Chapter 10

Summary and General Discussion
10.1. Summary of findings

In the past decades, researchers have characterized the processes seen both in aging and stress as a wear and tear of the body. It is increasingly suggested that psychological and biological stress might be linked with the aging process. While these factors cannot accelerate actual chronological aging, they might play an important role in cellular aging.

The main aim of this thesis was to examine: the link between A) cellular aging and biological stress, and B) cellular aging and psychological stress, including the potential mediators that underlie this association. Biological stress factors consisted of three physiological stress systems (i.e. inflammation, hypothalamus-pituitary adrenal (HPA)-axis and autonomic nervous system (ANS)) and dysregulations of the metabolic syndrome (MetS) components. Using data from the Netherlands Study of Depression and Anxiety (NESDA), we first tested the associations between these biological stress factors and telomere length (TL), the main cellular aging marker investigated in the present thesis. Within the Coronary Artery Risk Development in Young Adults Study (CARDIA), we extended these analyses to investigate the associations between metabolic dysregulations and an alternative cellular aging marker, mitochondrial DNA copy number (mtDNAcn). Consequently, we assessed the link between TL and psychiatric disorders, namely major depressive disorder (MDD) and anxiety disorders. Then we tested potential mediating mechanisms between psychopathology and short TL, such as lifestyle and biological stress. To conclude, we systematically investigated which psychological or biological stress factors are the main drivers of cellular aging.

The current chapter will summarize and discuss the findings of the Chapters 2-9, and present them in a broader context of the existing literature. Furthermore, this chapter will comment on some of the methodological considerations relevant for this thesis, give recommendations for future research and clinical practice, and finish with the general conclusions. In this thesis, we conducted cross-sectional analyses in order to determine the associations between cellular aging and biological and psychological stress, and extended these findings with longitudinal analyses to explore the changes over time.

In Chapter 2, we tested the cross-sectional associations between TL and three major physiological stress systems: the inflammatory system, HPA-axis and ANS. We hypothesized that a dysregulated physiological response commonly activated during chronic stress could be associated with cellular aging. For this purpose, we used data from the NESDA study (N=2981). First, we looked at the separate physiological stress markers, and found that higher levels of the inflammatory markers CRP and IL-6, higher cortisol awakening response (AUCi) and higher HR were associated with short TL. Next, we found that there was a dose-response relationship between cumulative dysregulations of these four stress
markers and shorter TL. This supports the concept that a dysregulated physiological stress response, thought to be present during chronic stress, accompanies the cellular aging process.

In **Chapter 3**, using NESDA data again, we first tested the cross-sectional associations between metabolic dysregulations and TL, and found significant associations between short TL and the presence of MetS, as well as the separate components: abdominal obesity, dyslipidaemia (high triglycerides and low HDL cholesterol), and hyperglycaemia. Again, we found a dose-response relationship between a high number of metabolic dysregulations and short TL. Next, we investigated the longitudinal link between MetS and TL. We started by looking at the effects of baseline TL on MetS components over a 2-year and 6-year follow-up, and found that shorter baseline TL was still associated with unfavourable metabolic profiles (i.e. abdominal obesity, dyslipidaemia and hyperglycaemia) at follow-up.

Vice versa, in **Chapter 4** we looked whether baseline MetS components predicted telomere attrition over six years of time, and saw consistent patterns: unfavourable metabolic profiles at baseline consistently predicted shorter TL, although the strength of this effect was decreasing over time. In addition, we explored whether 6-year changes in MetS run parallel with 6-year changes in TL. We observed a significant link between increased waist circumference and more telomere attrition over 6-year of time, and although non-significant - pointing in the same direction for increasing triglycerides and glucose levels. There seems to be a bidirectional link between metabolic dysregulations and cellular aging that is mainly driven by the abdominal fat component of MetS.

In **Chapter 5**, we extended these analyses to the CARDIA study (N=989) by looking at the cross-sectional and longitudinal associations between MetS and two cellular aging markers, TL and mtDNAcn, over a 10-year follow-up period. MtDNAcn and TL both decreased over time and were positively, although weakly, correlated. Higher triglyceride levels were consistently associated with lower mtDNAcn, and only lower HDL cholesterol was associated with short TL. When looking at the longitudinal relationships, we found that larger baseline waist circumference, glucose, number of metabolic dysregulations and the presence of MetS predicted larger 10-year decrease in mtDNAcn, but none of these markers were significantly associated with the 10-year telomere attrition. Vice versa, baseline cellular aging markers did not predict 10-year metabolic deteriorations. Last, in order to investigate the parallel changes over the 10-year follow-up, an increase in waist circumference was associated with 10-year telomere attrition, in line with earlier NESDA findings.

Next, we examined the cross-sectional associations between TL and MDD and anxiety disorders. Patients with MDD and anxiety disorders have an increased onset risk of aging-related somatic diseases, and cellular aging could be an explanatory mechanism. Current and remitted MDD patients had significantly shorter TL
compared to healthy controls (Chapter 6). Furthermore, we demonstrated that depressed patients had shorter TL according to a “dose-response” gradient: those with the most severe and chronic MDD showed the shortest TL.

Patients with current diagnoses of anxiety disorders (i.e. diagnosed with panic disorders with agoraphobia, social phobia or generalized anxiety disorder) also had significantly shorter TL compared to healthy controls (Chapter 7). Patients with a remitted diagnosis did not differ from controls, although the time since remission was positively related with TL. We now found a dose-response relationship between TL and severity of anxious symptoms, but not with disease duration. Cellular aging was not affected by antidepressant medication use or benzodiazepines.

Then, in Chapter 8, we tested whether the associations between depressive and anxiety disorders and short TL might be explained by physiological stress systems, metabolic dysregulations or lifestyle factors, which are often shown to be deteriorated in these patients. With mediation modelling, using the baseline sample of the NESDA study, we found that high C-reactive protein, interleukin-6, waist circumference, triglycerides, low HDL cholesterol and more cigarette smoking were significant mediators of the link between psychiatric disorders and short TL, and reduced the association between psychopathology and TL with approximately 35%. This means that part of the association runs indirectly through increased inflammation, abdominal obesity and dyslipidemia and smoking, although there is still a considerable direct association present that is not explained by biological stress factors.

To conclude, in Chapter 9 we systematically investigated 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. Cross-sectionally, short baseline TL was associated with older age, male sex, non-European ancestry, cigarette smoking, recent life events, and higher triglycerides and glucose, and longer pre-ejection period. This wide variety of determinant factors altogether explained 11% of the variance in TL. Longitudinally, 6-year telomere attrition was strongly associated with baseline TL: subjects with long TL at baseline had higher chance of attrition as compared to subjects with short TL. Baseline TL explained 52% of the variance in telomere attrition, whereas few other added factors, such as older age, long sleep, multiple childhood traumas and gastrointestinal disease explained an additional 4% of this variance.
Table 1. Summary of findings from this thesis of the associations between biological and psychological stress and telomere length (TL, single measurement) and telomere attrition (repeated measurement).

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<tr>
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<th>Telomere length</th>
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*Footnotes: * Confirmed associations; +/- Inconsistent findings; - No associations confirmed; NA not measured.
Table 1 presents a summary of the described associations between biological and psychological stress factors and cellular aging: either a one-time TL measurement or telomere attrition between repeated measurements. This table indicates that associations were either confirmed (+), inconsistent (+/-) or not confirmed (-) within this thesis. Cross-sectional associations with TL were confirmed for various inflammatory mediators, HPA-axis function measures, ANS markers, metabolic dysregulations, and psychological stress factors. Longitudinal associations with change in TL over six years were confirmed for baseline TL, some psychological stress factors: childhood trauma, marriage or partner status, and social network size at baseline predicted telomere attrition, as well as baseline abdominal obesity. Moreover, an increase in waist circumference was found to parallel telomere attrition over time, and we found some evidence that increases in triglycerides and glucose might go along with accelerated cellular aging too.

10.2. Interplay between biological stress and cellular aging

10.2.1. Physiological stress systems

An increasing body of evidence points towards a complex and bidirectional link between TL and inflammatory mediators, and few studies have found a link between TL and HPA-axis measures and ANS function as well. These three physiological stress systems are said to be dysregulated in chronic stress. Therefore, the supposed link with short TL might explain why chronic stress could accelerate the aging process, and eventually increase the onset risk of aging-related diseases. This thesis explored the role of these physiological stress systems in cellular aging in the NESDA study, a large-scale cohort study with data collected in patients with MDD and/or anxiety disorders and healthy controls. Within NESDA, we had data available of baseline and 6-year TL, and of baseline inflammatory mediators, HPA-axis measures and ANS markers.

Inflammation – We found that high baseline levels of CRP and IL-6 were cross-sectionally associated with short TL, and higher CRP was borderline significantly associated with 6-year telomere attrition. Inflammatory markers are highly reactive to various triggers in the body, and this might be an explanation why we did not observe a strong association between baseline levels of inflammation and accelerated cellular aging over a longer time period. However, one relatively small study found a parallel increase in CRP and telomere attrition over a 2-year follow-up. Nevertheless, although we could not investigate both directions of this reciprocal relationship, our findings support the link between inflammation and short TL. The aging of the immune system remains paradoxical: despite a decrease in immune responsiveness to infection and vaccination, an increase in systemic inflammation is visible during the aging process, sometimes called ‘inflammaging’.
On one hand, inflammation could lead to short TL by promoting cell turnover and replicative senescence, and by inducing the release of reactive oxygen species that damage telomeric DNA. Inflammatory markers could also inhibit programmed cell death (apoptosis), thereby contributing to the accumulation of senescent cells. In the opposite direction, senescent cells with short TL secrete factors to communicate their compromised state to the surrounding tissue, whether this is cellular damage or pathogenic invasion, thereby contributing to elevated inflammation. Accumulated DNA damage and telomere attrition in lymphoid hematopoietic stem cells could also induce differentiation into mature lymphocytes, thereby depleting the stem cell pool and increasing the number of damaged effector cells in the circulation, which in turn leads to the production of inflammatory markers.

**HPA-axis** – We found that short TL was cross-sectionally associated with hypersensitivity of the HPA-axis, or more specifically, a high cortisol awakening response, but not with high evening cortisol levels or a blunted dexamethasone suppression ratio. This suggests that TL is not influenced by the basal activity of the HPA-axis or its regulatory feedback system. However, we did not find a longitudinal link between baseline HPA-axis hyperactivity and 6-year telomere attrition, whereas some studies do suggest a causal relationship. In vitro, HPA-axis hyperactivity has shown to result in reduced telomerase activity and higher levels of oxidative stress, with subsequent shortening of TL. In a mouse study, the HPA-axis was activated by administering corticosterone over four weeks, and a subsequent telomere attrition was observed. Nevertheless, in humans there is less evidence for a causal role of cortisol and accelerated cellular aging. Perhaps, these diurnal cortisol indices reflect day-to-day fluctuations, suggesting state-like properties, rather than stable trait-like influences. That might explain why we only found a cross-sectional link with short TL, and not a longitudinal link. Conversely, shorter TL might lead to higher cortisol levels, possibly through inflammation and oxidative stress, but to our knowledge, this direction is not explored either.

Most of the earlier studies have shown significant associations between cortisol levels and TL, but it is hard to compare findings due to the large variety in measurement methods (i.e. 12-hour nocturnal urine, first morning urine, or salivary samples). Within NESDA, the levels of cortisol throughout the day have been measured in saliva. Advantages of salivary cortisol measurements are the easy application at home, under normal conditions and in a stress-free environment. Moreover, this method is non-invasive and can be performed multiple times during the day, also immediately after awakening. For these reasons, it is often used in large-scale studies. However, these measurements also have their drawbacks. Participants might forget to use the salivettes on the exact time points, might not use them correctly (by not filling the tubes sufficiently with saliva) and at home no assistance can be provided by the researchers. Within NESDA, the cortisol awakening response could be calculated in 62%, whereas the evening levels of cortisol were measured in
67% of the participants. This is a relatively good response rate, when compared to other studies.  

**ANS function** – We hypothesised that TL would be shorter in subjects with high sympathetic activity (indicated by short PEP) and low parasympathetic activity (indicated by low RSA) at baseline. As higher HR reflects a combination of low parasympathetic and high sympathetic activation, we further hypothesized that high HR would be associated with short TL. Only the association with high HR was confirmed in this thesis, supporting earlier observations that higher resting HR is strongly correlated to shorter lifespan, possibly due to higher basal metabolic rate, increased oxidative stress and accelerated deterioration of the cardiovascular system. TL may also have an impact on cardiovascular function, as functional telomeres are required for viable cardiovascular cells in vitro and for cardiomyocyte renewal. The extent to which TL reflects telomeres in heart tissue remains largely unclear, although a high correlation between leukocyte and aortic wall tissue TL has been reported.

Low parasympathetic activity (RSA), however, was not associated with TL, but we unexpectedly found that subjects with low sympathetic drive (i.e. long PEP) had shorter TL. Two earlier relatively small-sampled studies investigated the link between ANS and TL, but also did not find any associations between TL and basal ANS measures. They found an association between lower parasympathetic activation in response to stress and low telomerase activity, and between higher sympathetic activation in response to stress and short buccal cell TL. It remains unclear what could have driven our anomalous finding of long PEP being associated with short TL. This might have been a chance finding, although an earlier NESDA investigation has shown that long PEP is associated with low SBP, and we also found that there was a trend between short TL and low SBP. This suggests that there might be other factors involved in the unexpected link between cellular aging and long PEP. An intriguing possibility is that chronic stress with concurrent high levels of plasma (nor)epinephrine may have led to a down regulation of the cardiac beta-adrenergic receptors. This would lead to a prolongation of the PEP, even in the presence of high cardiac SNS activity. Desensitization of beta-adrenergic receptors has earlier been associated with anxious mood, caregiving status and heart failure. Another study found that in individuals with chronic congestive heart failure, the sustained activation of the adrenergic nervous system (i.e. increased circulating catecholamines) resulted in downregulation and desensitization of myocardial beta-adrenergic receptors. In general, it is hypothesized that with increasing age there are decreases in cardiac beta-adrenergic receptor density and in the efficiency of postsynaptic beta-adrenergic signalling, both contributing to the lower threshold for the development of cardiovascular disease. If the desensitization develops in an age-dependent manner, this could imply that the absolute length of the PEP loses its ability to reflect the actual sympathetic drive with
aging. Studies that measure both PEP and cardiac beta-receptor status are needed to test this speculative idea. However, this proposed beta-adrenergic receptor desensitization does not offer a full explanation for the inconsistencies in the PEP findings, as earlier research has – in line with hypotheses – confirmed that short PEP in the NESDA sample is related to unfavourable health factors such as high BMI, antidepressant use, and with baseline metabolic syndrome components, as well as the increase of metabolic abnormalities over time. Data on actual synaptic activity, cardiac beta-receptor status and catecholamine levels could yield additional information about the exact processes of chronic stress and aging.

Cumulative dysregulations – In addition to the associations between TL and the separate physiological stress system markers, we found that the cumulative dysregulations of these markers was associated with short TL in a dose-response manner. This was a novel finding, as some studies have indicated that combinations of physiological stress system dysregulations may have the strongest impact on cellular aging, but no study examined all these markers simultaneously before. In the meantime, another study investigated the cumulative dysregulation of biological and psychological stress responses to a laboratory stressor, and found that subjects with short TL and high telomerase activity had impaired physiological stress responses and impoverished psychosocial resources, particularly in the older male subgroup. It seems that the hypothesized ‘allostatic overload model’ is better described by a dose-response relationship between accumulated biological stress markers and cellular aging (i.e. the progressive ‘wear and tear’), instead of a ‘dysregulation threshold’, in which no deteriorating gradient is visible once the threshold is passed.

10.2.2. Metabolic dysregulations

Large epidemiological studies and reviews have focused on the interplay between chronic stress and the occurrence of aging-related diseases, such as CVD, and have identified abdominal obesity, dyslipidaemia, hypertension and hyperglycaemia as modifiable high risk factors. Metabolic Syndrome (MetS) cover five of these interrelated CVD risk factors, and therefore, we focused extensively on the five MetS components. MetS diagnosis requires the presence of three or more of the following dysregulations: 1) abdominal obesity; 2) hypertriglyceridaemia; 3) reduced HDL cholesterol; 4) hypertension; 5) hyperglycaemia. In this thesis we focused on the links between these metabolic dysregulations and TL, both cross-sectionally and longitudinally, as both we measured repeatedly in the NESDA and the CARDIA studies. Overall, even though these components are highly correlated, it seems that we can distinguish two domains: one consisting of obesity-related insulin resistance and dyslipidemia, and one domain with blood pressure.

Obesity-related insulin resistance and dyslipidaemia – We consistently found cross-sectional associations in the NESDA study between high waist circumference,
high triglyceride and glucose levels and reduced HDL cholesterol and short TL. There was weak evidence that baseline waist circumference predicted telomere attrition, or conversely, that baseline TL predicted increases in waist circumference and decreases in HDL cholesterol. We found that baseline differences progressively reduced over time (i.e. the lines were converging), but subjects with short TL at baseline still had a worse lipid and glucose profile after a 6-year follow-up. Conversely, subjects with high waist, dyslipidaemia or hyperglycaemia at baseline had consistently shorter TL over the follow-up. However, in both direction, baseline status did not predict a faster deterioration over time. Nevertheless, over time, increases in waist circumference, and in a lesser extent triglycerides and glucose, and larger telomere attrition were significantly associated.

Within the other longitudinal cohort study, CARDIA, we additionally investigated the associations between mitochondrial DNA copy numbers (mtDNAcn) and metabolic dysregulations. High triglycerides were consistently associated with low mtDNAcn, and low HDL cholesterol with short TL. Large baseline waist circumference and high glucose levels predicted larger 10-year decrease in mtDNAcn, but not TL attrition. Further, 10-year increase in waist circumference was associated with 10-year telomere attrition, thereby replicating the NESDA finding.

Results from this thesis strengthen the hypothesis of a link between telomere biology and abdominal adiposity and lipid disturbances. Adipose tissue, especially in the visceral domain, has been considered not only as a simple energy depository tissue, but also as an active endocrine organ. Intriguingly, significant associations in adipocytes were found between large cell size and short TL. One explanatory hypothesis in this field is the so-called ‘adipocyte overflow hypothesis’: this suggests that when adipocytes enlarge, they reach their fat storage capacities, causing an ‘overflow’ of fatty acids into sites such as the liver and muscle. These fatty acids not only promote deteriorations of insulin resistance, but they also increase systemic inflammation and oxidative stress, both catalysts of telomeric attrition. This ‘metabolic oversupply’ (i.e. high caloric intake and low physical activity) in cells is also shown to fragment mitochondria, increasing reactive oxygen species production promoting the accumulation of mtDNA damage, whereas an undersupply is shown to promote mitochondrial fusion and limit mtDNA damage. Therefore, maintenance of metabolic balance appears to be important to preserve mitochondrial function, and excessive mitochondrial damage may contribute to the pro-aging effects of MetS. The association between fasting glucose and mtDNAcn is also very interesting given the strong dose-response relationship between fasting glycaemia and all-cause mortality, and the damaging effects of hyperglycaemia on mtDNA. Conversely, when cells with short telomeres become senescent, they release inflammatory cytokines, inducing insulin resistance and defective HDL cholesterol. Another mechanism could be that telomeric DNA damage leads to compromised mitochondrial functioning through dampened p53 expression,
eventually causing less fatty acid oxidation and glucose utilization, and less protection against oxidative stress \(^{50,51}\). Additionally, shortened telomeres are also associated with adipocyte hypertrophy, which in turn are found to be linked to poor glycaemic and lipid control \(^{44}\).

**Hypertension** – Blood pressure can be measured by looking at the systolic blood pressure (SBP), but also diastolic blood pressure (DBP) or pulse pressure (PP), the difference between the systolic and diastolic pressure readings. We have not found any significant associations between high SBP, DBP or PP with short TL. This was in line with some of the earlier research \(^{14,52,53}\), although other review papers summarized studies that did report significant associations in animal and human studies \(^{54-56}\). Furthermore, one study found that lower blood pressure levels were also associated with slower rate of telomere shortening, but only in men \(^{57}\). Results from the current thesis did not confirm an association between blood pressure and cellular aging, and even showed a trend towards lower SBP in the subjects with short TL. In NESDA, low SBP was also found in depressed patients, whereas the antidepressant medication users had higher SBP \(^{58}\). Overall, blood pressure may reflect another pathway of metabolic dysregulations, as opposed to obesity-induced insulin resistance and dyslipidemia, and the latter pathway is suggested to be different in men and women \(^{59}\). This might be illustrated by the fact that studies in men found associations between short TL and SBP \(^{60,61}\), but various studies including both sexes did not replicate this \(^{53,62,63}\). However, not all studies confirm this sex-specific theory \(^{64}\), and in this thesis, we did not find that sex moderated the association between TL and hypertension. Other studies have also suggested that perhaps not the overall mean blood pressure is deleterious to cellular aging, but that mainly the arterial segments with higher hemodynamic stresses show more telomere attrition, due to enhanced endothelial cell turnover and accumulation of senescent cells \(^{60,65-67}\). Unfortunately, it is not feasible to analyse such specific arterial segments in large human cohorts.

**Metabolic syndrome** – There is international consensus about the assessment of MetS \(^{68}\), and it is based on the US National Cholesterol Education Program–Adult Treatment Panel III guidelines, requiring the presence of three or more of the following criteria: 1) high waist circumference; 2) high triglycerides or medication for hypertriglyceridemia; 3) low HDL cholesterol or medication for reduced HDL cholesterol; 4) high systolic blood pressure or antihypertensive medication; 5) high fasting glucose or antidiabetic medication \(^{36}\). The presence of MetS itself was associated with short TL as well, but not as strongly as the separate components of MetS. This might also be explained by the fact that the link between hypertension and TL diverges from the link between MetS and the other MetS components.

Furthermore, we analysed the SBP, HDL cholesterol, triglycerides and glucose, while adjusting the continuous measures for medication use (i.e.
antihypertensives for SBP, fibrates for lipids, and antidiabetics for glucose). Firstly, medication use cannot be ignored in these analyses, so either it has to be included as a confounder or the separate components have to be adjusted for it, in concordance with the guidelines that defined MetS. By investigating the ‘clean’ exposure to lipids, glucose and blood pressure, without taking medication into account, we might miss important information that is partly covered in utilization of medication. That is because persons on these medications are likely having metabolic dysregulation levels that are more disadvantageous than would emerge when you would use their raw MetS levels. Analyses with the uncorrected separate components yielded similar results and associations with TL. However, by incorporating these medications into our components, we feel we would have to add much more information regarding the specific effects of this medication on the MetS components itself and on telomeres.

10.3. Interplay between psychological stress and cellular aging

10.3.1. Psychiatric disorders

Within the current thesis, we found short TL both in MDD and anxiety disorders patients. Compared to healthy controls, TL was shorter among remitted and current MDD patients, in line with previous findings. Our results demonstrate that depressed patients show accelerated cellular aging according to a “dose-response” gradient: those with the most severe and chronic MDD showed the shortest TL. Comorbid anxiety disorder did seem to increase the chance of having shorter TL, consistent with a “dose-response” relationship, as comorbidity can be seen as a psychiatrically more severe condition. Current psychoactive medication use was not associated with TL either, similar to the results of other studies. Patients with a current anxiety disorder also had significantly shorter TL compared to healthy controls and remitted patients, aligned with earlier research. Although TL in the remitted patients did not differ from TL in healthy controls, the time since remission was positively related with TL, suggesting a process of reversal of cellular aging after remission. Furthermore, high anxiety symptom severity was associated with short TL, in line with a dose-response association.

Overall, this suggests that MDD is a disorder with more chronic cellular aging consequences, possibly leaving a ‘biological scar’ after each episode, whereas the deleterious consequences of anxiety disorders are more reversible after remission. Alternatively, this difference might have been due to the relatively higher levels of sub-threshold symptoms and shorter remission time found in the remitted MDD group as compared to the remitted anxious subjects. Moreover, as we did not observe that these psychiatric disorders predict faster telomere attrition over time, there might also be underlying genetic or other disposition factors in persons that have short TL and that develop MDD or an anxiety disorder. These psychiatric disorders and the other psychological stress factors are used in this thesis as estimates for the
amount of (chronic) stress, but it should be kept in mind that they are not exact measures of how much the individual suffers or how much psychological stress the person truly experiences.

### 10.3.2. Underlying explanatory mechanisms

As proposed in the Introduction of this thesis, the long-term perceiving of stress, as seen in psychiatric disorders, can cause chronic stress-related biological dysregulations, that in turn might accelerate the biological aging process. In this thesis, we confirm this hypothesis, as we found that elevated inflammation levels, abdominal obesity, dyslipidaemia and smoking can partly explain the association between MDD and anxiety disorders and short TL (Figure 1).

These factors are highly intertwined, as increased inflammation in these patients may be due to adaptation of an unhealthy lifestyle, including physical inactivity, smoking and a bad diet, that in turn all promote inflammation, creating a deleterious vicious cycle for physical and mental health. Elevated levels of inflammations were found in patients with MDD or anxiety disorders, more specifically in the atypical depression subtype, and in sleep disturbances, a well-known symptom of MDD. Various reviews have suggested bidirectional links between depression, anxiety disorders and inflammation.

After acute psychosocial stressors, catecholamines are released that can activate inflammatory signalling in immune cells. Repeated stressors are shown to induce imbalances in the endocrine-immune interplay. During chronic stress the homeostasis of cytokines can be distorted even further through gradual HPA-axis ‘fatigue’ after hyperactivation, resistance to catecholamines, and various inflammation-related pathways, such as the nuclear-factor kappa-B (NF-κB).

In general, chronic stress is accompanied by the activation of the HPA-axis and the ANS. Of the HPA-axis measures, in particular high cortisol awakening response is more often associated with psychiatric disorders, with vulnerability to the onset of MDD and the recurrence of MDD. Chronic stress is perceived by the brain, where the hypothalamus releases corticotrophin-releasing hormone onto pituitary receptors, ultimately resulting in release of cortisol into the peripheral blood stream. Furthermore, depression is hypothesized to involve a state of more sympathetic and less parasympathetic nervous system activation. However, in NESDA, the ANS indices have been associated more with antidepressant medication use, which is not associated with short TL, rather than with the psychiatric disorders themselves. Altogether, we found that the HPA-axis and ANS indices were not explaining the association between psychiatric disorders and short TL, maybe because the associations are less strong between HPA-axis measures and TL, and between psychopathology and ANS.
Waist circumference and dyslipidaemia did partly explain the link between depression and cellular aging. The link between metabolic dysregulations and psychological stress seems generally rather solid in the literature, as various studies reported bidirectional associations between MetS and MDD\textsuperscript{77,96} or anxiety disorders\textsuperscript{97,98}, and the separate MetS components\textsuperscript{77,99-102}. The obesity-related components (waist circumference, triglycerides and HDL cholesterol) seem more important drivers in the link between MetS and psychopathology, as compared to glucose and blood pressure\textsuperscript{77}. Increased adipose tissue and dyslipidaemia in these patients is associated with elevated inflammation and resistance to leptin, an anti-obesity hormone that would normally regulate nutritional intake and energy expenditure\textsuperscript{77,103}.

\textbf{Figure 1.} Mediating mechanisms that are found to link psychological stress and cellular aging in the current thesis.
10.4. Telomere length as marker of cellular aging

Several factors are said to contribute to TL and to the rate of telomere attrition. In the current thesis, we found a relatively small decline in TL over time, and the attrition rate was slightly increasing with older age. TL was cross-sectionally associated with multiple domains: sociodemographic factors (e.g. sex, ancestry), lifestyle (e.g. smoking), psychological stress (e.g. recent life events, MDD and anxiety disorders) and biological stress markers (e.g. high inflammation, high cortisol awakening response, high heart rate, abdominal fat, and high triglycerides and fasting glucose), although the effect sizes were small. However, larger telomere attrition was only associated with older age, childhood trauma, not being married or not having a partner, and the strongest predictor of telomere attrition was baseline TL. Persons with long TL at baseline showed more attrition over the six years than persons with already short TL. Therefore, from our findings, it seems that TL is a better marker for the current state of biological or psychological stress than a dynamic marker that fluctuates along with biological or psychological stress dysregulations.

**TL as a state marker** – On the cellular level, telomere attrition is viewed as a ‘mitotic clock’ that counts down the number of cell divisions, and might even predict the replicative capacity of a cell. During the early life phases, there is a faster attrition rate as compared to attrition rates seen in adults, suggesting that TL is more dynamic and modifiable during early life as compared to adulthood. In a large study that measured TL in salivary DNA specimens in 110,266 individuals, TL only showed a negative correlation with age up to 75 years, and in those older than 75 years, age positively correlated with longer telomeres, indicative of an association of longer telomeres with more years of survival in those older than 75 years. An additional novel finding was that the variance of TL between individuals increased with age. This supports the notion that ‘external’ factors occurring throughout the lifespan, such as psychological or biological stress, might influence the rate of telomere attrition. This thesis did not measure TL during the early or late life phases, so we could not investigate the linearity of telomere attrition over the entire lifespan. However, we have shown that during midlife, the TL is relatively stable and is not strongly influenced by ‘external’ factors, but seems to be dominated by the homeostatic system.

**TL as a trait marker** – There is a large inter-individual variation in TL, and approximately 64-70% of TL is explained by genetic and epigenetic factors, suggesting that (epi)genetics and early life environment are the main determinants of TL. Some earlier studies have also found that TL in offspring is associated with paternal age at conception, with parental TL and with sperm TL. Potentially, TL is thus already determined genetically, in utero or in the first life phases. We observed that TL was relatively stable throughout adulthood in the majority of the NESDA sample. Moreover, when relating baseline TL to the 6-
year change in TL, telomeres seemed to be stable around approximately 5500bp. Persons that started above this TL showed more attrition over time, whereas persons with TL below the 5500bp showed lengthening. The fact that baseline TL was the strongest predictor of TL change over time might be explained by a strong homeostatic system in the body that regulates TL. The key player in this homeostatic system seems to be telomerase. It has been reported before that telomerase tends to lengthen the shortest telomeres more than the longer ones. Collectively, our findings suggest that a person’s TL is more a trait marker that in general declines with aging, but oscillates around a certain homeostatic value, while reflecting the levels of psychological and biological stress factors in that person.

**Alternative cellular aging markers** – This thesis focused mainly on TL as a cellular aging marker, but there are various other potential molecular characteristics of aging that might lead to increased disease susceptibility. In general, these markers can mirror the amount of cellular damage, whether this damage has occurred in specific organelles, the entire DNA or parts of it or other parts of the cell. In the CARDIA study, we looked at a proxy for mitochondrial function, mtDNAcn, that has been less extensively investigated as compared to TL. In the current thesis, we found only little evidence that low mtDNAcn reflects a bad current metabolic status of individuals, although baseline metabolic dysregulations did predict a larger 10-year decline in mtDNAcn. Earlier studies also found significant associations between low mtDNAcn and metabolic dysregulations. However, at the same time, the findings are inconsistent for psychopathology, with some studies reporting low mtDNAcn in depression, whereas others found high mtDNAcn in psychiatric patients, and another study failed to find any association. Furthermore, low mtDNAcn has been reported in several health outcomes, such as Parkinson’s disease, chronic obstructive pulmonary disease, cancer and overall mortality, but high mtDNAcn in mitochondrial diseases. Recent research indicates that TL and mitochondrial processes are co-regulated, and are both associated with the aging process. Nevertheless, the correlation between TL and mtDNAcn was not strong in the current thesis, and the literature around mtDNAcn is less consistent as compared to TL. Some studies even suggested that a higher mtDNAcn may represent a marker of poor mitochondrial health, and mtDNAcn might be increased to compensate for DNA damage or mitochondrial dysfunction.

Other potential and promising biomarkers of aging that have more recently been introduced in the literature are DNA methylation, DNA damage markers, protein and gene expression, metabolite levels, or circulating cell-free DNA. These markers are not widely investigated in relation to chronic stress (either psychological or biological) yet, have not been validated extensively for health and disease outcomes, or their measurement might not be feasible in large-scale cohort studies in humans. Altogether, the current thesis significantly adds to the evidence that TL is a valid marker that is associated with multiple domains of...
sociodemographics, lifestyle, psychological stress and biological stress markers. In addition, mean TL is often associated with chronic diseases and mortality, so TL seems to be an important, although very general, indicator of aging and health, integrating the cumulative effects of biological and psychological stress.

10.5. Methodological considerations

Some methodological considerations have already been made in each separate chapter and throughout the current chapter. These include the measurement methods of TL, the choice for the cell type in which TL is measured, the assessments of biological and psychological stress factors, and more generally, the drawbacks of observational cohort studies and selection bias.

10.5.1. TL specific considerations

**TL measurement methods** – Several methods are available to measure TL, all varying in their pros and cons in different research settings \(^{145,146}\). In both studies of the current thesis, NESDA and CARDIA, TL was measured with quantitative polymerase chain reaction (qPCR), in which pure telomeric repeats (T) and a single copy locus (S) are amplified using primers, measured quantitatively and compared to a reference DNA sample. The T/S ratio is demonstrated to be proportional to the average TL in a cell \(^{147}\). This method allows comparisons between individuals but only gives an estimation of the actual TL. This technique has a widespread and popular application, especially in large-scale studies, due to its short timeline and low costs, but variability within and between samples remains relatively high and larger amounts of DNA are needed \(^{146}\). Problems that arise from this method are that different research centres might not use the same reagents or single copy loci, and are therefore not easily and directly comparable \(^{146}\). Also, qPCR measures the overall mean TL, whereas it has been suggested that the amount of critically short telomeres, rather than the mean bulk TL, is determinant for telomere dysfunction, and, thus, for cell and tissue dysfunction \(^{145}\).

The traditional ‘gold standard’ method of measuring TL by Southern blotting determines a mean terminal restriction fragment length \(^{148}\). This method also gives an estimate of the average genomic TL by comparison to a DNA ladder size standard, requires large amounts of DNA and is time-consuming, and inter-individual variation is large due to included subtelomeric DNA and telomere variant repeats \(^{146}\). Another technique is the single telomere elongation length analysis (STELA) \(^{149}\). This method displays single telomeres from single chromosome ends, requires low amounts of DNA and is highly accurate. However, STELA is labour-intensive, not all chromosome ends have been well-characterized, and the analysis of a single chromosome end may not be representative of the TL status within the entire cell \(^{146}\). Last, quantitative fluorescent in situ hybridization (Q-FISH) provides high resolution TL measurements at specific chromosome ends \(^{150}\). Flow FISH is similar to Q-FISH, but can also be used
to measure TL in distinct cell populations within a single sample by antibody staining. Flow-FISH requires low amounts of DNA, and was shown to be more accurate, reproducible, sensitive, and specific in the measurement of human leukocyte TL as compared to qPCR, but is labour-intensive.

Overall, no single technique has all the advantages together: high accuracy, easy and fast application and low costs. Therefore, the methodology should be selected based on the scientific question that needs to be addressed. Whereas Southern blotting and flow-FISH can be applied in medium-sized samples, Q-FISH and STELA are applicable to small samples (N<10), in particular in fundamental research. In large-scale studies, like used in the current thesis, qPCR is the only high-throughput strategy. Although most studies have used qPCR and this technique proved to be distinctive enough for several health and mortality measures, subtle changes in TL can be harder to detect with qPCR in the longitudinal investigations. For instance, we have seen that TL was relatively stable in the NESDA study, in which roughly half of the sample kept stable TL over time (<5% change). This distribution is similar to what is found by earlier studies, except for one study that found a much higher proportion of shorteners as compared to lengtheners after 10 years in older subjects. Also in the CARDIA study, we observed telomere attrition in a large proportion of the sample, and the attrition rate was considerably larger than in NESDA, with a slight increase in TL between baseline and 5-year follow-up, and a sharp drop between 5- and 10-year follow-up. With the qPCR measurement method, we unfortunately cannot rule out whether these discrepancies occurred due to inaccuracy of the technique, storage duration, amplification problems or other factors, like the redistribution of cell types.

TL in cell subtypes – The distribution of immune cell types and subsets can be highly informative to shed light on the changes during chronic stress and aging. The majority of the telomere research that is conducted lately in larger cohort studies, including the current thesis, did not have the resources (time and finances) to measure the exact distribution of cell subtypes with flow cytometry, and thus, cannot distinguish whether changes occur due to redistribution of cell types or actual loss of telomeric DNA repeats. The current thesis has examined TL in leukocytes that consist of cells from the myeloid lineage (i.e. neutrophils, monocytes, eosinophils and basophils) and the lymphoid lineage, or lymphocytes (i.e. CD4+ helper T cells, CD8+ cytotoxic T cells, B cells and natural killer cells). Have we missed any valuable information by not sorting the cells into the different subtypes?

Few studies have investigated and compared TL in these separate subsets. One study determined the longest TL and highest telomerase activity in the B cells, followed by CD4+, CD8+ cells, and the shortest TL and lowest telomerase activity in the replicatively senescent CD8+ cells. The same study also found that the TL in the total number of PBMCs was mostly correlated with the TL in CD8+ T cells, in
concordance with a theory of early senescence of CD8$^+$ T cells with aging\textsuperscript{159}. This information can aid large-scale epidemiological studies, by suggesting that the mean measured TL probably mostly reflects the CD8$^+$ T cells. There are also various factors that might shift the redistribution of leukocyte subsets towards more highly differentiated cell types, such as cytomegalovirus infections\textsuperscript{160}, MDD\textsuperscript{161}, and poor sleep quality combined with high perceived stress\textsuperscript{162}. This could perhaps partly explain why we find associations between short TL and inflammation, MDD and various other biological or psychological stress factors.

\textbf{10.5.2. Study design}

\textit{Cross-sectional vs. longitudinal analyses} – Various chapters in the current thesis have been based on baseline data of the NESDA study, thereby investigating the cross-sectional associations between biological and psychological stress and cellular aging. Studies with a cross-sectional nature capture only one moment in time, and fail to shed light on changes during aging. In the current thesis, we extended the cross-sectional results with data from two large-scale cohort studies that had repeated measurements available.

Major advantages of cohort studies are their prospective nature and the ability to measure the natural course of diseases or processes of aging, while adjusting for several confounding factors that have been measured at each follow-up as well. However, one limitation of this study design is the restricted ability to draw conclusions about causality. In many analyses, it remains largely unknown which was there first: the chicken or the egg? Or in some cases, the phenomenon of reversed causation could bias the results. For instance, when investigating and interpreting the explanatory mechanisms of the association between psychopathology and cellular aging, we might imply that these factors can only function as intermediary explanatory variables. However, with a cross-sectional and observational design, no such conclusions can be drawn, as opposed to experimental studies or randomized clinical trials that are designed specifically to test the causal effects of the manipulation of certain factors (e.g. weight loss interventions, lipid-lowering therapies, smoking cessation) on cellular aging markers. Ideally, such experimental trials should follow participants from birth until death in order to draw conclusions about causes, pathways and consequences on the short and long term. Unfortunately, these trials are hardly feasible, and knowledge should be gathered from large-scale epidemiological studies, middle-scale trials and small-scale fundamental mechanistic studies, all with a limited follow-up duration. However, hypothesized causal pathways can gain more confidence if analyses based on observational data yield consistent associations at multiple measurement time points across independent cohorts.

\textit{Selective drop-outs} – Within large-scale cohort studies, some participants will always drop out of the study during the follow-up period. In these type of studies,
follow-up assessments will be completed by participants that are more motivated, likely to be healthy and fit. This is also the case in the NESDA study that consists of a large group of depressed and/or anxious patients. Patients with the most severe symptoms will have a higher risk to drop-out due to a lack of motivation, fear or lack of fitness. This phenomenon should be taken into account for a comprehensive interpretation of the association between risk factors and cellular aging. It is indeed possible that for certain risk factors associations are no longer found to be predictive of cellular aging, solely because those who were both exposed and affected by this risk factor are excluded from the study. However, if selective drop-out tends to decrease the strength of investigated associations, it suggests that when associations are found (as in our studies) in reality the relationship might be even stronger. Moreover, in some analyses, we carefully checked the impact of this form of bias by applying multiple imputation techniques (replacing missing data with substituted values based on existing information). We checked the value of imputation and found that analyses with non-imputed data did not yield different results as compared to analyses including imputation.

10.6. Clinical implications and public health

The results of this thesis showed that short TL is a valid cellular aging marker, and is associated with a broad array of factors: physiological stress markers, metabolic dysregulations, psychiatric disorders and other psychological stress factors. According to the World Health Organization, the global population is aging rapidly, and many people are affected by stress-related diseases (e.g. depression, anxiety), and aging-related diseases (e.g. CVD, obesity and diabetes). It becomes increasingly important to understand the underlying mechanisms of chronic stress and aging. Even though there are no norm values for TL, and inter-individual variation is too large to use it as a diagnostic or prognostic tool in clinical practice, TL measurement could become an important tool to understand more about the complex web of biological and psychological stress markers in the body during the aging process.

However, we found that TL is not strongly affected by any of the factors in this thesis and seems to be a rather static system over time. Therefore, TL seems to be more a trait marker instead of a state marker. An important implication that arises from these results is that there should be more scepticism regarding intervention trials that target TL and its attrition rate. Potential goals for interventions could be lifestyle improvements, weight loss and psychological stress reduction. In an overview paper, we have seen that earlier interventions report inconsistent effects on TL, and that baseline TL is the strongest predictors of change over time. Earlier interventions have not seen any effects on telomere attrition by exercise, exercise and weight loss, but some studies found a beneficial effect of sedentary time, weight loss or a dietary intervention. To our knowledge, no earlier interventions have investigated the effects of smoking cessation on TL. A recent study
found dose-response effects of smoking on gene expression, and saw that most of the identified changes in gene expression were reversible after smoking cessation, thereby, raising hope for intervention programs. Potentially, if multifaceted interventions are designed that target multiple domains of biological or psychological stress, they might have more chance of affecting the entangled processes of aging and chronic stress, and thereby, reaching larger effectiveness. For example, psychotherapeutic treatments in psychiatric patients might be more beneficial in combination with lifestyle improvements (e.g. smoking cessation), and abdominal fat reduction, if the compliance can be kept high for a long period. The latter unfortunately forms a big problem, as long-term behavioural changes are extremely hard to accomplish.

Conceivably, there might also be another factor that determines whether a person gets entangled in the complex web of having a dysregulated physiological stress response, a deleterious metabolic profile and a lower resilience to psychological stress factors, in other words an ‘evolutionarily unfavourable profile’. Genetics and epigenetics may play a role in this determination, or in utero influences (e.g. chronic stress in the mother), or perhaps parental influences and early life environment. In the early life phase, cascades could be created of maladaptive coping strategies and inner (our outer) disturbances. The potential genetic base and complex interrelatedness of metabolic dysregulations, physiological stress markers and cellular aging could also pinpoint another obstacle. It has been hypothesized that these factors may cooperate in order to create a biological maintenance of high fat storages and work to restore the highest sustained body weight, thus precluding the long-term success of behavioral weight loss. A recent study also found two genetic variants influencing gene expression and making subjects vulnerable for smoking behaviour.

Altogether, if telomere attrition is strongest during the first 20 years of the lifespan, and body composition is important during these first years as well for later life, the starting ‘negative spiral’ between cellular aging and deleterious metabolic profile might already be prevented in the early life phase. Stressful events, negative emotions and problems during the early life phase can cause emotional eating behaviours, a more unhealthy dietary pattern and could thus contribute to the development of overweight. In 10-year old girls, slower BMI gain occurred when girls were less stressed and more physically active, whereas the most rapid and largest growth occurred in girls who were more stressed and less active. This once again highlights the importance of the early life environment in the determinants of healthy aging.
10.7. Future research

The current thesis adds epidemiological ground for the associations between biological and psychological stress and cellular aging in two large-scale studies, and elucidates potential explanatory mechanisms that link mental health to somatic health. However, it now seems that TL and its attrition rate are hard to influence. First of all, future investigations should aim to disentangle the precise homeostatic mechanisms of TL in humans by measuring both TL and telomerase activity at multiple time points, combining data spread over various life phases. Perhaps large prospective studies can more often bundle their forces to gain more statistical power, and to be able to compare multiple generations, like the group of Holohan et al. did [111]. The measurement of various proposed cellular aging markers (as described in Section 10.4 “Alternative cellular aging markers”) is found to be feasible in large-scale studies, and combining these in the same cohort could shed light on the links between the separate markers, and the adaptability of each marker. An extensive overview paper has also suggested high interconnectedness between all aging biomarkers, and they propose a hierarchical relation between them [177].

In the current thesis we did not have multiple measurements of the inflammatory markers, so their changeability should be investigated as well, and whether a change in inflammation is in concordance with changes in cellular aging. Various metabolic dysregulations, in particular increased abdominal obesity, are quite consistently found to be accompanied by accelerated cellular aging, so this is a promising focus for future studies as well. Within our cohorts, smoking behaviour was quite stable over time, and therefore, we could not investigate parallels between changes in smoking (onset or cessation) and changes in cellular aging either.

Furthermore, perhaps a thorough evaluation of the existing interventions could shed light on the optimal window in which the largest effects can be evoked on TL or biological and psychological stress factors by answering the following questions; in which life phase should the intervention be offered, what type of persons could benefit from these interventions; which interventions have the best balance between high compliance and multifaceted targeting?

Last, the usefulness of TL measurement might be investigated in alternative settings as well. For instance, one study showed that sperm TL was predictive of embryo quality during in vitro fertilization, implicating that TL can exert an effect on overall health and survival already in the first phases in utero [178]. Another potential use of TL has been reported by a study, that found that telomere attrition and telomerase subunits in the mucosal cells surrounding cancer tissue predicted the advancing of the tumour, and was a prognostic marker of mucosal failure [179]. These findings would imply that short TL or telomere attrition could also be markers of local tissue failure, instead of systemic processes.
10.8. General conclusions

The results of this thesis indicated that there is a link between cellular aging and proposed chronic stress-related factors, such as psychological stress factors, physiological stress systems, metabolic dysregulations and poor lifestyle. Short TL was found to be associated with cigarette smoking, inflammatory markers, a hyperactive HPA-axis, ANS imbalances, increased abdominal obesity, dyslipidaemia and hyperglycaemia. Furthermore, short TL was found in patients diagnosed with depressive and anxiety disorders, showing a dose-response relationship between cellular aging and the severity or chronicity of these psychiatric disorders. The associations between depression and anxiety disorders and TL were partly explained by the presence of elevated inflammation, abdominal obesity, dyslipidaemia and cigarette smoking. Although TL is found to be a valid cellular aging marker that reflects multiple dysregulations in the body, it is under strong homeostatic control as illustrated by the large impact of baseline TL on subsequent TL attrition. As we only found a few stress-related factors to be associated with TL attrition over time, changing TL over a longer term through intervention may not be an easy task. Future researchers should critically evaluate the effectiveness of interventions programs that aim to improve smoking behaviour, body composition and to reduce stress over the longer term. The results presented in this thesis contribute significantly to the reappraisal of the close link between somatic and mental health, providing directions for future research, and extending the knowledge about the biological processes of cellular aging.
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