Chapter 1

General introduction

“Every stress leaves an indelible scar, and the organism pays for its survival after a stressful situation by becoming a little older.”

Hans Selye (1907-1982)
1.1. AGING

Aging was defined by Alex Comfort in 1979 as “a progressive increase throughout life, or after a given stadium, in the likelihood that a given individual will die, during the next succeeding unit of time, from randomly distributed causes” ¹. Later in 1991, a new definition of aging was elaborated: “a persistent decline in the age-specific fitness components of an organism due to internal physiological deterioration” ². Overall, aging thus seems to occur due to an increase in age-specific mortality and a decrease in age-specific reproductive rate in a time-progressive manner ³.

We all age whether we want it or not, and unfortunately our loved ones age too. Aging is a process that starts already before we are born and in later stages of life it is commonly associated with adverse changes in human physiology, determining the development of degenerative and chronic conditions. There are various theories of why we age, and what exactly happens in the body. For instance, August Weismann introduced the aging theory of “wear and tear of the body” ⁴. He described that the body and its cells were damaged by overuse, and worn down by many internal and external stressors to which we subject our bodies. Recently, various cellular aging markers have been identified that give more detailed insight into the aging process, in addition to chronological aging (i.e. the exact time from birth). Two such cellular aging markers are leukocyte telomere length (TL, Section 1.1.1.), which will be the main focus of this thesis, and leukocyte mitochondrial DNA copy number (mtDNACn, Section 1.1.2.).

1.1.1. Cellular aging: telomere length

One theory at the cellular level is that the lifespan of each cell is genetically programmed. At each DNA strand terminus telomeres are located, several repeats of the sequence 5’-TTAGGG-3’ (Figure 1). They protect the DNA from breaks and fusions. At birth, human telomeres are approximately 11,000 nucleotides long, and they lose 50-100 nucleotides per DNA replication cycle ⁵;⁶. This loss of telomeric DNA is due to the ‘end-replication-problem’: DNA polymerase can only extend DNA with a preformed primer, and therefore it cannot copy the end of the strand ⁷. Since the RNA primer must always attach prior to the synthesis of the lagging strand fragments, and since the RNA primer must base pair to complementary nucleotides on the leading strand, the 5’ end of lagging strand will always be shorter than the 3’ end of the leading strand. Telomere length (TL) is a proxy for the age of a cell, as older mitotic cells tend to have shorter TL than younger cells, and the cell goes into a senescent state once a critically low TL is reached ⁸-¹⁰. Leonard Hayflick described this phenomenon in the “Hayflick limit theory of aging”, hypothesizing that human cells are able to divide only a limited amount of times (approximately 50 times), after which they stop dividing and die ¹¹. Variations in the number of telomeric repeats at birth are partly inheritable: approximately 64-70% of TL is explained by genetic factors ¹²;¹³. The rate of telomere attrition is shown to be heritable to a lesser extent.
than the initial TL (24-32%), and seems to take place largely in the first 20 years of the lifespan. The presence and activity of the unique telomere-synthesizing enzyme telomerase also influences the rate of cellular aging, as it can reverse the attrition of telomeres (Figure 1). Variations in the genes encoding the major subunits of telomerase, telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC), determine the speed of telomeric repair, and impact the inter-individual variation in TL. It has been reported that telomere attrition might be accelerated by (the cumulative exposure to) various stress-related factors in the body, such as psychological stress, physiological stress, metabolic dysregulations and oxidative damage.

Short TL has been associated with the onset of various aging-related somatic diseases, such as cardiovascular disease, cancer, dementia and premature death. Therefore, telomere attrition could potentially be seen as an early red flag of aging-related deteriorations, and it could explain partly why some individuals do develop aging-related disease, whereas others experience aging in good health. TL is often measured in leukocytes due to its easier access in the body, and it is shown to reflect systemic TL in other tissues.

Figure 1: Telomeres and their lengthening by telomerase.
1.1.2. Cellular aging: mitochondrial DNA copy number

Another theory of cellular aging is the ‘mitochondrial theory of aging’. This theory proposes a vicious cycle, in which oxidative stress accumulates due to free radicals, such as reactive oxygen species, making cells unstable and less functional throughout the aging process. In turn, mutations in mitochondrial DNA impair the cell’s respiration mechanisms, and further enhance the production of free radicals. Mitochondria are cellular energy-generating organelles that play an important role in the cell’s metabolic homeostasis, proliferation, differentiation and apoptosis. All cells contain numerous mitochondria in their cytoplasm, each containing multiple copies of mitochondrial DNA. The number of mitochondrial DNA molecules per cell – or mitochondrial DNA copy number (mtDNAcn) – can thus vary independently from the number of mitochondria in one cell. Aging-related mitochondrial changes are for instance increased free radicals and mitochondrial DNA mutations, and mtDNAcn is shown to decrease with aging. Although some studies have found increased mtDNAcn in childhood adversity and lifetime psychopathology, or no association at all between mtDNAcn and cancer, most studies seem to agree that a lower mtDNAcn is cross-sectionally associated with chronic somatic diseases, such as hyperlipidemia, Parkinson’s disease, metabolic syndrome, and longitudinally with a higher risk of cognitive and physical performance problems and all-cause mortality. As TL and mtDNAcn decrease with aging, and are reported to be positively correlated in various studies, they are used as two separate cellular aging markers in this thesis.

1.2. STRESS

Hans Selye (Selye János, a Hungarian endocrinologist) defined the word, “stress,” in 1936 as the “non-specific response of the body to any demand for change”. He first described this concept as the General Adaptation Syndrome in experiments in rats where he administered damaging agents, or stressors (i.e. cold exposure, surgical injuries, intoxications with sublethal doses of drugs, excessive muscular exercise), and found that these stressors evoked a biological stress response, or so-called “general alarm reaction of the organism”, resulting in various diseases, such as cardiovascular events (e.g. strokes and heart attacks), atherosclerosis, allergic reactions and ulcers.

However, stress became a word that people used interchangeably to describe either their daily strains, their physical reactions to them, or the final result of persistent strains, such as chronic diseases. In the British Medical Journal, this confusion was summarized with: “Stress in addition to being itself, was also the cause of itself, and the result of itself”. Selye tried to find a fitting definition for stress to eliminate the confusion, and later he redefined stress as “the rate of wear and tear on the body”, which also fits into the description of biological aging.
There are many classifications of stress, as it can be divided into good stress (eustress) and bad stress (distress), in which the good stress can be beneficial for the organism to be motivated and achieve more. In this thesis, we will mostly focus on the bad form of stress. Distress can also be divided into acute, traumatic and chronic stress. Acute stress, such as a lion attack or a car accident, is an immediate danger that activates the fight-or-flight response of the sympathetic nervous system, whereas traumatic stress, such as war or natural disasters, is a life-threatening event that evokes fear and helplessness. These stress types lead to short-term adaptations in the body's stress systems, while the body tries to keep constant conditions. Walter Bradford Cannon developed the concept of homeostasis, in which the body tries to keep constant conditions in the internal environment based on a set point. Two examples of the body's healthy reaction to acute stress are the boost of glucose levels in the blood in order to enable the organism to fight or flee from a threat, and the activated storage of body fat in case of food scarcity.

Chronic stress, on the other hand, is a persistent stressor throughout everyday life that is said to disharmonize the stress systems. The term allostasis refers to the active process in the brain to maintain homeostasis in the body by constantly adapting. Whereas homeostasis attempts to keep the stress response accurate by providing local feedback, allostasis anticipates based on prior knowledge, and adapts the local feedback to meet future predicted demands. Chronic stress and aging lead to 'allostatic overload': the dysregulation of allostasis, and in turn chronic activation of mediators that are triggered by stress. From an evolutionary point of view, these alterations are central to the survival and wellbeing of the organism in its natural environment. However, in the case of chronic stress, these alterations become dysregulations. Chronically elevated blood glucose then eventually leads to the onset of diabetes mellitus type 2, and the unnecessary fat storage, even in the presence of sufficient nutritional resources, leads to obesity.

Early psychobiologists, such as Cannon already found a connection between psychological stress and physiology. With the capacity to learn, remember and anticipate stressful situations in higher level organisms, including humans, psychological stress can create chronic states of vigilance in the body, even in the absence of evident stressors. In humans, the “cognitive appraisal” of the stress stimulus becomes more important, as proposed by Lazarus and Folkman (1984) in a model of stress as a two-way process; the environment produces stressors, and the individual finds ways to deal with these. If the individual perceives a lot of stimuli and perceives these as threatening and uncontrollable, this can eventually determine a chronic activation of the stress response systems. Cognitive evaluations biased toward a generalized perception of environmental challenges as threatening and uncontrollable are an hallmark of psychiatric disorders (Section 1.2.1). The long-term perceiving of stress can also cause chronic stress-related biological dysregulations. Overall, we distinguish two types of biological stress in this thesis (Section 1.2.2): at
the physiological level the body's main stress systems can be impaired (Section 1.2.2.1), and at the metabolic level the body's composition, blood lipids and glucose levels can be dysregulated (Section 1.2.2).

1.2.1. Psychological stress

The onset of psychiatric disorders, such as depressive and anxiety disorders, marks a loss of resilience to stressors from the environment. In the Netherlands, it has been reported that the lifetime prevalence of depressive and anxiety disorders is both 20% with a high comorbidity, whereas depression alone is said to affect 350 million people worldwide.

According to the Diagnostic and Statistical Manual of Mental Disorders (4th edition, DSM-IV-TR) major depressive disorder (MDD) is defined as having a depressed mood and/or a loss of interest or pleasure in daily activities for more than two weeks. Together with an impaired functioning, five out of these nine symptoms are present in these patients most of the day nearly every day: 1) depressed mood or irritable; 2) decreased interest or pleasure in most activities; 3) significant weight or appetite change (5%); 4) change in sleep; 5) change in activity levels; 6) fatigue or loss of energy; 7) guilt/worthlessness; 8) concentration problems; 9) suicidality. Anxiety disorders are a category of mental disorders characterized by feelings of anxiety and fear. There are a number of anxiety disorders, such as generalized anxiety disorder, social phobia, agoraphobia, and panic disorder. Generalized anxiety disorder is characterized by excessive or disproportionate worrying almost every day for at least six months about several aspects of life, such as work, social relationships, or financial matters. Social phobia and agoraphobia are psychiatric disorders with an excessive and unreasonable fear of social situations or public (open) spaces, respectively. At last, panic disorders arise when a person encounters panic attacks (i.e. sudden short overwhelming feelings of fear and anxiety), and consequently anticipates on these attacks with behavioral changes in order to prevent another panic attack.

These psychiatric disorders all have in common that the person is experiencing chronic stress. Depression, as described in Beck's “cognitive triad”, is characterized by negative evaluations about the self, the environment and the future, and it has been associated with the so-called "learned helplessness": the perceived absence of control over the outcome of a situation. In anxiety, the exposure (or simply its anticipation) to several common stimuli of every-day life is perceived as highly threatening. Patients with depressive or anxiety disorders not only have a loss of quality of life, but in addition, they have a higher risk of developing various aging-related somatic diseases. The link between these psychiatric disorders and somatic diseases might be explained by accelerated cellular aging and biological stress dysregulations.
1.2.2. Biological stress

1.2.2.1. Physiological stress systems

The body’s major physiological stress systems are the immuno-inflammatory system, the hypothalamus-pituitary-adrenal (HPA)-axis and the autonomic nervous system (ANS). Chronic stress can lead to systemic dysregulations of these systems, resulting in systemic inflammation, hyperactivity of the HPA-axis, sympathetic activation and parasympathetic withdrawal 75-77.

Inflammation – Inflammation is a response triggered by damage to tissues, and it serves as a defense mechanism against injury and infectious agents. Inflammation can be acute or chronic. The acute variant lasts a few days, and is a healthy process in the body, unless its regulatory mechanisms are defective, resulting in allergies and autoimmune diseases. Acute inflammation can become chronic, if the infectious organisms are able to resist host defenses and persist in tissues for an extended period. When inflammation lasts longer, even when the threat is gone, chronic low-grade inflammation can be present in the entire body. Leukocytes play an important role in the initiation and propagation of inflammation, and they can release a myriad of pro-inflammatory and anti-inflammatory mediators. Important pro-inflammatory mediators are the pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), both mediators of the onset of the acute phase response with c-reactive protein (CRP) production in the liver.

Hypothalamus-pituitary-adrenal axis – Another essential effector of the stress response is the hypothalamus-pituitary-adrenal (HPA) axis. During the stress response, neurons in the hypothalamus secrete corticotrophin-releasing hormone, which enters the anterior pituitary gland and binds to its receptor. This in turn induces the release of adrenocorticotropic hormone into the systemic circulation, which subsequently stimulates glucocorticoid synthesis in the adrenal cortex 78. An important glucocorticoid is cortisol; a steroid hormone that regulates metabolic, cardiovascular, immune, and behavioral processes 76;77;79;80. Under normal conditions cortisol levels follow a diurnal rhythm. In the early morning cortisol starts to peak, the so-called cortisol awakening response (CAR), reaching an apex around 30 minutes after awakening 81;82. The CAR is suggested to represent a distinct measure of the HPA-axis, and might be sign of the anticipation for the upcoming demands of the day 83. After the morning peak, cortisol declines throughout the day, and evening cortisol levels are indicative of basal HPA-axis activity 84;85. The HPA-axis is tightly regulated by feedback mechanisms that maintain homeostasis through glucocorticoid and mineralocorticoid receptors in the pituitary and hippocampus 86-88.

Autonomic nervous system – The autonomic nervous system (ANS) controls the homeostasis of various unconscious body functions in order to keep physiological processes adequate in response to changing internal and external factors. The ANS controls processes such as heart rate (HR), sweating, pupil reflexes, energy
expenditure and metabolism regulation. The two branches of the ANS are the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS). The SNS prepares the body for danger in times of stress by e.g. increasing the HR and blood pressure by creating vasoconstriction and increasing contractility of the cardiac muscles, and by increasing blood flow and energy availability, and is also called the ‘fight or flight’ state. The PNS, on the other hand, regulates the resting state of the body by increasing intestinal and gland activity, decreasing HR and increasing heart rate variability, and is known as the ‘rest and digest’ state. ANS activity can be monitored with electrocardiography (ECG) and impedance cardiography (ICG). HR is an indicator of combined SNS and PNS activities, and can be extracted from the ECG signal. To separately index the cardiac effects of the SNS, the pre-ejection period (PEP) is a measure of cardiac sympathetic control, as it can reliably index β-adrenergic inotropic drive to the left ventricle, with long PEP reflecting low cardiac sympathetic control. The PNS branch can be indexed by various measures of heart rate variability, such as the respiratory sinus arrhythmia (RSA), reflecting cardiac parasympathetic (vagal) control, with high RSA reflecting high cardiac vagal control.

1.2.2.2. Metabolic dysregulations
Stress can also lead to metabolic imbalances that are clustered in metabolic syndrome (MetS): various metabolic alterations that can occur as a healthy response to stress, or become dysregulated during chronic stress. These dysregulations are at the same time interrelated risk factors for developing cardiovascular diseases and type 2 diabetes mellitus.

The National Institute for Public Health and the Environment (RIVM) reported in a monitoring study (2009-2010) that MetS is diagnosed in 24% of women and 34% of men in the Netherlands, with the prevalence increasing with higher age and with lower education. In general, it is estimated that one-quarter of the world’s adult population has MetS, and this might range widely, depending on sociodemographic, regional and urbanization factors. MetS consists of the following dysregulations: abdominal obesity, dyslipidemia including low HDL cholesterol and high triglycerides, hypertension and hyperglycemia. Diagnosis of MetS requires the presence of three or more of the following criteria: 1) waist circumference ≥102cm in men and ≥88cm in women; 2) triglycerides ≥1.7mmol/L or medication for hypertriglyceridemia; 3) high-density lipoprotein (HDL) cholesterol <1.03mmol/L in men and <1.30mmol/L in women or medication for reduced HDL cholesterol; 4) blood pressure: systolic ≥130mmHg and/or diastolic ≥85mmHg or antihypertensive medication; 5) fasting plasma glucose ≥5.6mmol/L or antidiabetic medication.

Although genetics and lifestyle are important determinants of metabolic dysregulations, these metabolic dysregulations could also be partly considered as a maladaptive consequence of an initially successful adaptation to high environmental
demands \textsuperscript{93}. Aging is also associated with metabolic changes, and chronic stress might accelerate these age-related changes \textsuperscript{17;94}.

\textbf{1.3. THIS THESIS: THE INTERPLAY BETWEEN STRESS AND AGING}

The previous sections have described how both aging and stress lead to several dysregulations in the body. However, it is increasingly suggested that stress-related factors might accelerate the aging process, as is expressed by the well-known statement “stress gives you grey hair”. While stressful conditions cannot accelerate the progression of chronological aging, there have been studies linking accelerated cellular aging to psychological and biological stress.

Various cross-sectional studies have investigated the link between short TL and psychological stress \textsuperscript{15;17;95-98} and biological stress: increased inflammation \textsuperscript{20;99-103}, hyperactive HPA-axis \textsuperscript{16;104-106}, ANS stress responsivity \textsuperscript{16;106;107} and metabolic dysregulations \textsuperscript{21-28;108-111}. However, not all studies confirmed the associations with inflammation \textsuperscript{105;112;113}, HPA-axis measures \textsuperscript{114}, or MetS components \textsuperscript{16;99;115}. Longitudinal studies with multiple measurements of TL are scarce. The studies that did have repeated TL measurements agreed that baseline TL is the strongest predictor of telomeric attrition \textsuperscript{116;117}. Only few studies examined the longitudinal associations between psychological stress and TL. One longitudinal study found no association between depression and telomere attrition rate \textsuperscript{118}, whereas another study did find accelerated attrition in depressed and anxious men as compared to healthy men, but not in the women \textsuperscript{98}. Longitudinal studies investigating biological stress and TL are also scarce. One study showed that short baseline TL is associated with worse MetS outcomes at follow-up \textsuperscript{119}, or vice versa, that baseline MetS components predict shorter TL over time \textsuperscript{120}. Only two longitudinal studies have measured both MetS and TL at multiple time points, and reported that changes in TL paralleled the changes in obesity measures \textsuperscript{120;121}. Furthermore, no longitudinal study has ever examined the associations between the physiological stress systems and TL.

The other cellular aging marker is investigated less frequently. Lower mtDNAcn has been associated with psychiatric disorders \textsuperscript{45;52;122-124} and MetS components \textsuperscript{49;125-130}. Only one study examined the cross-sectional associations between MetS and both mtDNAcn and TL \textsuperscript{50}. No longitudinal study has yet investigated mtDNAcn and stress, or the combination of TL and mtDNAcn within one research frame.

Overall, numerous studies have contributed to the current knowledge about the interplay between stress and cellular aging, but these studies had their shortcomings and have led to new research questions. Many of these studies have been performed in relatively small study samples (<150) or in restricted samples (e.g. only women, narrow age range). Within the current thesis, we investigated these biological stress factors and cellular aging markers in large-scale studies. Hereby the effects of multiple confounders could be taken into account, such as sex, race, sociodemographic factors, lifestyle and clinical factors. Moreover, there is a lack of
longitudinal studies that could elucidate whether stress significantly predicts TL over time, or maybe even lead to accelerated telomere attrition. The analyses within this thesis were conducted with data from longitudinal studies with repeated measurements of the cellular aging markers and many of the psychological and biological stress factors. The larger sample sizes used within this thesis provided the statistical power to enter a broad array of predictors of TL into our models simultaneously, whereas the earlier studies looked at these predictors separately. This empowered us to look at the effects of cumulative dysregulations on TL as well. Additionally, we investigated potential pathways between psychological stress and cellular aging with mediation models in the current thesis. For this purpose we had access to a large-scale dataset, in which patients with depressive and anxiety disorders were overrepresented. Thereby, high levels of psychological stress were present and the effects of these mediating processes could be examined optimally.

1.3.1. General aims
The main aims of this thesis are to examine the associations between cellular aging markers and A) biological stress and B) psychological stress in two large-scale longitudinal cohort studies. Another aim is to test a hypothetical pathway, in which the association between psychiatric disorders and TL is mediated by dysregulation of physiological stress systems and MetS components.

By examining a broad array of biological and psychological stress factors, we aim to identify the main drivers of cellular aging. Findings presented in this thesis can propose directions for future research focused to understand the mechanism behind these associations. Furthermore, future clinical studies could be designed to modulate these new biological risk factors of cellular aging and thus, impact on aging-related complications. Moreover, by increasing our understanding of the pathways connecting psychological stress with biological dysregulations and cellular aging, findings from the present thesis may help improving treatment of depressive and/or anxious patients in order to prevent their disadvantageous consequences on morbidity and mortality.

1.3.2. An epidemiological perspective
As opposed to small-scale in vitro or animal experiments, epidemiological research in humans is performed to describe patterns, causes, and effects of health and disease in defined populations on a larger scale. This can be achieved, as in the present thesis, using data from observational (cohort) studies, preferably in a large sample and with a long follow-up period. Epidemiologists aim to inspect health and disease processes with statistical analyses, while taking into account all the relevant confounding factors, such as sociodemographic, lifestyle and clinical characteristics.
1.3.3. Studies used in this thesis

The current thesis is based on information from two large-scale longitudinal observational cohort studies: 1) the Netherlands Study on Depression and Anxiety (NESDA) and 2) the Coronary Artery Risk Development in Young Adults (CARDIA) study.

**NESDA** – The NESDA study is an ongoing multi-site naturalistic cohort study among 2981 participants (18-65 years old) that aims to describe the long-term course and consequences of depressive and anxiety disorders. The respondents were recruited between 2004 and 2007 from the community, primary care, and specialized mental health care, in order to recruit persons reflecting various settings and developmental stages of psychopathology. Baseline data collection consisted of a medical examination, collection of blood and saliva samples, extensive information about health outcomes and demographic, psychosocial, clinical, biological and genetic determinants, administered by specially trained research staff. Follow-up examinations were conducted at years 2, 4 and 6. ([http://www.nesda.nl/](http://www.nesda.nl/))

**CARDIA** – Within the CARDIA study 5115 participants (18-30 years old) balanced by race, sex and education, were randomly recruited from the general population between 1985 to 1986, in order to trace the development of risk factors for coronary heart disease in the United States. Baseline data collection included standardized measurements of major CVD risk factors, as well as psychosocial, dietary, and exercise-related characteristics. Follow-up examinations were conducted at years 2, 5, 7, 10, 15, 20, and 25. ([http://www.cardia.dopm.uab.edu/](http://www.cardia.dopm.uab.edu/))

1.3.4. Outline of this thesis

Based on knowledge that is presented in the General Introduction, the outline of the current thesis is presented in Figure 2. All studies have been performed in the NESDA study, except for Chapter 5 that has used data from the CARDIA study. In Chapter 2, we tested the cross-sectional associations between the three physiological stress systems (inflammation, HPA-axis and ANS) and TL. First we looked at the separate physiological stress markers, and then examined the effects of cumulative dysregulations on TL. In Chapter 3, we first tested the cross-sectional associations between metabolic dysregulations (abdominal obesity, dyslipidemia, hypertension and hyperglycemia) and TL. Next, the longitudinal associations have been investigated between baseline TL and the MetS components over a 6-year follow-up. Vice versa, Chapter 4 shows whether baseline MetS components predict telomere attrition over time, and whether changes in MetS run parallel with changes in TL. Chapter 5 extends these analyses to the CARDIA study, in which the cross-sectional and longitudinal associations are presented between MetS and two cellular aging markers, TL and mtDNAcn, over a 10-year follow-up period. Chapter 6 and 7 present the cross-sectional associations between baseline TL and psychological stress: MDD and anxiety disorders, respectively. Then, in Chapter 8, the hypothesis is tested
whether the associations between depressive and anxiety disorders and short TL might be mediated by physiological stress systems or metabolic dysregulations. Chapter 9 then systematically investigates all the psychological and biological stress factors as predictors of baseline TL and 6-year telomere attrition, in order to shed light on the main drivers of cellular aging. Finally, Chapter 10 summarizes the knowledge that is gained from Chapters 2-9, and integrates these findings within a broad framework, in pursuance of a greater understanding of the aging process and the role of psychological and biological stress.

Figure 2: Outline of the current thesis with the numbers indicating thesis chapters.
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