissue specificity (II), we used the GENE2FUNC tool in FUMA exploiting GTEx v7 53 tissue types. Table 5 summarizes the bioinformatics tools used in the Publications I-III, as well as the methods of assessment of the statistical significance, and corresponding citations.

Publication	Tool	Statistical significance	Source
I	GO-based enrichment: GoMiner, Panther, g:Profiler	FDR-corrected P < 0.05	[300]-[306]
	Pathways: IPA	Uncorrected P < 0.05	
	Other: Ensembl release 90, RGD, UniProt		
II	Enrichr pathways: Reactome 2016	FDR-corrected P < 0.05	[228],
	Enrichr ontologies: GO 2018	FDR-corrected P < 0.05	[229],
	Other: FUMA, Ensembl, release 93	Bonferroni-corrected P < 0.05	[307], [308]
III	Pathways: IPA	Uncorrected P < 0.05	[303],
	Other : Ensembl		[304]

Table 5: Bioinformatics tools used in Publications I-III.

4.5 Statistical analyses and data visualization

All statistical analyses in Publications I-III were done in R. Multiple testing correction was performed whenever needed using Benjamini-Hochberg (BH) correction method (EWAS, gene set enrichment analyses and pathways, correlation analyses) or Bonferroni method (FUMA, association studies). *P* value < 0.05 (after the multiple testing correction) was used to determine statistical significance, except for all methylome-wide analyses (I-III) where

uncorrected *P* value < 0.05 was defined as a threshold for a DMP. Correlation analyses (II) were performed using Spearman correlation. Statistical t-test (III) was two-sided. Association studies (III) involved linear regression model without covariates. The enrichment (a) and hypomethylation (b) scores (II) for the given Reactome 2016 pathway were calculated from the EWAS results, as follows:

(a) N, DMPs / N, all 450K CpGs corresponding to the pathway genes, %

(b) N hypomethylated DMPs / N, all 450K CpGs corresponding to the pathway genes, %

Visualization of the results was performed using R packages: *qqman, karyoploteR, RCircos* (I), and *ggplot2, ggpubr* (II).

5 Results

5.1 DNAm pattern underlying insufficient sleep (I)

To explore DNAm changes associated with insufficient sleep and SWD, we performed methylome-wide analyses in DILGOM, a population-based sample of 79 men (17 cases suffering from insufficient sleep), and in AIRLINE I, an occupational sample of 26 male shift workers (17 SWD cases). After the identification of significant CpGs with uncorrected *P* values < 0.05 (DMPs), we compared the results and found 720 DMPs common for both samples. Further analysis revealed the set of 317 genes (327 DMPs) that was subjected to the pathway analyses and to the study of genomic locations. A schematic illustration of the discovery of the overlap set is shown in Figure 10.

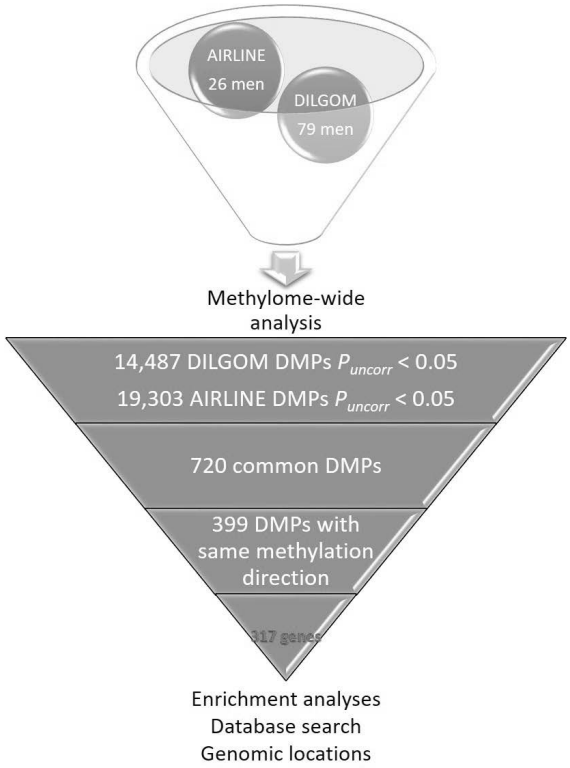


Figure 10. Schematic of the study in Publication I.

5.1.1 Loss of DNAm associated with loss of sleep

The overlap set included 317 genes annotated to 399 DMPs common for both samples. The majority of these DMPs (78%, 248/317) were significantly hypomethylated in cases, as compared to the controls, suggesting that loss of sleep may be associated with the loss of methylation.

5.1.2 Gene set ontology enrichment analyses

To identify the affected biological pathways, we explored GO libraries in four online resources (GoMiner, Panther, g:Profiler) and in commercially available pathway analysis tool IPA (QIAGEN Inc.). The gene set enrichment analyses of 317 genes resulted in two major groups of pathways, with ”Nervous system development” (NSD) being common for all pathway tools. Table 6 lists the *P* values identified for two groups of pathways in four pathway tools and corresponding GO terms.

	Nervous system development GO:0007399	Cellular processes GO:0009987, GO:0044763
GoMiner	0.039	0.014
Panther	0.014	-
g:Profiler	0.001	0.0015
IPA	1.545E-5	-

Table 6: Top results of the pathway analyses for 317 genes: numbers are FDR-corrected *P* values (GoMiner, Panther, g:Profiler) and uncorrected *P* value (IPA).

The consensus NSD pathway included 89 genes (92 corresponding DMPs) after we removed the duplicates and unified the gene names in all pathway tools.

5.1.3 The database search and study of the genomic locations

To search for the additional information on the genes included in the overlap set, we investigated the phenotype associations using the Ensembl genome browser (Human

GRCh38.p10). Of these 317 genes, 59 had no information about phenotype; 182 did not relate to any specific pathological condition; 19 were related to sleep or circadian rhythm, for example, sleep duration, sleep deprivation, chronotype or abnormal sleep behavior; and 50 genes were associated with a genetic syndrome. After a close look at the genetic syndromes, we discovered that 13/50 syndromes involved some type of disturbances in visual processing, such as retinal abnormality, retinal dystrophy, retinitis pigmentosa or photoreceptor degeneration. Thus, we conducted an additional check in Ensembl and found eight genes which phenotypes were related with disturbances in visual processing. In total, we assessed 79/317 genes with phenotypic descriptions involving “sleep disturbance”, “circadian rhythm”, “visual disturbance”, and/or “genetic syndrome”.

To inspect the genomic locations of these 79 genes, we created a circular representation of the genome (ideogram) and mapped these genes according to the chromosome location and phenotype groups (Figure 11). The ideogram revealed several clusters of DMPs on various chromosomes, out of which the most prominent one was located on chromosome 17 comprising 15 genes. Other clusters were observed at the following chromosomal locations: 1p36.12, 2p23.3, 3p21.31, 7q11.23, 11q13.1, and 19p12.

The cluster located on chromosome 17 numbered fifteen genes, of which twelve have been earlier associated with SMS in rats or humans (*ACLY*, *ANAPC11*, *AP2B1*, *BECN1*, *CPD*, *DCAKD*, *DLX4*, *DYNLL2*, *FAM195B*, *PCGF2*, *RAC*, *SLC9A3R1*) and three genes related to retinopathies both in animals and humans (*UNC119, CDK5R1*, *TSEN54*), according to the RGD. All of those were found to situate at the q-arm of the chromosome 17.

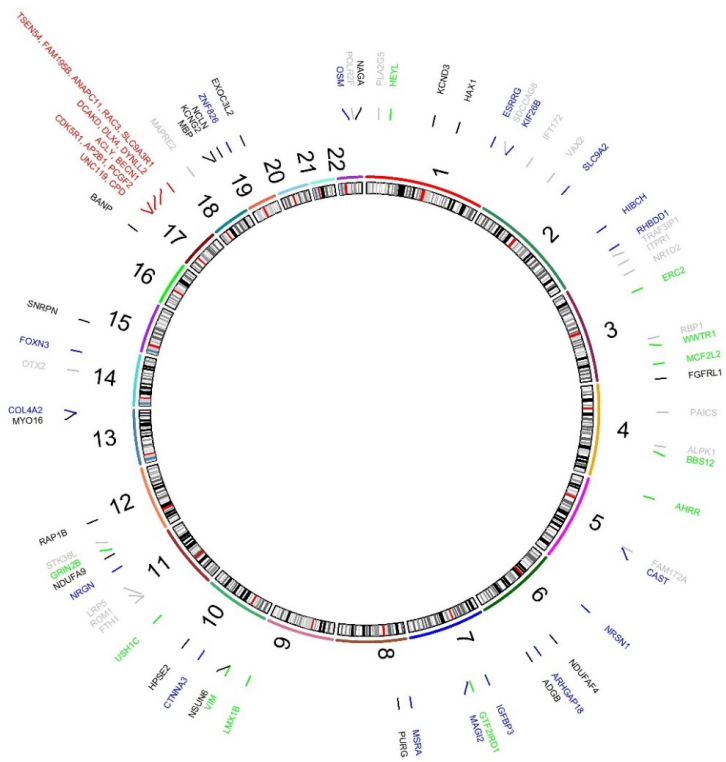


Figure 11. Circular representation of genome with mapped 79/317 genes. Genes marked in purple are associated with the sleep disturbances; in light grey – with disturbances in visual processing, in green – with both sleep and disturbances in visual processing. Genes marked in black are associated with the genetic syndromes. Red color shows genes located on chromosome 17. Figure is modified from my Master's thesis, unpublished.

5.2 DNAm pattern underlying recovery in shift workers (II)

To investigate the DNAm changes resulted from being on vacation versus working period, we performed paired methylome-wide analysis in AIRLINE II, an occupational sample of shift workers including 32 individuals (7 women, 21 SWD cases). The strongest effect of vacation was discovered for the SWD group, with identified DMPs numbering 6.5% (28,419/433,479). Considering the remarkable effect of vacation in the group with SWD, we concentrated on top ranked DMPs from SWD group in the subsequent enrichment

analyses. We investigated the biological relevance of the top findings, using in-depth pathway analysis, and evidenced SWD-specific DNAm changes in the genes *CREB1*, *GRIN2C*, and *CAMK2B* that might be crucial for the recovery from the shift work. A schematic illustration of the major findings of the Publication II is shown in Figure 12.

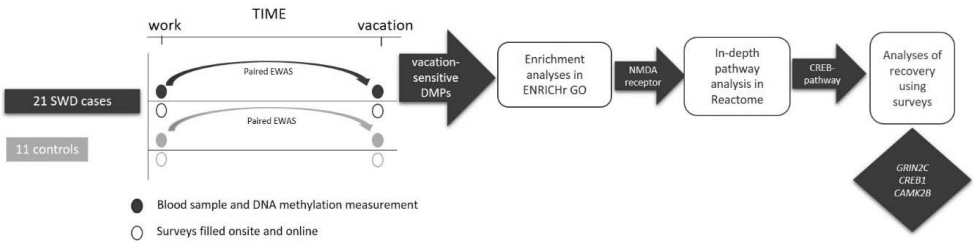


Figure 12. Schematic of the study in Publication II.

5.2.1 Restoration of DNAm associated with vacation

We found the differences in the direction of genome-wide methylation in the SWD group versus control group: in SWD, 67% (290,430/433,479) of CpGs were significantly hypomethylated during working period as compared with vacation (40% in the control group). Among the DMPs, the percentage of the hypomethylated sites numbered 78 (22,166/28,419), being 52 in the control group, proposing that vacation promoted restoration of DNAm in individuals suffering from SWD.

5.2.2 Enrichment analyses of the vacation-sensitive gene set

To explore the biological relevance of the top findings from the methylome-wide analysis in the SWD group, we ranked genes corresponding to the DMPs by the statistical significance (uncorrected *P* values). First 30 genes (uncorrected *P* value < 1E-5, FDR-corrected *P* value ≤ 0.16), identified as vacation-sensitive genes, served as input for the following enrichment analysis tools: FUMA [227] (tissue specificity), Enrichr GO 2018 (Ontology terms), and Enrichr Reactome 2016 (Pathways) [228], [229].

FUMA GTEx v7. Among the 53 specific tissue types available in GENE2FUNC tool, a significant enrichment at Bonferroni corrected *P* value ≤ 0.05 was observed for the brain tissue "Substantia nigra". Top three nominally significant results at uncorrected *P* value < 0.05 included two other brain tissues "Amygdala" and "Hippocampus". Altogether, we observed eight brain tissues among the top ten tissues with nominally significant enrichment, suggesting that the vacation-sensitive set may be differentially expressed in the brain.

Enrichr Gene Ontology 2018. The enrichment analyses of the vacation-sensitive gene set performed in GO libraries "Biological Process", "Molecular Function", and "Cellular Component" agreed on the same biological process as the top finding – NMDA glutamate receptor complex/ activity. Table 7 summarizes top three findings ranked by the statistical significance (uncorrected *P* values) from each library.

Name	GO term	*P* value, uncorr.	*P* value, BH-corr.
GO Biological Process 2018			
excitatory chemical synaptic transmission	GO:0098976	0.00004546	0.232
calcium ion transport into cytosol	GO:0060402	0.0003661	0.934
excitatory postsynaptic potential	GO:0060079	0.0003661	0.6227
GO Molecular Function 2018			
NMDA glutamate receptor activity	GO:0004972	0.00004546	0.05233
ionotropic glutamate receptor activity	GO:0004970	0.0002576	0.1483
ligand-gated calcium channel activity	GO:0099604	0.0004064	0.1559
GO Cellular Component 2018			
NMDA selective glutamate receptor complex	GO:0008328	0.00007779	0.03469
ionotropic glutamate receptor complex	GO:0034703	0.001557	0.3472
cation channel complex	GO:0034703	0.004266	0.6343

Table 7: Top 3 results from GO 2018 libraries identified for the vacation-sensitive set of 30 genes in the SWD group. Highlighted in green is the GO term found statistically significant at $P < 0.05$ after the correction for the multiple testing.

Enrichr Reactome 2016. In-depth pathway analyses with Reactome revealed similar findings as we showed using GO libraries: top ten findings related to the activity of NMDA receptor. For each of the top ten Reactome pathways ranked by statistical significance, we calculated the DMP enrichment and hypomethylation scores in the SWD and control group. In SWD group, we found the highest DMP enrichment score of 8.3% (38/458) for the pathway "CREP phosphorylation through the activation of CAMKII" (further referred as "CREB-pathway"). Since Reactome pathways are organized in hierarchical structures [309], we were able to display specific molecular processes included in the parent NMDA-associated pathway. This full hierarchical structure is shown in Figure 13.

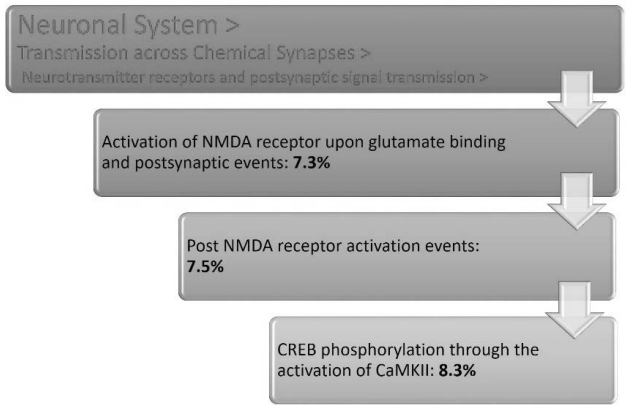


Figure 13. Reactome 2016 hierarchical representation of pathways: starting from the parent term "Neuronal System" to the major finding identified in the SWD group "CREB phosphorylation through the activation of CAMKII". Numbers are DMP enrichment scores calculated for SWD.

CREB-pathway was specific for the SWD group, as the DMP enrichment score numbered 4.1% (19/458) in the control group. We also observed hypomethylation in 74% of the DMPs in the SWD group, while in the control group the hypomethylation score was 26.3%. Thus, our findings evidenced that vacation may result in the specific DNAm changes in the genes from the CREB-pathway, which might be important for the recovery from SWD.

5.2.3 Identifying putative DNAm biomarkers of recovery from SWD

CREB-pathway identified at the previous step comprised 15 genes which corresponded to 458 CpGs in Illumina 450K array. In SWD, we found 13/15 genes affected by vacation corresponding to 38 DMPs. We further investigated the correlations between methylation values (M-values) of these 38 DMPs and the degree of recovery (changes in SWD symptoms after the vacation). Three DMPs were found to correlate at P <0.05 (not significant after FDR-correction) with the degree of recovery: cg13823003 (*GRIN2C*), cg05019488 (*CREB1*), and cg18848222 (*CAMK2B*). Since these three sites showed the biggest absolute changes in the M-values for the shift workers with the highest degree of recovery from SWD, we proposed them as possible important indicators of a restoration of the healthy state.

5.3 Studying adolescents with depression and sleep disturbances (III)

5.3.1 Synaptic long-term depression pathway associated with depression and sleep disturbances in adolescents

To investigate the effect of sleep disturbances and depression on DNAm, we performed methylome-wide analyses in ADSLEEP, a selected sample of 17 male adolescents (8 cases suffering from depression and sleep problems, 9 healthy controls). None of CpGs showed statistically significant difference in the methylation levels between cases and controls. We ranked the genes corresponding to the CpGs by the statistical significance (uncorrected P values) and selected top 500 DMPs (332 corresponding genes) with uncorrected P values < 0.05 that differed the most between cases and controls. This list of genes served as input for the analysis of canonical pathways in IPA. Three canonical pathways reached statistical significance: “Synaptic long-term depression” (LTD) with uncorrected P value = 0.00045, followed by “NOS signaling in skeletal muscle cells”, and “Netrin signaling”, both with P values = 0.0042. The top canonical LTD pathway comprised ten genes: *CACNG1*, *CACNG6*, *GRM6*, *IGF1R*, *ERK12*, *PLA2G16*, *PLA2R1*, *PPP2R5C*, *PRKG1*, and *RYR3*. Among the corresponding CpGs, all but three (cg1684179 *CACNG1*, cg08364956 *GRM6*, cg05110803 *IGF1R*) showed hypermethylation in cases versus controls.

5.3.2 Association studies between methylation levels of 10 LTP loci and symptoms of depression and sleep

Ten CpGs corresponding to ten genes from the LTD pathway were included in the association studies aiming to explore the correlations between the M-values and symptoms of depression and sleep. The results of the linear regression analyses for five sites correlating at $P < 0.05$ (significant after Bonferroni correction) are summarized in the Table 8.

CpG (gene)	Symptom (tool)	P_{uncorr}	Methylation
cg12066398 (*PLA2G16*)	depressed mood (BDI-19)	0.00031	hyper
cg04367351 (*PLA2R1*)	tiredness (PDSS)	0.00047	hyper
cg05110803 (*IGF1R*)	tiredness (PDSS)	0.00029	hypo
cg25405123 (*RYR*)	sleep (AIS)	0.00041	hyper
cg02263165 (PPP2R5C)	sleep (SWA dissipation)	0.004	hypo

Table 8: Results from the linear regression secondary analyses in ADSLEEP. *P* values are uncorrected *P* values; methylation is referred as "hypo" standing for "hypomethylation" and "hyper" standing for "hypermethylation"; SWA, slow wave dissipation during 1st episode of the night.

5.3.3 A comparative study of the results from ADSLEEP and AIRLINE II

At last we explored ten CpGs corresponding to ten genes from LTD pathway identified in ADSLEEP, aiming to compare a) direction of methylation in SWD group (hyper or hypomethylation at work), and b) effect of vacation in SWD group (uncorrected *P* value from paired methylome-wide analysis). Table 9 presents a summary of the results.

		ADSLEEP cases		AIRLINE II SWD	
Gene	CpG	*P*	Methylation, cases	*P*	Methylation
CACNG1	cg16841761	0.000381	hypo	0.036	hypo
CACNG6	cg22025854	0.000527	hyper	0.877	hypo
GRM6	cg08364956	0.000838	hypo	NA	NA
IGF1R	cg05110803	0.000306	hypo	NA	NA
MAPK1	cg19161850	0.000663	hyper	0.039	hyper
PLA2G16	cg12066398	0.000540	hyper	0.624	hyper
PLA2R1	cg04367351	0.000659	hyper	0.909	hyper
PPP2R5C	cg02263165	0.000207	hyper	0.025	hyper
PRKG1	cg18823846	0.000903	hyper	0.210	hyper
RYR3	cg25405123	0.000427	hyper	0.243	hyper

Table 9: Results from the cross-check of ten CpGs corresponding to ten genes from LTP pathway identified in ADSLEEP. *P* values are uncorrected *P* values; methylation in SWD group of AIRLINE II is referred to the data point "work" with "hypo" standing for "hypomethylation" and "hyper" standing for "hypermethylation"; two sites with NA were not assessed in the AIRLINE II sample due to the issues with quality control.

Of the ten sites, eight were included in the AIRLINE II study and all but one (cg22025854 *CACNG6*) showed the same direction of methylation. The significant effect of vacation at uncorrected $P < 0.05$ was observed for three sites: cg16841761 (*CACNG1*), cg19161850 (*MAPK1*), and cg02263165 (*PPP2R5C*).

6 Discussion

The three studies presented in this thesis aimed to explore DNAm modifications in blood leukocytes associated with sleep loss. In Publication I, we identified a distinctive methylation pattern in relation to subjective sleep insufficiency in a population cohort and in an occupational shift work sample of men. Publication II focused on the same shift work sample, but this study design involved paired data in combination with information from sleep diaries and questionnaires from both genders. The study enabled us to explore dynamic nature of DNAm during work and after the vacation, at the genome-wide scale. Publication III provided insights into the effects of disturbed sleep and depression on methylation patterns in adolescent boys compared to age-and gender-matched controls. Both Publication I and II investigate either self-reported or SWD-related insufficient sleep in cohorts of adults, while Publication III involves a case-control sample of young males with and without depressive disorder and comorbid insomnia and experiencing a crucial period of prominent changes in sleep patterns. As adolescence is characterized by sleep loss and circadian misalignments due to phase delay, this study allowed us to explore DNAm changes occurring in critical period of developmental changes. The findings of the three studies and their implications exemplify wide range of insights that can be obtained from the analysis of DNAm microarray data.

The major finding of the first study is the hypomethylated set of 399 DMPs/ 317 genes that appeared to be common for both cohorts. The loss of methylation associated with a loss of sleep is concordant with a study of Bhatti *et al*., which observed significantly and consistently decreased DNAm in blood of nightshift workers compared to dayshift workers [245]. It is noteworthy that the effect of hypomethylation on transcription is difficult to estimate due to different kinds of CpG locations, i.e. promoter region or gene body. We found that among 317 genes, only 10% showed nominally significant correlations between methylation and gene expression levels indicating that possibly DNAm is just one of the epigenetic mechanisms regulating transcription.

The observed pattern of 317 genes showed an enrichment of associations with the NSD pathway, which, according to GO database, comprises various processes underlying changes in nervous tissue. These processes include neurogenesis, synapse maturation, nerve development, and regulation of nervous system development. This finding is in agreement

with the DNAm study in brain tissues of rodents conducted by Massart *et al*., which showed that that DNAm changes in sleep-deprived animals occurred in genes involved in synaptic plasticity and neuritogenesis [25].

The analysis of genomic locations of 317 genes led us to the discovery of several interesting clusters. One of them, located in chromosome 3, included genes associated with cellular responses to such stresses as hypoxia (*LIMD1*[310], *SCAP*[311]), DNA damage (*CDC25A*[312]*, NEK4*[313]) and dietary K^+ depletion (*SLC38A3*[314]). At least two earlier studies [315], [316] highlighted the association of sleep deprivation with cellular stress, and it is possible that we disclosed epigenetic mechanisms behind this association. A cluster in chromosome 7 comprised few genes related previously to autism and anxiety (*STX1A*[317], *GTF21RD1*[318], *MAGI2*[319]). A cluster possibly involved in regulation of transcription was located in chromosome 19 and included zinc finger transcription factors *ZNF441*, *ZNF709*, *ZNF506*, *ZNF826*, and *ZNF43*. Of 317 genes, we found 9 hypomethylated DMPs corresponding to zinc fingers and hypothesize that this the loss of methylation of transcription factors might be sleep-loss specific and contribute to overall changes in gene expression, as was noted in two transcriptome studies of insufficient [293] and mistimed sleep in humans [8].

However, the most intriguing finding of the first study related to the cluster of 18 DMPs located in chromosome 17. In rodents twelve of these DMPs corresponded to genes earlier associated to SMS, a rare genetic disorder caused by mutations in *RAI1* or more complex rearrangements of long arm of the chromosome 17 [320], [321]. *RAI1* plays an important role in the regulation of circadian rhythmicity and its mutations in SMS patients are known to be responsible for the inversion of melatonin cycle [322]. The genetic variation in loci located within or near *RAI1* were recently linked to OSA in men [323] and sleepiness in a large UK Biobank cohort [324]. Since one of the two studied cohorts in the first study consisted of shift workers suffering from SWD, we hypothesize that circadian misalignments, induced by the shift work, are connected to DNAm changes in the same regions in chromosome 17 that are involved in the melatonin disruptions of SMS. It is plausible to assume that region in chromosome 17 may play a role in the regulation of sleep and circadian rhythm via epigenetic mechanisms.

The second study continued from the Publication I, incorporating a longitudinal aspect: here we studied DNAm methylation data in the same cohort of shift workers of both genders, with SWD and without, during work and after the vacation. We observed a prominent effect of vacation in the SWD group, indicating that firstly, a relatively small fraction of sites exhibits short-term dynamic changes across the genome, 6.5%. This number agrees with the earlier studies showing that human methylome is rather stable than dynamic and that only a small percentage of genomic CpG undergo dynamic changes in a non-pathological context [204], [205] . Secondly, the gain of sleep occurring during the vacation corresponded to the gain in methylation, and the DNAm restoration was more remarkable for the SWD group. Since our study, to the best of our knowledge, is the only longitudinal EWAS on recovery from a circadian sleep disorder, we could not compare our finding to other studies. We suggest that sleep restoration in shift workers may be accompanied by genome-wide DNAm increase but, importantly, our EWAS is restricted by the CpGs included in Illumina 450K and this finding cannot extend to the level of human methylome including 28 million CpGs.

With the longitudinal design of the second study, we were able to narrow down a wide group of nervous system-associated pathways to the brain-specific pathways related to the activity of a glutamatergic NMDA receptor. The largest DNAm changes in relation to the degree of recovery in SWD group occurred in the genes from "CREB phosphorylation through the activation of CAMKII" pathway, including cAMP responsive element binding protein 1 *(CREB1)*, calcium/calmodulin dependent protein kinase II beta *(CAMK2B)*, and glutamate ionotropic receptor NMDA type, subunit 2C *(GRIN2C)*. The key players of CREB-CAMKII pathway belong to the Ca^{2+}-dependent hyperpolarization pathway that earlier has been indicated in the regulation of sleep duration [325]. Several animal studies have shown that the activity of GRIN-subunits and CAMK-kinases is associated with the cortical capacity to evoke slow-wave oscillations, which is related to sleep length [326]-[328].

Based on the gene enrichment analyses and studies of recovery, we propose a mechanism, according to which recovery during vacation affects Ca^{2+}-dependent hyperpolarization pathway (Figure 14). Furthermore, such vacation-induced changes in gene activity may be regulated via DNA methylation and involve specific CpGs in *CREB1*, *CAMK2B*, and *GRIN2C*. Thus, methylome of shift workers was affected by the two-week period of vacation, with distinct changes occurring in genes involved in the activity of NMDA glutamate receptors.

The CpG sites in *CREB1*, *CAMK2B* and *GRIN2C* can serve as putatively important indicators of recovery in a shift worker with SWD symptoms. One of them, cg13823003 from the CpG Island located at the promoter region of *GRIN2C,* demonstrated statistical significance through all our analyses and might be a particularly sensitive indicator of the restoration of a healthy state and sleep recovery.

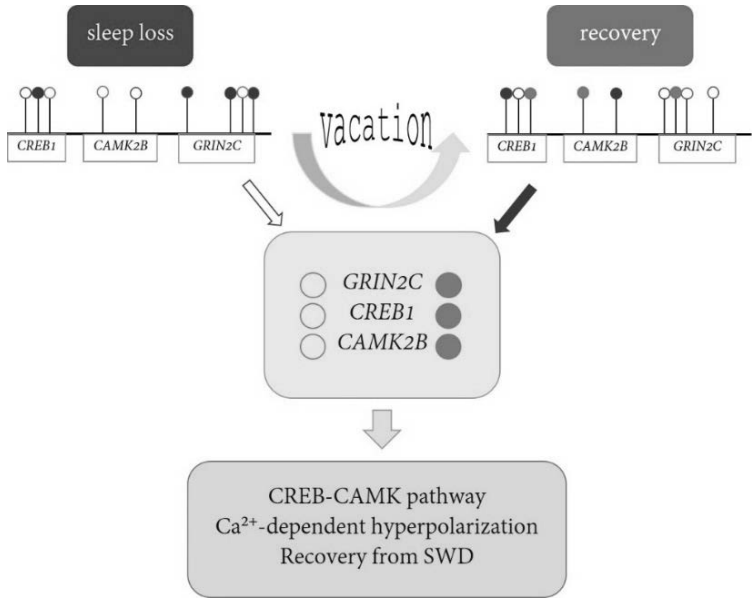


Figure 14. The mechanism of vacation-induced changes in the CREB-CaMKII pathway underlying recovery in shift workers with SWD. The schematic depicts the changes at the DNAm level (white circles, hypomethylated DMPs, dark circles, hypermethylated DMPs) in *CREB1*, *CAMK2B*, and *GRIN2C*. Orange dots represent three DMPs identified in the correlation analyses with the degree of recovery (shallow circles, hypomethylated, orange circles, hypermethylated)).

Publication III aimed to investigate the effect of sleep difficulties on DNAm in subjects suffering of sleep problems and depression. Though no genome-wide significant differences emerged from EWAS, we conducted pathway analyses for the top 500 sites that differed most between cases and controls. The top canonical pathway was the LTD pathway, with the largest DNAm changes in genes involved in the activity of calcium voltage-gated channel (*CACNG1*, *CACNG6*), metabotropic glutamate receptor (*GRM6*), as well as

signaling kinases (*ERK12*, *MAPK1*, *PRKG1*) and phospholipases (*PLA2G16*, *PLA2R1*). The synaptic LTD pathway is essential for learning and memory consolidation and refers to the process of weakening of the individual synapses. Both LTD and LTP are linked to sleep in the synaptic homeostasis hypothesis proposed by Tononi and Cirelli [21] and contribute to memory consolidation. In rats, sleep deprivation can significantly increase LTD at glutamatergic synapses, with possible mechanisms of phosphatase activation, presynaptic regulation of glutamate release and postsynaptic glutamate receptor endocytosis [329], [330]. Publication III gave evidence that adolescent boys with sleep difficulties and chronic depression show changes in DNAm in the LTD pathway indicating compromised synaptic plasticity.

Synaptic LTD pathway identified in the study of ADSLEEP is not defined in the Reactome 2016 library; however, synaptic LTP pathway is situated under "Post NMDA receptor activation events", neighboring CREB-pathway, along with several others. Considering both identified pathways might be related, we explored whether any of the CpGs identified in LTP pathway in ADSLEEP showed significant changes in AIRLINE II after the recovery period. Firstly, we observed the same direction of methylation for all but one CpG, indicating that for *MAPK1*, *PLA2G16*, *PLA2R1*, *PPP2R5C*, *PRKG1*, and *RYR3* the hypermethylated CpG during sleep loss undergoes hypomethylation during the recovery phase. Secondly, three CpGs corresponding to *CACNG1*, *MAPK1*, and *PPP2R5C* replicated in the study of recover, suggesting that some of the genes associated with LTP might be involved in the recovery from sleep loss.

Limitations. One of the obvious limitations of the three studies is the sample size. Publications I and III failed to yield genome-wide significant differences in methylation levels between cases and controls. However, in Publication I we combined the results from two independent cohorts matched by age and gender, which was a major strength of this study. In Publication III we conducted analyses in a carefully selected and homogenous sample of young, non-medicated boys and carried out careful clinical assessment of the cohort. Publication II used a relatively small sample of shift workers, with SWD status assessed by both objective and subjective measurements, as required by ICSD-3 criteria [99].

The second common limitation of the three studies is the use of blood samples for the study of sleep. DNAm is tissue-specific, so findings from blood leukocytes need to be interpreted with caution. However, sleep and circadian rhythms disturbances have a systemic effect on the human body, as described earlier [8], and all three studies agreed in the DNAm changes observed in brain-specific pathways, indicating that blood samples are informative source for changes induced by sleep loss.

Publications I and III are conducted in the male samples which limits the generalizability of the results. Both sleep and DNAm are known to be largely affected by gender, therefore we limited out studies to the male gender in Publication I. Publication II included both genders and resulted in using gender as a covariate in EWAS.

The fourth limitation, common for Publication II and III is the lack of gene expression data for AIRLINE and ADSLEEP. The functional significance of the dynamic sites identified in Publication II is of a great interest and integrative analysis of DNAm and gene expression data would enhance our understanding of epigenetic regulation of gene expression. In Publication I we attempted to explore the correlations between DNAm level and gene expression and found a rather small fraction of sites with nominally significant correlations. No significant findings were observed once we explored the correlations between DNAm levels and gene expression in DILGOM gene expression data for three DMPs from Publication II and ten DMPs from Publication III.

Conclusions and future prospects. The findings presented in this thesis shed light on some of the DNAm changes corresponding to sleep insufficiency and recovery from it. Many interesting aspects of this dynamic nature of human methylome still remain to be clarified. Both Publications I and III utilizing cross-sectional designs enabled us to show DNAm pattern in relation to subjective sleep insufficiency, and objectively assessed sleep difficulties in men. Both studies highlighted that lack of sleep is associated with DNAm alterations in brain-specific nervous system-related processes. The studies in well-powered independent cohorts or pooled data sets could improve our understanding of biological pathways behind insufficient sleep. Publication II revealed the dynamic nature of these alterations, indicating that synaptic events occurring at glutamatergic NMDA receptor underlie the process of recovery from sleep debt. Prospective studies in larger cohorts, possibly in expanded groups of shift workers, are necessary to confirm the observed DNAm

changes in the molecular processes of synaptic plasticity. Investigations of the epigenetic changes associated with insufficient sleep may lead to the discoveries of important indicators of sleep loss in shift workers with circadian rhythm disturbances and may be of practical use for prevention of chronic adverse health effects of the shift work.

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This book is dedicated to the memory of my Dad who passed away on the 5th of March of 2020. Had he lived long enough to see my public examination, it would have made his day, I am sure.

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Appendix

Some common human diseases with studied DNAm patterns (Figure 5 in the main text)

Autoimmune	
Rheumatoid arthritis	[1]-[9]
Systemic lupus erythematosus	[10]-[16]
Multiple sclerosis	[17]-[24]
Type 1 diabetes	[25]-[30]
Autoimmune thyroid disease	[31], [32]
Inflammatory bowel disease	[33]-[41]
Psoriasis	[42]-[45]

Metabolic	
Type 2 diabetes	[46]-[55]
Obesity	[56]-[61]
Adiposity	[62]-[64]
Body-mass index (BMI)	[65]-[68]
Metabolic syndrome	[69]-[71]
Metabolic traits	[72]-[76]

Cardiovascular	
Cardiovascular disease	[77]-[79]
Myocardial infarction	[80]-[83]
Ischemic stroke	[84]-[88]
Coronary artery disease	[89], [90]
Atherosclerosis	[91]-[95]
Hypertension	[96]-[98]
Lipid levels	[99]-[103]

Neurological	
Alzheimer's disease	[104]-[111]
Parkinson's disease	[112]-[115]
Amyotrophic lateral sclerosis	[116], [117]
Epilepsy	[118]-[123]
Pain	[124]-[128]
Migraine	[129], [130]
Chronic fatigue syndrome	[131], [132]

Psychiatric	
Depression	[133]-[137]
Anxiety	[138]-[141]
Bipolar disorder	[142]-[148]
Schizophrenia	[149]-[158]
Psychosis	[159]-[161]
Post-traumatic stress disorder	[162]-[166]
Eating disorders	[167]-[171]
Personality disorders	[172]-[175]

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