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PRENATAL, CHILDHOOD, AND ADOLESCENCE SLEEP AND STRESS

Association with DNA methylation and telomere length

Antti-Jussi Ämmälä

DOCTORAL DISSERTATION

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ABSTRACT

The Developmental Origins of Health and Disease (DOHaD) hypothesis states that several prenatal, perinatal, childhood, and adolescence factors may program the future health of an individual. These preprograming factors include maternal stress, anxiety, depression, or sleep during pregnancy or adverse life experiences in childhood or stress during adolescence. The programming processes may be changes in deoxyribonucleic acid (DNA) methylation or shortening of leukocyte telomere length (LTL). DNA methylation refers to an epigenetic mechanism that affects gene expression and is modified by external conditions. Telomere shortening is an event where the end of a chromosome shortens slightly in each cell division, ultimately leading to programmed cell death. Therefore, LTL is considered a marker for biological age. We studied this with a large birth cohort of newborns and calculated based on existing literature that our sample size was sufficient to detect previously reported findings. Despite sufficient statistical power, we could not replicate previous findings. Several reasons for this are discussed.

Childhood is a phase of rapid brain development, and adverse events in early life are linked to a wide range of adverse health outcomes in adulthood. Several mechanisms behind this association have been proposed, among them LTL shortening. In turn, this shortening is also affected by current mental health disorders, stress, and lifestyle factors. We studied the effect of adverse life events (ACE) during childhood on adult LTL in a large, population-based nationally representative cohort of adults. Current mental disorders, stress, sleep, and various lifestyle and socioeconomic variables were considered. While current stress or mental health did not affect LTL, early adverse experiences had a cumulative effect on adult LTL, even when confounding factors were considered. This suggests that programming of cellular age can occur during childhood and persist into adulthood independent of later health and lifestyle.

Adolescence is another phase where rapid brain development occurs and thus the brain is vulnerable to external and internal stressors. We explored this by studying epigenome-wide methylation in a sample of adolescent boys with or without depression and sleep disturbances. Due to the small sample size, we could not identify any significant genome-wide results. However, when the 500 best differentially methylated positions (DMP) were explored, a pathway related to synaptic pruning, the long-term depression (LTD) pathway, was identified as the most significant pathway. In a post-hoc analysis, a flattened slow-wave sleep dissipation, tiredness, and depression correlated with several individual sites in that pathway, suggesting that methylation changes in the

LTD pathway may be one potential mechanism behind widespread adverse effects of sleep disturbances.

Biological programming may occur in rapid phases of brain development and these effects may last for longer periods of time. However, careful methodological consideration is required to detect these effects.

Keywords; DOHaD-hypothesis, stress, sleep, prenatal stress, early adverse experiences, mental disorders, insomnia, epigenome-wide association (EWAS), telomere, adolescents, depression, DNA methylation, long-term depression, newborn leucocyte telomere length.

TIIVISTELMÄ

Terveyden ja hyvinvoinnin kehitykselliset juuret- hypoteesi esittää, että useat syntymää edeltävät sekä syntymän jälkeiset sekä lapsuuden ja nuoruuden aikaiset tekijät voivat ohjelmoida yksilön tulevaa terveyttä. Näihin ennalta ohjelmoiviin tekijöihin voivat lukeutua esimerkiksi äidin raskaudenaikainen stressi, ahdistus, masennus tai univaikeudet. Myös lapsuuden epäsuotuisat kokemukset tai nuoruudessa koettu stressi voivat ohjelmoida terveyttä tulevaisuuteen. Näitä ohjelmoivia tapahtumia voivat olla muutokset DNAn metylaatiossa tai valkosoluista mitatun telomeerin lyheneminen. DNA metylaatiomuutokset viittaavat epigeneettiseen säätelymekanismiin, jossa ulkopuoliset tekijät muuttavat perintötekijöiden ilmenemistä. Telomeerien lyheneminen puolestaan viittaa tapahtumaketjuun, jossa jokaisen solujakautumisen yhteydessä kromosomien päässä oleva telomeeri lyhenee hieman, johtaen lopulta ohjattuun solukuolemaan. Tästä syystä telomeerien pituutta on pidetty biologista ikää kuvaavana tekijänä. Tutkimme äidin raskausajan voinnin vaikutusta syntyvän lapsen telomeeripituuksiin suuressa syntymäkohortissa, ja tekemiemme voimalaskelmien perusteella aineistomme koon pitäisi riittää vähintään aiemmin raportoitujen havaintojen toistamiseen. Huolimatta riittävän suuresta aineistosta, emme kyenneet toistamaan aiempia havaintoja. Tälle on useita mahdollisia selityksiä, joita pohdimme työssämme.

Lapsuus on aivojen nopean kasvun ja kehityksen vaihe ja siksi lapsuuden epäsuotuisien kokemusten onkin osoitettu olevan yhteydessä erilaisiin terveyden tilan heikkenemisiin aikuisuudessa. Tälle on ehdotettu useita mahdollisia välittäviä tekijöitä tai merkkejä, joista yhtenä on kuvattu valkosolujen telomeerien lyhentyminen. Myös aikuisuuden mielenterveysongelmien ja stressin on osoitettu olevan yhteydessä telomeeripituuteen. Tutkimme lapsuuden vastoinkäymisten merkitystä aikuisiän telomeeripituuteen suurella yleisväestöä edustavalla kohortilla. Ajankohtainen mielenterveys, stressi, uni, sosioekonominen tilanne tai elintavat eivät selittäneet yhteyttä telomeeripituuteen, sen sijaan lapsuuden kokemuksilla oli yhteys, vaikka kaikki em. tekijät huomioitaisiinkin. Lapsuuden epäsuotuisten kokemusten vaikutus näyttääkin yltävän pitkälle aikuisuuteen riippumatta aikuisiän tekijöistä.

Nuoruus on vaihe, jossa aivot kehittyvät nopeasti ja ovat siten haavoittuvia sisäisille ja ulkoisille kuormitustekijöille. Tutkimme masennuksen ja univaikeuksien vaikutusta valkosoluista mitattuun koko perimän laajuiseen DNA metylaatioon vertaamalla nuorilla pojilla masennuksesta ja univaikeuksista kärsiviä terveisiin verrokkeihin. Liittyen aineiston pienuuteen emme havainneet perimänlaajuisia eroja, mutta eniten metylaation suhteen

eroavat alueet rikastuivat geeneihin, jotka polkuanalyysissä liittyivät synapsien muovautuvuuteen, niin sanottuun Long Term Depression-polkuun. Syväunen määrän muutokset, väsymys ja masennus olivat jälkikäteisanalyysissä yhteydessä moniin tuon polun geenien metylaatiokohtiin viitaten siihen, että metylaatiomuutokset tuon polun geeneissä voisivat olla yksi mahdollinen tekijä masennukseen ja unihäiriöihin liittyvien laaja-alaisten vaikeuksien taustalla.

Herkkyytemme biologiamme muovautumiselle saattaa olla lisääntynyt niissä kasvun ja kehityksen vaiheissa, joissa aivojen kehitys on nopeimmillaan ja nämä muovautumiset saattavat vaikuttaa pitkänkin aikaa eteenpäin. Näiden muutosten havaitseminen vaatii kuitenkin menetelmien osalta huolellisuutta ja tarkkuutta.

Avainsanat; DOHaD- hypoteesi, stressi, uni, raskaudenaikainen stressi, varhaiset epäedulliset kokemukset, mielenterveyden häiriöt, unettomuus, Perimänlaajuiset epigeneettiset yhteydet, telomeerit, nuoret, masennus, DNA metylaatio , vastasyntyneen valkosolujen telomeeripituus.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Antti-JussiÄmmälä, Emma I. K. Vitikainen, Iiris Hovatta, Juulia Paavonen, Outi Saarenpää-Heikkilä, Anneli Kylliäinen, Pirjo Pölkki, Tarja Porkka-Heiskanen and Tiina Paunio. Maternal Stress or Sleep During Pregnancy Are Not Reflected On Telomere Length of Newborns. Sci Rep. 2020 Aug 19; 10(1):13986.

II Antti-Jussi Ämmälä, Jaana Suvisaari, Laura Kananen, Jouko Lönnqvist, Samuli Ripatti, Sami Pirkola, Tiina Paunio and Iiris Hovatta. Childhood adversities are associated with shorter leukocyte telomere length at adult age in a population-based study.
Psychoneuroendocrinology.2021 Aug; 130:, 105276

III Antti-Jussi Ämmälä, Anna-Sofia Urrila, Aleksandra Lahtinen a, Olena Santangeli, Antti Hakkarainen, Katri Kantojarvi , 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 symptoms of insomnia. Sleep Med. 2019 Sep; 61:95-103

The publications are referred to in the text by their Roman numerals.

ABBREVIATIONS

ACE	Adverse childhood experience
ACHT	Adrenocorticotropic hormone
AIS	Athens Insomnia Scale
BDNF	Brain-derived neurotrophic factor
BH	Benjamini-Hochberg procedure
BMI	Body mass index
BNSQ	Basic Nordic Sleep Questionnaire
CES-D	Center for Epidemiological Studies Depression scale
CNS	Central nervous system
CORT	Cortisol
CpG	Methylated cytosine-guanine dinucleotide
Creb1	CAMP Responsive Element Binding Protein 1
CRH	Corticotropin-releasing hormone
DMNT	DNA methyl transferase
DNA	Deoxyribonucleic acid
DOHaD	Developmental Origins of Health
e.g.	exempli gratia
EEG	Electroencephalograph
EMG	Electromyogram
EOG	Electrooculogram
etc.	et cetera
EWAS	Epigenome-wide analysis
FDR	False discovery rate
GHQ	General Health Questionnaire
HPA axis	Hypothalamic-pituitary-adrenal axis
Hrs.	Hours
Hz	Hertz
i.e.	id est
IPA	Ingenuity Pathway Analysis
K-SADS-PL	Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version
LTD	Long-term depression
LTL	Leukocyte telomere length
LTP	Long-term potentiation
M-CIDI	Munich Composite International Diagnostic Interview

N1,2,3	Stage 1 of NREM sleep, etc.
NREM	Non-REM sleep
OECD	Organisation for Economic Co-operation and Development
PDSS	Pediatric Daytime Sleepiness Scale
PSG	Polysomnography
PSS	Perceived stress scale
PTSD	Post-traumatic stress disorder
PVT	Psychomotor Vigilance Task
QC	Quality control
qPCR	Quantitative real-time polymerase chain reaction
REM	Rapid eye movement
Rest	Neuron Restrictive Silencer Factor
ROS	Reactive oxygen species
S.D.	Standard deviation
STAI	State and Trait Anxiety Scale
SWAdiss	Slow-wave dissipation
SWS	Slow-wave sleep
TSS	Transcription start site
WW2	Second world war

1 INTRODUCTION

In the early days of medicine, there was an active discussion concerning whether it is biology or environment that has a pivotal role in disease development, revolving around what was called the "Nature versus Nurture" question [1]. In the last century, a more comprehensive approach was formulated, binding these two together into a complex, interactive canvas of factors that modify each other [2], known as The Developmental Origins of Health and Disease (DOHaD) hypothesis [3]. The origins of this hypothesis lie in the observations from a naturalistic cohort that was formed in the second world war in The Netherlands, where severe malnutrition plagued a distinct area during a relatively short period of time; this was also known as "The Dutch Hunger Winter" [4]. Later it was observed that the children whose fetal growth coincided with that period had significantly more morbidity (especially cardiovascular morbidity) and schizophrenia than children who were born just before or later after the hunger period [5]. This led to a hypothesis of prenatal programming of biology that affects the later health and disease of an individual exposed to a preprogramming factor. This was first presented by Baker and thus this hypothesis was called Baker's hypothesis [6-9]. This was later expanded and reformulated into the DOHaD hypothesis.

Since the original presentation of this hypothesis, the DOHaD hypothesis has been expanded from physical adversities (such as lack of food) to more complex social and psychological adversities, such as maternal stress, anxiety, or depression during the prenatal period [10-12]. Along with prenatal factors, factors during early childhood were also related to future health [13]. These factors, commonly called adverse childhood experiences (ACE), represent any factor that occurs during a critical and sensitive time related to the child's development. These include potentially traumatic or disruptive elements, such as neglect, maltreatment, or loss of a caregiver.

Another critical time for development is adolescence, when a rapid maturation of many brain areas occurs [14]. Different adverse factors, such as prolonged stress, might influence this process in a long-lasting way similar to the programming effects of prenatal and childhood factors [15, 16].

It is worth noting that similar different stressors in critical developmental periods have also been observed in social mammals other than humans. For example, yellow baboon females who had several early life adversities had a shorter life span than baboons without such experiences [17]. These findings have also been observed with other social animals, such as hyenas, whose life span was shorted by years due to cumulative adverse experiences in childhood [18]. These findings are important, as many childhood adversities in humans

are intercorrelated with each other, such as childhood socioeconomic status and living with only one parent [19]. However, such correlations might be weak or nonexistent in wild animal populations such as hyenas [17].

The biological mechanisms involved in the interactions between environmental factors and changing biology are numerous and include hormonal, immunological, and nervous-system developmental factors. More recently, gut microbiota-related factors have also been considered [20-28]. Epigenetic factors are also widely studied in this context, mostly in mothers or in offspring, but also increasingly in fathers [29-31]. Only a fraction of our genes is expressed in somatic cells and activation and silencing of genes occurs via different mechanisms. One of these mechanisms are epigenetic regulation processes, which can directly affect deoxyribonucleic acid (DNA) and transcription factors and associated protein complexes, many of which have complex interactions [32]. One of the most studied epigenetic regulation mechanisms is DNA methylation [33]. DNA methylation of an infant has indeed been associated with maternal care [34].

Chromosomal changes can also drive these complex interactions between biology and environment. Chromosomal instability can lead to accelerated apoptosis, if replication stress exceeds the capacity of chromosome-stabilization mechanisms [35]. Chromosomal integrity is maintained mainly with telomeres, which are short tandem repeats at each end of a chromosome that shorten in each replication, thus eventually wearing out and leading to apoptosis [36]. Telomeres serve as a marker for cellular oxidative stress [37] and thus act as a marker for cellular aging. For example, telomere shortening has been linked to cardiovascular morbidity and mortality [38]. Telomere length is associated with prenatal programming, thus forming a link between stressor and outcome [39].

2 REVIEW OF THE LITERATURE

2.1 SLEEP

Normal sleep

To our knowledge, all vertebrate animals sleep [40, 41]. Sleep has a crucial role in maintaining brain homeostasis [42]. Despite the brain's high energy consumption during sleep, sleep is necessary to restore energy repositories in the brain [43]. Sleep is needed to prune unnecessary synapses [44] and to remove metabolic byproducts from brain tissue [45]. Sleep is a period where the body acts as in a resting state [46]. Awareness of environment is altered and often diminished, metabolism is altered, body temperature is lowered, and electric activity in the brain cortex follows typical patterns [47]. Sleep consists of cycles where certain phases of sleep follow each other [48].

Sleep phases are defined by electrical activity patterns in the brain cortex, as measured by an electroencephalograph (EEG) [47]. These phases are divided into the categories rapid eye movement (REM) sleep or non-REM sleep (NREM). The first phase of sleep, the so-called phase 1 or N1 sleep, is characterized by rhythmic electric activity with an approximate frequency of 4-7 hertz (Hz). This is called light sleep, as people typically are somewhat aware of their environment during this phase. In the N2 phase, electrical activity is 12-14 Hz, and sleep spindles or K-complexes (a certain type of EEG activity) may appear. Gradually, awareness of environment decreases in this phase. In so-called deep sleep or N3 sleep, there is typically 0.5-2 Hz rhythmic electric activity; this phase is therefore often also called slow-wave sleep (SWS). The REM sleep phase is characterized by irregular electrical activity and irregular autonomic nervous system activity, with phasic sympathetically driven periods and tonic parasympathetically driven periods with muscle relaxation and rapid eye movements, from which this phase is called. Most people dream during this period, but dreams appear also in other phases, mainly in N3 phase. One sleep cycle is defined by a series of N1-N2-N3 phases and a REM phase.

Cycles are dissimilar through the sleep period. During the first cycles in the sleep period, there are relatively more N3 sleep phases compared to later sleep cycles in the same period, as N3 phases typically tend to get shorter towards the end of the sleep period [47]. This is called SWS dissipation and was previously linked to depression [49]. On the other hand, REM periods tend to become longer towards the end of the sleep period. Normally, a short moment of wakefulness appears during the sleep period typically between sleep cycles,

but usually these moments are not recalled afterwards or are recalled only partially.

Many hormonal secretions follow sleep and circadian periods [50]; the autonomic nervous system, control of body metabolism [51], and the immune system [52] also follow such patterns. Thus, this periodic behavioral, metabolic, and other biological activity is carefully coordinated in the body.

Sleep is regulated by two main mechanisms, a circadian mechanism, and a homeostatic mechanism [53]. The circadian mechanism refers to the oscillating rhythm that approximately follows a 24-hour period. All cells in the body roughly follow this rhythm [54]. Central phasing of these rhythms occurs in the suprachiasmatic nuclei in the occipital cortex, where certain types of cells monitor the amount of light entering the retina of the eye and phases the body via secretion of the hormone melatonin [55]. This hormone in turn phases all other cells and attempts to maintain a coordinated rhythm in the body [56]. Both regulatory systems seem to be at least partially regulated by genetic factors [57] and uses multiple modulating agents [58]. Types of circadian preference, especially eveningness, are associated with many general medical conditions and psychiatric conditions, such as type II diabetes and depression [59-61].

Another mechanism behind the regulation of sleep-wake cycles is the homeostatic mechanism [62]. During waking periods, a homeostatic pressure for sleep gradually accumulates and peaks when sleep initiation is occurring [63]. Thereafter, this pressure is gradually lowered during sleep, eventually reaching its lowest point at the time of awaking. These two mechanisms are presented in Figure 1.

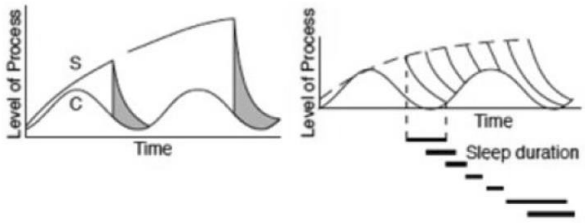

FIG. 1. Two-process model of sleep regulation. S=Process S, the sleep homeostatic factor; C=Process C, the circadian rhythm process; Grey areas=time of sleep. When an individual self-selects their sleep onset (one of the two vertical lines), the level of homeostatic drive is at the Process S level (upper line); homeostatic drive then declines in an exponential manner until it reaches the Process C level. The person then awakens and the level of homeostatic drive begins to rise again (not shown). (Right) Sleep durations associated with different self-selected sleep onsets. Reproduced with permission from Elsevier publishing [53].

A newborn human sleeps most of the time and is awake for only short periods [64]. The sleep of a newborn is not initially consolidated but consists of different active and quiet sleep phases [65]; consolidation begins gradually. As children develop, the need for sleep gradually diminishes from approximately 12 hours (hrs) at 3 years to 9 hrs at 12 years [64]. During puberty, the need for sleep remains at approximately 9 hrs, but circadian rhythm starts to advance [66] and the amount of SWS starts to dramatically decrease [67]. Adults in Finland sleep approximately 7.39 hrs per night (standard deviation [S.D.] 0.89) and the amount of sleep appears to have gradually diminished from 1975 to 2011 [68]. The need for sleep can vary within an individual based on general medical condition, stress level, and other factors. Nutrition, substance use (and especially abuse), and exercise cause alterations to sleep quantity and quality. During old age, sleep typically becomes more fragile [69], although gender differences appear.

Normal sleep is a prerequisite for maintaining synaptic plasticity in the brain [70, 71]. Synaptic plasticity refers to the constant alterations in the nervous system where frequently used connections are strengthened and less frequently used connections are pruned. This plasticity uses several mechanisms. From the perspective of sleep, the main interest has been on two opposing mechanisms, namely synaptic long-term potentiation (LTP) and synaptic long-term depression (LTD) [72]. Sleep, and especially SWS, have also been linked to plasticity modulation with several mechanisms other than LTD or LTP [73]. For example, SWS increases after cortical injection of brain-derived neurotrophic factor, a major growth factor in the central nervous system (CNS) [74].

Normal sleep is required not only to maintain brain homeostasis and energy repositories, but also to maintain regulation of mood, learning, and other cognitive functioning and even to maintain the body's metabolism and immune system. This is best understood in humans when the consequences of sleep disturbances are observed [75-78].

Disordered sleep

Sleep can be disturbed in many ways. One of the most common disturbances is likely insufficient sleep, often defined as <7 hrs of sleep in adults. Excessive sleep is at the other extreme, where sleep is excessive to the point that it consumes considerable time over other activities. Although some general medical conditions are associated with insufficient sleep, excessive sleep is in many cases related to a general medical condition [79, 80].

Insomnia is defined as difficulty initiating sleep and is one of the most prevalent sleep disorders. Other types of sleep disorders, such as waking in the middle of the night and excessively early awakenings, are also common in the population. In Finland, one out of three persons suffers from at least temporary insomnia each year, and approximately 10% of the population suffer from a chronic sleep disorder, most often insomnia [81].

Fragmented sleep is a disorder where the subject wakes several times and typically needs time to fall asleep again. Both sleep quality and quantity are jeopardized in this disorder.

Early morning awakenings means that sleep is disrupted too early, typically after one or two sleep cycles, and is followed by difficulty or inability to fall asleep again.

Sleep can also be non-restorative, meaning that a person experiences tiredness despite sufficient sleep. This is often related to poor sleep quality, which can mean insufficient N3 sleep, too little REM sleep, excess N1-N2 sleep, or very fragmented sleep cycles.

Disordered sleep has many consequences to individual health. It increases the risk of cardiovascular diseases, type II diabetes, and metabolic conditions [82-84]. Poor physical performance is also linked to insufficient sleep [85].

Disordered sleep affects the immune system and can make an individual more prone to infectious diseases [86]. Chronic autoimmune disorders are linked to sleep disorders, emphasizing the role of sleep as an immune-system modulator [87].

Many psychiatric disorders are associated with poor sleep, most notably depression [88]. Sleep disorders are hallmarks and typical clinical symptoms of depression, but sleep disorders more likely lead to depression than vice versa [89]. Disordered sleep is also associated with other psychiatric disorders, such as schizophrenia [90], post-traumatic stress disorder (PTSD) [91], substance abuse [92], and neuropsychiatric disorders [93]. Sleep deprivation has been used to treat depression with some success, emphasizing the crucial role of sleep in mood regulation [88].

Disordered sleep affects cognition in several ways; it influences vigilance and attention, which is typically observed in psychomotor tasks and attention tasks in neuropsychological studies. Disordered sleep also impairs memory and learning [94, 95]. Although most learning takes place while awake, when vigilance is high, memory consolidation occurs during sleep [96].

Disordered sleep seems to affect synaptic plasticity processes that are important components of this learning and memory consolidation, namely synaptic LTP and synaptic LTD. Both are complex processes involving alterations in gene expression, altered protein production, and eventually altered potentiation in the cell. This involves hundreds of different molecules, several of which possess rate-limiting qualities [97]. They are linked to sleep, as sleep deprivation alters plasticity in the brain using these mechanisms, which consequently affects spine density, synaptic strength, or downscale synapses [76] [70]. This is hypothesized as one possible mechanism that connects depression and sleep together [98].

2.2 STRESS

Stress can be either a positive force that drives goal-driven behavior (called eustress), or a negative force that strains resources and makes it more difficult to cope (called distress). Stress in the context of this thesis refers to distress. Stress is in its broadest definition whatever situation where external and internal demands exceed the individual's resources [99].

Stress can be further be divided into systemic stress, which concerns the entire body, or cellular stress, which refers to metabolic events occurring inside one cell or in a certain tissue, which is called oxidative stress [100].

Systemic stress effects the autonomic nervous system and activates its functioning as a rapid response to external stimuli [101]. Via this system, systemic stress can influence virtually all organs and be an etiologic factor in health and disease. For example, stress-mediated autonomic nervous system hyperarousal is associated with cardiovascular diseases [102].

2.2.1 Stress and Hormone system

Stress also activates the hypothalamic-pituitary- adrenal system, called the HPA axis [103]. The HPA axis is initiated by secretion of corticotrophin-releasing hormone (CRH) from the hypothalamus in the basal brain. Thereafter, this hormone in turn primes a cascade leading to release of adrenocorticotropic hormone (ACHT) from the hypophysis. A series of negative feedback loops regulate these actions.

Corticoid hormones in turn have a widespread effect on various tissues. They enter the cellular nucleus and affect gene transcription. Corticoid hormones have pleiotropic effects on various metabolic developmental and immune mechanisms. Synthetic hormones have long been used to modulate immune response in various diseases and conditions [104].

2.2.2 Stress, sleep, and psychiatric disorders

Stress as a psychological phenomenon can be described as an individual evaluation of how threating a stressful situation is and how well the available resources can be used to cope with the situation [105].

Stress and sleep are linked together. In particular, people with a highly reactive sleep system react to stress with sleep difficulties, most often with difficulties initiating sleep and awakenings during night [106]. Stress-related sleep difficulties are linked to several mechanisms common to both stress reactivity and sleep regulation, such as the autonomic nervous system and HPA axis.

Stress and depression are closely linked together. Chronic stress can lead to development of depression. Depression is one of the most common mental health disorders, with a lifetime prevalence in the Organization for Economic Co-operation and Development (OECD) countries of approximately 20%. The annual incidence in Finland is 3-5%. Women are more prone to depression than men. One of the hallmark symptoms of depression is sleep disturbances, most commonly early morning awakenings.

Depression and sleep are linked together, as sleep disorders are known to increase the risk of depression [107-110] and both share partially common mechanisms. These mechanisms include serotonin and dopamine dysregulation, altered regulation of neural circuits, altered cytokine regulation, and biased memory consolidation [107]. Depression and sleep also affect various cognitive functions, and both sleep symptoms and cognitive difficulties are hallmarks for depressive disorder, making these two tightly interwoven [111]. Besides cognition, the immune system also appears to be similarly affected by both conditions. Both induce a low-level systemic inflammation, which broadly affects leukocytes, such as through gene expression [112, 113].

Anxiety disorders are also associated with various forms of stress. Anxiety disorders are even more common than depression in adult and adolescent populations, and approximately one out of four persons will suffer from anxiety disorders at some point in life. Anxiety is also connected to immune system functioning [114].

2.3 DNA METHYLATION AS A REGULATORY MECHANISM

Epigenetics refers to regulatory events that alter DNA functioning without altering the nucleotide sequence of the genome [115]. This can include alterations in DNA-binding proteins, which in turn affects the availability of DNA for transcription factors and associated complexes [116]. Epigenetics can also refer to alterations in nucleotides and associated proteins such that the transcription complex cannot bind to DNA and transcription thus cannot be initiated (Fig.2) [117]. DNA methylation refers to a dynamic procedure where a methyl group is attached to a cytosine nucleotide in a cytosine-guanine dinucleotide, forming methylated dinucleotide (CpG) [118]. This methyl group is transferred by a group of enzymes called methyltransferases [119]. In particular, the transcription binding region is enriched with these dinucleotides and general methylation of these regions tend to silence the expression of a gene by preventing binding of the transcription complex to the transcription start site (TSS). Methylation has indeed been shown to affect gene expression, even when the changes appear to be minor [120-122]. Most genes are permanently methylated and thus silenced, but dynamic changes do appear when a gene is activated, such as in response to a change in the environment. Several well-known diseases are associated with abnormal DNA methylation, such as Rett's syndrome [123] and Angelman's syndrome [124].

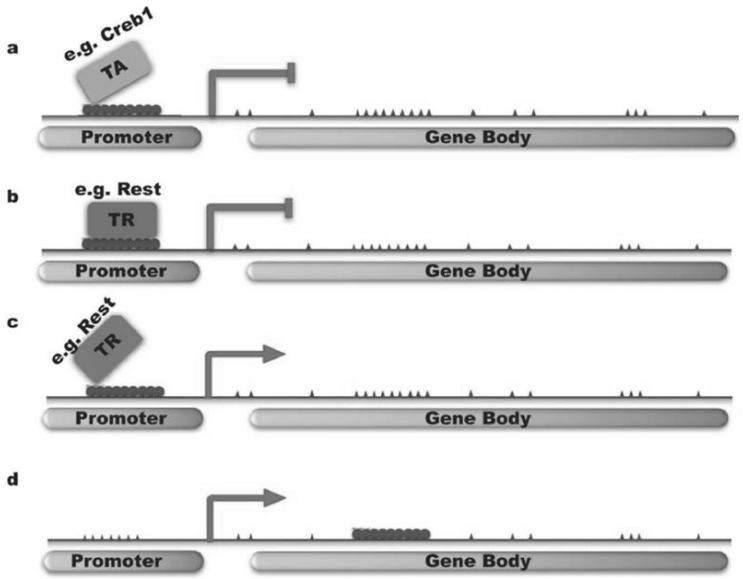

Figure 2. DNA methylation and neuronal gene transcription. (a,b) DNA methylation can inhibit gene transcription by preventing the loading of a TA (transcription activator e.g., CAMP Responsive Element Binding Protein 1(Creb1)) or by facilitating transcription repressor binding; (c,d) DNA methyl transferases (DNMT) can activate gene transcription by inhibiting the binding of TR (transcriptional repressor e.g., Neuron Restrictive Silencer Factor (Rest)) or by gene body methylation. Reproduced with permission from MDPI, Basel, Switzerland[117].

DNA methylation is tissue-specific to some extent [125, 126], but certain parallels can be found between peripheral-blood DNA methylation and methylation in the CNS [127]. Several lifestyle factors and diseases are associated with DNA methylation, such as smoking and diabetes [128, 129].

DNA methylation is widely connected with different cancer-cell populations [130]. It has also been linked to various complex disorders, such as depression, anxiety, PTSD, psychoses, and suicidality [131-136].

Leucocyte telomere length as a marker for cellular age and stress

In each cellular replication, chromosomes open and a replication fork appears, which separates the two strands from double-helix DNA to a leading strand and a lagging strand. Replication proceeds on both strands simultaneously, resulting in two identical double helixes, one for each cell [137]. This replication is driven by the DNA polymerase enzyme and can only proceed from 5′ to 3′ direction.

In the leading strand, this is not a problem since replication proceeds in this direction anyway. However, in the lagging strand where the template is a mirror image of the leading strand (from 3′ to 5′), this creates a problem. This is solved in a way that the replication complex moves in advance and then builds short blocs from 5′ to 3′, known as Okazaki fragments. These fragments are then united by the enzyme DNA ligase to form a continuous DNA strand. This solution, however, creates a new kind of problem, the so-called end-replication problem, since last base pairs at the 5′ end of the lagging strand cannot be replicated (Figure 3) [36]. Thus, in each cellular replication, the chromosomes progressively become shorter. To protect chromosomal integrity, there is short tandem replication sequence TTAGGG at each end of a chromosome, called a telomere [138]. Telomeres also forms loops that prevent tangling of chromosome ends [36]. Telomere shortening is not linear; new telomere is created by enzymes called telomerases [139].

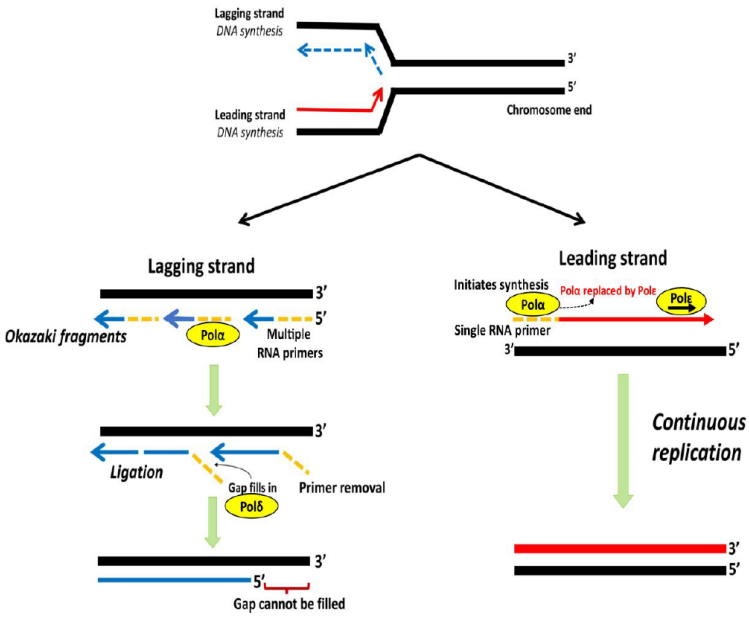

Figure 3. Schematic representation of lagging and leading strand replication. DNA polymerase Polα with a single RNA primer initiates synthesis of the leading strand, which is subsequently replaced by Polα" for further elongation. The lagging strand is copied through discontinuous Okazaki fragments from multiple primers. RNA primers are degraded and the gaps are filled by Polα followed by ligation of discontinuous fragments. The gap at the 5′ end remains unfilled, leading to a non-replicated terminal region. Reprinted with permission from Cold Spring Harbor Laboratory Press. Adapted from [140].

Telomeres are highly conserved tandem repeat sequences at each end of a chromosome. The length of telomere varies from species to species; typical telomere length in humans ranges from 10 to 15 kilobases (Kb) [141]. Although the telomerase enzyme recovers loss of telomere in cell division [142], this recovery is not sufficient to cover losses and thus telomeres are shortened in each cellular division, which eventually leads to cellular senescence [143].

Many external factors are associated with telomere shortening, such as reactive oxygen species (ROS) and other cytotoxic agents [144-146]. This led to the hypothesis that telomere length serves as a proxy measurement of overall cellular stress and a marker for cellular age [147, 148]. Several genetic factors are associated with regulation of LTL biology [149, 150].

Lifestyle factors are associated with shorter LTL, including smoking, lack of exercise, and overweight [151-153]. Socioeconomic factors, such as education, occupational status, marital status, living conditions, and income are associated with LTL, thus emphasizing its role as a marker for cumulative stress from various sources. Although regulation of LTL biology is highly

heritable (estimates are as high as 70%), only a part of telomere length is predicted by genetic factors, which emphasizes the role of other regulatory factors [149, 154, 155].

2.4 PRENATAL MATERNAL STRESS, SLEEP, DEPRESSION, AND ANXIETY

Developmental programming can be defined as a cascade of events, primed by an environmental factor, which acts during a sensitive developmental period and affects structure, function, or both of tissues that ultimately leads to changes that persist throughout life [3].

Development of the CNS is a complex process that starts after the eighteenth day from fertilization via folding of the neural plate into the neural tube [156]. This includes neuronal cell generation, cell proliferation, cell migration, and further transformation of cells [157]. This complex process is astonishingly rapid. Normal pregnancy is divided into three trimesters; by the end of day 56 from fertilization (that is during the first trimester), all essential structures are formed [158].

Thereafter, generating new neurons (neurogenesis) proceeds along with growing of connections [157]. In the second trimester, brain structures enlarge exponentially in cortical areas (up to over 20-fold increase in volume). During the third trimester, growth continues (but at a significantly lower rate) and the brain-cortex volume doubles during the third trimester compared to second [159].

Maternal stress during pregnancy is linked to the offspring's brain development in several studies [160-164]. Maternal stress during pregnancy is most often related to depression, anxiety, worry about the health of the child, and various events in life that are perceived as stressful [165]. There are several maternal-related factors that are associated with increased stress during pregnancy, such as lower level of education, more children, younger age, living without a spouse, and coping style [166-168]. Stress during pregnancy is quite common; approximately 7-20% of women suffer from prenatal stress in various forms [167, 169-173]. Diagnosable depression or anxiety disorders affect approximately 8-12% of pregnant women [174-176]. It is worth noting that these findings are from western countries and might not be applicable to other regions. There is great discrepancy among studies whether a certain period in pregnancy is more related to adverse effects on the newborn. There is currently no consensus on which period is most important for stress-related effects on offspring [10].

In addition to brain development, there are several possible mechanisms that may mediate the association between maternal stress and the offspring's future health.

Prenatal maternal stress has been shown to contribute to the offspring's HPA-axis functioning, but results vary according to which time point maternal stress is measured during pregnancy, what kind of stress the mother is experiencing, and at what age the offspring's HPA axis functioning is measured and how [177, 178].

Maternal cortisol reactivity is predictive of the offspring's cortisol reactivity. However, results are mixed; in some studies, higher maternal cortisol reactivity predicted a higher cortisol reaction in the offspring [179-181], whereas in some studies contrasting results were observed [182, 183]. Similarly, findings describing the effect of maternal basal cortisol secretion on offspring basal cortical secretion were also mixed[183, 184]. One study reported that even the offspring's gender can be a modifying factor [185].

Other confounding factors include pregnancy-induced changes in maternal cortisol; typically maternal cortisol levels rise during pregnancy towards the end of pregnancy [186]. Further, the fetal cortisol level is dependent on how cortisol passes the placenta, and this is regulated by the enzyme 11-beta-hydroxystreoid-dehydrogenase-type 2 (11-β-HSD-2). There is variability in the activity of this enzyme [186], which creates an additional source of variation.

Prenatal stress is linked to postnatal cognitive performance of a child [187]. This effect was statistically significant but modest. Concerns have been expressed regarding the mediating effects of maternal depression and anxiety and their effect on caregiving, responsiveness, sensitivity to the child's needs, and bonding with the child [188, 189].

Prenatal maternal depression also has an effect on offspring via similar mechanisms described earlier concerning the effects of maternal prenatal stress [190, 191], although it appears that the evidence concerning the effect of maternal prenatal depression and HPA-axis functioning of the offspring is limited. Studies exploring the effect of maternal prenatal anxiety and offspring brain structure and function are very heterogenous, but alterations in both structure and function in frontal and temporal lobes and limbic regions have been reported [192].

Prenatal maternal stress, sleep, anxiety and depression, and leukocyte telomere length of a newborn

Telomere length is linked with cortisol reactivity, suggesting that telomere length may be a reflector of stress [193-195]. Leukocytes treated with cortisol

also exhibit decreased telomerase activity [196], thus linking stress and telomere length together.

Fetal growth is a period when several factors affect development and preprogramming of the future health of the child. It can also be conceptualized as a special environment that shapes both growth and preprogramming [197]. This environment is constructed by many external factors, such as environmental factors (e.g., nutrition), and by many factors related to maternal health and wellbeing. The fetal period is a phase of rapid growth and most cellular divisions occur during this period. Accordingly, events that affect cell division, such as telomere attrition, can be seen in the LTL of a newborn. Indeed, several studies support this reasoning and have given this phenomenon the name "The Fetal Programming of Telomere Biology Hypothesis" [197-203]. This can be considered as a special example of the DOHaD hypothesis, which focuses on a certain developmental period (fetal period) and a certain biological process that is programmed (leukocyte telomere biology).

Maternal stress during pregnancy is one of the most studied external factors in The Fetal Programming of Telomere Biology Hypothesis [204-207]. Interestingly, maternal pregnancy-related stress did not influence maternal LTL, but newborn LTL was negatively associated with maternal stress during pregnancy [204]. In addition to maternal stress, maternal lifetime psychiatric morbidity did not affect maternal LTL but was associated with newborn LTL, suggesting a specific vulnerability of the fetus to maternal stress [204].

Other factors associated with newborn LTL are maternal pre-pregnancy weight, education, depression, and maternal smoking [208, 209]. There may be a significant gender effect in some of the factors; for example, lower maternal education and higher weight were observed in male offspring only [208, 210]. Ethnicity may also play a role. In one study on black infants, black female infants in particular had longer LTL at birth compared with white infants, even when birth-related and demographic factors were controlled for [211]. Maternal diabetes [212] and folate concentration [213] are also associated with newborn LTL.

Similar to stress, disrupted sleep is an environmental factor during pregnancy and is possibly very prominent, as sleep disturbances are relatively common during pregnancy [214, 215]. While sleep disturbances can appear in all trimesters, these seem to be more prominent towards the end of pregnancy. Indeed, there are some studies describing the effect of maternal sleep during pregnancy and newborn LTL [214, 216]. Sleep apnea is associated with shorter offspring LTL, whereas maternal daytime sleepiness is not statistically significantly associated with LTL, although a trend towards shorter LTL was

observed [214]. Sleep apnea is associated with many pregnancy-related and many child-related outcomes [215].

2.5 CHILDHOOD ADVERSITIES AND HEALTH AND DISEASE

The American Heart Association's scientific statement declares that "despite a lack of objective agreement on what subjectively qualifies as exposure to childhood adversity and a dearth of prospective studies, substantial evidence documents an association between childhood adversity and cardiometabolic outcomes across the life course" [217]. Indeed, several studies support this statement. For cardiovascular diseases, childhood adverse experiences are associated with myocardial infarction, ischemic heart disease, coronary artery disease, hypertension, and stroke [218-221]. Of cardiovascular risk factors, obesity is linked to ACEs [222-225]. Type 2 diabetes is not only a medical condition on its own but is also a major risk factor for cardiovascular disease, and there is evidence that it too is linked to ACEs [219, 226]. ACEs are connected to all-cause mortality in the Finnish population [227].

Adverse experiences can include a wide range of different experiences and are typically threatening bodily, familial, or social security or safety situations [228, 229]. These may include economic difficulties or familial dysfunction or precise categories, such as bullying [222]. This phenomenon is quite common; up to nearly 60% of U.S. households reported experiencing at least one kind of adversity during childhood [230].

It appears that rather than concentrating on single adverse events, the cumulative effect of these events is more important. Several studies have demonstrated a dose-dependent effect of adverse events and health outcomes [229, 231-236]. However, some have suggested a threshold effect, for example cardiovascular disease risk was elevated in one large study only in a group with four or more adverse experiences [226, 233]. The possibility of a mixed and combined effect of the type of adversity, intensity of experience, and severity of event has been suggested [237]. The chronicity of the experience has also been suggested as crucial factor for the increase in adverse health consequences [238]. Finally, gender-based differences may exist, although there is some evidence that at least for diabetes this might not be the case [239].

In addition to many somatic conditions, many psychiatric conditions are also related to ACEs. Psychosis and psychotic experiences [240-242], depression [243], anxiety disorder [244], and bipolar disorder [245] are all linked to ACEs. Similar to somatic disorders, a dose-dependent pattern was also observed in the mental health consequences of ACEs [246].

Recall bias and omitting experiences are caveats when using self-reported measurements in adults, and this poses a challenge for studying the effects of ACEs. For example, a depressed mood can make an individual more prone to recall negative memories, and a person suffering excruciating anxiety might omit all painful memories just to avoid becoming overwhelmed [247].

There are several biological systems that may be involved in mediating the adverse effects between ACE and health. Cortisol levels are associated with ACE. For example, decreased diurnal variation in cortisol levels has been observed in imprisoned women with a history of sexual abuse in childhood [248] or elevated total cortisol in urine in women sexually abused in adulthood [249]. For example, early-morning cortisol levels are lower in maltreated children [250]. Timing of the stressor may also have an impact on cortisol response and cortisol reactivity [251]. In a study where both number of ACE exposures and timing of exposure where considered, ACE occurring between the age of 3 to 7 years had more profound effect on cortisol reactivity than ACE at other timepoints, thus highlighting the importance of when the exposure occurred. This in turn can affect how certain brain regions and circuits are developed [252].

Neural circuits that participate in threat-related stimulus processing react differentially in children exposed to ACEs [253]. For example, the volume of the amygdala, a central brain region in processing emotions and threats, is smaller in children exposed to ACEs [254, 255]. However, other studies have not found differences in the amygdala or even reported increased amygdala volume, making the evidence somewhat contradictory [256]. There are several possible reasons for this discrepancy, including critical timing of ACE regarding amygdala neurodevelopment, types of ACE explored, and imagining methodology differences among studies. The reactivity of the amygdala also seems to be altered in these children [257, 258]. Even the pace at which the median prefrontal cortex matures during adolescence can be affected by ACEs [259].

Inflammation is another widely studied mechanism that links ACEs to negative health outcomes [260-272]. For example, elevated interleukin-6 and tumor necrosis factor α levels have been observed in cases of childhood maltreatment [261, 262, 266, 272]. Other inflammation markers, such as C-reactive protein, are linked to ACEs [273]. This makes it plausible to explore ACE effects on peripheral blood.

Childhood adversities and telomere length

As shown in a seminal work of Epel et coworkers, self-perceived psychological stress is connected to shorter LTL [274]. Childhood adversities have also been widely linked to LTL attrition and health. For example, childhood cognitive development and sleep are associated with shorter LTL in adulthood [275].

Several meta-analyses emphasize these findings connecting childhood adversities to shorter LTL in childhood and in adulthood [276-278]. However, some meta-analyses point out the fact that there is considerable heterogeneity in studies and results, and most studies were conducted on relatively small sample sizes. The use of clinical samples instead of population-based samples may introduce bias, as some phenomena may be enriched in clinical samples and therefore findings may not be directly generalizable to other populations [278, 279]. Some studies have even reported *longer* LTL connected with ACEs [280].

There are several types of stressors, such as violence, neglect, emotional and sexual abuse, and bullying. Different types of stressors have different effects on health and disease. LTL has also been shown to react differently depending on the type of stressor. Stress can either be a single event or repeatedly occurring stress that accumulates the effect of single events. In a sample of children repeatedly exposed to violence, cumulative stress is associated with not only shorter initial LTL, but also faster LTL shortening [281]. Stress can also effect several biological processes, such as DNA methylation, LTL shortening, or accelerated pubertal timing simultaneously; thus a pleiotropic effect might exist [282].

2.6 DEPRESSION AND SLEEP IN ADOLESCENCE

Adolescence is associated with major changes in sleep patterns [283]. Bedtime is later, sleep onset may be delayed, and a greater discrepancy between weekdays and weekend days appear, especially in boys [284-286]. Total sleep time decreases, and daytime sleepiness is reported more frequently. Adolescence is a period of rapid growth and also a period with elevated risk for both depression and sleep disorders [287]. Sleep architecture also changes during adolescence. The most remarkable change is the drastic decrease in SWS [288], indicating rapid brain maturation and synaptic decline associated with puberty [289, 290]. Disordered sleep affects many young people. Estimates for different types of sleep disorders among adolescents are as high as 13.6-23.8%, with girls affected more often than boys [284].

Sleep affects cognition in adolescents in many ways, impairing memory, concentration, learning, vigilance, mood regulation, and attention [93, 291-294].

Depression is also a common disorder and its prevalence increases from prepuberty to late puberty. In 2016 in the U.S., 5% of 12-year-olds reported having a depressive episode in the past 12 months, and 17% of 17-year-olds reported having a depressive episode in the past 12 months [295]. Depression

in adolescents is associated with many different domains of cognitive functioning, such as memory [296] and learning [297].

Sleep, depression, methylation, and cognition in adolescence

Sleep is also associated with DNA methylation. Inadequate sleep is associated with methylation changes and a rest period can induce further changes [298, 299]. Interestingly, methylation changes in sleep may be acting via LTD, an important part of synaptic plasticity [300, 301]. Sleep deprivation both in young men and in older women altered methylation detected in peripheral blood leukocytes, suggesting that sleep-induced alterations in methylation can also be observed from peripheral blood samples [300, 302]. Furthermore, depression in adolescents is also associated with inflammatory processes [303]. Depression is associated with C-reactive protein and interleukin-6, which in turn predicted depression.

2.7 ADULT STRESS, SLEEP, MENTAL HEALTH, AND LEUKOCYTE TELOMERE LENGTH

LTL in adulthood has been widely studied in the context of many chronic conditions, such as cardiovascular disease and diabetes [304-306]. LTL is associated with both excess morbidity and mortality [147, 199, 274, 307, 308].

Complex disorders, such as depression, anxiety, psychoses, PTSD, and substance abuse are also associated with shorter telomeres both in population-level samples and in clinical samples [307, 309-318]. In addition, sleep and stress are connected to shorter LTL [319, 320]. The results are somewhat mixed; in population-based cohorts, anxiety disorders, but not depression, are associated with LTL [315]. In another population-based study, both depression and internalizing problems were not associated with LTL [316, 317]. On the other hand, PTSD was not associated with shorter LTL at the population level [318].

Alcohol abuse, a major mental health disorder and a significant risk factor for other mental health problems, is also related to LTL. The results are somewhat mixed, but it appears that alcohol consumption does not have an effect on LTL or the rate of shortening [307, 311, 321]. It is worth noting that concurrent mental health was not considered in these studies.

3 AIMS OF THE STUDY

3.1 GENERAL AIMS

According to the DOHaD hypothesis, a child's future health can be shaped by several prenatal environmental factors, such as mother's weight, perceived stress, and mother's poor sleep during pregnancy. The systemic prenatal programming effect of these factors could potentially be detected from the peripheral blood sample of a newborn. In later stages of life, specifically in adolescence and in adulthood, stress such as adverse life events or poor sleep could also execute similar programming. Most likely, this programming occurs in a timeframe that extends from early childhood through adolescence, as neurodevelopment is most rapid during this phase of life. Such changes could be methylation of CpG sites or differences in LTL. Sleep as an environmental factor has been studied in several different developmental phases, such as maternal sleep as an environment for fetal development, in adolescence, and in adulthood. We examined the effect of prenatal stressors on newborn LTL in a large birth cohort and the effect of depression and sleep on DNA methylation in a small sample of unmedicated adolescent boys with or without depression and sleep disorders. Finally, the effect of adverse life experiences in childhood on LTL was examined in a large population-based nationally representative sample of adults.

3.2 SPECIFIC AIMS OF EACH STUDY

Study I: To study the effect of prenatal maternal stress, weight, anxiety, and sleep quality on newborn LTL.

Study II: To study the effect of childhood adversities and current sleep, stress, psychiatric diagnoses, and lifestyle factors on LTL in adulthood in a population-based nationally representative cohort.

Study III: To study the effect of subjective and objective sleep difficulties and depression on DNA methylation in medication-free adolescents with or without depression.

4 MATERIAL AND METHODS

4.1 STUDY SAMPLES

Child sleep cohort

The Child Sleep (CS) cohort [322] was a large prospective birth cohort collected from the Pirkanmaa region between 2011 and 2013. All participants provided informed consent, and parents' consent to collect data from newborns was acquired. Mothers completed a study questionnaire during the last trimester of pregnancy, and an umbilical cord blood sample was collected at birth from the newborn. The Hospital district of Pirkanmaa ethical committee approved the study plan a priori. Some key characteristics of the cohort are presented in table 1.

Table 1. Characteristics of Child Sleep (CS) sample

Variable	Mean	S.D.	n
Maternal age	30.64	4.57	1323
Maternal BMI (kg/m^2)	28.43	4.42	1351
Child's gestational age at birth, days	280.70	8.51	1340
Birth weight (g)	3597	449	1414
Apgar score, 5 min	8.64	1.40	1337
Non- continuous variables			n
Maternal smoking	Yes, 79 (5.7%)	No, 1308 (94.3%)	1387
Child's gender	M=693 (51.7%)	F=647 (48.3%)	1340
Vaginal birth	82.4%		1165
Vacuum-assisted vaginal birth	7.5%		106
Elective cesarean section	2.9%		41
Acute cesarean section	7.2%		102

Modified from [323] Ämmälä AJ, Maternal stress or sleep during pregnancy are not reflected on telomere length of newborns. Scientific Reports. 2020 Springer Nature.

Health 2000 cohort

The Health 2000 cohort (Terveys 2000 in Finnish) is an unbiased representation of the entire Finnish mainland population aged ≥30 years. It was collected by The National Public Health Institute (currently National Institute for Health and Welfare) [324] to explore the health and wellbeing of the Finnish population. It also collected information on the functional capacities of the population and associated factors. A total of 8028 individuals participated in study, and data were collected from questionnaires, interviews, and health examinations. All participants provided their written informed consent to participate, and the study protocol was approved by both National Health Institutes and the Hospital district of Helsinki and Uusimaa ethical committees.

To obtain national representativeness, a two-step clustered stratified sampling was performed using five university hospital districts, which each had a population of 1 million as a stratum. In the initial step, a sampling from the 15 largest cities in Finland with probability of 1 was taken, followed by a second sampling from 65 areas, 13 from each university hospital district using the probability proportioned to population size (PPS) method [324]. In the final step, from each of these 80 areas (15 cities and 65 other areas), a random selection of subjects was drawn using the National Population Register as a data source. Again, the total number of subjects selected from each area was proportional to the population size of the area where they were selected from.

ADSLEEP cohort

The ADSLEEP cohort consists of a case-control sample of adolescent boys suffering from depression and sleep disorders and healthy controls. Cases (N=10) were collected from an adolescent psychiatric outpatient clinic in the Helsinki University Hospital. Controls (N=10) were enrolled via a newspaper ad published in the Helsinki University Hospital staff journal. All participants were medication free and were aged 14.7-17.4 years (mean age 16.1 years). Some participants withdrew after enrolment at the DNA-sampling phase. One control and 2 cases left the study, resulting in 8 cases and 9 controls for a total of 17 subjects. Exclusion criteria included medication and age >17.5 or <14.5 years. Subjects with any chronic medical condition or mental retardation were excluded. The possibility of a somatic condition was excluded with standard laboratory testing prior to enrolment, including glucose, liver, kidney, thyroid functioning, and blood cell counts. Subjects with any substance abuse were also excluded. Since all questionnaires and interviews were in Finnish, sufficient Finnish language skill was required. Any psychiatric disorder other than depression and sleep disorder were exclusion criteria for cases, and controls did not have any diagnosis. All subjects abstained from any psychotropic or any other medication throughout the entire study. This

sample was a part of larger project studying brain maturation [49, 325, 326], and thus any contraindication for Magnetic Resonance Imaging (MRI) was an exclusion criterion. None of the subjects had any structural pathologies in brain MRI. Cases and controls did not differ as a group by body mass index (BMI), caffeine use, serum testosterone levels, or age. The study protocol was approved by the ethical committee of Helsinki and Uusimaa hospital districts, and written informed consent was acquired from the legal guardians of all subjects.

4.2 MOLECULAR GENETIC ANALYSES

Telomere length

LTL used in studies I and II was analyzed using quantitative real-time polymerase chain reaction (qPCR) [327-330]. Telomere length is a relative measurement where the length of a single-copy gene (S), in this case the β-hemoglobin gene, is compared to an absolute amount of telomere DNA (T), resulting in a T/S ratio as a measurement of telomere length.

DNA was extracted from peripheral blood and was performed at THL Biobank. A total of 7364 DNA samples were analyzed in triplicates in the Health 2000 sample and 1405 samples in the CS sample.

A standard curve with known genomic concentrations was generated by including a seven-point standard quantity in each plate. Next, we compared the single-copy genomic results and telomeric results, yielding a correlation from 0.993-0.999 between sample measurements and standard curve, thus leading to sufficient PCR reaction efficiencies ranging from 77.8-100.4%.

Finally, a quality-control procedure was performed and included removing samples where the correlation between triplicates deviated more than 0.5 S.D. Samples that did not fit the standard curve or samples where the PCR reaction was not successful for any reason were discarded from the analysis. We also considered the plate effect and normalized telomere and reference-gene signals with a signal obtained from the same control sample used in every plate.

DNA methylation

For DNA methylation analyses (study III), DNA samples used in analyses were obtained from peripheral venous samples and DNA was extracted using standard methods. There was some delay between clinical interviews and cognitive tests and sampling (on average 25 days). The range was significant, varying from 3 days up to 218 days. However, the clinical evaluator remained

the same and no clinical change was observed in the subjects, thus giving reason to assume this delay did not influence the results. Cases remained cases and controls remained controls.

For cases, the average length of depression before sampling was 415 days, but again there were significant variation from 78 to 1078 days (S.D. 377 days). This again raised the question of whether length of depression would be a confounding factor and therefore we separately analyzed methylation status of each significant loci used in secondary analysis against the length of depression. Only one locus had nominal correlation between length of depression and methylation status; we decided not to consider depression length as a covariate in further analysis, as this would have led to loss of controls with a resultant sample of insufficient size.

After extraction, a bisulfide conversion was performed using an EZ-methylation kit (ZYMO research, Irvine, CA, USA). In this procedure, bisulfite treatment turns any *un*methylated cytosines (C) to uracils (U), whereas *methylated* cytosines remains cytosines. Followed by a PCR reaction, uracils are read as thymine (T) and the C/T ratio thus reveals the relative methylation status of the CpG site explored [331].

After conversion, samples were analyzed with the Illumina Infinium HumanMethylation450 BeadChip kit (Illumina Inc. San Diego CA, USA). The analysis was performed in the Estonian Genome Center in Tartu, Estonia.

Quality control was done using R-software (version 3.0.1) using packages "minfi", "limma", and "IlluminaHumanMethylation450Manifest" [332]. First, probes with detection p-value >0.01 were excluded from analysis, followed by exclusion of probes known to cross-react in more than one genomic location, probes having single-nucleotide polymorphism (SNP) in the CpG site or single-nucleotide extension at the CpG site, and probes with no signal. Probes in X or Y chromosomes were also excluded. This resulted in exclusion of 46 384 probes, and thus 439 128 probes were qualified for analysis. The next step was normalization of signals, which was performed with R-function "SWAN" in "minfi" package. This was necessary as the 450K chip is actually constructed from two different probe sets using slightly different chemistry. Normalization makes signals from different probe sets comparable [333, 334]. We also computed white blood cell counts in cases and controls and compared them with *t*-test to determine whether cell composition between groups could be confounding factors, as different white blood cell populations have slightly different methylation patterns [125, 126]. There were no significant differences and thus methylation patterns could be compared. In the final step, all raw signal values were transformed into so-called M-values by taking the $\log_2$ ratio from signal intensities from methylated and unmethylated probes. This was necessary as raw signals in the array follow a Bernoulli-type

distribution, meaning that it is linear only in the mid values and tends to be sigmoidal in extremes [335]. A positive M-value indicates that more than half of all probes investigated were methylated.

4.3 STUDY TRAITS

Maternal stress, BMI, sleep, depression, and anxiety during pregnancy

Maternal stress was evaluated during pregnancy by using a short version of the Perceived Stress Scale(PSS) [336]. This is a five-point scale that measures perceived stress, ranging from 0 to 17 points in total. In our sample, Cronbach's α for total score was 0.67.

Maternal self-reported weight before pregnancy was divided with self-reported height^2 resulting in BMI before pregnancy, which was then used as a measurement of weight affecting the fetus.

The Basic Nordic Sleep Questionnaire (BNSQ) [337] was used to assess sleep quality. The BNSQ contains 21 questions covering essential parts of sleep quality, such as self-reported length of sleep, sleep latency, awakenings, snoring, and daytime tiredness. First, we formed an index for insomnia by calculating the sum of questions 1, 3, 4, 5, and 6, which all measure different facets of insomnia. The sum score had a range from 5-25; Cronbach's α was 0.70 in our sample. To evaluate sleep-related breathing disorder, we dichotomized BNSQ question 16 "Do you snore?" such that snoring at least once a week qualified as a case.

Maternal depression during pregnancy was measured with the Center for Epidemiological Studies Depression Scale (CES-D) [338]. The CES-D contains 10 items with a total score ranging from 2-23. In our sample, Cronbach's α was 0.78.

Maternal anxiety was measured during pregnancy by using the State and Trait Anxiety Scale (STAI), Short version [339]. STAI has six questions and total score ranges from 6-21; Cronbach's α was 0.78 in our sample.

Childhood adverse experiences, socioeconomical status, and specific stressors

Childhood adversities were evaluated with a questionnaire containing 11 different adversities that might have occurred before age 16 [340, 341]. This was performed during home interview and all questions started with the

sentence “When you think about your growth years, i.e., before you were age 16…”. Questions were the following:

1. Did your family have long-term financial difficulties?
2. Was your father or mother often unemployed although they wanted to work?
3. Did your father or mother suffer from some serious disease or disability?
4. Did your father have alcohol problems?
5. Did your mother have alcohol problems?
6. Did your father have any mental health problem, e.g., schizophrenia, other psychosis, or depression?
7. Did your mother have any mental health problem, e.g., schizophrenia, other psychosis, or depression?
8. Were there any serious conflicts within your family?
9. Did your parents divorce?
10. Were you yourself seriously or chronically ill?
11. Were you bullied at school?

The answer choices were “Yes”, “No”, and “Cannot say”; only “Yes” was coded as positive. We categorized the answers to form the four following groups: no childhood adversities (N=4316), one adversity (N=1374), two adversities (N=1120), and three or more adversities (N=2057).

Childhood socioeconomic status was evaluated by self-reported parental education level; if the parents had different levels, the higher was chosen.

Specific stressors during childhood included self-reported parental death before age of 16 years.

Sleep, depression, and cognition in adolescents

Sleep in adolescents was evaluated as a part of the Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version (K-SADS-PL) interview attachment of affective disorders. This attachment that evaluates affective disorders also has six questions that evaluate sleep. They cover issues of initial insomnia, insomnia in mid-sleep, terminal insomnia, disturbed sleep-wake rhythm, hypersomnia, and non-restorative sleep. The clinical interviewer evaluated each of these facets using standard criteria [342]. None of the controls had any sleep disorders according to this evaluation.

In addition to clinical evaluation of sleep disorders, self-reported measurements were also used. Insomnia was evaluated using the Athens Insomnia Scale (AIS) [343]. AIS has eight items and total score ranges from 0-24. It covers essential symptoms of insomnia, such as total sleep duration,

difficulty falling asleep, early awakenings, daytime sleepiness, waking during night, daytime functioning, daytime well-being, and sleep quality.

Daytime sleepiness was evaluated separately using the Pediatric Daytime Sleepiness Scale (PDSS) [344]. The PDSS is especially appropriate for evaluating adolescent sleep and consists of eight items covering questions related to daytime sleepiness and alertness. The total sum of scores ranges from 0-32.

Objective measurements were also used for sleep architecture. This was performed with polysomnography, which combines recordings from EEG, electrooculogram (EOG), and electromyogram (EMG). Measurements were performed in the adolescent's home over two consecutive nights; the first night was for gaining familiarity with measurements and the second was used for analysis. We used a portable device (Embla, Flaga, Hf. Medical Devices, Reykjavik, Iceland). Electrodes were positioned according to the international 10-20 system. Our main interest was SWS, which was calculated from EEG with power spectral analysis [49]. A particular emphasis was paid to frontal electrodes, as SWA dissipation was altered in the frontal cortex in previous studies in the same cohort [49].

Depression was assessed with a semi-structured diagnostic interview covering the Diagnostic and statistical manual for mental disorders fourth edition (DSM-IV) axis I disorders K-SADS-PL [345]. An experienced clinician performed all interviews and confirmed the diagnoses after discussion with a professor of adolescent psychiatry. Six cases had first-time depression, one had a second lifetime episode, and one case had a circadian rhythm disorder with mild depressive symptoms. None of the controls were diagnosed with any axis I disorder. None of the subjects suffered from psychotic disorder or bipolar disorder. Two cases had comorbid diagnoses, one with anxiety disorder and one with behavioral disorder. Symptom severity of depression was evaluated using the Beck Depression Inventory (BDI) [346-348], a validated instrument for assessing depression symptom severity also in adolescents. The BDI has 21 items with a sum score ranging from 0-63. To evaluate the effect of depression in secondary analysis, we used a shorter version where two questions related to sleep and tiredness were removed.

From cognitive functions, we concentrated on psychomotor vigilance, which was evaluated using the Psychomotor Vigilance Task (PVT), as the effect of sleep disorders in this domain has been very consistently shown [95, 349]. The PVT is a neurobehavioral test widely used to test sustained attention. This was a part of a larger test battery, from which a PVT-192 unit was used (Ambulatory Monitoring Inc. Ardsley, NY, USA).

Adult mental health, current stress, sleep, socioeconomic status, and health behavior

In adults, current mental health was evaluated using a structured psychiatric interview that uses DSM-IV criteria [111] and the Munich Composite International Diagnostic Interview (M-CIDI) [350]. This interview covers psychotic disorders, major depression and dysthymia, substance abuse or dependence, and anxiety disorders.

Current stress was evaluated using the General Health Questionnaire (GHQ) [351], which is a 12-item self-reported measurement of recent psychosocial distress.

Sleep was evaluated with a self-reported questionnaire containing questions about difficulties initiating sleep, maintaining sleep, fatigue, and tiredness [352]. A self-evaluation of total sleep time was also performed and was dichotomized to short sleep (<6 hrs) and to normal sleep (6-9 hrs). Subjects sleeping >10 hrs were excluded from analysis, since it is possible that such individuals have a health condition that could affect secondary analysis. There were 189 subjects excluded due to long sleep.

The following sociodemographic factors were collected from questionnaires: age, gender, marital status, education (highest achieved), and current employment status. University hospital district was also considered.

For health-related behavior and factors related to health, we included BMI, smoking, and physical activity.

4.4 STATISTICAL ANALYSES

For study I, we used a linear regression model where LTL served as a dependent variable and maternal stress, sleep, anxiety, depression, and BMI were an explanatory variable. qPCR plate, maternal smoking during pregnancy, child's gender, and gestational age at birth were also entered as explanatory variables. Perceived stress (PSS), anxiety (STAI), and depression (CES-D) were analyzed in a separate analysis since they had high intercorrelations to minimize the risk for collinearity bias. STAI and CES-D were transformed into natural logarithms to reduce skewness and to fit them better into the linear regression model. These analyses were performed with SPSS v. 24 (IBM, Armonk, NY, USA).

Power analysis utilized effect-size estimates as reported previously [204, 208, 209, 216]. The α error level was set to <0.05 and the same parameters were

also used when calculating the minimum detectable effect size with 80% likelihood. Analysis was performed with R-software (Version 3.5.1) with package "pwr" [353]. To evaluate the false discovery rate (FDR), we used R-package "p.adjust" [354].

In study II, we again used SPSS multiple linear regression modeling where in each model LTL acted as the dependent variable and psychiatric diagnoses, stress, adversities, socioeconomic factors, lifestyle factors, and sleep served as explanatory factors. All analyses were adjusted for the effects of age, gender, and qPCR plate. In addition, a population weight factor was added to adjust the sampling effect detected after collection of the cohort was finished.

Study III used several approaches. In epigenome-wide analysis (EWAS), an empirical Bayes moderated *t*-test was applied to achieve probe-wise comparisons of methylation status in cases and controls. This approach was especially designed for the 450K chip and considers the fact that it contains both one- and two-channel microarrays [355] and relatively small sample size. Multiple testing is a true challenge in any genome-wide array, and thus the Benjamini-Hochberg (BH) procedure was applied using $p<5.0 \times 10^{-8}$ as a significance threshold [332, 354] using the R-package "limma".

In the next phase of analysis, a pathway analysis was performed using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood city, CA, USA). We used the 500 most significantly differentially methylated sites in the original case-control analysis. After QC, a total of 332 sites was mapped to a known genomic location and were entered into pathway analysis. This software compares this set of genes to known biological pathways and uses Fisher's exact test (right-tailed) to determine whether a given set of genes is enriched into some biological pathway more than could be expected by chance. It does not correct for multiple testing, but correction can only correct the magnitude of likelihood, but the pathways would still be the same and appear in the same order as in uncorrected analysis. The results yielded the top canonical pathways, which refer to pathways that are generalized and represent the most common form of a pathway across different animal species and different tissues [356].

Finally, a linear regression was used in secondary analyses for methylation values for identified loci in the pathway and for mood, sleep, SWS, and vigilance. One could expect that original case-control status could influence these analyses, and we also performed a separate analysis using case-control status as a covariate. The BH procedure was applied to consider multiple testing. These analyses were performed with SPSS v. 24 (IBM, Armonk, NY, USA).

4.5 ETHICAL CONSIDERATIONS

Our study involved pregnant mothers and fetuses. They should be considered as especially vulnerable groups and thus particular care should be applied in designing a study for these groups. Adolescents can also be considered as a vulnerable group. The aim of our study was to increase understanding of stressors and their effects on biology and thus provide insight into what groups are at special risk and on when the risk is and what kind of risk is present. This knowledge may be used to design screening programs for populations at risk, support programs, or planning policies in society in general that favors risk reduction, improves access to support, or minimizes risk factors. This leads to the notion that considering consequentialism ethics it is justified to conduct research that generates knowledge to guide service design and politics. This implies that participants in this study do not benefit directly from participating in the study and may only get disadvantages, such as time lost to completing questionnaires or possible adverse events from blood sampling. On the other hand, they might later benefit from results during a new pregnancy or when the children participating in this study themselves become parents. Considering virtue ethics, it is justified to study pregnant women and unborn and newborn children for future benefit of children and their mothers.

There was careful ethical consideration in the design phase of each study and preauthorization from the Committee on ethics was obtained for each study before the protocol was executed (THL 407/E3/2000, Helsingin ja Uudenmaan hospital district ethical committee 137/13/03/03/2011 and Pirkanmaan hospital district ethical committee 3/2011 (22.2.2011), Tiede 67§ R1 1032). All participants provided informed consent. For unborn children, consent was obtained from parents. The adolescents' legal guardians provided informed consent to participate.

5 RESULTS AND DISCUSSION

5.1 THE EFFECT OF MATERNAL SLEEP OR STRESS DURING PREGNANCY AND LTL OF A NEWBORN (STUDY I)

In study I, we explored the association between maternal stress, sleep, anxiety, and depression on newborn LTL following the Fetal Programming of Telomere Biology hypothesis.

Before analyzing the data, we performed a power analysis for each variable of interest to verify that our sample size is sufficient to replicate previous findings based on sample size for each variable of interest and previously reported effect size. In conclusion, the likelihood to detect at least a previously reported effect was >99% and the minimum detectable effect size with 80% likelihood was very low (0.07).

After analyzing stress, sleep, depression, and weight, only anxiety (STAI) and pre-pregnancy BMI had nominal significance with newborn LTL. For STAI, β was -0.09 (p=0.04) and for BMI β was -0.01 (p=0.04). We then applied FDR correction, after which neither remained significant ($STAI_{FDR}$ p=0.12, BMI_{FDR} p= 0.12). It is worth noting that detected estimates of effect (β) were very low and were below the minimum detectable value estimated in power analysis for BMI (>0.07).

Although our sample size was twice as large as the largest sample size reported thus far, we could not replicate previous findings [204-206]. There may be several different reasons for this.

The methodology for evaluating both the dependent variable (LTL) and explanatory variables (such as stress, sleep, anxiety) may be different in different settings, which would lead to different results. The most common method used is qPCR, but other methods, such as restriction enzyme based methods, may yield slightly different results [357]. Even tissue of origin can lead to differences in results. For example, if there is a different mixture of white blood cell types, differences in LTL may reflect this instead of a true difference caused by the explanatory variable [358]. Contrasting findings also exist, where there are no marked differences between LTL in different tissues [209]. Unfortunately, despite accumulating knowledge about the effect of prenatal factors on LTL, only a few previous studies have utilized umbilical cord blood samples [277]. The statistical models and covariates used also varied, and this may create difficulty when comparing different studies. For example, some studies used BMI as a covariate while others did not. Use of

several explanatory factors can induce a collinearity problem [359]. Measurement of stressors can also induce undesired variance. The timing of stress, type of stressors, or single event or chronic type can all have a modifying effect. For example, concentrating on trajectories instead of single-point measurements may yield different results [360].

This study had several strengths, such as large sample size and population-based sampling from certain geographical regions, which allows for generalizing the findings to the general population. There were also limitations. In a population-based sample, a minority of participants suffered from severe stress, anxiety, or depression; severe sleep disorders were also scarce. It is possible that there was a minor selection bias, as mothers were recruited via the prenatal maternal care system, which is a publicly provided, free-of-charge service available in every community in Finland. Despite this accessibility, it is possible that mothers with the most stressful life situations and poorest supporting network did not use this service and thus were excluded from the study. This is general challenge in every population cohort when recruiting those who are most ill to participate. Therefore, stressors may be more readily detected in a clinical sample or in a case-control setting.

5.2 THE EFFECT OF CHILDHOOD ADVERSITIES ON LTL IN ADULTHOOD (STUDY II)

The aim of the second study was to explore whether ACE's influence LTL in adulthood. This was explored in a large, nationally representative population cohort. In the initial analysis, both age (β=-0.322, p=4.23x10^{-6}), gender (β=0.104, p=1.0x10^{-6}), and qPCR plate (β=0.105, p=2.6x10^{-25}) had a significant association with LTL, and thus were always entered into models as covariates.

Interestingly, none of the adversities alone were significantly associated with LTL in adulthood when analyzed separately. However, when adversities were grouped into four categories (none, single, two, and three or more adversities) an association was detected. More adversities were associated with shorter LTL in adulthood (β=-0.006, p=0.005).

Losing a parent before the age of 16 years (β=0.009, p=0.430) and childhood socioeconomic status (β=-0.016, p=0.216) were not associated with LTL.

We explored whether the effect of cumulative ACE on LTL could be explained by current psychiatric diagnosis, but this did not appear to be true (β=-0.030, p=0.02). Lifestyle factors affecting LTL (β=-0.030, p=0.013) and known socioeconomic factors during adulthood (β=-0.033, p=0.006) also did not

explain this effect. Thus, the association remained significant even when considering known factors that affect LTL in both childhood and in adulthood.

Our finding is consistent with previous findings that a cumulative effect of stressors, rather than any single episode, is associated with LTL in adulthood [361, 362]. Puterman et al found in nationally representative population cohort in United States that no single childhood adversity was related to adulthood LTL, but cumulative ACE explained the difference in LTL in adulthood. They also found that it was childhood adversities, not adulthood adversities, that contributed to shortening of LTL. In their systematic review and meta-analysis, Hughes et al found that having four or more risk factors were associated with shorter LTL, although their risk factors were factors occurring in adulthood and were not ACEs.

Our main limitation was that the ACE questionnaire used has not been validated externally, and some severe forms of adversities (such as sexual abuse) were absent. Despite these limitations, this questionnaire is a useful instrument based on previous reports utilizing this and very similar questionnaires [276, 361, 363-367]. We could not replicate at the population level the previous findings related the effect of mental disorders on LTL. However, there were relatively few mental health cases in a population-based sample. Similar to the findings from maternal factors, different results may appear from cohorts with enrichment of cases or at-risk subjects. A strength of this study was that we used a large nationally representative population cohort, which emphasizes that these effects do not appear only in risk populations or clinical samples.

5.3 THE EFFECT OF DEPRESSION AND SLEEP INTO DNA METHYLATION IN ADOLESCENTS (STUDY III)

In this study, we used a case-control setting where we first explored whether there are epigenome-wide (EWAS) differences in methylation status between cases and controls. We set the significance threshold to 5.0×10^{-8} and found no epigenome-wide differences between these two groups.

Next, we performed a pathway analysis to explore whether there is enrichment of genes that differentiate the most into known canonical pathways. We chose for this analysis the top 500 sites that differentiated the most to obtain a manageable number of genes. Of these, 332 unique sites mapped to known genes were identified and were subsequently entered into IPA pathway analysis software. Our top canonical pathway was the LTD pathway ($p=0.00045$), followed by the nitric oxide signaling pathway (NOS) and netrin signaling pathway. Both had a p-value of 0.0042, which is 10-fold larger than the LTD pathway. We thus decided to continue with the LTD pathway in

secondary analysis. There are 165 different genes involved in the LTD pathway; of these, our top-list genes included 10. Out of these 10 sites, seven were relatively hypermethylated in cases vs controls and three were relatively hypomethylated. Table 2 presents the genes, their original p-value in the case-control setting, relative methylation status, and difference in M-values.

Table 2. Relative methylation status of LTD pathway methylation sites that appeared in the 500 sites that differed the most.

Locus	**Gene**	**p-value in 450K array**	**Methylation level in cases vs. controls**	**Average M-valuefor cases / controls**
cg1684176	*CACNG1*	0.000381	Lower	3.18 / 3.49
cg22025854	*CACNG6*	0.000527	Higher	-1.17 / -1.57
cg08364956	*GRM6*	0.000838	Lower	3.29 / 3.56
cg05110803	*IGF1R*	0.000306	Lower	1.47 / 1.95
cg19161850	*ERK12*	0.000663	Higher	-3.88 / -4.13
cg12066398	*PLA2G16*	0.000540	Higher	-3.70 / -4.07
cg04367351	*PLA2R1*	0.000659	Higher	-4.14 / -4.46
cg02263165	*PPP2R5C*	0.000207	Higher	-3.14 / -3.35
cg18823846	*PRKG1*	0.000903	Higher	-3.95 / -4.20
cg25405123	*RYR3*	0.000427	Higher	-3.61 / -3.89

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In secondary analysis, we explored the association between methylation of each locus and the variables sleep, depression, and vigilance. Only one out of

10 sites had an association between methylation and length of depression (β 0.860, p=0.013, uncorrected with multiple testing). We thus decided not to perform covariate analysis with depression length, as this would have led to abolishing controls and would make the sample very small.

We found several associations between sleep, mood, and SWA dissipation (Table 3).

Table 3. Linear regression results between LTD pathway methylation sites and mood, vigilance, and tiredness and sleep.

Locus (Gene)	Mood BDI-19 B p-value	Vigilance and Tiredness Median RT B p-value	PDSS B p-value	Sleep AIS B p-value	SWA Dissipation B	p-value
cg1684176 (*CACNG1*)	-0.69 0.002	-0.44 0.08	-0.58 0.02	-0.68 0.003	0.38	0.14
cg22025854 (*CACNG6*)	0.44 0.08	0.40 0.11	0.19 0.49	0.55 0.02	-0.25	.36
cg08364956 *(GRM6)*	-0.47 0.06	-0.26 0.31	-0.60 0.01	-0.75 0.001	0.38	0.15
cg05110803 *(IGF1R)*	-0.50 0.04	-0.28 0.29	-0.79 0.00029*†	-0.68 0.003	0.24	0.38
cg19161850 *(ERK 12)*	0.55 0.023	0.21 0.43	0.72 0.002*	0.65 0.005	-0.47	0.67
cg12066398 *(PLA2G16)*	0.77 0.00031*†	0.66 0.004	0.33 0.21	0.70 0.002	-0.32	0.23
cg04367351 (*PLA2R1*)	0.48 0.053	0.17 0.51	0.77 0.00047*†	0.68 0.003	-0.30	0.26
cg02263165 *(PPP2R5C)*	0.64 0.006	0.51 0.035	0.32 0.23	0.65 0.005	-0.67	0.004*
cg18823846 (*PRKG1*)	0.57 0.018	0.49 0.045	0.50 0.051	0.60 0.010	-0.16	0.56
cg25405123 *(RYR)*	0.64 0.006	0.42 0.095	0.44 0.091	0.76 0.00041†	-0.54	0.031

† denotes significant association after Bonferroni correction. * denotes significant association at < 0.05 level when case-control status is controlled for (nominal). BDI-19 = Beck Depression Inventory with questions concerning sleep and tiredness removed, PDSS = Pediatric Sleepiness Scale, Median RT = Median reaction time in psychomotor vigilance task (PVT, AIS = Athens Insomnia Scale, SWAdiss = Slow wave dissipation during first episode of the night measured from frontal electrodes). [368], reproduced with permission from Elsevier publishing.

Of these associations, PLA2G16 and BDI score (β=0.77, p=0.00031), *IGF1R* (β=-0.79, p=0.00029), *PLA2R1* (β=0.77, p=0.00047), and tiredness and *RYR3* (β=0.76, p=0.00041), and insomnia also passed correction with multiple testing.

The small sample size limited the statistical significance of the results. Nevertheless, the results also passed correction with multiple testing. The results are also biologically plausible. For example, in a case-control setting, a lower methylation was observed in cases for the *IGF1R* site, and tiredness was negatively associated with methylation values in this site. Similar findings were observed with *PPP2R5C* and SWA dissipation.

Vigilance among cases was moderately lower, consistent with previous findings connecting depression and sleep difficulties to vigilance [369, 370]. This might reflect the developmental period ongoing in the CNS [371] and thus the ability to compensate for comprehensive cognitive difficulties.

This study was designed to be a hypothesis-generating study rather than a hypothesis-verifying study. The small sample size did not support finding any EWAS-level observations but served as a tool to identify important DMPs for further analysis. Enrichment of genes into the LTD pathway is consistent with evidence connecting sleep to memory processes in the developing brain [372]. LTD and LTP act as balancing components of synaptic plasticity related to memory consolidation and are connected to sleep [373-375]. LTD is particularly connected to SWS sleep [376], whereas LTP has previously been connected to REM sleep [377, 378]. In our sample, there were no marked differences in REM sleep [49], which might explain the emphasis on LTD-related genes rather than LTP-related genes.

Some genes in the LTD pathway exhibited fairly good correlation between peripheral blood leukocyte methylation level and methylation in the CNS, such as *PPP2r5C* [379]. However, for most genes explored, such knowledge was not available. We did observe an association between *ERK12* and tiredness, and this enzyme was indeed previously reported to associate with LTP, a balancing counterpart of LTD [380]. It is postulated that during daytime, LTP is favored over LTD, which is prominent during SWS. *ERK12* is mediated by phosphatases (such as PPP2R5C) and phospholipases (such as *PLA2G16*), creating a balancing synaptic homeostasis between strengthening and pruning of synaptic connections [381-383]. *IGF1R* also plays a role in regulation of LTD, as blocking *IGF1R* in the mouse cerebellum appears to cause cessation of LTD [369].

A limitation of our study was that we lacked expression data to correlate methylation changes to changes in expression. Methylation can be either an active modifier of gene expression or a byproduct of another mechanism regulating the expression, but nevertheless methylation serves as a proxy measurement of altered expression [120, 121]. Even small changes in methylation have been linked to altered phenotype [384]. Another shortcoming was the lack of genotype data; it is possible that there could be methylation quantitative loci (mQTL) that could influence the methylation status of the sites detected. The strengths of our study included a well selected and homogenous sample (all male, non-medicated, narrow age range, and no somatic or psychiatric comorbidities). The use of polysomnography brought objective measurements of sleep quality in addition to subjective evaluations.

Based on preliminary findings in a small sample, our conclusion is that there may be a connection between sleep and depression and synaptic plasticity that is at least partially mediated via methylation alterations detectable in peripheral blood leukocytes. The brain's lymphatic drainage systems connects sleep, CNS, and peripheral leukocytes together, thus making these observations plausible [385].

5.4 THE EFFECT OF CURRENT PSYCHIATRIC DIAGNOSES, CURRENT STRESS, AND SLEEP ON LTL IN ADULTHOOD (STUDY III)

Present psychiatric diagnoses, including major depression, any anxiety disorder, any psychotic disorder, and substance abuse were not associated with LTL. The explored current sleep difficulties and current psychosocial stress measured with the 12-item GHQ questionnaire were also not associated with LTL (table 4).

Table 4. Association between mental health disorders and adult LTL.

Variable	Unstandardized Coefficients	Standardized Coefficients	p-value	95.0% Confidence Interval for β	
	β	β		Lower Bound	Upper Bound
Sex	0.045	0.104	$1.0\ 10^{-6}$	0.034	0.056
age	-0.005	-0.322	$4.2\ 10^{-6}$	-0.005	-0.004
PCR plate	0.001	0.105	$2.6\ 10^{-25}$	0.001	0.001
Any anxiety disorder	-0.006	-0.006	0.618	-0.029	0.017
Depression or dysthymia	-0.011	-0.012	0.356	-0.036	0.013
Any psychotic disorder	-0.001	0.000	0.997	-0.031	0.031
Any substance abuse	-0.005	-0.020	0.125	-0.012	0.001
GHQ total score	-0.001	-0.013	0.326	-0.021	0.034
Difficulties falling asleep	-0.006	-0.021	0.110	-0.012	0.001
Early morning awakenings	-0.002	-0.009	0.482	-0.009	0.004
Tiredness	0.005	0.017	0.176	-0.002	0.013
Sleep length	0.000	-0.001	0.945	-0.014	0.013
BMI	-0.001	-0.017	0.163	-0.002	0.000
Physical activity	0.001	0.003	0.805	-0.005	0.007
Smoking	0.003	0.017	0.168	-0.001	0.008
Marital status	0.000	0.003	0.836	-0.003	0.004
Education	0.001	0.003	0.535	-0.001	0.002
Hospital district	0.001	0.015	0.202	-0.005	0.001
Occupational status	-0.006	-0.029	0.045	-0.013	-0.001

Results from 16 different independent linear regression models are shown, each including PCR plate, age, and sex as covariates. GHQ=General health questionnaire, BMI=body mass index, LTL=Leukocyte telomere length [386] with permission from Elsevier publishing.

All explored lifestyle and socioeconomic factors, except occupational status, did not have an independent association on LTL (Table 4). Being in full-time employment was associated with longer LTL, although the association was modest (β=-0.03, p=0.01).

Previous findings concerning the association [279] between ACEs and LTL in adulthood were confirmed in a large, population-based nationally representative Finnish cohort. Interestingly, we did not find support for previous findings concerning the effect of mental disorders and LTL. The association between current occupational status and LTL is interesting, as previous studies have revealed somewhat conflicting results. In one study it was reported that women currently employed had shorter LTL compared to those not working [387], whereas in men the opposite was observed [388]. Studies that reported no association between these two have also been published [389]. One possible explanation might be the fact that mental health and occupational status are often linked, and thus a collinearity problem might exist [359]. A study utilizing the same cohort as ours revealed an association between work-related exhaustion and LTL [390], and this may be such a collinearity-based example. The strengths of our study included a large sample size and strong representation of the general population. There may also be limitations; it is possible that in clinical samples or high-risk subjects an association between mental disorders and LTL would have been detected.

6 GENERAL DISCUSSION AND CONCLUSIONS

Our study on ACEs confirmed that the effect of early adversities can be observed in LTL in adulthood, thus expanding the Prenatal Programming of Telomere Biology hypothesis from prenatal stress to postnatal stressors. It is worth noting that this programming effect can be observed at the population level, confirming this phenomenon from special at-risk populations to cover all subjects at the national level.

On the other hand, we could not replicate at the population level the effect of prenatal stress on newborn LTL. While this suggests that there may be special populations at risk where a notable effect may play a role, these findings cannot easily be transferred to the general population. These factors combined lead to the conclusion that stress recognition and management is needed both at the population level and in at risk- groups. When the effect of stressors (such as a mental health condition, in this case depression and sleep disturbances) was explored in a high-risk group (depressed adolescents with sleep disorders), an association was observed despite the relatively small sample size, thus demonstrating the ability to explore vague signals in high-risk groups that are carefully described and thoroughly examined and diagnosed. These well-characterized cohorts also enable generation of new hypotheses concerning possible biological mechanisms behind these hypotheses.

Several caveats may explain the inconsistent findings regarding different studies. As mentioned, different populations might yield different results. The methodology is also very different for both LTL measurements and methylation-level measurements, and even this might create some discrepancy among findings. Even the tissue used for sampling may lead to marked differences and should be considered when possible. Statistical models (especially which covariates are used) play an important role, as does measurement of phenotype. Timing of measurements (such as those related to pregnancy) can vary and stressors can be diverse, as some are short-term drastic events, and some are more subtle and chronic.

Unfortunately, we could not consider resilience factors in our studies. This would be important to implement in future studies, as it is possible that personal resilience factors can play a major modifying role in stress biology. More research is also needed for specific timing of different stressors and their impact on DOHaD. Studies with interventions directed towards occurrence of ACE should be designed to explore whether interventions may lead to changes at the DOHaD-biology level.

In conclusion, we expanded the previous preprogramming hypothesis into a wider perspective that also covered other important growth phases. Our results also emphasize the importance of vigorous methodological consideration, as methodological aspects can have a profound effect on results in this field.

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