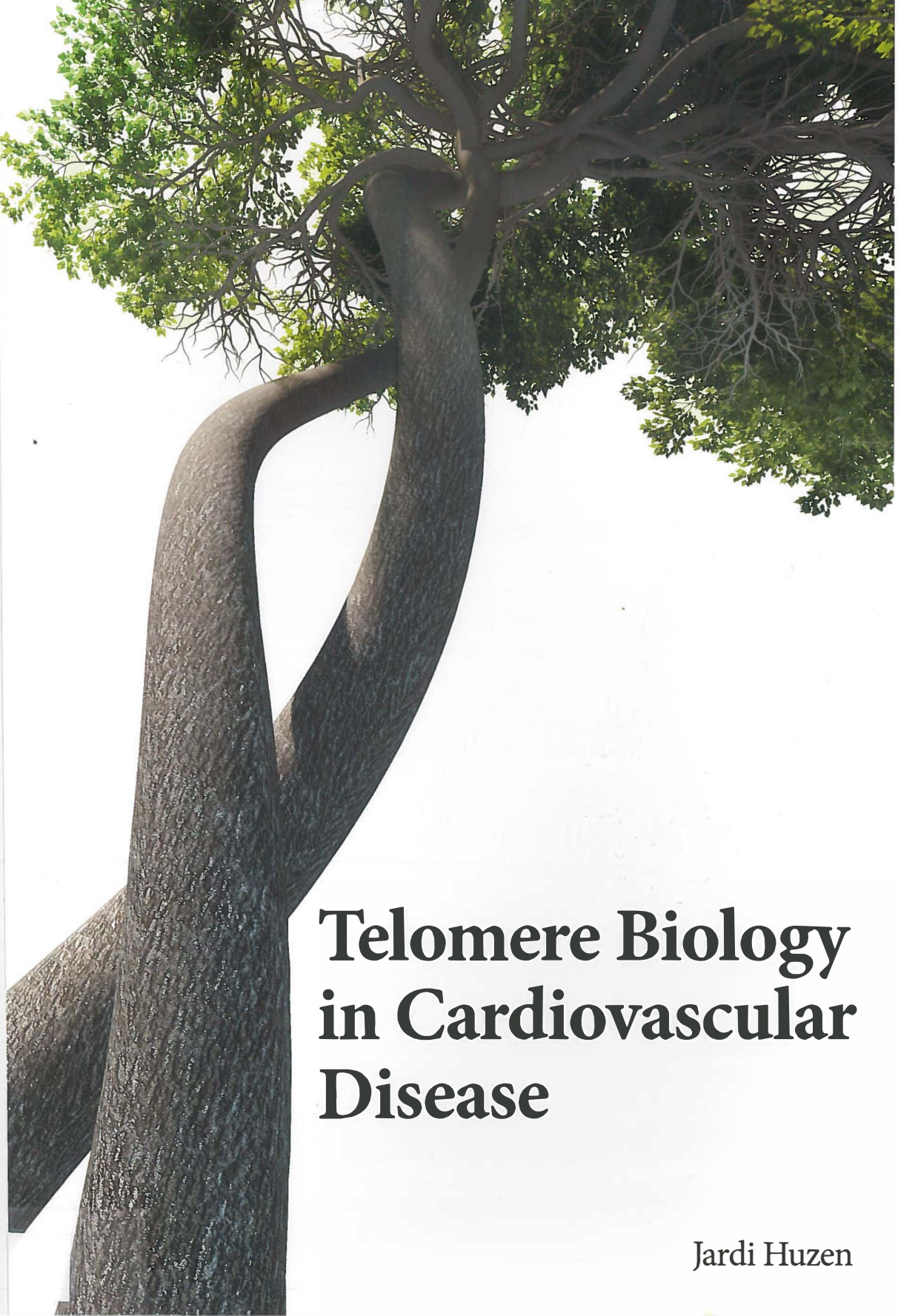
Telomere biology in cardiovascular disease

Huzen, Jardi



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STELLINGEN

Behorende bij het proefschrift *Telomere Biology in Cardiovascular Disease* door Jardi Huzen

1. Bij patiënten met hartfalen is een betere psychische gezondheid geassocieerd met langere telomeren. (dit proefschrift)

2. Leukocyt telomeerlengte is een matige afspiegeling van locale telomeerlengte in atherosclerotisch weefsel. (dit proefschrift)

3. De aanwezigheid van inflammatoire, lipidenrijke plaques is geassocieerd met kortere leukocyt telomeren, maar juist langere plaque telomeren (dit proefschrift)

4. Kortere telomeren in atherosclerotische plaques zijn geassocieerd met een grotere kans op restenose van het geopereerde bloedvat. (dit proefschrift)

5. Bij het ouder worden verkorten telomeren op populatieniveau geleidelijk, op individueel niveau zijn er echter diverse telomeerlengte trajecten mogelijk, waaronder telomeerverlenging. (dit proefschrift)

6. De mate van telomeerverkorting is geasscocieerd met verscheidene markers van het metabool syndroom en rookgewoonten. (dit proefschrift)

7. Telomeerverkorting treedt reeds vóór het optreden van cardiovasculaire events op, en stabiliseert na een event. (dit proefschrift)

8. Telomeren en polonaise vertonen een opvallende gelijkenis: als ze te kort worden functioneren ze niet meer.

9. Een goede syntax zegt vaak meer dan 1000 woorden (dr. P. van der Harst)

10. Mensen die angst hebben dat de wereld ooit wordt overgenomen door robots zouden eens wat vaker met ze moeten (proberen te) werken.

11. Nothing will benefit human health and increase the chances for survival of life on Earth as much as the evolution to a vegetarian diet. (Albert Einstein)

12. Als de hele wereld gek lijkt, kijk dan nog eens kritisch naar jezelf.

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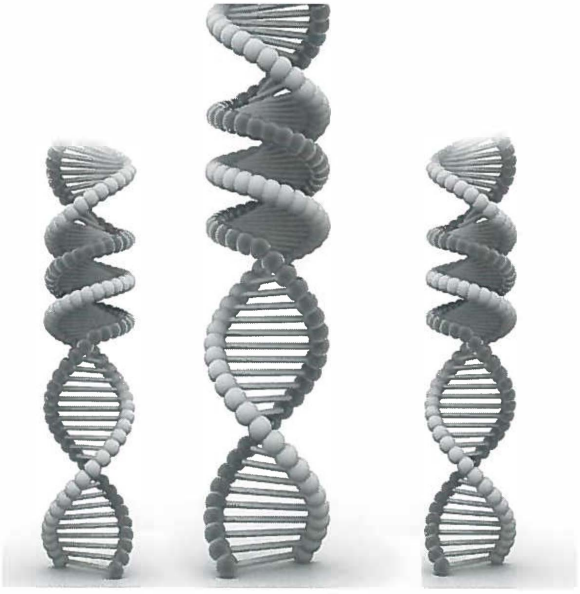
CHAPTER 1 Introduction



The average age of the Dutch population is increasing and so is the prevalence of age-related diseases including coronary artery disease and chronic heart failure. Cardiovascular diseases (CVD) form one of the largest clusters of age-related diseases and are among the primary causes of death in the Netherlands.[1]
The age-relation is based on the age as defined by the date of birth. This age is referred to as chronological age and is solely derived from subtracting the date of birth from the present date, ignoring how the time in between is spent. However, there exists a large variation in chronological age and onset, if any, of CVD. In addition to chronological age, environmental and genetic factors have been identified as risk factors for the development and progression of CVD. To some extent, this intra-individual variation in the risk of CVD might be explained by differences in biological age and the pace of biological aging.[2] One potential marker of biological age is telomere length.

TELOMERES

Telomeres form the distal ends of our chromosomes and consist of numerous repeats of a specific DNA sequence, which is TTAGGG in mammals. The main function of telomeric DNA is to maintain the genomic stability and to protect the coding DNA. Already in 1931, Barbara McClintock observed that after X-irradiation broken chromosome ends fused while this did not occur in intact chromosome ends (the telomeres).[3] Intact telomeres end in a three dimensional structure called the T-loop which is capable of hiding the ends of the chromosome.[4] In this way the chromosomal ends are protected from being recognised as double-stranded DNA breaks hereby preventing activation of DNA-repair mechanisms, detrimental end-to-end fusion and chromosomal instability which will lead to senescence or apoptosis.[4,5]
During life telomeres get shorter due to the "end replication problem". This phenomenon was almost simultaneously described in the early 1970s by the American James D. Watson, at that time already a Nobel prize laureate for the discovery of the molecular structure of DNA and the Russian Alexey Olovnikov who observed an analogy between the railway track that represented the DNA and the train that represented DNA polymerase in a Moscow subway station. The end replication problem is caused by the inability of DNA polymerase to completely replicate the distal ends of the chromosome resulting in loss of telomeric basepairs during every mitosis.[6,7]

Other factors like oxidative stress can cause additional telomere attrition.[8] Telomere length can thus be regarded as a reflection of the replicative history of cells and its exposure to detrimental factors.
When reaching a critically short length telomeres lose their protective properties and the cell enters a state called senescence. In this state the cell is unable to divide, and mostly becomes apoptotic.[9] Due to morphological changes and the decreased or altered functional capacity, a high percentage of senescent cells can disrupt tissue architectures resulting in dysfunctional tissue or organs.[10]

TELOMERE LENGTH AND CARDIOVASCULAR DISEASE

In comparison to healthy subjects, patients with coronary artery disease have telomeres which are on average 300 base pairs shorter. This corresponds to telomere lengths of almost nine year older control subjects.[11] Also patients with chronic heart failure, which is the final common endpoint of cardiac disease, have shorter telomeres than age matched control subjects.[12]
In chapter 2 we give an overview of the literature available on the associations of telomere length with cardiovascular diseases and cardiovascular risk factors. In chapter 3 we investigate the association of one particular cardiovascular risk factor, namely psychological well being on leukocyte telomere length.
Besides telomere attrition in leukocytes, patients with atherosclerosis also have shorter telomeres in their coronary endothelial cells than subjects without atherosclerosis.[13] In arteries, spots with increased hemodynamic stress, display increased cellular turn-over and telomere attrition.[14,15] In a small scale study it was reported that diseased as well as healthy abdominal aorta wall telomere length correlated with leukocyte telomere length.[16] However, our knowledge of telomere status of diseased tissue itself and its relation with presence and progression of disease is still limited. We therefore investigate the correlation of atherosclerotic plaque tissue telomere length with leukocyte telomere length in chapter 4. In this chapter we also evaluate the capabilities of plaque tissue telomere length and leukocyte telomere length in predicting recurrence of atherosclerotic disease in patients with carotid atherosclerosis.
Besides telomere shortening in the presence of overt cardiovascular disease, also a striking amount of cardiovascular risk factors are associated with shorter telomeres. A limitation of these associations is the cross-sectional nature of the concerning studies. In chapter 5 we investigate whether or not the degree and length of exposure to certain factors are associated with telomere length dynamics in a longitudinal study design. In previous cross-sectional studies,

even after adjustment for known risk factors, the relation between coronary artery disease and telomere length remains.[17,18] This suggests that the relation between telomere shortening and cardiovascular disease is not merely formed by the summation of conventional risk factors but that telomere shortening plays an independent role. The big question whether this role is causal or not remains a subject of debate. Hence, we evaluate telomere length trajectories prior and after the occurrence of cardiovascular events in chapter 5. This question bears great importance since insight into the temporal sequence is necessary to eventually accept or reject the hypothesis with regard to the causal involvement of telomere length in the development of cardiovascular disease.[19] Consequences of a causal relation would be that it potentially opens the door for pharmacological and lifestyle interventions and perhaps even gene modification in the battle against the development and progression of cardiovascular diseases.

AIMS OF THIS THESIS

The aim of the present thesis is threefold:

1) To investigate novel modifiers of telomere length. Chapter 3 focuses on psychological factors and chapter 5 on physical, biochemical and behavioral characteristics.

2) To determine whether telomere length is similar among different tissues involved in cardiovascular disease. Chapter 4 describes influences on telomere length of atherosclerotic plaque tissue and its relation with progression of disease.

3) Gain more insight in the question whether telomeres are causally involved in the development of cardiovascular disease or not. To this end we evaluated telomere dynamics prior and after the occurrence of cardiovascular events in chapter 5.

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CHAPTER 2

The emerging role of telomere biology in cardiovascular disease

This chapter is based on:

The emerging role of telomere biology in cardiovascular disease
J. Huzen, R.A. de Boer, D.J. van Veldhuisen, W.H. van Gilst, P. van der Harst

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Telomeres and biological ageing in cardiovascular disease
J. Huzen, D.J. van Veldhuisen, W.H. van Gilst, P. van der Harst

Nederlands Tijdschrift voor Geneeskunde 2008; 152(22):1265-1270

ABSTRACT

A striking variability exists in the susceptibility, age of onset and pace of progression of cardiovascular diseases. This is inadequately explained by the presence or absence of conventional risk factors. Differences in biological aging might provide an additional component of the observed variability. Telomere length provides a potential marker of an individual's biological age, shorter telomeres reflect a more advanced biological age. Telomere length at birth is mainly determined by genetic factors. Telomere attrition occurs as a consequence of cellular replication and can be accelerated by harmful environmental factors such as oxidative stress. When telomeres reach a critical threshold the cell will enter senescence and becomes dysfunctional. Telomeres are remarkably shorter in patients with aging associated diseases, including coronary artery disease and chronic heart failure. In addition, numerous conventional cardiovascular risk factors are associated with shorter telomere length. If telomeres can be proven to be not only associated but also causally involved in the pathogenesis of cardiovascular disease it might provide exciting new avenues for the development of future preventive and therapeutic strategies.

Cardiovascular disease (CVD) is the leading cause of death and chronic heart failure (CHF) is the main cardiovascular discharge diagnosis in the United States.[1,2] In particular after the necessity of hospital admission, CHF is associated with a high mortality rate and is a growing economic burden for society.[3,4] Both the incidence and prevalence of coronary artery disease (CAD) and CHF drastically increase with chronological aging (defined by the date of birth). Nevertheless, there exists a striking variability in the susceptibility, age of onset and pace of progression of both CAD and CHF. This variability cannot completely be attributed to the presence of conventional risk factors. Although chronological age is important, we also have to consider biological age as a contributing factor in the development of CVD. Unfortunately, the pace of biological aging and its interindividual variation is not easily quantified. Telomere length fulfils a number of criteria to be considered a robust biomarker of biological aging. Telomere length is largely heritable and is affected by biological processes fundamentally involved in aging, including the number of cell divisions and exposure to external stressors.[5-7] In this review we will focus on the properties and functions of telomeres, describe the process of telomere shortening and elongation and the association of telomere length with cardiovascular diseases, cardiovascular risk factors and prognosis. This review provides a timely fundament for our hypothesis that telomere length is an appropriate marker of biological age and might even be involved in cardiovascular disease manifestations. We will conclude this chapter with potential future preventive and therapeutic strategies targeting telomere biology.

TELOMERES AND TELOMERASE

Telomeres

Telomeres are located at the distal ends of our chromosomes (Figure 1). Their primary function is to maintain genomic stability by protecting the integrity of the coding DNA sequence.[8] Telomeres consist of numerous repeats of a specific nucleotide sequence; TTAGGG in vertebrates. In conjunction with several telomere specific and essential proteins the telomere can form a complex three dimensional structure, named the T-loop, (Figure 2) which conceals the terminal single stranded end of the chromosome.[9] This is important as it prevents it from being recognised as double stranded DNA breaks consequently leading to activation of DNA-repair mechanisms.[10] The following non-homologous

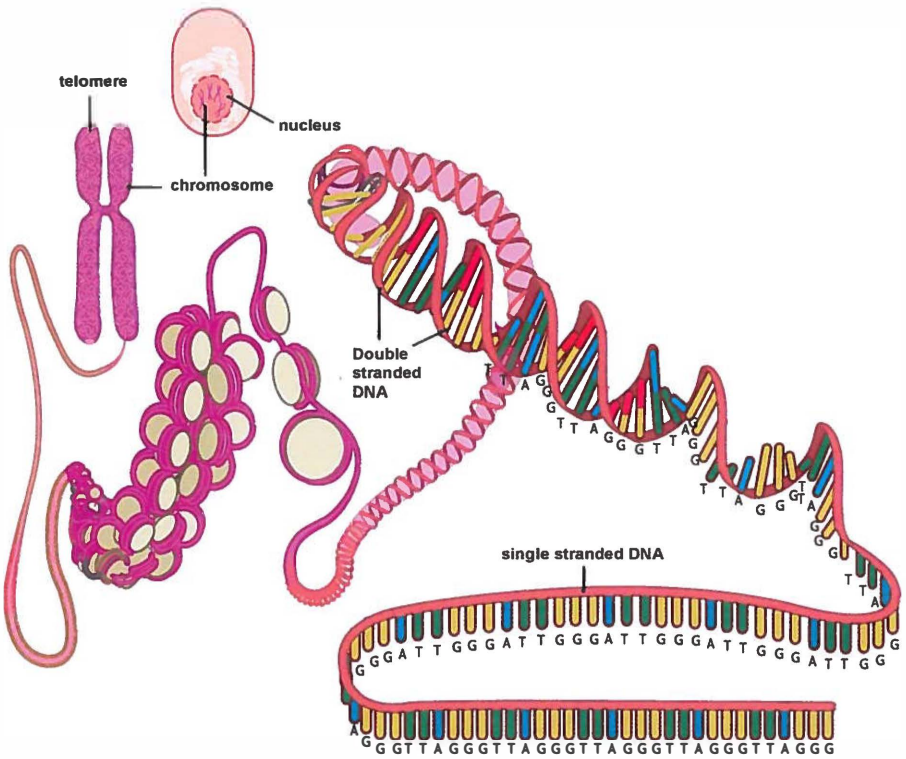

Figure 1. Localisation of the telomere in the cell. The telomere is located at the distal end of the chromosome.

end joining will cause chromosomal instability and will lead to senescence or apoptosis.[9] The stability of the T-loop is largely dependent on the integrity of the associated telomere specific proteins (Figure 2). Telomere repeat binding factor (TRF) 1 and TRF 2 directly bind to the TTAGGG sequence and facilitate the formation of the protective T-loop. In the absence of TRFs telomeres will lose their protective T-loop structure.[11] Furthermore, the capability to form this protective T-loop is influenced by the length of the single strand overhang at the end of the telomere itself. However, in the presence of high levels of TRF2 even short telomeres can form T-loops.[10,12,13]

It is estimated that telomere length is for 80% dependent of the parental telomere length and the age of the father.[5,14] Remarkable in this respect is that a higher paternal age at conception is associated with longer telomeres in the offspring. It is tempting to speculate that this is related to the duration of telomerase activity and the selection of cells with long telomeres in the male germ-line stem cells.[15]

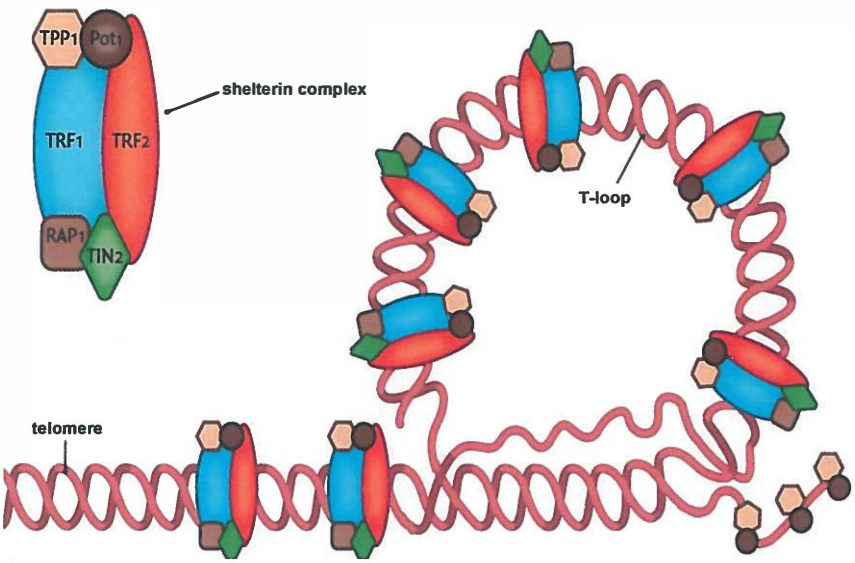

Figure 2. T-loop and the shelterin complex. The extreme end of the telomere is single stranded and ends in the T-loop. In this configuration the telomere is not recognized as a DNA break. Essential for the formation of the T-loop are the shelterin complexes. Shelterin consists of the following proteins: 'TTAGGG repeat binding factor' (TRF) 1 and 2, 'TRF1 interacting nuclear protein 2' (TIN 2), 'repressor activator protein 1' (RAP 1) and the 'protection of telomere 1' (POT 1)-TTP1 heterodimer which can form several complexes in different configurations.

During life telomeres get shorter due to the so called end replication problem. This problem is caused by the inability of DNA polymerase to completely replicate the lagging DNA strand resulting in loss of telomeric base pairs during every mitosis. Besides the end replication problem there are also external factors which cause telomere attrition. For example, oxidative stress and smoking are associated with increased telomere attrition.[6] Telomere length reflects both the replicative history of a cell and its exposure to detrimental factors such as oxidative stress.

When telomeres reaches a critically short length they lose their protective properties and the cell enters a non-dividing state called senescence.[16] The faith of the majority of senescent cells is to enter apoptosis, although this is not necessarily true for all senescent cells. Due to morphological changes and the decreased or altered functional capacity, including the excretion of growth factors, cytokines or enzymes, a high percentage of senescent cells can disrupt tissue architecture and can result in dysfunctional tissues or even dysfunctional organs.[17]

Telomerase

To avoid extinction of species there must be mechanisms to upkeep the length of telomeres of certain cells (e.g. the germ cell line). The most important mechanism involves the specialized ribonucleoprotein enzyme telomerase. This enzyme consists of two molecules of Telomerase RNA Component (TERC), two molecules of Telomerase Reverse Transcriptase (TERT) and one molecule of dyskerin.[9,18] The TERC component is complementary to the telomeric DNA and functions as a template for the new to be formed telomeric repeats which are formed by the TERT component (Figure 3). The main function of dyskerin is to stabilize the telomerase complex.[19] In the foetal phase, and later on in life in stem- and germ line cells, telomerase adds new TTAGGG sequences to the telomere during mitosis. The essential function of telomerase becomes especially evident when it fails. This is the case in the rare condition Dyskeratosis Congenita. Dyskeratosis Congenita is a progressive bone marrow failure syndrome that is characterized by abnormal skin pigmentation, nail dystrophy and leukoplakia. Most patients with Dyskeratosis Congenita become grey or get alopecia at an early age and die young mainly due to progressive bone marrow failure or malignancies.[20] Telomerase deficient mice suffer progressive telomere shortening which becomes more evident in every subsequent generation. In later generations they show premature aging symptoms like infertility, grey hair or alopecia, hypertension, and decreased tissue regeneration.[21] Reduced telomere length in these mice is also associated with attenuated myocyte proliferation, increased apoptosis and cardiac myocyte hypertrophy. Eventually, left ventricular failure and pathological cardiac remodelling is seen in these mice and is comparable to dilated cardiac myopathies in humans.[22]

TELOMERES IN CARDIOVASCULAR DISEASES

Telomere length in Coronary Artery Disease

Only recently telomere biology caught the attention of cardiovascular researchers. The first study in humans on telomere length in cardiovascular disease originates from 2001.[23] In this study patients with coronary angiography proven three vessel disease were compared to patients without angiographic abnormalities. Patients with CAD had approximately 300 base pairs shorter telomere lengths. Considering the yearly attrition rate, the observed difference translates back to almost nine years difference in age.[23] In a case control study with 203 cases and 180 controls it was concluded that patients with premature (before 50 years of age) myocardial infarction had

telomere lengths comparable to 11.3 years older healthy controls.[24] Telomere length has also been associated with the severity of disease. In a subgroup of 437 ischemic heart failure patients leukocyte telomere length was associated with the number of atherosclerotic disease manifestations. Patients with more affected vessels had shorter telomere lengths.[24,25]

The association between telomere length and CAD is not limited to leukocytes but can also be observed in vascular cells. Endothelial cells in coronary arteries of patients with atherosclerosis have shorter telomeres than in patients without atherosclerosis.[26] Wall biopsies of abdominal aortic aneurysms taken during surgery show shorter telomeres than healthy abdominal aortic biopsies of diseased organ donors.[27] In arteries, spots with increased hemodynamic stress, display increased cellular turn-over.[28] Endothelial cells at these spots (Iliacal Artery) show increased telomere attrition compared to the Internal Thoracic Artery.[29] In atherosclerotic patients, the telomeres of coronary endothelial cells are shorter than those of non-affected vessels.[26] Senescent human aortic endothelial cells exhibit increased levels of intercellular adhesion molecule (ICAM)-1 which stimulates the adhesion of monocytes.[30] In addition senescent vascular endothelial cells show upregulation of plasminogen activator inhibitor I, have reduced production of nitric oxide (NO) and endothelial NO syntase activity.[30,31] Taking this all together senescent endothelial cells seem to promote an atherogenic environment.

Whether leukocyte telomere length can be used as a good reflection of telomere status in diseased tissue can be disputed. In a recent small-scale study among 32 subjects a positive correlation has been reported between leukocyte telomere length and the telomere length of abdominal aortic wall tissue biopsies.[27] These promising results will need to be confirmed in large scale studies.

Telomere length in Chronic Heart Failure

In a study conducted in 19 patients with dilated myopathy and 7 healthy age matched controls endomyocardial biopsies of the diseased hearts were characterised by shorter telomeres, increased cellular senescence and cell death.[32] In an independent set of cardiac muscle biopsies of 8 failing hearts, telomere length was reduced by 25% compared to hearts of 8 healthy or 8 hypertrophic, non-failing obstructive cardiomyopathy subjects.[33] In a large study leukocyte telomere length was substantially shorter in patients with CHF compared to age and gender balanced controls.[25] This observation accounts for patients with ischemic as well as non-ischemic aetiology of heart failure. Moreover, the clinical severity of CHF was related to the degree of telomere shortening.[25]

Figure 3. Telomerase. Active telomerase is formed by two RNA-complexes (TERC, one depicted here), two telomere reverse transcriptase complexes (TERT, one depicted here) and dyskerin. Dyskerin is essential for the stability of telomerase. The TERC functions as a template for the newly formed TTAGGG sequences.

Ejection fraction is strongly associated with telomere length in subjects without evidence of previous myocardial infarction.[34] One standard deviation longer telomere length was associated with 5% higher ejection fraction. Telomere length alone accounted for 12% in the observed variability in ejection fraction in these elderly subjects.[34]
In older patients findings between the presence and absence of cardiovascular disease is in general less well consistent related to telomere length.[35-37] In 193 subjects over 70 years of age telomere length was not associated with the presence of CAD, but an association with aortic valve calcification was reported.[36] In 190 persons over 85 years of age shorter telomere length was related to the presence of self reported heart disease and ischemic changes on electrocardiography.[35] However, studies in elderly are likely to suffer from important selection biases.

Telomere length and cardiovascular risk factors
Besides the relation with cardiovascular diseases, telomere length has also been associated with a striking amount of cardiovascular risk factors. A good example is the male gender. Male gender is an important risk factor for cardiovascular disease as well as it is associated with shorter telomere length.[38,39] In men the pace of telomere shortening during life is also faster than in women.[40] Possibly due to the protective properties of estrogen on telomerase.[41] A positive family history of cardiovascular disease is one of the most important risk factors. Offspring of parents with premature CAD already have shorter telomeres than children of parents without cardiovascular disease.[42,43]
In vitro as well as *in vivo* there is clear evidence that oxidative stress reduces telomere length.[44,45] Consistent is also the dose-dependent relation of smoking with reduced telomere length.[46,47] In cross-sectional studies shorter telomeres have also been associated with diabetes, increasing body weight and increasing insulin resistance.[45,46,48]

Increased pulse pressure in men and increased carotid artery internal media thickness (ICA-IMT) is also associated with shorter telomere length.[38,47] In the Framingham heart study, subjects with increased circulating biomarkers of the renin-angiotensinaldosterone system had shorter telomeres.[49] Subjects with decreased renal function are at increased risk to experience cardiovascular events and also have shorter telomeres.[50-52] Recently an inverse correlation between plasma homocysteine levels and telomere length was found.[53] Besides these physical and biochemical risk factors there are also psychological and environmental risk factors associated with telomere length. Psychological stress, chronicity of stress, depressive symptoms and decreased social status are associated with having shorter telomeres.[54-59] In a recent large survey among 1,502 subjects self-perceived early aging was associated with abdominal obesity, poor self-rated health, lower education and shorter telomere length.[60] An overview of the associations between telomere length and cardiovascular risk factors can be found in Table 1.

Interestingly, the relation between CAD and telomere length cannot be fully explained by classical risk factors.[24,61] This suggests that the relation of telomere shortening on cardiovascular disease is not only through classical pathways, but might be an independent factor as well. Taken together, these data supports the hypothesis that telomere shortening is involved in the pathogenesis of cardiovascular diseases.[43]

THE PROGNOSTIC VALUE OF TELOMERE LENGTH

When telomere length is considered a biomarker of biological aging and associates with cardiovascular pathology the question arises whether telomere length conceals prognostic value as well. The first study aimed to answer this question was undertaken in 143 patients without selection on presence or absence of diseases. Adjusted for age, and after 20 years of follow-up, patients with telomere lengths shorter than the median had a three times higher risk of cardiovascular death than patients with telomeres longer than the median. Subjects with telomere length in the lowest quartile had an eight times increased risk of dying from infectious diseases than persons from the three higher quartiles.[62]

In a case-control study a three times increased risk of getting a premature myocardial infarction (before 50 years of age) was observed in persons with telomere lengths shorter than average compared to persons with telomere lengths in the highest quartile.[24] In another case-control study, nested in the West of Scotland Primary Prevention Study (WOSCOPS), 485 patients who

reached the primary endpoint (myocardial infarction or cardiovascular death) after a follow-up of 5 years where compared to 1058 matched controls who did not reach the endpoint. Patients with shorter telomeres had an almost doubled risk of reaching the primary endpoint than patients with long telomeres.[61] In 870 patients with stable CAD it was observed that patients in the lowest quartile of telomere length had a 1.9 increased risk of dying compared to patients in the highest quartile after adjusting for age, clinical, inflammatory and echocardiographical risk factors.[63] In the elderly this association is again less clear. In 812 persons aged 73-101 years old and in 598 persons over 85 years

Table 1. Overview of the association of cardiovascular risk factors and leukocyte telomere length

Risk factor	Association with telomere length	Reference
Gender	Females have longer telomeres	38,39
	Men have higher telomere attrition rate	39,14
Family history	Offspring of fathers with premature myocardial infarction have shorter telomeres than offspring with healthy fathers	42,43
Diabetes	Diabetics have shorter telomeres than non-diabetics. Adequate glykemic control prevents telomere shortening	73
	Insulin resistance is associated with increased telomere attrition	45,48
Blood pressure	Negative correlation between telomere length and pulse pressure	38
	Hypertensives have shorter telomeres than normotensives	45
	Telomere length is associated with circulating biomarkers of the renin-angiotensin-aldosterone system	49
Renal function	Telomere length correlates positively with estimated glomerular filtration rate in chronic heart failure patients	51
ICA-IMT	Telomere length is inversely associated with ICA-IMT	47,67
Homocysteine	Increased homocysteine levels are associated with shortened telomeres	53
Obesity	Obesity is negatively correlated with telomere length	46
	Telomere length is inversely associated with body mass index	47
Life style	Telomere length is positively associated with physical activity	78
	Subjects with moderate physical activity have longer telomeres compared to low and high levels of physical activity	79
Smoking	Smoking females have shorter telomeres	46
	Smokers have shorter telomeres than never-smokers	47
Psychological stress	Psychological stress is associated with lower telomerase activity and shorter telomeres	54,55
	Patients with mood disorders have shorter telomeres	59

Abbreviations: internal carotid artery intima media thickness, ICA-IMT

of age no association between telomere length and survival was observed.[64,65] However in 195 stroke survivors over 75 years of age longer telomeres were associated with a better survival and in 412 healthy persons over 65 years of age with a median age of 74.4 years telomere shortening was associated with an increased risk for myocardial infarction and stroke.[66,67] Again in the elderly telomere length may be a less accurate marker of biological age due to an important selection bias or confounders.

FUTURE PERSPECTIVES AND CONCLUSIONS

Potential Intervention strategies

If telomere biology is proven to be involved in the development and progression of cardiovascular disease it will pave the way for new therapeutic or preventive strategies. For example, statins have, besides their many influences on conventional risk factors, proven to have a protective effect on telomeres by preventing the loss of the essential TRF-2 protein in endothelial progenitor cells.[68-71] In addition, statins promote DNA repair and prevent telomere shortening and senescence in cultures of vascular smooth muscle cells.[72] In diabetic patients adequate glycemic control prevents additional telomere shortening.[73] A different possible target for therapy can be through telomerase. Human telomerase can also be regulated by erythropoietin.[74] Evidence is accumulating for a beneficial role of erythropoietin in endothelial function, independent of increasing haemoglobin levels.[75,76] Increased levels of telomerase could be involved in this process. Changes in telomerase activity can also be achieved by comprehensive changes in lifestyle.[77] In short these changes included a low-fat diet, moderate aerobic exercise, stress management and food supplements and resulted in an increased telomerase activity in peripheral blood mononuclear cells after a period of three months.[77] Increased levels of physical exercise are by itself associated with longer telomeres and can thus potentially have a decelerating effect on biological aging.[78,79]

Besides pharmacological and lifestyle interventions, gene modification can also provide new opportunities. Over-expression of telomerase can counteract telomere dependent replicative senescence.[80] Transfecting cells with the human TERT increases telomerase activity resulting in delay of replicative senescence.[80] In vitro immortalized TERT overexpressing swine umbilical vein epithelial cells produced normal levels of NO, endothelin and prostacylin indicating their biological functioning and metabolic capacities are similar to mortal cells.[81] Porquine ventricular endocardial endothelial cells (EEC) also

exhibit phenotypic and functional characteristics similar to primary EEC.[82] Bovine microvascular endothelial cells exhibit an endothelial phenotype similar to that of wild-type endothelial cells. Specifically, they had the typical cobblestone morphology, expressed endothelial cellspecific markers and vascular endothelial growth factor receptor-2 (VEGFR-2). Besides, they expressed receptors for low density lipoprotein (LDL) and were able to form tubular structures.[83]

In humans several trials have been undertaken to evaluate the safety and efficacy of infusion of bone marrow derived mononuclear cells.[84] The results this far look promising however there are still some concerns. One of them is the viability and the proliferative potential of the cells transfused.[84] Homologues transplantation of cells of various tissues and progenitor cells, of which the telomeres have been elongated in vitro, may in the future help to more effectively repair damaged endothelium or alter the remodelling processes leading to CHF. However one should remember that telomere dependent senescence also has a protective function, namely to prevent cells from unlimited cell divisions. A possible solution for this potentially dangerous process is to bring selective expression of telomerase under control by a specific substance sensitive promotor. In the presence of this substance telomeres can then be elongated in vitro and after having them transfused back, telomerase expression will cease again.

Conclusions

Exponential increasing evidence is suggesting that telomere length is associated with cardiovascular diseases. Shorter telomeres are not only associated with the presence of cardiovascular risk factors and established cardiovascular diseases but also the degree of telomere shortening is related to the severity of the disease.[25] In addition, telomere length also predicts the occurrence of clinical manifestations of cardiovascular disease and outcome. The major limitation of most previous studies present lays in its cross-sectional nature. The key question, whether or not telomere shortening is causally involved in the development and progression of cardiovascular diseases, remains a target for future studies. In these studies telomere dynamics over time need to be related to the development of cardiovascular diseases and events. In addition, this design will give more insight in other potential cardiovascular risk factors or modifiers of telomere length. With a longitudinal design interactions of risk factors or efficacy of interventions on telomere length and clinical outcome can be identified better. The aforementioned telomerase deficient mice with short telomeres could serve as a model to study the vulnerability of having short telomeres to specific factors. These factors can be genetic, for example

deficient DNA repair mechanisms, pathologic, for example diabetes and hypertension or environmental factors like smoking, stress or diet. Combining these different scientific approaches more insight can be gained in the causal role telomere shortening potentially plays in cardiovascular disease.

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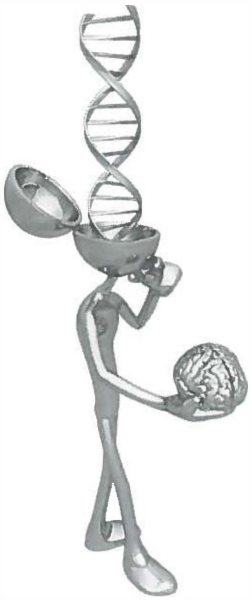
CHAPTER 3

Telomere length and psychological well-being in patients with chronic heart failure



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ABSTRACT

Objectives
To asses the association between telomere length and psychological functioning in patients with chronic heart failure.

Background
Shorter telomere length indicates a more advanced biological age. Several studies have implicated psychological stress and depressive symptoms with accelerated aging and increased disease progression. It is unknown whether mental health is associated with telomere length in patients with the chronic somatic condition of heart failure.

Methods
Telomere length was determined by quantitative polymerase chain reaction. We evaluated the perceived mental health by the validated RAND-36 questionnaire. Depressive symptoms were assessed by the Centre for Epidemiologic Studies Depression scale (CES-D) and the presence of type- D personality was evaluated by the DS14.

Results
We studied 890 patients with New York Heart Association (NYHA) functional class II to IV heart failure. Telomere length correlated well with age ($r= -0.19$, $p < 0.0001$). A lower perceived mental health on the RAND-36 score was associated with shorter telomere length and adjustment for age and gender did not change our findings (standardised beta 0.11; P-value, 0.002). Telomere length was not associated with the CES-D or DS14 score.

Conclusion
Decreased perceived mental health is associated with shorter leukocyte telomere length in patients with chronic heart failure. Future work should determine whether psychological stress accelerates biological aging.

Telomeres are the protective caps at the ends of linear chromosomes. Telomeres play an important role in the preservation of the genomic integrity and stability by preventing them from being recognized as double-stranded DNA breaks.[1] During every cell division the very final part of the telomere fails to be replicated and as a consequence telomeres become progressively shorter. Telomeres are considered a marker of cumulative cell-divisions and biological aging. In addition to this replicative stress, other biological stressors can cause additional telomere attrition. In this regard, oxidative stress is among the best studied factors causing telomere erosion.[2] Eventually, telomeres will reach a critical short length which will prevent further cell divisions and can cause decreased cellular functioning.[3] Various aging associated cardiovascular disease entities, including Chronic Heart Failure (CHF), have been associated with reduced telomere length in humans.[4-6]

Several small-scale studies have implicated shorter telomere length with mood disorders.[7] In addition, increased levels of perceived psychological stress and the chronicity of stress are associated with shorter leukocyte telomere lengths in apparently healthy subjects.[8-10] The biological basis for this association is not known, but speculated to originate from neurohormonal activation and increased oxidative stress.[9-11]

Patients with cardiovascular diseases are particularly susceptible to depressive symptoms and increased perceived psychological stress.[12-14] The presence of co-morbidity frequently associated with CHF (diabetes, Chronic Obstructive Pulmonary Diasease (COPD) and stroke) have an additional deteriorating effect on quality of life.[13] Decreased mental health and the presence of depressive symptoms in patients with cardiovascular disease is associated with faster disease progression and worse outcome.[15,16] It is unknown whether the association between telomere length and mental health continues to persist in the presence of the severe chronic somatic condition of CHF. Therefore, we investigated the potential association of telomere length with psychological functioning using well validated questionnaires in subjects with CHF.

METHODS

Patients

DNA was collected from 890 CHF patients who participated in the Coordinating study evaluating Outcomes of Advising and Counseling in Heart failure (COACH) study.[17]

Patients were 18 years or older and had evidence of an underlying structural heart diseases shown by cardiovascular imaging. Patients were in New York Heart Association (NYHA)class II-IV.[18]

Telomere length

Telomere length was determined in duplicate in leukocytes using a real time quantitative polymerase chain reaction as described in detail previously.[4, 19] We determined the relative ratio of the Telomere repeat copy numbers (T) to the Single-copy reference gene (S; gene 36B4). Telomere length is expressed as the T/S ratio. All samples were compared to the same reference DNA pool.

Assessment of psychological functioning

During index hospitalization psychological functioning was measured by well validated questionnaires; the RAND-36 (perceived mental health), the CES-D (depressive symptoms), and the DS14 (type D personality).

RAND-36

The RAND-36 was used to evaluate perceived mental health, one of the domains of the Medical Outcome study 36-item General Health Survey. This is a validated self report questionnaire consisting of 36-items on general health summarized into nine health concepts that represent dimensions of quality of life. One of the dimensions is Mental Health. This domain has a score between 0-100 and a higher score means better mental health.[20] The RAND-36 was completed by 847 (95%) of participating patients.

CES-D

The CES-D is a validated self-report questionnaire for the general population and the medical ill to measure depressive symptoms.[21] A cut-off point of 16 out of maximum 60 points is commonly used to define the presence of depressive symptoms.[22] For the COACH study, a third category was created based on the median CES-D score of patients with depressive symptoms which was 24. In creating this third category (no, moderate and severe depressive symptoms) a graded effect of depressive symptoms could be examined.[15] Of all patients 835 (94%) completed the CES-D questionnaire.

Type-D

Presence of type-D personality was assessed with the validated type-D scale 14 (DS14) questionnaire. This questionnaire consists of 14 items with a response scale ranging from 0 (false) to 4 (true), of which 7 items refer to negative affectivity and 7 to social inhibition. The presence of type-D personality is

defined as having a score of at least 10 points on both subscales.[23]
Type-D denotes the synergistic effect of negative affectivity (tendency to experience negative emotions) and social inhibition (tendency to inhibit self-expression). Type D personality is considered a rather stable trait and persons with this personality are believed to experience more stress.[23] The type-D questionnaire was completed by 820 (92%) of the patients.

Statistical analysis
Telomere length was log-transformed to improve the normality of the distribution. Differences between groups were tested by students T-test, one way analysis of variance and Chi2 test when appropriate. In order to adjust for potential confounders we used standard linear regression models. All tests were performed in SPSS, version 14.0 (SPSS inc. Chicago, Illinois). A two sided p-value of 0.05 or less was considered to indicate statistical significance.

RESULTS

We studied 890 patients (39% female) with a median age of 73 and an interquartile range (IQR) of 64–79. Baseline characteristics are presented in Table 1. At time of inclusion, 51% of patients were in NYHA class II, 46% in class III and 3% in class IV. The median telomere length was 0.69 (IQR = 0.59–0.85) and, as expected, was correlated well with age ($r = -0.19$, $p < 0.0001$).
As expected, perceived mental health score correlated negatively with CES-D score ($r^2 = 0.39$, $p < 0.001$). Having a type D personality was associated with lower perceived mental health ($r^2 = 0.13$, $p < 0.001$) and a higher CES-D score ($r^2 = 0.13$, $p < 0.001$).

The median score on the mental health scale was 69 (IQR: 48–84) out of 100. Patients in the lowest tertile of the mental health domain in the RAND-36 questionnaire had shorter telomeres (median T/S ratio = 0.67 (0.55–0.81)) than patients in the second tertile (T/S = 0.70 (0.61–0.86); P = 0.01) and third tertile (T/S = 0.70 (0.60–0.88); p = 0.02) (see Figure 1).
Using standard linear regression analysis, the relation between telomere length and mental health score persisted after adjustment for age and gender (standardised beta = 0.11, p = 0.002; Table 2) and also after further adjustment for variables known to be associated with the severity of heart failure (NYHA class, left ventricular ejection fraction and estimated glomerular filtration rate) or otherwise affecting quality of life (presence of COPD, diabetes and history of stroke) (standardised beta = 0.08, p= 0.022; Table 2).

Table 1. Baseline characteristics

Patient characteristics	(n=890)
Age (years)	73 [64–79]
Male (n (%))	535 (61)
eGFR (mL/min/1.73 m2)	53 [40–68]
Creatinine (µmol/L)	113 [90–144]
Age of onset CHF (years)	71 [61–77]
Body mass index (kg/m2)	26 [24–30]
LVEF (%)	30 [23–44]
NYHA class (n (%))	
II	438 (51)
III	397 (46)
IV	27 (3)
Haemoglobin (mmol/L)	13.5 [12.2–14.8]
Heart rate (beats/min)	72 [64–80]
Systolic blood pressure (mm Hg)	115 [101–130]
Diastolic blood pressure (mm Hg)	70 [60–76]
Medical history (n (%))	
Myocardial infarction	369 (42)
Hypertension	367 (41)
Diabetes mellitus	246 (28)
Atrial fibrillation/flutter	388 (44)
Stroke	88 (10)

Data is presented as 'median [interquartile range]' or 'number (%)'. The body mass index is the weight in kilogrammes divided by the square of the height in metres; LVEF, left ventricular ejection fraction; eGFR, estimated glomerular filtration rate

The median CES-D score of this population was 13 out of 60 (IQR: 7–21). In total, 299 patients (36%) had depressive symptoms (CES-D score ≥ 16) of whom 154 (18%) had severe depressive symptoms (CES-D score ≥ 24). In total, 536 patients (64%) had no depressive symptoms. There were no significant differences in telomere lengths among these groups ($p = 0.51$). We also did not find a relation between the CES-D score as a continuous variable and telomere length.

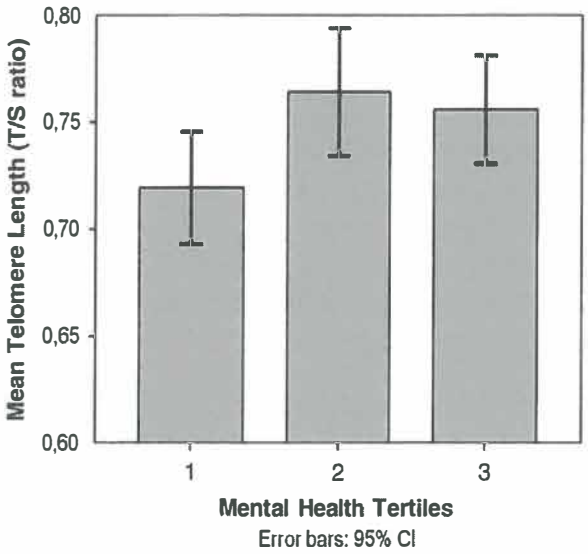

Figure 1.
Mean telomere length per tertile of mental health.

Of all patients, 105 (13%) had a type D personality versus 715 patients (87%) who did not have this personality trait. Patients with type D personality had a median telomere length of 0.68 (IQR: 0.56–0.82) and patients without the type D personality had 0.70 (IQR: 0.60–0.86). There was no statistical significant difference in telomere length between these groups (p = 0.32).

Table 2. Multivariate linear regression analysis

Characteristics	Mental health		
	Beta	95% CI	p-value
Model 1			
Telomere length	8.29	2.96 to 13.62	0.002
Age (years)	0.38	0.25 to 0.52	< 0.001
Female gender	−5.0	−8.08 to −1.90	0.002
Model 2			
Telomere length	6.79	0.98 to 12.60	0.022
Age (years)	0.45	0.29 to 0.60	< 0.001
Female gender	−4.61	−8.06 to −1.16	0.009
NYHA class	−4.70	−7.68 to −1.72	0.002
LVEF	−0.01	−0.13 to 0.11	0.865
eGFR (mL/min/1.73 m2)	2.61	−1.57 to 6.80	0.221
Presence of COPD	−3.53	−7.34 to 0.26	0.068
History of stroke	−4.09	−9.56 to 1.38	0.142
Presence of diabetes	1.19	−2.50 to 4.88	0.526

COPD, chronic obstructive pulmonary disease.

DISCUSSION

This is the first study to show that reduced perceived mental health, as assessed by the RAND-36 questionnaire, is associated with shorter telomere length in patients with CHF. The mechanism by which psychological well-being is associated with telomere length remains to be clarified. However, our observation is consistent with the evidence suggesting that chronic stress may accelerate biological ageing.[27] Stress has been associated with chronic increased neurohormonal activation and elevation of blood pressure, which adversely affects outcome in CHF.[28,29] In addition, psychological stress has also been directly associated with increased levels of oxidative stress.[28] Epel et al. suggested that increased oxidative stress explains the association between psychological stress and telomere length in presumably healthy subjects.[9,10] Thus reduced levels of perceived mental health may have physical repercussions that translate back into progression of CHF. We previously showed that performance score (6-min walk test) of CHF patients is strongly associated with reduced perceived mental health.[30] Telomere length and psychological well-being are both related to the severity of CHF.[4,31] However, the observed association between telomere length and psychological well-being persisted also after adjustment for several measures of heart failure severity (ejection fraction, NYHA functional class and renal function). Taken together, reduced perceived mental health clusters with shorter telomere length and is associated with decreased functional status. Because of the cross-sectional design, we cannot draw definite conclusions on whether decreased telomere length is a cause or a consequence of reduced perceived mental health.

Others have also reported circumstantial proof of the relationship between stress and telomere biology. Recently, a higher level of pessimism was associated with shorter telomere length in leukocytes of healthy post-menopausal women.[32] In this study, pessimism was also associated with higher basal levels of IL-6, an indicator of systemic inflammation and possibly immune system aging. Interestingly, also activation of the renin-angiotensin-aldosterone system is associated with increased oxidative stress and inflammation.[33] Recently, activation of the reninangiotensin-aldosterone system has been associated with reduced leukocyte telomere length in participants of the Framingham Heart Study.[34]

Our findings further support the notion that psychological well-being might be implicated in accelerated biological aging. Given the importance of psychological well-being in determining cardiovascular disease and outcome, our findings might have potential clinical relevance.[18,35] However, as we do not have longitudinal telomere or psychological stress measurements, we are not

able to draw conclusions regarding the causality of the observed relationship. Future studies are needed to 1) replicate our findings and 2) to study the nature of the observed associations.

Although a clear association seems to exist between telomere length and reduced perceived mental health in general, we were unable to confirm previously reported associations with depressive symptoms and reduced telomere length in leukocytes.[7] This might be due to the simple fact that telomere length is a reflection of cumulative environmental factors over time, and the CES-D score is based on the situation of the patient in the past week only. In addition, this null finding could be due to the population under study as in CHF the additional influence of depressive symptoms on telomere length might be relatively small compared to the condition of CHF itself.

In conclusion, shorter telomere length was associated with lower perceived mental health in patients with CHF. Future replication and longitudinal mechanistic studies will be needed to address the causality of this relationship.

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CHAPTER 4

Circulating leukocyte and carotid atherosclerotic plaque telomere length: interrelation, association with plaque characteristics, and restenosis after endarterectomy.

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ABSTRACT

Objective

Shorter leukocyte telomeres are associated with atherosclerosis and predict future heart disease. The goal of the present study was to determine whether leukocyte telomere length is related to atherosclerotic plaque telomere length and whether it is associated with plaque characteristics or recurrence of disease.

Methods and Results

Telomere length was measured by real-time quantitative polymerase chain reaction in atherosclerotic plaques and leukocytes in patients with carotid atherosclerosis undergoing carotid endarterectomy (n=684) and of leukocytes in age- and gender-balanced subjects without clinical atherosclerosis (n=780). Leukocyte telomere length was shorter in patients versus controls (0.99 [interquartile range (IQR): 0.79 to 1.26] versus 1.06 [0.80 to 1.39]; $p<0.001$). Plaque telomeres were longer than leukocyte telomeres (1.42 [IQR: 1.21 to 1.77] versus 1.01 [IQR: 0.75 to 1.34]; $p<1.00 \times 10^{6}$) and independent of age. Leukocyte and plaque telomere length were only weakly correlated (correlation coefficient $r^2= 0.04$, $p= 0.03$). Patients, whose plaques showed marked macrophage infiltration and large lipid core, had longer plaque telomeres (1.61 [IQR: 1.32 to 2.04] versus 1.40 [IQR: 1.15 to 1.57]; $p= 0.006$) and shorter leukocyte telomeres (0.88 [IQR: 0.75 to 1.20] versus 1.03 [IQR: 0.83 to 1.34]; $p= 0.02$). Plaque telomere length was associated with restenosis 1 year after endarterectomy (OR 1.580.206; $p= 0.026$ per SD decrease of plaque telomere length).

Conclusion

Leukocyte telomere length is associated with the presence of atherosclerotic carotid plaques but is not a proxy for local plaque telomere length. Plaque telomere length is related to plaque characteristics and development of restenosis following endarterectomy.

Telomere length is an indicator of replicative history and is considered a biomarker of aging.[1] In addition to cell divisions, external factors such as oxidative stress can cause additional telomere attrition in vitro.[2,3] When telomeres reach a critically short length, the cell enters a nondividing state called senescence and becomes dysfunctional.[4] Atherosclerosis is characterized by a high percentage of senescent endothelial and vascular smooth muscle cells.[5,6] Two small studies have observed reduced telomere length in atherosclerotic lesions compared with healthy vascular tissue of the same subject.[6,7] However, the majority of clinical data relating telomere length to atherosclerosis, coronary heart disease, myocardial function, cardiovascular risk factors, and clinical events are derived from telomere lengths measured in circulating leukocytes.[8–16] Only 1 study of 32 subjects evaluated the association between leukocyte telomere length and abdominal aortic vascular tissue, and this study reported a strong association irrespective of the presence of vascular disease.[17] This contradicts earlier reports observing major differences in telomere length between atherosclerotic plaque and healthy vascular tissue derived from a single subject.[5,6]

Whether leukocyte telomere length is a reliable reflection of atherosclerotic plaque telomere length remains to be established. Therefore, we determined telomere length in atherosclerotic carotid plaques and of circulating leukocytes from subjects undergoing carotid endarterectomy. We also studied the associations of telomere length with biochemical and histopathologic characteristics of disease. Previous research has shown that inflammatory, lipid-rich plaques on histopathologic examination were associated with a lower risk of restenosis,[18] possibly because of remodeling and cell turnover induced by plaque remnants. We therefore also evaluated the relation between telomere length and recurrence of local disease, measured by carotid restenosis, after 1 year of follow-up.

METHODS

Design and Subjects

We performed a case-control study to determine the association between circulating leukocyte telomere length and the presence of carotid artery stenosis. A detailed description is provided in the Supplemental Data, at the end of this chapter. In brief, subjects undergoing carotid endarterectomy (cases) were derived from the Athero-Express study (the Netherlands).[18,19] Age

and gender-balanced presumably healthy subjects (controls) were derived from the population based PREVEND study (the Netherlands).[20] In cases, blood was drawn before surgery. In addition, plaques were harvested in cases during carotid endarterectomy, and plaque telomere length was compared intraindividually to that of circulating leukocytes.
Telomere length of both circulating leukocytes and plaques were studied in cases in relation to clinical, biochemical, and histopathologic characteristics of disease. Recurrence of disease and clinical outcome was evaluated by duplex follow-up at 1 year after endarterectomy to determine the presence of restenosis. Study protocols were approved by the appropriate institutional review boards and comply with the declaration of Helsinki. All subjects provided written informed consent.

Atherosclerotic Plaque Characterization
During surgery, atherosclerotic plaques were freshly harvested and divided into sections of 5 mm thickness. The segment with the greatest plaque burden was stained for histological examination with hematoxylin-eosin, CD-68 immunostain, alpha-actin, and picrosirius red. For details, see Supplemental Data.

Telomere Length
Telomere length was measured in triplicate using a real-time monochrome multiplex quantitative polymerase chain reaction method using a single-well strategy to measure telomere (T) relative to a single reference (S) signal based on the albumin gene.[21,22] A single fluorescent DNA-intercalating dye (SYBR) was used to collect the T signals in early cycles, before S signal rises above baseline, and the S signals was collected at a temperature that fully melts the T product, sending its signal to baseline.[21] The ratio of telomere and reference gene content (T/S ratio) is a relative measure of telomere length. All experimental DNA samples were assayed in triplicate. Seven concentrations of a reference DNA sample (standard) spanning an almost 12-fold range (5.2 to 60 ng) of DNA concentrations were prepared by serial dilution and analyzed in triplicate in every 384-well plate. Good linearity was observed across this range (R^2= 0.99). Two wells received water as the no-template control, two wells were loaded with a human control sample, and two were loaded with DNA of a human leukemia cell line (1301) with extreme long telomeres (kindly provided by Dr Cesaro, IST, Genova) as a positive/maximum control. For quality control, all samples were checked for concordance between triplicate values. The final coefficient of variation for the T amplicon was 1.52%, for the S amplicon it was 1.17%, and for T/S it was 3.3%. Reproducibility data were

obtained for 216 subjects from PREVEND, and good agreement between T/S ratios, measured on different days, was observed (r^2= 0.99, P< 0.0001; interrun coefficient of variation, 3.9%). For details, see Supplemental Figure I and other supplemental material.

Follow-Up

The primary end point of the Athero-Express study was the occurrence of 50% or greater restenosis measured 1 year after intervention as determined by duplex ultrasound on the basis of the recommendations of the Society of Radiologists in Ultrasound.[18,23] For details, see Supplemental Data.

Statistical Methods

Because of the skewed distribution, telomere length (T/S ratio) was log transformed. Controls were matched on age and gender with a propensity score matching algorithm (psmatch2)[24] without other clinical, biochemical, or telomere length knowledge. Differences in patient characteristics between groups were tested by t tests and chi-square test when appropriate. A paired sample t-test was used to compare intraindividual plaque versus leukocyte telomere length. Pearson and standard linear regression techniques were used to associate telomere length with individual factors and to make adjustments. One primary clinical end point was defined: occurrence of 50% restenosis after 1 year. Logistic regression models were used to estimate the odds of experiencing the primary end point. Covariates that were introduced were univariately associated with restenosis (lipid core size and macrophage infiltration) or associated with plaque telomere length (diastolic blood pressure, diabetes). Analyses were performed using StataMP, version 10.1 (StataCorp) and SPSS, version 14.0 (SPSS Inc, Chicago, IL). A 2-sided probability value of <0.05 was interpreted to indicate statistical significance. An expanded description of the methods is provided in the Supplemental Data.

RESULTS

Population Characteristics

The median age of patients with carotid atherosclerosis was 73 years (interquartile range [IQR]: 66 to 79), and for control subjects without clinical atherosclerosis, it was 72 years (IQR: 66 to 78). In both groups, 69% of subjects were male.

Clinical and biochemical baseline characteristics of cases with carotid atherosclerosis (n=684) are presented in Table 1.

For cases, 785 DNA samples were collected from atherosclerotic carotid plaques or leukocytes; from 101 subjects, both leukocyte and plaque samples were available. Clinical and biochemical characteristics for subgroups of cases are presented in Supplemental Table I. Detailed histological plaque characteristics are presented in Supplemental Table II.

Table 1. Clinical and biochemical baseline characteristics of patients and their associations with leukocyte and plaque telomere length

Characteristics	Baseline Value	ß for Leukocyte TL (95% CI)	P	ß for Plaque TL (95% CI)	P
Age (years)	73 (66-79)	-0.007 (-0.012 to -0.003)	0.001	-0.001 (-0.004 to 0.001)	0.258
Male gender (%)	69.0	-0.072 (-0.150 to 0.007)	0.072	-0.067 (-0.180 to -0.016)	0.010
Diabetes (%)	22.9	-0.002 (-0.090 to 0.086)	0.965	-0.065 (-0.123 to -0.007)	0.028
Antihypertensives (%)	90.4	0.044 (-0.093 to 0.181)	0.532	0.031 (-0.064 to 0.125)	0.520
Statin use (%)	75.5	0.038 (-0.044 to 0.120)	0.361	-0.007 (-0.064 to 0.051)	0.821
Current smoker (%)	27.6	0.043 (-0.037 to 0.124)	0.288	0.017 (-0.040 to 0.074)	0.553
Systolic BP (mm Hg)	155 (140-172)	0.000 (-0.001 to 0.002)	0.605	0.00 (0.000 to 0.001)	0.628
Diastolic BP (mm Hg)	80 (75-90)	0.001 (-0.002 to 0.004)	0.611	0.002 (0.000 to 0.004)	0.019
(hs)CRP (mg/L)	3.2 (1.37-6.93)	0.001 (-0.002 to 0.003)	0.279	-0.001 (-0.004 to 0.003)	0.219
Hemoglobin (mmol/L)	8.7 (8.1-9.3)	-0.015 (-0.052 to 0.023)	0.446	0.015 (-0.008 to 0.039)	0.192
HDL cholesterol (mmol/L)	1.07 (0.86-1.33)	0.021 (-0.016 to 0.059)	0.268	-0.001 (-0.043 to 0.040)	0.950
LDL cholesterol (mmol/L)	2.65 (2.05-3.35)	-0.031 (-0.133 to 0.071)	0.549	0.050 (-0.062 to 1.161)	0.379
Triglycerides (mmol/L)	1.38 (0.99-2.01)	-0.013 (-0.053 to 0.027)	0.530	-0.027 (-0.078 to 0.023)	0.291

Shown are the median (IQR) values or percentages.
TL indicates telomere length; BP, blood pressure; hs, high-sensitivity; CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Leukocyte Telomere Length in Cases and Controls

Circulating leukocyte DNA was available from 390 cases. For each case, we selected 2 controls. A total of 780 presumably healthy age and gender balanced controls were included. Circulating leukocyte telomere length was considerably shorter in subjects with carotid atherosclerosis compared with controls (0.99 [IQR: 0.79 to 1.26] versus 1.06 [0.80 to 1.39]; p= 0.0007) (Figure 1).

This difference was independent of age and gender. Leukocyte telomere length was associated with age (ß = -0.11, p= 0.0002). Addition of squared or cubed age terms was nonsignificant, indicating a linear relationship. In addition, adding an interaction term for age*case-control status was not significant (p= 0.216), indicating a similar age-telomere length correlation in leukocytes for cases and controls.

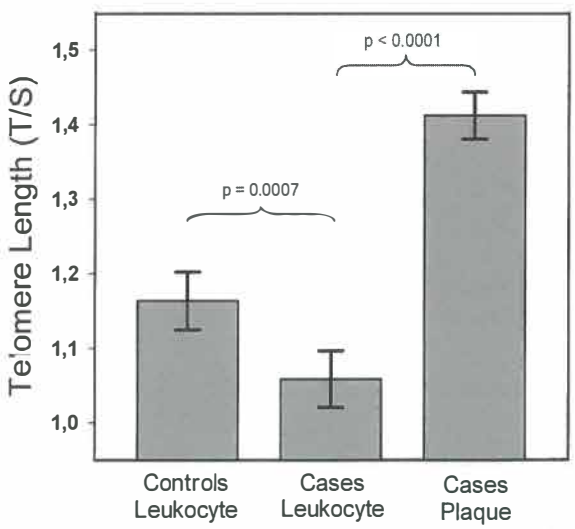


Figure 1. Leukocyte and plaque telomere length in cases and controls. Shown are mean leukocyte and plaque telomere length in carotid artery stenosis cases and leukocyte telomere length in healthy controls. Whiskers represent 95% confidence intervals.

Comparison of Leukocyte Telomere Length and Plaque Telomere Length

We observed a remarkably large difference between telomere lengths of circulating leukocytes compared with plaques for the total group (Figure 1). This did not change when we considered only the subgroup of which both telomere lengths were available (Supplemental Figure II). Median leukocyte telomere length was 1.01 (IQR: 0.75 to 1.34), and median plaque telomere length was 1.42 (IQR: 1.21 to 1.77), $p< 1.0x10^{-6}$. This difference was not explained by age and gender.

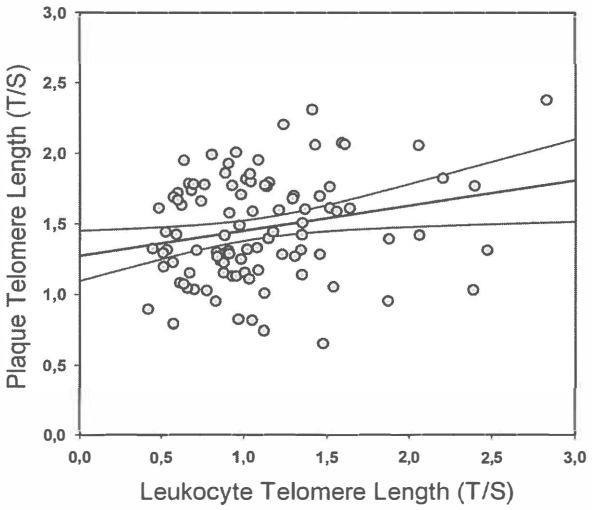


Figure 2. Correlation between leukocyte and plaque telomere length within the same subject for 101 subjects of whom both these measurements were available. Partial correlation coefficient controlling for age and gender = 0.215, p= 0.033.

To determine whether leukocyte telomere length is a reliable reflection of plaque tissue telomere length, we evaluated their pairwise correlation. We observed a positive, though weak, correlation (r= 0.210, p= 0.034) (Figure 2). After we controlled for age and gender, the partial correlation coefficient remained similar (0.215, p= 0.033).

Telomere Length and Clinical and Biochemical Characteristics

Associations of leukocyte and plaque telomere length with clinical and biochemical characteristics for patients are presented in Table 1. In contrast with leukocyte telomere length, atherosclerotic plaque telomere length was not associated with age (Figure 3). Male patients tended to have shorter telomeres, although this did not reach statistical significance in leukocytes. Patients with diabetes had shorter plaque telomere length compared with nondiabetic patients (median T/S ratio: 1.32 [IQR: 1.10 to 1.48] versus 1.41 [IQR: 1.19 to 1.67], P= 0.028; Table 1). Finally, plaque telomere length was associated with diastolic blood pressure. These associations persisted after adjustment for age and gender; ß (+95% confidence interval) was as follows: for male gender, -0.067 (-0.118 to -0.016), p= 0.01; for diabetes, -0.063 (-0.121 to -0.005), p= 0.032; and for diastolic blood pressure, 0.002 (0.001 to 0.004), p= 0.038.
Clinical presentation of the patient was not associated with leukocyte or plaque telomere length (see Supplemental Table III).

Between asymptomatic and symptomatic patients, there was also no difference in leukocyte telomere length (median T/S ratio: 1.06 [IQR: 0.82 to 1.30] versus 0.98 [0.79 to 1.26], p= 0.52) or plaque telomere length (1.39 [1.09 to 1.67] versus 1.38 [1.18 to 1.65], p= 0.26).

Telomere Length and Histopathologic Characteristics

When analyzing plaque content, we noticed that plaque telomere length was shorter in restenosis-prone noninflammatory, fibrous plaques compared with the more restenosis resistant inflammatory atheromatous plaques: 1.40 (IQR: 1.15 to 1.57) versus 1.61 (IQR: 1.32 to 2.04), p= 0.006 (Table 2). However, telomere length in leukocytes of subjects with inflammatory atheromatous plaques were shorter compared with the noninflammatory fibrous plaques: 0.88 (IQR: 0.75 to 1.20) versus 1.03 (IQR: 0.83 to 1.34), p= 0.022 (Table 2). Adjustment for age, gender, blood pressure and diabetes did not change these findings (Figure 4). Plaque collagen or smooth muscle cell content was not associated with telomere length.

Figure 3. Association of leukocyte and plaque telomere length with age, visualized by the regression line and its 95% confidence interval. Plaque telomere length is longer than leukocyte telomere length and does not decline with increasing age.

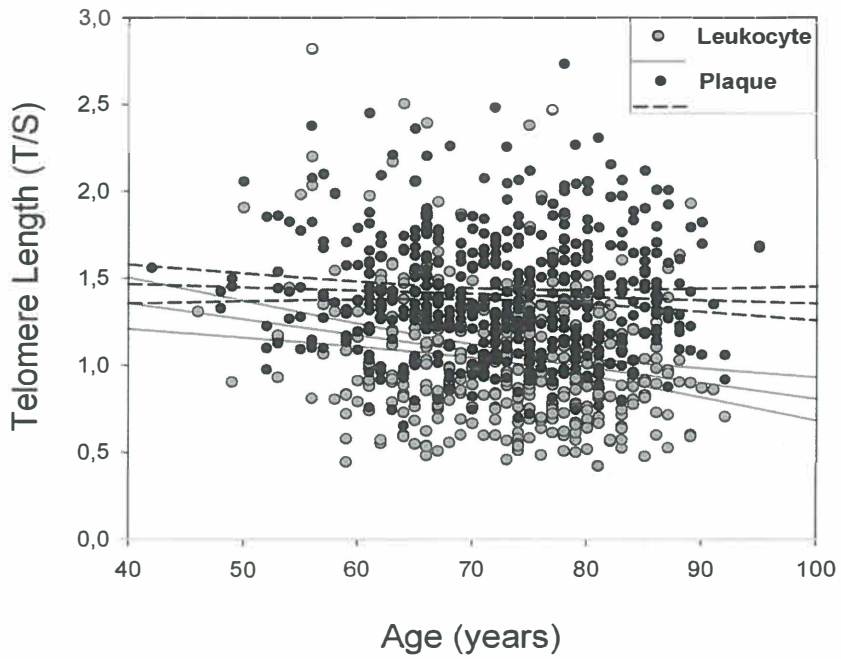
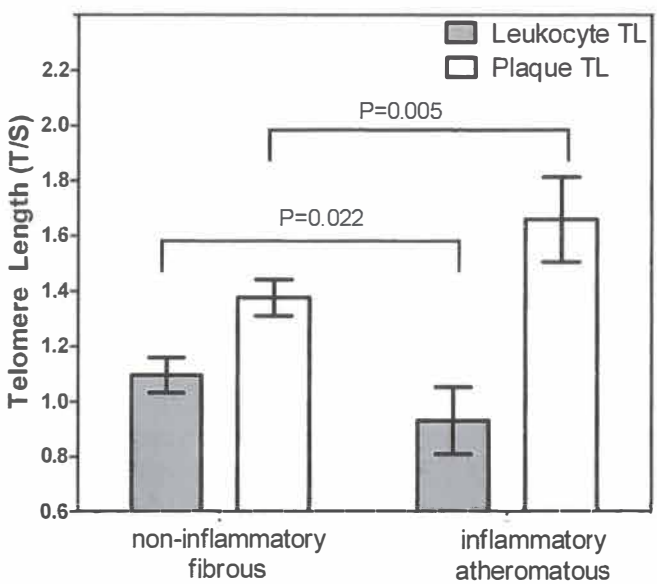


Table 2. Histopathological Plaque Characteristics and Telomere Length

	Leukocyte Telomere Length				Plaque Telomere Length			
Characteristic	**N**	**T/S***	**IQR**	**P**	**N**	**T/S***	**IQR**	**P**
Lipid inflammation								
Noninflammatory fibrous	330	1.03	0.83-1.34	0.02	307	1.40	1.15-1.57	0.006
Inflammatory atheromatous	61	0.88	0.75-1.20		85	1.61	1.32-2.04	
Smooth muscle cell								
None/minor	115	0.96	0.76–1.23	0.79	110	1.42	1.15-1.67	0.59
Moderate/heavy	276	1.02	0.81–1.29		282	1.37	1.18-1.64	
Collagen								
None/minor	80	0.92	0.76-1.28	0.68	66	1.35	1.18-1.69	0.62
Moderate/heavy	311	1.02	0.82-1.28		326	1.39	1.17-1.64	

* median T/S

Figure 4. Adjusted leukocyte and plaque telomere length (TL) and histopathologic characteristics. Whiskers represent 95% confidence intervals.



Telomere Length and Incidence of Restenosis at 1 Year

The incidence of restenosis of 50% or greater 1 year after carotid endarterectomy was evaluated with duplex ultrasound, and the overall restenosis incidence in our study was 17%. A plaque telomere length that was 1 SD shorter was associated with a 58% increased restenosis risk (p= 0.026; Table 3).

This observation persisted after adjustment for age, gender, and other covariates associated with plaque telomere length (diabetes and diastolic blood pressure) or occurrence of restenosis (lipid core size and macrophage infiltration; p= 0.038, Table 3). Circulating leukocyte telomere length was not an independent predictor of carotid restenosis at 1 year (Supplemental Table IV).

Table 3. Logistic Regression Analysis for 50% or Greater Restenosis (Primary End Point) for Plaque Telomere Length

	Univariate		Full adjusted model	
Characteristic	**OR (95% CI)**	**P**	**OR (95% CI)**	**P**
Plaque telomere length, per SD shorter	1.58 (1.06 to 2.37)	0.026	1.65 (1.03 to 2.65)	0.038
Age, per 10 years	0.81 (0.62 to 1.06)	0.126	0.68 (0.41 to 1.11)	0.125
Male gender	1.22 (0.74 to 2.00)	0.433	0.99 (0.36 to 2.73)	0.978
Lipid core size*				
10% to 40%	0.60 (0.34 to 1.04)	0.070	0.30 (0.10 to 0.88)	0.028
>40%	0.32 (0.17 to 0.62)	0.010	0.32 (0.09 to 1.13)	0.077
Macrophage infiltration	0.44 (0.27 to 0.70)	0.010	0.49 (0.19 to 1.27)	0.142
Diastolic blood pressure	1.01 (0.99 to 1.03)	0.590	1.02 (0.98 to 1.06)	0.361
Diabetes	0.93 (0.52 to 1.65)	0.810	1.36 (0.44 to 4.18)	0.597

*Compared with <10%.

DISCUSSION

In the current study, we observed that leukocyte telomere length in subjects with carotid atherosclerosis is shorter compared with age- and gender-balanced controls without clinical atherosclerosis. Previous research already reported no association between leukocyte telomere length and preclinical atherosclerosis,[8,13] whereas the association between telomere length and clinically significant atherosclerosis is well established.[9-14,25] We now confirm this association specifically in carotid atherosclerosis. In contrast to leukocyte telomere length, little is known about the potential differences in telomere length

of cells originating from different tissues. We therefore measured telomere length in circulating leukocytes, as well as in atherosclerotic plaques, and made 4 important observations. First, we showed that telomeres in atherosclerotic plaques are considerable longer than those in circulating leukocytes. Second, in contrast to leukocyte telomere length, plaque telomere length was not associated with age. Third, although we did observe a statistically significant correlation between the 2 samples obtained from the same individual, the correlation was weak and explained less than 5% of the variance. Finally, we showed that leukocyte and plaque telomere length are differently related to atherosclerotic plaque characteristics.
In contrast to the study by Wilson et al,[17] who reported an r ranging from 0.44 in subjects with asymptomatic abdominal aortic aneurysms to 0.68 in normal aortas, we found only a weak correlation between leukocyte and tissue telomere length. The difference is not likely to arise from telomere length measurement method, as both our study and that of Wilson et al[17] used quantitative polymerase chain reaction. Also, the age of the studied subjects was fairly similar. It is possible that the severity of local vascular disease might obscure the correlation with blood leukocyte telomere length. Wilson et al reported the strongest correlation in vessels from normal aortas of subjects who experienced intracerebral hemorrhage.[17] The correlation in asymptomatic vascular disease was less strong. In our population, consisting of subjects with significant and symptomatic carotid atherosclerosis, the correlation observed was weak. In addition, our sample size was considerably larger that that of Wilson et al,[17] who studied 32 vessel wall and blood leukocyte pairs.

The weak correlation that we observed suggests that telomere length in 1 cell type does not necessarily provide a good surrogate for the relative telomere length in other tissues. This implies that the extrapolation of telomere length associations observed in leukocytes to that of the atherosclerotic vessel merits careful consideration. There are several potential explanations for the absence of a strong association between leukocyte and plaque telomere length. We not only have to realize that both samples consist of a heterogeneous population of cells; we also need to appreciate the difference in replicative history of leukocytes compared with vessel wall derived cells. The replicative history might be very different as the cells circulating leukocytes originate from are thought to divide far more frequently than vascular cells. Also specifically in carotid atherosclerotic plaques, a very long turnover time has recently been reported.[26] Furthermore, telomere length can also be modified by the telomere elongating enzyme telomerase.[1] There is evidence that the activity of this enzyme differs between different types of tissue and in different situations.[27] In

healthy rat carotid arteries, telomerase activity is barely detectable. However, after balloon injury there is a 10-fold increase of telomerase activity.[28] In patients with unstable angina, inflammatory cells present in the coronary artery plaque express considerably higher telomerase activity compared with circulating leukocytes.[29] Local leukocyte telomerase reactivation in plaques is thought to prolong the lifespan of inflammatory cells, which might make it possible to maintain the inflammatory response. This phenomenon could be an explanation for the longer plaque telomeres we found in inflammatory lipid-rich plaques, as well as for the lack of correlation of plaque telomere length with age. These telomere length modifying factors and their interplay together could be held responsible for the seemingly paradoxical difference in telomere biology we observed between leukocyte and plaque tissue. Unfortunately, we did not have live cells available from the Athero-Express study to measure telomerase activity.

Recently, data from the Athero-Express study showed that the marked presence of inflammation and lipid-rich plaques reduced risk of restenosis during follow-up.[18] With the current study, we add the observation that these plaque characteristics are also related to telomere length, but differently for leukocytes compared with plaque tissue. Circulating leukocyte telomere length was strongly associated with the presence of carotid atherosclerosis in the current study. However, among patients experiencing carotid atherosclerosis and undergoing endarterectomy, we did not find an association between leukocyte telomere length and restenosis at 1 year. This might indicate that the involvement of circulating leukocyte telomere length is more relevant early in the process of atherosclerosis and that local processes are more relevant in the restenosis process. In previous studies, it has been suggested that the plaque composition at baseline is related to the dynamic vascular remodeling process after the endarterectomy procedure.[18] Inflammation and protease activity in the media and adventitia (the layers remaining after endarterectomy) might give rise to production of matrix metalloproteinases, which leads to thinning of the media and expansive remodeling as opposed to luminal narrowing.[18] One could speculate that the atherosclerotic plaques in which we measured shorter mean telomere lengths leave behind also inflammatory cells with shorter telomere lengths in their media/adventitia, which is related to an impaired inflammatory capacity. This could be related to decreased media thinning and remodeling and therefore a higher risk of restenosis. These are all indicators that we should not use telomere length of circulating leukocytes as a proxy for that of the plaque, or more generally, target tissue of interest.

Telomere length of leukocytes was shorter in subjects with carotid atherosclerosis and associated with age. Interestingly, in cases with atherosclerosis, plaque

telomere lengths were similar among different ages. It is tempting to speculate that a subject becomes a case at a certain threshold determined by his or her biological age as estimated by leukocyte telomere length and possibly unrelated to their date of birth.

There are some limitations to this study. Because ourcontrol cohort had a median age of 72 and its members were not specifically screened for presence or absence of carotid atherosclerosis, subclinical atherosclerosis is to be expected to be omnipresent. Consequently, the observed difference between cases and controls might be an underestimation of the true difference between cases and truly healthy subjects. Also, the use of flow velocity in the definition of restenosis could be considered a limitation as these criteria are based on consensus not commonly accepted and might have their limitations in categorizing stenosis. Finally, we studied mean telomere length of DNA isolated from the overall leukocyte population and the overall cell population of the plaque. We cannot exclude the possibility that telomere length of the different cell populations within leukocytes or tissue is different. Plaque tissue harvested consisted mainly of intima, though traces of media cannot be excluded. A future major methodological challenge remains to analyze the individual component of the plaques (eg, with laser dissection techniques or magnetic beads), which will provide additional insights.

In conclusion, in contrast to what has previously been suggested, we have demonstrated that telomere length of circulating leukocytes does not provide a good surrogate for telomere length of the atherosclerotic plaque. Circulating leukocyte and atherosclerotic plaque telomere lengths are associated differently with plaque composition. Restenosis at 1 year appears to be associated with plaque telomere length. The current findings justify further research to determine differences among cells characterized in more detail.

ACKNOWLEDGEMENTS

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SUPLLEMENTAL MATERIAL

Study Design and Population

To determine the association between circulating leukocyte telomere length and carotid artery stenosis we performed a case-control study. Cases were subjects undergoing carotid endarterectomy. Cases were derived from the Athero-Express study, of which the design has been reported in detail previously.[1,2] The criteria to perform carotid endarterectomy were based on the recommendations by the Asymptomatic Carotid Atherosclerosis Study and Asymptomatic Carotid Surgery Trial studies for asymptomatic patients and the North American Symptomatic Carotid EndarterectomyTrial and European Carotid Surgery Trial studies for symptomatic patients.[2] In brief, blood and carotid plaques of patients undergoing primary carotid artery endarterectomy were collected. After one year patients underwent duplex follow-up to determine target vessel patency. The primary endpoint of the study was the presence of restenosis which was defined as > 50% restenosis of the operated carotid artery after one year.

Presumably healthy subjects were derived from the population based Prevention of REnal and Vascular ENd stage Disease (PREVEND)[3] study, an ongoing prospective study investigating the natural course of increased levels of urinary albumin excretion and its relation to renal and cardiovascular disease in a large cohort drawn from the general population. Details of this protocol have been described elsewhere.[3,4] The PREVEND study includes a total of 8,592 subjects. To account for major differences in age and gender, we selected two age and gender balanced controls for each case using the psmatch2 algorithm[5] (STATA).

Differences between telomere lengths of cells derived from circulating leukocytes were compared with cells derived from plaques in subjects undergoing carotid endarterectomy and of whom both samples were available (within subject comparison). Finally, in cases telomere lengths of both circulating leukocytes and plaques were studied in relation to clinical, biochemical and histopathological characteristics.

Clinical outcome was evaluated by duplex follow-up at one year after endarterectomy to determine the presence of restenosis.

Atherosclerotic Plaque Characterization

During surgery, directly after excision, the atherosclerotic plaque specimenswere taken to the laboratory. Plaques were divided in segments of 5-mm thickness along the longitudinal axis. The thickest plaque segment

was fixed in formalin for immunohistochemical staining and remaining parts were freshly snap frozen to study protease activity and future protein and RNA expressions. The segment with the greatest plaque burden area was defined as the culprit lesion and used for histological examination.[1] Semiquantitative scores were made for macrophage inflitration using CD-68, smooth muscle cell infiltration with alpha-actin, the amount of collagen with Picro-Sirius Red and the lipid core size with hematoxylin and eosin and Picro-Sirius Red stains. The scores for collagen and smooth muscle cells were composed as follows: 1: none or minor, 2: moderate or heavy staining. Inflammatory atheromatous plaques were categorized as follows: 1: no or minor macrophage infiltration and lipid core <40%, 2: moderate or heave macrophage infiltration and lipid core >40%. Histological observations were made blinded for clinical data and performed by two independent observers. The inter-rater and intra-rater reproducibilitywas assessed in 100 specimens. Briefly, 100 specimens were assessed by two independent observers and the ratings of both observers were compared with statistics. To assess intra-observer reproducibility, the second observer reassessed the specimens two months afterwards with blinding for the previous assessments of the plaques. Both inter-observer and intra-observer reproducibilitywere found to be excellent (κ = 0.6-0.9).[6]

Telomere length measurements

Mean telomere length was measured with the recently modified QPCR protocol using a single well strategy to measure to the telomere (T) and single reference (S) signal.[7] All experimental DNA samples were assayed in triplicate.[7,8] The ratio of telomere and reference gene content (T/S ratio) is a relative measure of telomere length. PCR reactions were set up by alliquoting 8 µL of master mix into each well reaction of a 384-well plate compatible with our Bio-Rad CFX384 real-time system on a C1000 thermal cycler, followed by addition of 2µl DNA (~20ng), for a final volume of 10 µL per reaction. Seven concentrations of a reference DNA sample (standard) spanning a ~12-fold range (5.2 to 60 ng) of DNA concentrations were prepared by serial dilution and analyzed in triplicate in every 384-well plate. Good linearity was observed across this range (r^2 = 0.99). Two wells received water as the no template control (NTC), two wells were loaded with a human control sample and two with DNA of a human leukemia cell line (1301) with extreme long telomeres (kindly provided by dr. Cesaro, IST, Genova) as a positive/max control. The final concentrations of reagents in the PCR were 1U Titanium Taq DNA polymerase with the provided Titanium Taq PCR buffer, 0.75 x SYBR Green I (Sigma), 0.2 mM of each dNTP, 1 mM DTT, 1M betaine, 900nM of each telomere primers (Telg and Telc),

900nM of each albumin (Albu and Albd). The primers were; telomere, telg, ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT and telc, TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA, that generate a short, fixed length product (for a further explanation and details see Cawthon 2009).[7] The S albumin primers were albu: CGGCGGCGGGCGGCGCGGGCTGGGCGGaaatgctgcacagaatccttg albd: GCCCGGCCCGCCGCGCCCGTCCCGCCGgaaaagcatggtcgcctgtt. The predicted product size is 106 bp. Capitalized bases of the albumin primers are non-template 5' tag sequences that confer a high melting temperature on resulting PCR product (for a further explanation and details see Cawthon 2009).[7] The thermal cycling profile was Stage 1: 15 min at 95°C; Stage 2: 2 cycles of 15 s at 94°C, 15 s at 49°C; Stage 3: 5 cycles of 15 s at 94°C, 15 s at 66°C; Stage 4: 32 cycles of 15 s at 94°C, 10 s at 60°C, 15 s at 72°C with signal acquisition, 10 s at 85°C, and 15 s at 89°C with signal acquisition. Stage 5; for QC a final dissociation stage was performed from 60°C to 95°C in steps of 0.05 s. At stage 4; the 72°C reads provide Ct values for the amplification of the telomere template (in early cycles when the S signal is still at baseline); the 89°C reads provided the Ct values for the amplification of the S template (at this temperature there is no signal from the telomere PCR product, because it is fully melted). For the reference DNA sample, each DNA concentration the Ct for albumin occurred ~ 7.2 cycles later in cycling than the Ct for the telomere. The Bio-Rad CFX manager software was used to generate two standard curves for each plate as previously described.[7] For quality control all samples were checked for concordance between triplicate values. The final coefficient of variation for the T amplicon was 1.52%, and for the S amplicon 1.17% and for T/S 3.3%. Reproducibility data was obtained for 216 subjects from PREVEND and good agreement between T/S ratios, measured on different days, was observed (r^2=0.99, p<0.0001, inter-run CV 3.9%).[7,8] See figure II

Follow-up

Clinical outcome was evaluated by duplex follow-up at 1 year after endarterectomy to determine the presence of restenosis (defined as >50% restenosis).[2,9] Patients underwent follow-up with duplex ultrasound (PhilipsMedical Systems, Eindhoven, the Netherlands). The definition of occurrence of 50% or greater restenosis was defined as a peak systolic velocity of at least 125 cm/s at the ipsilateral bifurcation.[2,10] Duplex measurements were performed by investigators who were blinded for data regarding plaque phenotype and baseline characteristics.[1]

Supplementart table I. Clinical and biochemical baseline characteristics of cases divided for availability of DNA (plaque, circulating leukocytes, both).

Characteristics	Plaque only Group (n= 291)	Leukocyte only Group (n=292)	Combined Group (n= 101)	p-value (*)
Age (years)	73.0 (66.0–80.0)	73.5 (67.0-9.0)	73.0 (66.0–80.0)	0.846
Females (%)	30.6	31.5	31.7	0.440
Diabetes (%)	22.8	24.1	19.8	0.390
Antihypertensives(%)	91.1	90.1	89.2	0.420
Statins use (%)	79.7	72.7	73.3	0.022
Current smoker (%)	27.0	29.7	23.0	0.272
Systolic BP (mmHg)	154 (140–170)	155 (140–172)	160 (140–175)	0.176
Diastolic BP (mmHg)	83 (75–90)	80 (75–90)	80 (75–90)	0.160
Ln Hs-CRP (mg/dL)†	3.34 (1.68–5.96)	3.30 (1.34–7.73)	3.2 (1.31-5.60)	0.405
Hb (mmol/L)	8.7 (8.1–9.3)	8.7 (8.0–9.2)	8.8 (8.2–9.4)	0.660
Lipids (mmol/L)				
-HDL cholesterol ‡	0.94 (0.72–1.36)	1.06 (0.88–1.33)	1.11 (0.92-1.33)	0.001
- LDL cholesterol §	2.37 (1.92–3.05)	2.70 (2.12-3.39)	2.53 (2.06–3.21)	0.432
-Triglycerides	1.23 (0.97–1.89)	1.53 (1.07–2.10)	1.14 (0.90–1.66)	0.871

(*) p-value for difference between leukocyte and plaque group. † High sensitivity C-reactive protein, ‡ High Density Lipoprotein cholesterol, § Low Density Lipoprotein cholesterol. Presented are the median (interquartile range) values or percentages (%).

Supplementary tabel II. Histopathological characterisation of cases

Characteristics	Plaque only Group (n= 291)	Leukocyte only Group (n=292)	Combined Group (n= 101)	p-value (*)
Non-inflammatory fibrous	78.4	84.4	94.4	0.156
Inflammatory atheromatous	21.6	15.6	5.6	
None or minor smooth muscle cells	30.0	28.1	31.0	0.286
Moderate or heavy smooth muscle cells	70.0	71.9	69.0	
None or minor collagen	20.9	16.4	17.0	0.042
Moderate or heavy collagen	79.1	83.6	83.0	

(*) p-value for difference between leukocyte and plaque group. Presented are the percentages.

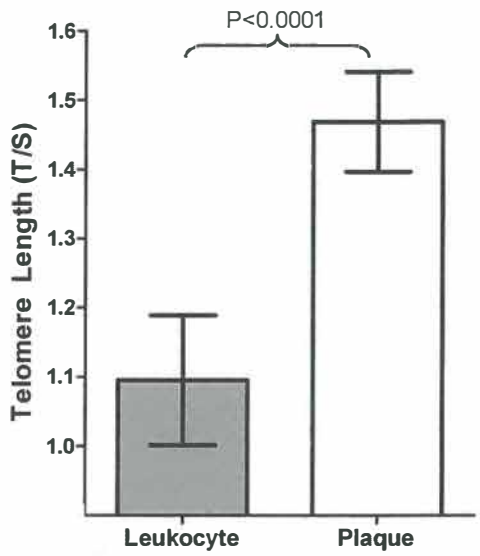
Supplementary table III. Clinical presentation and telomere length

Characteristics	Plaque			Leukocyte		
	n	Median T/S	IQR	n	Median T/S	IQR
Asymptomatic	67	1.06	0.82-1.30	47	1.39	1.09–1.67
TIA	152	0.98	0.76–1.29	185	1.35	1.15-1.60
CVA	100	1.01	0.79–1.26	98	1.42	1.18–1.63
Amaurosis fugax	46	0.98	0.86–1.20	49	1.47	2.23-1.73
Other	27	0.91	0.79-.25	14	1.32	1.23–1.69

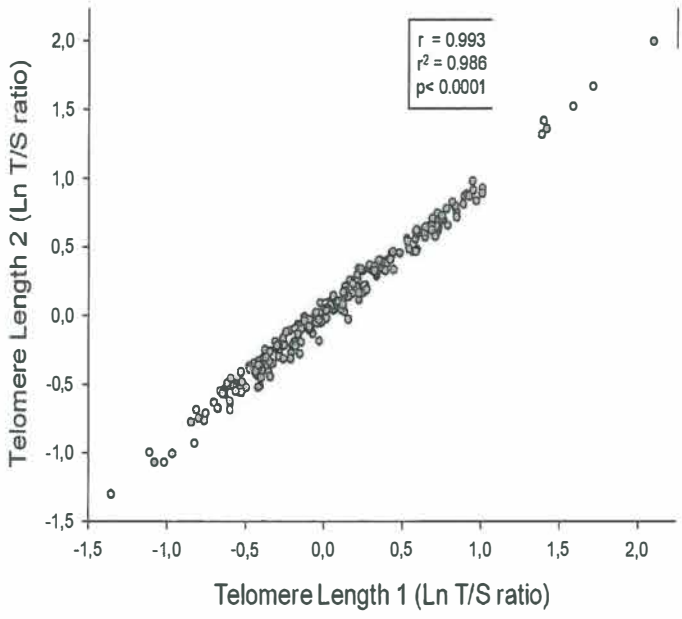
TIA: transient ischemic attack; CVA: cerebrovascular event; IQR: inter quartile range. P-values for differences among groups for leukocyte telomere length: p= 0.93 and for plaque telomere length: p= 0.23.

Supplementart table IV. logistic regression on 50% or greater restenosis (Primary endpoint) for circulating leukocyte telomere length

Characteristics	Restenosis > 50%	
	OR (95% CI)	p-value
Normalised Leukocyte Telomere Length	1.020 (0.764-1.361)	0.894



Supplementary figure I. Mean Telomere length with 95% confidence intervals for carotid artery stenosis cases with both plaque and circulating leukocyte telomere length available



Supplementary figure II. Correlation between telomere lengths of 216 subjects measured on two different occasions.

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CHAPTER 5

Human longitudinal telomere length dynamics

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submitted

ABSTRACT

Background
Human telomere length is considered a biomarker of aging and is associated with cardiovascular risk and events. The determinants of telomere attrition have not been established in a longitudinal study.

Methods
To study the temporal relationship between telomere length and its determinants we measured telomere length by multiplex monochrome real time quantitative polymerase chain reaction of 8,074 subjects of the Prevend study in all available DNA samples of three occasions (at baseline, ~4.3 years, and ~6.6 years of follow-up) in a total of 17,796 samples. Multilevel growth curve models were constructed to identify factors influencing telomere dynamics.

Results
We observed an attrition rate of 0.57 ± 0.078 relative telomere length units per year. Increasing age was associated with accelerated telomere shortening ($p<0.0001$). There was a significant age*gender interaction suggesting a stronger telomere attrition in ageing males (p for interaction 0.047). The major environmental factors determining telomere attrition rate were smoking (effect of smoking -0.67 ± 0.16; $p<0.0001$) and of most components of the metabolic syndrome (waist-hip ratio: -2.66 ± 0.99; $p=0.007$, HDL-cholesterol levels: 0.91 ± 0.24; $p< 0.001$, glucose levels: -0.76 ± 0.38; $p=0.045$). In exploratory analysis, we observed telomere attrition to occur prior, instead of after, the occurrence of a cardiovascular event ($p=0.021$).

Conclusion
The main determinants of accelerated biological aging in humans is smoking and obesity. We provide novel insights in the temporal relationship between telomere attrition and the occurrence of a cardiovascular event.

Biological aging has been suggested to play a role in cardiovascular diseases, one of the leading causes of death in the United States and Europe.[1,2] Telomeres are the terminal ends of linear chromosomes and consist of large numbers of tandem repeats of a simple DNA sequence (TTAGGG in humans). Telomeres are essential structures involved in maintenance of chromosomal stability and cell cycle control.

The length of telomeres have been considered to mark the inter-individual variation of biological aging for several observations: 1) in vitro, somatic cell telomeres appear to shorten progressively with repeated cell divisions, 2) there is a significant genetic determination and variation among individuals at birth and throughout life, 3) in vitro, the amount of telomere lost during each cellular division varies and is affected by environmental factors. In many cell types senescence and cell death often occur in vitro when telomere length reaches a critical value. A considerable number of studies have now shown an association between shorter telomere length and aging associated diseases, including atherosclerosis, coronary heart disease and heart failure.[3-6] Patients with coronary artery disease have telomere lengths comparable to that of ~10 year older healthy individuals.[4,5] In contrast to numerous cross-sectional associations, we know only very little about factors affecting the temporal associations of telomere attrition rate. Therefore we studied telomere length dynamics in a large population based study and aimed to identify the factors associated with temporal changes in telomere length.

METHODS

Study population

This prospective study was performed in the framework of the Prevention of Renal and Vascular End Stage Disease study (PREVEND). The PREVEND study is an ongoing longitudinal cohort study based on the general population aged 28-75 years in the city of Groningen, the Netherlands. For the present study, we excluded subjects of whom a venous blood sample for DNA isolation was not available at any time point.

Details of the study have been described previously.[7,8] In brief 8,592 subjects completed the baseline survey (1997-1998) and were invited to visit the outpatient department at intervals of approximately three years. At each visit, demographic, anthropometric and serum biomarkers were assessed. Detailed information about the measurements and definitions used can be found in

the supplementary methods section at the end of this chapter. Cardiovascular events were defined as the following: acute myocardial infarction (ICD-code 410) or acute- and subacute ischemic heart disease (411). Information about cardiovascular events wase obtained from Prismant, an organisation registering hospital discharge diagnoses.
The PREVEND study has been approved by the local medical ethics committee and is conducted in accordance with the guidelines of the Declaration of Helsinki. All participants provided written informed consent.

Telomere length
To avoid variation in the DNA extraction method, all samples for the current study have been re-extracted using a standard DNA extraction kit (QIamp 96 DNA blood kit, catalogno 51162 of Qiagen, Venlo, the Netherlands) with the original non-defrosted full-blood samples according to the instructions of the manufacturer. DNA from the three different collection points in time were randomized for DNA extraction to avoid batch-effects. Samples with a DNA concentration higher than 70 µg/mL (nanodrop) were diluted to reach a final concentration of 20 – 60 µg/mL. Detailed information on the telomere length measurement is provided in the supplements. In brief, mean leukocyte telomere length was measured without knowledge of clinical data using a novel multiplex monochrome real time quantitative polymerase chain reaction technique.[9] This technique, developed by Cawthon, allows performing the telomere specific duplication and the reference gene duplication in one reaction.[9] Because of specific primer properties double stranded DNA quantities can be evaluated at different temperatures to distinct between telomere and household gene duplicates.[9] Samples were compared to a calibration curve which ran in triplicate on every plate, with a known and fixed DNA concentration. As negative controls water was used and as positive controls DNA was used of a human leukemia cell line (1301) which is known to possess very long telomeres (provided by dr. Cesaro, IST, Genova) The ratio of telomere and household gene content (T/S ratio) is a relative measure of telomere length (RTL) and is expressed in arbitrary units. All samples have been measured in triplicate.
Based on earlier studies, we arbitrary defined three categories of telomere trajectories: 1) RTL shortening, when >10% decrease of RTL was present at T3 compared to baseline, 2) stable RTL for less than 10% change at T3 compared to baseline, and 3) RTL elongation for > 10% increase in RTL at T3 compared to baseline.[10,11]

Cardiovascular events

Cardiovascular events were defined as the following: acute myocardial infarction (ICD-code 410) or acute- and subacute ischemic heart disease (ICD-code 411). Information on non-fatal CVD and CVD-hospitalisation were obtained from Prismant the National Dutch registry of hospital discharge diagnoses.

Statistical analysis

To obtain a normal distribution telomere length was natural log transformed, and the 0,5% extreme values were characterised as outliers and omitted. Other continuous variables with a skewed distribution were also natural log transformed prior to further analysis.

Differences in telomere lengths between groups were tested using student's T-test or one way analysis of variance (ANOVA). Cross sectional associations of variables with telomere length were evaluated using standard linear regression models. Multivariate linear regression models were used to adjust for other relevant variables.

To investigate telomere dynamics across time, two-level hierarchical growth curve models were constructed. Continuous variables were centred around the grand mean (e.g. the mean was set to zero) in order to improve interpretation of the intercepts and other parameters, and to reduce potential collinearity between variables. Detailed information about the model building strategy can be found in the supplementary methods.

RESULTS

Baseline characteristics are presented in table A. The median age at baseline (T1) was 48 (range 28 to 75) years with 50.1 % females and 94.9% Caucasian. At baseline, telomere length could successfully be determined in 8,074 subjects (94% of available DNA samples). Shorter baseline telomere length was associated with a higher age, the male gender, increased levels of glucose and insulin, higher waist-to-hip ratio and body mass index (BMI), increased levels of cholesterol and triglycerides, decreased levels of high density lipoprotein (HDL) cholesterol, increased levels of CRP and cigarette smoking. (see table 1 and 2) These associations are all adjusted for of age and gender.

Table 1. Baseline values and association with baseline telomere length.

	Baseline-value (n=8074)	B (+ 95% C.I.)	St. B*	p-value†
Age (years)	48 [39 – 60]	-0.47 (-0.52 to -0.42)	-0.205	<0.001
Female Gender (%)	4047 (50.1)	2.51 (1.26 to 3.76)		<0.001
Syst BP (mm Hg)	126 [114 – 141]	-0.030 (-0.066 to 0.006)	-0.021	0.098
Diast BP (mm Hg)	73 [67 – 80]	-0.029 (-0.102 to 0.043)	-0.010	0.425
eGFR (‡)	78.8 [69.9 – 88.4]	0.0226 (-0.027 to 0.072)	0.011	0.368
Creatinin (µmol/L)	82 [74 – 92]	-2.36 (-6.76 to 2.04)	-0.014	0.293
Insulin (pmol/L)	8.0 [5.6 – 12.1]	-2.00 (-3.03 to -0.97)	-0.0424	<0.001
Glucose (mmol/L)	4.70 [4.30 – 5.10]	-9.79 (-13.37 to -6.20	-0.0620	<0.001
BMI (kg/m2)	25.6 [23.1 – 28.4]	-0.233 (-0.386 to -0.080)	-0.0339	0.003
Waist-hip ratio	0.88 [0.81 – 0.95]	-22.76 (-31.88 to -13.65)	-0.0735	<0.001
CRP (mg/L)	1.29 [0.56 – 2.99]	-1.82 (-2.38 to -1.26)	-0.0732	<0.001
Cholesterol (mmol/L)	5.55 [4.89 – 6.32]	-4.08 (-7.40 to -0.758)	-0.0279	0.016
HDL (mmol/L)	1.27 [1.03 – 1.56]	6.28 (3.99 to 8.57)	0.0647	<0.001
Cholesterol / HDL	4.36 [3.36 – 5.63]	-5.38 (-7.25 to -3.51)	-0.0679	<0.001
Triglycerides(mmol/L)	1.16 [0.85 – 1.69]	-2.94 (-4.18 to -1.71)	-0.0533	<0.001
Cigarettes / day <6 | 6-20 | > 20	447 (16.4) | 1786 (65.3) | 499 (18.3)	-2.20 (-3.37 to -1.03)		<0.001

Data is presented as "median [interquartile range]" or "number (percentage)"
* Standardized B
† p-value after adjustment for age and gender
‡ mL/min.x 1.73m2

Telomere dynamics

In addition to the baseline measurements we obtained in total 9,230 follow-up measurements on 2 occasions; 3,572 for the second time point (T2) on average at 4.3 years and 5,457 for the third time point (T3), which was on average 6.6 years after T1. Missing data points at T2 are mainly due to the absence of blood samples. The collection of these was added to the protocol while the follow-up visits were already on-going.

Using the unconditional growth model we observed an average telomere attrition rate of 0.57 ± 0.078 RTL per year. However, there existed some variation among individual telomere trajectories. In 44.1% of subjects RTL shortening occurred, 21.9% had a stable RTL trajectory and in 34.0% of subjects, we observed RTL elongation after 6.6 years.

Table 2. Differences in baseline RTL for different groups

	N (%)	Mean RTL (95% CI)	Median T/S (IQR)	p-value *	p-value †
Female	4047 (50.1)	3.75 (2.85 to 4.64)	1.03 (0.84–1.25)	<0.001	<0.001
Male	4027 (49.9)	0.16 (-0.74 to 1.06)	0.98 (0.82–1.20)		
Caucasian	7664 (94.9)	1.68 (1.03 to 2.33)	0.99 (0.83–1.22)		
Negroid	77 (1.0)	12.46 (5.86 to 19.06)	1.11 (0.91–1.44)	0.01	0.016 ‡
Asian	167 (2.1)	5.86 (1.06 to 10.66)	1.02 (0.85–1.30)	0.06	0.333 ‡
Others	166 (2.1)	5.89 (1.22 to 10.56)	1.07 (0.84–1.28)	0.07	0.289 ‡
Hypertension	2577 (31.9)	-3.57 (-4.67 to -2.47)	0.95 (0.79–1.14)	<0.001	0.013
Intermediate	3241 (40.2)	3.68 (2.69 to 4.66)	1.03 (0.84–1.24)		
No hypertension	2256 (27.9)	5.81 (4.59 to 7.03)	1.04 (0.87–1.29)		
Diabetics	203 (2.5)	-8.00 (-11.78 to -4.22)	0.92 (0.76–1.11)	<0.001	0.024
Non-diabetics	7871 (97.5)	2.22 (1.57 to 2.86)	1.00 (0.83–1.23)		
Obesitas	1259 (15.8)	-2.28 (-3.83 to -0.72)	0.96 (0.80-1.16)	<0.001	0.001
Intermediate	3255 (40.7)	0.39 (-0.61 to 1.40)	0.98 (0.81-1.21)		
Non-obesitas	3473 (43.5)	4.85 (3.88 to 5.82)	1.04 (0.86-1.27)		
HC	1682 (20.9)	-3.01 (-4.36 to -1.66)	0.96 (0.80–1.16)	<0.001	0.005
Intermediate	3713 (45.8)	1.58 (0.63 to 2.53)	0.99 (0.82–1.22)		
Non-HC	2679 (33.3)	5.73 (4.64 to 6.82)	1.05 (0.87–1.28)		
Smoking	2742 (34.1)	-0.44 (-1.53 to 0.65)	0.98 (0.81–1.20)	<0.001	<0.001
Stopped	2929 (36.4)	1.78 (0.72 to 2.84)	0.99 (0.83–1.22)		
Non-smoking	2373 (29.5)	4.93 (3.78 to 6.09)	1.04 (0.86–1.27)		

* for difference among groups, † adjusted for age, gender and ethnicity where appropriate ‡ compared with Caucasians,
IQR= interquartile range, HC= hypercholesterolemia

In addition to the association with baseline telomere length, increasing age and the male gender were also associated with a higher RTL shortening rate (figure 1). Furthermore increasing age had a stronger effect in males. In our final basic model the annual telomere shortening for subjects with an average age and baseline RTL was 0.221 ± 0.087 / year for females and 0.470 ± 0.209 for males. For every 10 years of increasing age, the telomere attrition rate increased with 0.642 ± 0.077 RTL for females and 0.850 ± 0.179 RTL for males. (table 3) Table S1 in the supplemental secion details the modelling of age, gender, ethnicity and baseline RTL for our basic model which was used as a starting point for the further exploration of covariates associated with telomere dynamics.

Table 3. Estimates of annual telomere attrition rate

Model	Variables	Estimate (95%)	p-value
Unconditional	Time (years)	-0.571 (-0.725 to -0.418)	<0.001
Basic model	Time (years)	-0.221 (-0.391 to -0.051)	0.011
	Baseline telomere length (RTL)	-10.124 (-10.451 to -9.796)	<0.001
	Age (years)	-0.064 (-0.074 to -0.054)	<0.001
	Male gender	-0.249 (-0.485 to -0.013)	0.097
	Age x male gender	-0.021 (-0.041 to -0.001)	0.043
Haemodynamics	Syst BP (10 mm Hg)	-0.074 (-0.143 to -0.004)	0.038
	Diast BP (10 mm Hg)	-0.150 (-0.286 to -0.014)	0.031
Metabolic	Insulin (pmol/L) *	-0.215 (-0.420 to -0.011)	0.039
	Glucose (mmol/L) *	-1.290 (-2.006 to -0.575)	<0.001
	Diabetes	-0.879 (-0.127 to -1.631)	0.022
	Body mass index	-0.035 (-0.063 to -0.007)	0.013
	Waist-hip ratio	-4.065 (-5.870 to -2.259)	<0.001
	HDL (mmol/L) *	1.264 (0.815 to 1.712)	<0.001
	Cholesterol-HDL ratio *	-0.835 (-1.224 to 0.447)	<0.001
	Triglycerides (mmol/L) *	-0.505 (-0.742 to -0.267)	<0.001
Smoking	Smoking†	-0.728 (-0.428 to -1.028)	<0.001
Multifactorial	Time (years)	-0.255 (-0.526 to 0.015)	<0.001
model	Baseline telomere length (RTL)	-10.703 (-11.039 to 10.367)	<0.001
	Age (years)	-0.048 (-0.064 to -0.032)	<0.001
	Male gender	0.313 (-0.007 to 0.634)	0.055
	Age x male gender	-0.021 (-0.042 to -0.001)	0.047
	Glucose (mmol/L) *	-0.762 (-1.506 to -0.018)	0.045
	Waist-hip ratio	-2.658 (4.591 to -0.724)	0.007
	HDL (mmol/L) *	0.912 (0.438 to 1.386)	<0.001
	Smoking †	-0.675 (-0.982 to -0.367)	<0.001

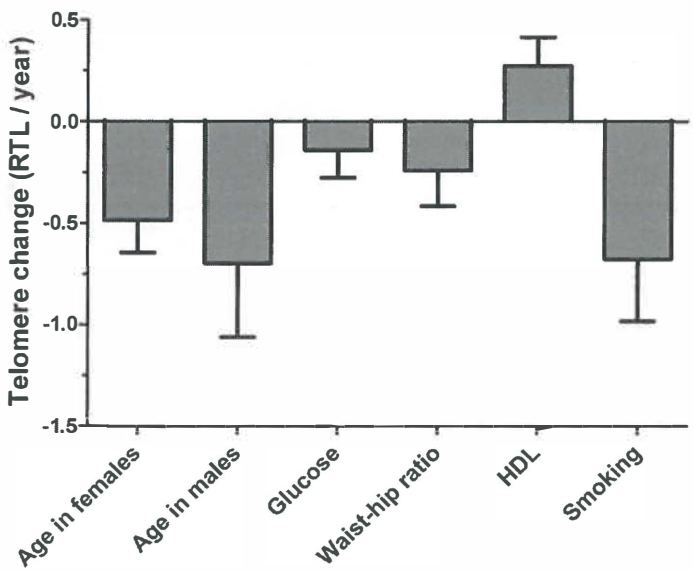
* estimate is for one increase in log transformed unit † compared to non-smokers

The effect of individual cardiovascular risk factors

We tested the role of individual cardiovascular risk factors by adding them to the basic growth model. Blood pressure: although presence of hypertension was associated with baseline telomere length (table 2), in the longitudinal analyses this binary trait was not associated with telomere dynamics. However, both higher systolic (p=0.038) and diastolic (p=0.031) blood pressures were related to an increased telomere attrition rate (table 3).

Lipids: On a longitudinal scale, hypercholesterolemia or elevated total and LDL-cholesterol level were not associated with increased telomere shortening. An increase of cholesterol/HDL ratio and triglyceride levels were related to increased telomere attrition rate (table 3). However a higher level of HDL-cholesterol was associated with a decreased telomere attrition rate (table 3, figure 1).
Diabetes: The presence of diabetes was associated with increased telomere attrition rate. Also higher levels of fasting glucose or insulin were associated with accelerated telomere attrition. (table 3, figure 1)
Obesity: Increasing BMI was associated with increased telomere attrition. The distribution of fat, as measured by waist-hip-ratio, was also associated with telomere attrition rate (table 3, figure 1).
Smoking: Smokers had a higher telomere attrition rate compared to than subjects who never smoked or who stopped smoking before the baseline measurement (table 3, figure 1).

Figure 1. Effect size on RTL change in the multifactorial model.

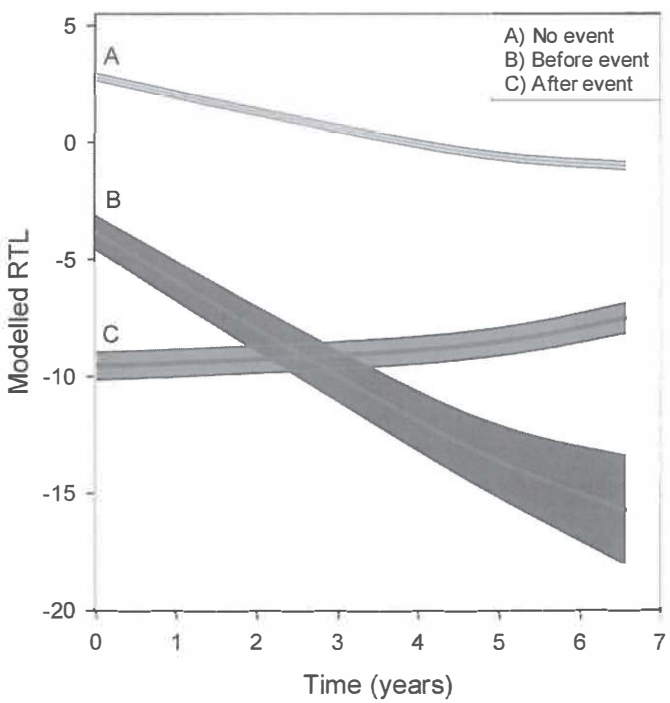


The effects of the variables present in the multifactorial model on RTL change. The 0-line represents the basic attrition rate for non-smoking subjects when all other variables are at the mean. The boxes represent the additional telomere attrition rate for an increase of respectively: 10 years of age, one standard deviation of glucose, waist-hip ratio and HDL. For smoking the box represents the additional telomere attrition for current smokers. The whiskers represent the 95% confidence interval for the estimates.

Multifactorial model

Next we tested a more complex model to explain telomere attrition rate by testing all the significant individual traits together. Non-significant variables were removed using a stepwise conditional backward strategy (for details see table S2). The final multifactorial model included baseline RTL, age, gender, age x gender, smoking, waist-hip ratio, glucose and HDL-cholesterol. In this

Figure 2. Peri-event telomere length trajectories.



Modelled telomere length trajectories for subjects who did not have a cardiovascular events (A), subjects who are prior to having a cardiovascular event (B) and subjects who already had their cardiovascular event (C). As described in the supplementary section, a time dependent variable was introduced which informed us whether the subject at that time point is prior to having his cardiovascular event or that the subject already experienced his cardiovascular event. Details about the numbers of subjects in every category are listed in table S3. This time dependent variable was added to the multifactorial model to observe peri-event telomere length trajectories. As can be seen, subjects who were on the course of getting a cardiovascular event had a higher annual telomere attrition rate of 2.15 RTL/year compared to subjects who already experienced their event. (p=0.021) Telomere dynamics post-event seem to have stabilised.

model we found an annual attrition rate of 0.255 ± 0.134 RTL for non-smoking females of average age (48 years). Every ten years of increasing age gave an additional shortening of 0.484 ± 0.082 RTL and 0.695 ± 0.188 for males. The other variables are gender independent and their estimates can be found in table 3 and figure 1.

Future cardiovascular events and telomere dynamics

We performed several exploratory models to study the temporal relationship between the occurrence of a cardiovascular event and telomere dynamics. Telomere attrition was accelerated in subjects prior to their cardiovascular event and remained relatively stable after the event (figure 2). This observation was independent of the variables present in the aforementioned multifactorial model.

DISCUSSION

Our baseline cross-sectional data is in line with previous studies suggesting an association with telomere length and age, gender, ethnicity,[12,13] smoking,[14,15] hypertension,[16] body mass index,[15,16] waist-hip ratio,[10,17] insulin,[3,16] glucose,[16,17] total cholesterol,[18] HDL-cholesterol,[19] triglycerides[18] and CRP levels.[3] Few studies evaluated telomere length changes in longitudinal designs. The previous longitudinal studies were, due to low numbers (up to 662 subjects[19]), insufficiently powered to adequately address the relationships of cardiovascular risk factors and longitudinal changes of telomere length. We now have evaluated over 8,000 subjects and measured more than 17,000 telomeres and present convincing data demonstrating that blood pressure, body composition, smoking habits and glucose- and lipid metabolism are all associated with telomere length attrition rate independent of age, gender and baseline telomere length.

Previous studies have reported associations of waist-hip ratio,[10] HDL cholesterol,[19] and smoking[20] but were unable to replicate each other's findings. With our multifactorial model we can demonstrate that these factors, and in addition glucose levels, are all independently associated with telomere attrition rate in the general adult population. We also observed a gender effect as was observed previously.[20] Since we modelled the yearly telomere attrition rate and made age time independent we could add to this the evidence that the effect of increasing age on telomere attrition is larger in males.

It is of great interest that, besides age and gender, the identified telomere length influencing factors are modifiable and suggest potential targets to decrease the telomere attrition rate and biological ageing process. One could hypothesize that modifying smoking behaviour and obesity would lead to a slower pace of biological aging, which is also in line with epidemiological evidence on life expectancy. One pilot study already provides suggestive evidence of increased telomerase activity after the implementation of comprehensive life style changes.[21] Recently, marine omega-3 fatty acids were identified to reduce the rate of telomere shortening.[11] These protective effects have been suggested to be caused by reducing oxidative stress on telomeric DNA[22] and/or telomerase.[23,24] Future studies will need to address the mechanisms of these interventions in more detail.

We performed some exploratory analyses to study telomere length before and after the clinical occurrence of a cardiovascular event. Our data suggest that telomere attrition is significantly accelerated prior to the occurrence of an event, and stabilizes afterwards. Both trajectories are substantially below the level of subjects not experiencing an event. The stabilisation after the event might be a reflection of changes in lifestyle and pharmacological interventions applied after the event. The observed pattern of accelerated telomere shortening in the period leading to an event with stability after the event supports the hypothesis that telomere shortening might be causally involved in the development of cardiovascular disease and it not merely a consequence of the event.

Based on cell cultures and cross-sectional measurements it has been assumed that telomere length of somatic cells can only become shorter in time. However, this dogma has recently been challenged by several studies measuring leukocyte telomere length on multiple occasions.[10,25,26] Our data supports earlier studies suggesting more dynamic individual changes, although the overall average length of the population is likely to decrease in time. This observation supports the notion of the previous suggested homeostatic process of telomere length. Nevertheless, considering some of the potential technical shortcomings is also appropriate. Unavoidable to the longitudinal nature, DNA was collected at different time points and we cannot exclude potential collection conditions or other artefacts that might have ultimately affected the technique to measure telomere length. Surprisingly little is known on how these factors might affect the telomere integrity or measurement error making it difficult to control for it. In addition, the relative contribution of cell types (potentially harbouring different telomere lengths) making up full-blood DNA might temporarily

change during the collection times. Nevertheless, these factors most likely will affect our quantitative estimates rather than our qualitative conclusions. A systematic bias in a narrow collection time period is likely to equally affect all samples of that time frame instead of selectively affecting subsamples of a particular trait under investigation.
Another recently published potential explanation for the still controversial telomere elongation is measurement error.[20] However this opinion is disputed by others.[25] Although all longitudinal studies conclude gradual telomere shortening on population level, most longitudinal data suggest telomere elongation in a percentage of subjects. Since telomere attrition rate is associated with various factors, characteristics of the population under study will probably influence this percentage. Whether leukocyte telomere elongation over time is only an artefact of measurement error or indeed truly a physiological phenomenon is still matter of debate and future research.

The major strength of our study is its large sample size and its longitudinal design which allowed us for the first time to model factors affecting telomere length changes in time. One of the limitations of our study is the large number of missing values in T2. Besides a natural decline in study subjects, the reason for our missing values is incomplete collection of whole blood for DNA isolation at T2. Because the date of visiting the outpatient clinic was not based on any of the subject's characteristics, we concluded that the missing values were random. This is important since it is one of the assumptions necessary to meet when using multilevel growth curve modelling.
The little variation in ethnicity could also be considered a limitation. Although we observed ethnicity specific differences in baseline RTL, we did not find differences in telomere attrition rates. This is in concordance with previous reports.[10] Aviv et al. reported faster telomere shortening in African Americans, which was probably caused by longer baseline RTL.[26] This is in line with our results if one considers that our longitudinal RTL changes are adjusted for baseline RTL.
A third potential limitation could be the low number of cardiovascular events experienced by our study subjects. One reasons for this is that we only used subjects who had one non-fatal cardiovascular event prior or during our study for our specific cardiovascular event model. Secondly subjects were recruited in a random fashion, which means there was no selection on presence of cardiovascular risk factors. Furthermore our cohort was, with a median baseline age of 48 years, rather young to experience cardiovascular events.

In conclusion, we present the largest study measuring telomere length to date and provide convincing data for a longitudinal association of increased telomere length attrition rate with smoking, higher waist-hip ratio, low HDL-cholesterol levels and high glucose levels. Our exploratory analyses indicate that accelerated telomere attrition takes place before the clinical occurrence of a cardiovascular event. Our observations support the hypothesis that telomere length is not merely an innocent bystander of cardiovascular disease but might be more closely involved in the development and progression of atherosclerotic disease.

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SUPPLEMENTAL MATERIAL

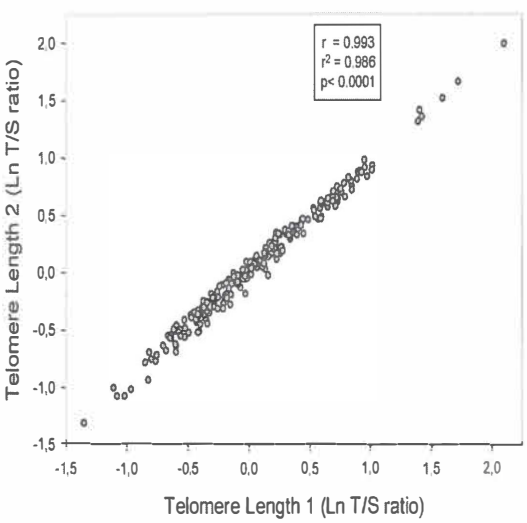
Telomere measurements

All laboratory work and PCR data analyses were performed without knowledge of clinical data. Mean telomere length was measured with the recently modified qPCR protocol using a single well strategy to measure both the telomere (T) and single reference (S) signal.[1] All experimental DNA samples were assayed in triplicates which were measured on different plates but in the same well position. Samples of the three different time points were equally divided over our PCR schedule to prevent potential time- or seasonal influences.[1,2] The ratio of telomere and reference gene content (T/S ratio) is a relative measure of telomere length. PCR reactions were set up by alliquoting 8µL of master mix into each well of a 384-well plate compatible with our Bio-Rad CFX384 real-time system on a C1000 thermal cycler, followed by addition of 2µl DNA (~20ng), for a final volume of 10µL per reaction. Seven concentrations of a reference DNA sample (standard) spanning a ~12-fold range (5.2 to 60 ng) of DNA concentrations were prepared by serial dilution and analyzed in triplicate on every 384-well plate. Good linearity was observed across this range (r^2 = 0.99). Two wells received water as the no template control (NTC), two wells were loaded with a human control sample and two with DNA of a human leukemia cell line (1301) with extreme long telomeres (kindly provided by dr. Cesaro, IST, Genova) as a positive/max control. The final concentrations of reagents in the PCR were 1U Titanium Taq DNA polymerase with the provided Titanium Taq PCR buffer, 0.75xSYBR Green I (Sigma), 0.2 mM of each dNTP, 1 mM DTT, 1M betaine, 900nM of each telomere primers (Telg and Telc), 900nM of each albumin (Albu and Albd). The primers were; telomere,
telg: ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT and
telc: TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA,
that generate a short, fixed-length product (for a further explanation and details see Cawthon 2009).[1] The S albumin primers were
albu:CGGCGGCGGGCGGCGCGGGCTGGGCGGaaatgctgcacagaatccttg
and albd: GCCCGGCCCGCCGCGCCCGTCCCGCCGgaaaagcatggtcgcctgtt.
The predicted product size is 106 bp. Capitalized bases of the albumin primers are non-template 5' tag sequences that confer a high melting temperature of the resulting PCR product (for a further explanation and details see Cawthon 2009).[1] The thermal cycling profile was Stage 1: 15 min at 95°C; Stage 2: 2 cycles of 15 s at 94°C, 15 s at 49°C; Stage 3: 5 cycles of 15 s at 94°C, 15 s at 66°C; Stage 4: 32 cycles of 15 s at 94°C, 10 s at 60°C, 15 s at 72°C with signal acquisition, 10 s at 85°C, and 15 s at 89°C with signal acquisition. Stage 5; for QC a final dissociation stage was performed from 60°C to 95°C in steps of 0.05

seconds. At stage 4; the 72°C reads provide Ct values for the amplification of the telomere template (in early cycles when the S signal is still at baseline); the 89°C reads provided the Ct values for the amplification of the S template (at this temperature there is no signal from the telomere PCR product, because it is fully melted). For each DNA concentration of the reference DNA samples the Ct for albumin occurred ~ 7.2 cycles later in cycling than the Ct for the telomere. The Bio-Rad CFX manager software was used to generate two standard curves for each plate as previously described.[1] For quality control all samples were checked for concordance between triplicate values. Samples with a coefficient of variation (CV) of ≥ 10% within the triplicate were re-run. If the CV remained ≥ 10% the sample was omitted from the statistical analyses. Samples were run in triplicate and the intra-assay coefficient of variation was 2.0% (T), 1.85% (S) and 4.5% (T/S ratio).

Reproducibility data was obtained for 216 subjects from PREVEND and good agreement between T/S ratios, measured on different days, was observed (r^2=0.99, p <0.0001, inter-run CV 3.9%, see figure S1).[1,2] T/S ratios of the three points in time were conformed to the great mean for every year of age, for interpretational and statistical reasons the median RTL was first centred around 1 and then log transformed. To improve readability each telomere length was multiplied by 100.

Figure S1. Correlation between telomere lengths of 216 subjects measured on two different occasions.



Measurements and definitions

Three different telomere trajectories were classified: 1) shortening, when RTL at T3 was decreased more than 10% compared to T1, 2) elongation in case of > 10% increase, 3) stable RTL for trajectories with ≤10% increase or decrease.[3,4] All participants completed a questionnaire on demographic profile, cardiovascular disease history, medication use and smoking habits. Smoking

was categorized as current smoking, stopped smoking and non-smoking. Additionally current smokers were divided in subjects smoking <6 cigarettes per day, 6-20 cigarettes/day and >20 cigarettes/day. Blood pressure was measured with an automatic device (Dinamap XL model 9300, Johnson-Johnson Medical, Tampa Florida) in a supine position on the right arm. Hypertension was defined systolic blood pressure (SBP) of ≥ 140 mm Hg or diastolic blood pressure (DBP) of ≥ 90 mm Hg or the use of antihypertensive medication. Blood pressures were defined as optimal when SBP < 120 mm Hg and DBP < 80 mm Hg. Subjects in between were categorised as having an intermediate blood pressure.
The measured weight was divided by the height in meters squared to calculate the body mass index (BMI). Obesity was defined as having a BMI above 30 kg/m2. Subjects were categorized as non-obese when BMI was below 25 kg/m2. All subjects in between were categorized as intermediate.
Plasma glucose, serum creatinine and total cholesterol were measured by dry chemistry (Eastman Kodak, Rochester, New York). High density lipoprotein cholesterol (HDL) was measured using a homogeneous method (direct HDL, Aerosat TM system, Abbott Laboratories, Abbott Park, Illinois). Triglycerides were measured enzymatically. High-sensitivity C-reactive protein (hs-CRP) was measured using nephelometry (BN II, Dade Behring, Marburg Germany). Insulin was determined with an AxSym® auto-analyzer (Abbott Diagnostics Amstelveen, the Netherlands).
Diabetes was defined as a fasting plasma glucose level of ≥ 7.0 mmol/L (126 mg/dL), non-fasting plasma glucose level of ≥ 11.1 mmol/L (200 mg/dL) or the use of oral antidiabetics. Hypercholesterolemia was defined as having a total cholesterol of ≥ 6.5 mmol/L (250 mg/dL) or the use of lipid lowering medication. An optimal cholesterol was defined as ≤ 5.13 mmol/L (200 mg/dL). Subjects in between were classified as intermediate. Estimated glomerular filteration rate (eGFR) was calculated using the Modification of Diet in Renal Disease study equation taking into account sex, age, race and serum creatinine levels.[5]

Model building strategy

The annual change in RTL over time was modelled as the dependent variable and variables of interest as covariates and factors. The hierarchy was modelled as follows: level one is the within-subject level consisting of the repeated telomere length measurements which are clustered in the second level which is the individual subject (between-subject level). This statistical technique is particular useful to explore longitudinal data, since the data of the repeated measures are clustered within the study subjects. In this way the first level is

the respective measurement occasion (point in time) and the second level the individual subject. The benefits of this individual growth curve analysis are 1) it takes the dependency of longitudinal data into account by clustering it in the second level. 2) the technique is capable in handling unbalanced data (provided that the data is missing at random) 3) numerous numbers of waves (measurement occasions) can be used.

The goodness of fit for the models constructed was evaluated using maximum likelihood comparison. The deviance of every model, defined as the -2 loglikelihood, was compared with the deviance of the former model. The model was classified statistically significant better when the change in deviance was greater than the critical value of the chi-square distribution for the relevant change in degrees of freedom.

First an unconditional growth model was constructed which was then extended by variables classified as very likely to influence telomere dynamics over time by biological reasons or previous publications. In this basic model baseline telomere length, age, gender and ethnicity were introduced and their validity tested as can be read above. After having constructed the basic model our variables of interest were added to the basic model. Also interaction terms of the variables of interest with the variables of the basic model were sequentially included. All non-significant interaction terms were left out of the tables in the results section. All variables associated with telomere dynamics were then introduced into the multi factorial model. This model was constructed in a stepwise conditional backward manner by removing each variable and test whether the change in deviance with one degree of freedom was statistically significant. The resulting final mulit factorial model then served as the starting point for evaluating the effect of cardiovascular events.

In order to evaluate peri-event telomere length trajectories subjects who experienced a non-fatal cardiovascular event were categorized as prior (1) or after (2) the event for every telomere length measurement occasion. Subjects without cardiovascular event were coded 0. Using this variable we were able to model three RTL trajectories: 1) leading to the event, 2) after having experienced the event and 3) no cardiovascular event at all. To differentiate trajectories before and after the event we included only subjects who experienced one cardiovascular event prior or during the study for this analysis.

Table S1. Modelling of the basic model

Variables	Decrease in deviance	Increase in df	p-value*
Unconditional growth			
Baseline RTL	2805.58	1	
Baseline RTL + age	152.03	1	6.24×10^{-35}
Baseline RTL + gender	8.04	1	4.66×10^{-3}
Baseline RTL + ethnicity	3.96	3	0.266
Baseline RTL + age + gender	4.27	1	0.039
Baseline RTL + age + gender + age x gender	4.11	1	0.043
Basic model	**3076.88**	**4**	

* p-value for significance of improvement of model. Df denotes degrees of freedom

Table S2. Conditional stepwise backwards modelling of the multi factorial model

Omitted variable	Increase in deviance	Decrease in df	p-value*
Insuline	0.03	1	0.890
SBP	0.04	1	0.834
BMI	0.09	1	0.760
Triglycerides	0.35	1	0.552
Diabetes	1.42	1	0.233
Cholesterol / HDL	0.96	1	0.327
DBP	0.94	1	0.332
Gender x age, gender	6.74	2	0.034
Final multi factorial model	3.85	6	0.797

* p-value > 0.05 indicates the previous model is not statistically significant better
Df denotes degrees of freedom

Table S3. Number of subjects prior to and after their cardiovascular events at the three time points.

	T1	%	T2	%	T3	%
Before event	329	4.1	69	1.9	39	0.7
After event	204	2.5	145	4.1	267	4.9
No event	7541	93.4	3358	94.0	5151	94.4

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4. Farzaneh-Far R, Lin J, Epel ES, Harris WS, Blackburn EH, Whooley MA. Association of marine omega-3 fatty acid levels with telomeric aging in patients with coronary heart disease. JAMA 2010; 303(3):250-257.

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CHAPTER 6

Summary, discussion and future perspectives

As telomeres are a reflection of the past ánd future of a cell, this chapter will give a reflection of the past and future of telomere biology in cardiovascular diseases. In the continuously moving layer in between, called the present, the results of this thesis will be summarized and put in a broader perspective. The first part of this chapter will focus on the scientific aspects of telomere biology. In the second part the focus will be on the potential clinical implementations of this research.

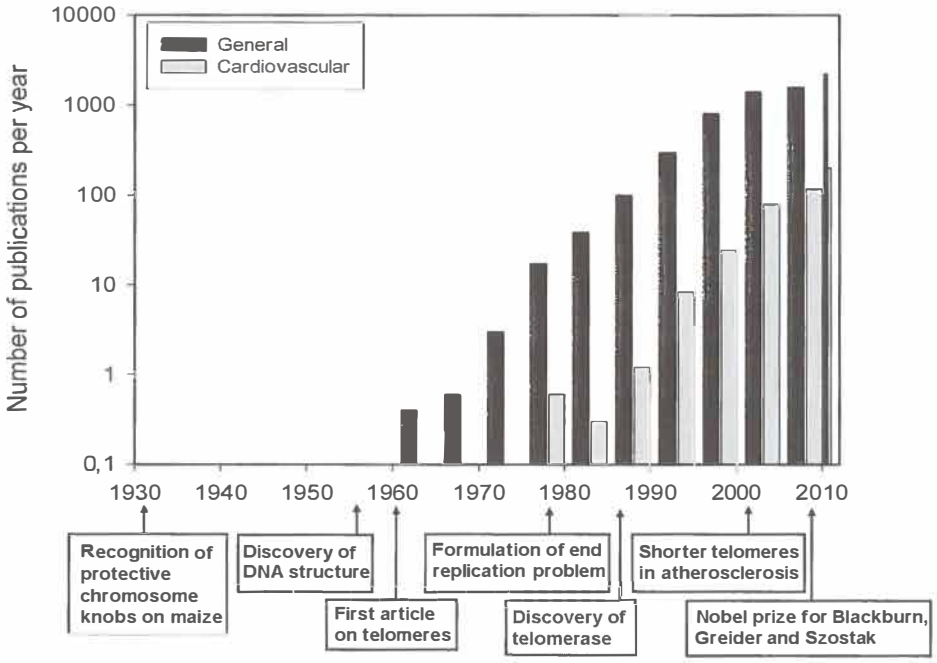
PART I

Perspectives on telomere research

About 80 years ago it was already recognized that chromosomes survived X-irradiation better when their 'knobs' on the end of the chromosomes were intact, compared to chromosomes with broken ends.[1] Later on, these 'knobs' appeared in articles as 'telomeres'.[2] After publication of the end replication problem[3,4] interest in telomeres rose, since it implicated that these protective chromosomal ends would get shorter with every cellular replication. In the meanwhile it also became clear that there is a limit to somatic cell divisions. Hayflick noticed that the number of human fetal cell divisions in vitro is limited to approximately 50 divisions. After reaching this Hayflick limit, as it is called now, the cell enters a non-dividing state called senescense.[5]

Although most breakthroughs were already forecasted by theoreticians, it took until the late 1980s when several theories came together and we were enriched with the knowledge that 1) telomere shortening leads to senescence [6-8], and 2) that telomeres can be elongated by telomerase[7,9] The first observation gave rise to the idea of telomere length as a marker of cellular ageing or the 'mitotic clock' which eventually limits further cellular renewal.[8] The latter observation, in contrast, was associated with unlimited cellular growth. This lead on the one hand to fear of malignant tranformation[10] while on the other hand fed fantasies of immortality and longevity.[11] After these breakthroughs, the number of publications on telomere biology expanded rapidly (see chapter 2 and figure 1). The number of telomere related articles in the field of cardiology also grew rapidly due to some important observations. It became apparent that patients with premature myocardial infarction[12], atherosclerosis[13] and chronic heart failure[14] have shorter telomeres than healthy individuals of the same age. Even children of patients with coronary artery disease already have shorter telomeres than children of healthy parents, suggesting a causal role for telomere length on the development of cardiovascular disease.[15, 16]

Figure 1. number of telomere related articles published per year on a logarithmic scale.



Up to the present day, telomere length has been linked to numerous diseases, internal- and external- but also environmental factors, while new scientific revolutions failed to materialize. For most observed associations, the exact mechanism is not yet identified or sometimes even suspected. Most association studies have a cross sectional design. Although this can generate new hypotheses, the reported association is observed on population level and does not necessarily imply that the same is true for the individual. We therefore studied telomere length dynamics in a longitudinal study design in chapter 5. We measured up to three consecutive telomere lengths in individual subjects over a mean follow-up period of 6.6 years. With the multilevel modeling technique used, we observed different patterns on the individual level. As expected, our population as a whole showed gradual telomere shortening over time. Confirming some smaller studies[17-19] we observed that a substantial proportion (~1/3) of the general population actually experienced telomere elongation over a mean period of 6.6 years. This observed telomere elongation is still matter of debate. Telomere elongation has been suggested

to be an artifact of measurement error,[20] while others suggest a biological feedback mechanism.[18] With our large data set we are confident to say that the old notion of inevitable gradual telomere shortening over time, based on the negative correlation between telomere length and age on population level, does not hold stand anymore. Can this observed individual variability in telomere dynamics be explained? In chapter 5 we strongly confirmed the observation that the main determinant of telomere attrition rate is baseline telomere length.[17-19] Also the meaning of this observation is still subject of debate. It has been suggested that the observation is merely due to mathematical coupling.[21] Others, however corrected for inflation of p-value and still found a highly significant association.[22] Strengthened by mathematical models of telomere shortening,[23] the presence of a negative feedback mechanism was suggested by others.[18] The results of our large dataset of over 17.000 samples also suggest that individual leukocyte telomere length could be monitored and maintained by mechanisms not yet completely elucidated.

To gain more insight into this possibility, more longitudinal studies with multiple repeated measurements (waves) are necessary in the future. The follow-up durations and sample sizes of the current studies are probably already sufficient; hence the most gain can be achieved by increasing the number of waves. More waves means a better resolution of the shape of the growth curve and more modeling possibilities. An even bigger challenge in longitudinal telomere research is the influence of various factors on individual telomere dynamics. In chapter 5 we observed that besides baseline telomere length, a higher baseline age, higher levels of glucose, larger waist to hip ratio and cigarette smoking were independently associated with a higher telomere attrition rate, while increasing levels of high density lipoprotein cholesterol were associated with decreased telomere shortening. This relatively small number of associations with telomere shortening contrasts with the many associations observed with baseline telomere length (cross sectional) presented in table 1 of chapter 5 and reviewed in chapter 2. When the epidemiological branch has established more factors associated with longitudinal telomere length dynamics, the potential mechanisms responsible for this can hopefully be elucidated. First, under the controlled circumstances of the laboratory but also intervention studies can be conducted in which pharmacological and/or lifestyle interventions can be applied using telomere length dynamics as one of the endpoints. In a pilot study, comprehensive lifestyle changes have already been associated with increased telomerase activity.[24] Another promising study in this respect is the longitudinal Asklepios study which focuses on the interplay between ageing, haemodynamics and inflammation in (preclinical) cardiovascular disease and also includes telomere length as a variable of interest.[25]

Based on our results of chapter 5 the emphasis in these studies, in first instance, should be on interventions in the cholesterol and glucose metabolism or in prevention or curation of central obesity and nicotine abuse.

Besides the cross-sectional design, another limitation of the majority of previously published articles is that they describe associations with leukocyte telomere length. The most likely reason for this is, that leukocytes are abundant and relatively easy to obtain. Furthermore, since they circulate throughout the whole body, they are seen as representatives of systemic processes. However, little evidence exists on the correlation between leukocyte telomere length and cardiovascular tissue telomere length. In 32 subjects it was observed that leukocyte telomere length correlated with vascular wall telomere length.[26] In chapter 4 we compared telomere lengths of atherosclerotic plaques with that of circulating leukocytes in 101 subjects, and concluded that leukocyte telomere length is substantially shorter compared to plaque telomere length and that there is only a weak correlation between the two. Leukocyte telomere length can therefore not just be used as a reflection of telomere status in the diseased tissue. In addition, plaque vulnerability was associated with shorter leukocyte telomeres but longer plaque telomeres. This suggests that there is an essential difference in telomere biology between leukocyte- and plaque tissue and illustrates the complexity of telomere biology among different tissues, which is, to a large extent, still unknown. Future research should therefore also focus more on telomere biology in the diseased tissue itself to get a better idea of the potential mechanisms involved.

PART II

Clincical perspective

As reviewed in chapter 2, several cardiovascular diseases have been associated with shorter leukocyte telomeres.[12-14] The prognostic value of telomere length has extensively been described, although sometimes with contradictory conclusions. Several studies have reported an inverse association of leukocyte telomere length with survival [27-30], myocardial infarction[12, 31] or a combination of clinical outcome parameters.[32,33] In other reports, no association between telomere length and survival was found.[34-36]

In the present day, leukocyte telomere length has not yet been implemented as a prognostic- or diagnostic tool in clinical cardiology. One of the uncertainties, in this question, is how leukocyte telomere length can foresee progression of cardiovascular disease and whether leukocyte telomere length is a reflection of tissue telomere length or not. To gain more insight into this, we measured telomere lengths of leukocytes and atherosclerotic plaques in chapter 4 and evaluated their associations with presence, characteristics and recurrence of disease. First, we observed shorter leukocyte telomeres in patients with carotid atherosclerosis. This was not a surprise since atherosclerosis is a systemic disease and is mostly present throughout the whole body.[37-39] We also observed that patients with plaques which were more prone to restenosis have shorter leukocyte telomeres, but longer plaque telomeres than patients with less vulnerable plaques. Furthermore, we observed only a weak correlation between plaque and leukocyte telomere length, and only the latter was associated with the patient's age. This suggests an essential difference in telomere biology between leukocyte- and plaque tissue which needs further exploration. Based on chapter 4 one could hypothesize that shorter leukocyte telomeres are associated with presence and severity of disease, but, in the case of atherosclerosis, probably more to the systemic effects of atherosclerosis and its risk factors. Once the atherosclerosis has developed into a plaque, this local plaque could, as previously suggested, have its own telomere biology which is focused on maintaining telomere length in order to maintain the inflammatory response and the proliferative capacity of the plaque.[40] This is in line with the observation that inflammatory cells in coronary atherosclerotic plaques have higher telomerase activity than systemic inflammatory cells within the same patient.[40] Therefore, this local tissue telomere length could well be more informative for local disease status than leukocyte telomere length. Indeed, in chapter 4 we also observed that plaque telomere length was associated with restenosis after endarterectomy while leukocyte telomere length was not.

In the future, telomere length is not likely to become a new diagnostic- or prognostic marker in the setting of clinical cardiology. As a general diagnostic marker of cardiovascular disease, telomere length is probably not specific enough and due to a large inter-individual variation also not sensitive enough. There are, however, some potential implications for the use of telomere length as a marker of biological age. In chapter 3 we describe the association of additional mental health problems with shorter telomeres in a large group of heart failure patients. In the prevention of coronary heart disease, it has been reported that statin treatment is more effective in subjects with short telomeres.[32] Further knowledge of the associations between telomere length and multimorbidity, medication and their interactions may in the future lead to a more tailored medication regime based on a person's biological age measured by telomere length. In chapter 4 we observed that shorter plaque tissue telomere length is associated with an increased risk of restenosis after endarterectomy. In this harvested plaque it is relatively easy, cheap and quick to measure its telomere length, although its added value to individual risk stratification and follow-up regime is not yet established.

Figure 2.
Telomere length as a potential future marker in clinical setting

		27-08-2010 10:34
Hemoglobin	mmol/l	8.7
Telomere Length	T/S	0.411
Sodium	mmol/l	137
Potassium	mmol/l	4.4
Urea Nitrogen	mmol/l	6.9
Creatinin	umol/l	72
eGFR	ml/min * 1.73 m2	97
Triglycerides	mmol/l	2.72
Cholesterol	mmol/l	5.70
HDL-Chol	mmol/l	0.90
LDL-Chol	mmol/l	3.90

Besides the potential roles telomere length could play in the fields of diagnostics, prognostics and risk stratification one could also consider a therapeutic role if we can establish a causal link. In chapter 5 we specifically investigated the longitudinal telomere length trajectories before and after the occurrence of cardiovascular events in order to understand the sequence. We observed that telomere shortening already occurred in the pre-clinical phase before the event. Surprisingly, telomere length remained stable after the cardiovascular event. This adds temporal evidence to the hypothesis that telomere length is

causally involved in the development of cardiovascular disease. When this potential causal role has been definitively established and it is confirmed that telomere dependent senescence contributes to the development of cardiovascular diseases this would open the door to new gene-based therapies. There is increasing evidence that senescent cells accumulate in aging tissue and by disrupting tissue architecture contribute to age-related pathology.[41] Homologues transplantation of various (progenitor) cells to restore disrupted tissue architecture looks promising. However, one concern is the limited viability and proliferative capacity of these transplanted cells.[42] This could be an effect of aging since the transplanted cells originate from the same, and thus affected, person. This effect may in the future be bypassed by transplanting cells which have their telomeres lengthened. Cells, immortalized by transfecting them with the inducible telomerase RNA complex, have an increased activity of the telomere elongating enzyme telomerase. Overexpression of telomerase can counteract or even reverse telomere dependent replicative senescence.[43] These cells exhibit normal phenotypes and their biological function and metabolic capacities are similar to mortal cells.[44-46] Transplanting these cells, after for example myocardial infarction, could be more effective in repairing damaged endothelium or alter the remodelling processes leading to chronic heart failure than normal, biologically aged cells. However, one should remember that telomere dependent senescence also has a protective function, namely to prevent cells from unlimited cellular divisions. Future research should evaluate the safety and efficacy of these gene-based therapeutical interventions.

In the present thesis we have investigated and discussed the role of telomeres in cardiovascular disease. We have elucidated more aspects of telomere biology in atherosclerotic tissue and described longitudinal leukocyte telomere dynamics. Based on this dynamic process and its mediators, we have described and discussed potential new tools in the detection and prevention of cardiovascular disease. We have added evidence for a causal role for telomere shortening in the development of cardiovascular diseases. However, in order to fully understand and appreciate this role, mechanistic studies should be undertaken as well as large epidemiological studies and eventually interventional studies with telomere length as surrogate endpoint. These studies could help in the development of new therapeutic and preventive agents for patients with cardiovascular diseases.

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Nederlandse samenvatting

Hart- en vaatziekten vormen de belangrijkste primaire doodsoorzaak in Nederland. Coronaire hartziekten (kransslagadervernauwing) en hartfalen (verminderde pompfunctie van het hart) komen beide vaker voor op een hogere leeftijd. Toch bestaat er een grote variatie in chronologische leeftijd (bepaald door geboortedatum) en het al dan niet ontstaan van hart- en vaatziekten, die niet geheel verklaard kan worden door reeds bekende risicofactoren.
Naast chronologisch, verouderen we ook biologisch. Er bestaan grote verschillen in de snelheid van biologische veroudering tussen individuen. De lengte van chromosoomuiteinden, ook wel telomeren genoemd is in opkomst als biomarker van deze biologische leeftijd. Telomeerlengte voldoet aan een aantal criteria van een robuuste biomarker voor biologische veroudering. Allereerst wordt de beginlengte grotendeels erfelijk bepaald en bestaat er een aanzienlijke variatie tussen individuen. Deze variatie neemt toe met de leeftijd. Daarnaast wordt de telomeerlengte door cellulaire processen beïnvloed, zoals het aantal celdelingen en de blootstelling aan schadelijke factoren zoals oxidatieve stress. Van deze processen wordt verondersteld dat ze fundamenteel betrokken zijn bij het verouderingsproces.

Het doel van dit proefschrift was drieledig. Allereerst wilden we onderzoeken welke risicofactoren voor hart- en vaatziekten van invloed zijn op telomeerlengte. Een tweede doel was telomeerlengtes tussen verschillende weefsels te vergelijken en hun relatie met aanwezigheid en vooruitgang van ziekte te bepalen. Als derde doel wilden we meer inzicht krijgen in de vraag of telomeerverkorting een oorzaak of een gevolg is van (het ontstaan van) hart- en vaatziekten.

Telomeren en Telomerase
Telomeren vormen de uiteinden van chromosomen en zijn essentieel voor de bescherming van het coderend DNA. Aangezien het DNA-polymerase niet in staat is het laatste stukje DNA van een chromosoom (dus de telomeer) bij elke celdeling te kopiëren verkorten telomeren geleidelijk. Schadelijke omgevingsfactoren leiden tot extra telomeerverkorting. Telomeerlengte kan dus gezien worden als een afspiegeling van het aantal voorafgegane celdelingen en de blootstelling aan schadelijke factoren.

Als telomeren te kort worden kunnen ze hun beschermende functie niet meer uitoefenen en treedt scenescentie op. Dit is een toestand waarin de cel niet meer kan delen. Een hoog percentage van zulke cellen resulteert in slecht functionerende weefsels of organen. Dat de mens toch al vele duizenden jaren bestaat betekent dat er mechanismen bestaan om telomeerlengte te handhaven

of herstellen. De belangrijkste is het gespecialiseerde enzym telomerase. Voor de geboorte, en daarna in stam- en kiemcellijnen, voegt telomerase nieuwe TTAGGG sequenties toe.

Telomeren en cardiovasculaire risicofactoren

Zoals in hoofdstuk 2 staat beschreven zijn er, naast leeftijd, opvallend veel cardiovasculaire risicofactoren geassocieerd met kortere telomeren. In hoofdstuk 3 wordt de relatie tussen psychische gezondheid en telomeerlengte, gemeten in 890 patiënten met hartfalen, besproken. Hieruit blijkt dat een slechtere psychische gezondheid is geassocieerd met kortere telomeren. Depressie en type-D persoonlijkheid hebben geen relatie met telomeerlengte. In hoofdstuk 5 worden de resultaten beschreven van telomeerlengtemetingen in 8074 mensen uit de Prevend studie. Uit deze resultaten wordt duidelijk dat telomeerlengte, onafhankelijk van leeftijd en geslacht, is geassocieerd met roken, hypertensie, body mass index, buikomvang, insuline, glucose, cholesterol, HDL-cholesterol, trygliceriden en CRP.

Een nadeel van veel studies is dat men kijkt naar de invloed van een factor (bv leeftijd of bloeddruk) op een eenmalige telomeerlengtemeting, terwijl het eigenlijke doel is te bepalen welke factoren zijn gerelateerd aan de snelheid van telomeerverkorting. Om hier meer inzicht in te krijgen hebben wij ook telomeerlengtes gemeten van dezelfde personen op drie verschillende tijdspunten die samen gemiddeld 6,5 jaar bestrijken. Hieruit blijkt dat een hogere leeftijd, het mannelijke geslacht, roken, grotere buikomvang en hogere bloedsuikerspiegels onafhankelijk zijn geassocieerd met snellere telomeerverkorting gedurende de studie. Een hogere HDL-cholesterolspiegel in het bloed was geassocieerd met een langzamere telomeerverkorting.

Telomeren en hart- en vaatziekten

De eerste studie naar de relatie tussen coronaire atherosclerose en telomeerlengte dateert uit 2001. Patiënten met coronairlijden hadden gemiddeld ruim 300 basenparen kortere telomeren in hun witte bloedcellen. Dit kwam overeen met de telomeerlengte van bijna 9 jaar oudere controlepatiënten. Deze eerste aanwijzing dat patiënten met hart- en vaatziekten mogelijk 'biologisch' ouder zijn werd later in verschillende studies bevestigd zoals in hoofdstuk 2 wordt besproken. In hoofdstuk 4 voegen wij aan deze kennis toe, dat ook patiënten met een vernauwing in de halsslagader kortere telomeren hebben dan gezonde mensen van dezelfde leeftijd. Bij patiënten met hart- en vaatziekten zijn niet alleen de telomeren in witte bloedcellen korter, maar ook die van bijvoorbeeld endotheelcellen. Deze cellen, betrokken bij het ontstaan van atherosclerose, bekleden de vaatwand. Omdat witte bloedcellen gemakkelijk

te verkrijgen zijn via een weinig belastende venapunctie, wilden we bekijken of telomeerverkorting in witte bloedcellen en ziek vaatweefsel hand in hand met elkaar gaan. In hoofdstuk 4 wordt de relatie tussen telomeerlengtes van witte bloedcellen en zieke vaatwandcellen vergeleken in 684 patiënten die zijn geopereerd aan een vernauwing van de halsslagader. Hieruit blijkt dat er wel een relatie bestaat tussen beide celtypes, maar dat het verband zwak is. Patiënten bij wie de geopereerde vaatvernauwing werd gekarakteriseerd door veel ontstekings- en vetcellen hadden kortere telomeren in hun witte bloedcellen, maar juist langere telomeren in hun vaatweefsel. Deze twee observaties wijzen er op dat er verschillende patronen in telomeerverkorting bestaan tussen verschillende celtypes.

De relatie tussen hart- en vaatziekten en telomeerlengte wordt overigens niet simpelweg bepaald door eerdergenoemde risicofactoren, maar blijft ook bestaan na correctie hiervoor. Dit suggereert dat de telomeerlengte niet alleen afhangt van een opsomming van klassieke risicofactoren. In hoofdstuk 5 laten we zien dat mensen in aanloop naar het krijgen van een cardiovasculaire ziekte (hartinfarct of zuurstofgebrek van het hartspierweefsel) al telomeerverkorting laten zien ten opzichte van de periode na het ontstaan van de ziekte. Wij hebben ook hier rekening gehouden met andere persoonskarakteristieken die van invloed zijn op telomeerverkorting. Deze observatie ondersteunt het idee dat telomeerlengte een causale rol speelt in het ontstaan van hart- en vaatziekten.

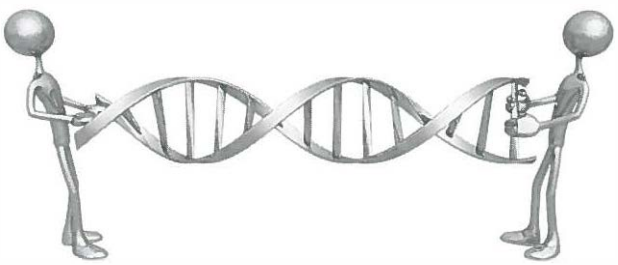
Toekomstperspectief

In de toekomst zou telomeerlengte, als maat voor biologische veroudering, mogelijk kunnen worden gebruikt om een risico-inschatting te maken op het krijgen of verergeren van hart- en vaatziekten. In hoofdstuk 4 wordt beschreven dat de telomeerlengte van het aangetaste vaatwandweefsel voorspellend is voor het terugkeren van een vernauwing na de operatie. Door het meten van telomeerlengtes op meerdere tijdspunten in hoofdstuk 5 is duidelijk geworden dat bepaalde factoren ook daadwerkelijk met de mate van telomeerverkorting zijn gerelateerd. Het lijkt dan ook voor de hand te liggen te concluderen dat verandering van deze factoren zoals stoppen met roken, afvallen, verlagen van het bloedsuikergehalte en verhogen van het HDL-cholesterol zal leiden tot minder snelle biologische veroudering. Om dit definitief te bevestigen is meer onderzoek nodig waarin mensen gedurende de studie hun levensstijl aanpassen.

In hoofdstuk 5 hebben we ook laten zien dat telomeerverkorting al optreedt voordat de ziekte zich openbaart. Hiermee hebben we meer aannemelijk gemaakt dat telomeerverkorting een oorzakelijke rol speelt in het ontstaan

van hart- en vaatziekten. Toch is deze rol met onze huidige studieopzetten niet helemaal te bewijzen. Wanneer definitief blijkt dat telomeerverkorting betrokken is bij het ontstaan van hart- en vaatziekten zou dit nieuwe wegen voor preventie en behandeling kunnen openen. Zo zouden mogelijk telomeerstabiliserende of verlengende gentherapieën gebruikt kunnen worden in de strijd tegen hart- en vaatziekten.

Dankwoord



The Road goes ever on and on.
Down from the door where it began.
Now far ahead the Road has gone,
And I must follow, if I can,
Pursuing it with eager feet,
Until it joins some larger way
Where many paths and errands meet.
And whither then? I cannot say.

[J.R.R. Tolkien, Lord of the Rings]

Gebaseerd op eigen ervaring en de resultaten van hoofdstuk 3 durf ik te beweren dat promoveren zijn weerslag heeft op je telomeren. Gelukkig zijn er vele mensen die het proces én de promovendus op dreef houden. Deze wil ik dan ook graag op deze plek bedanken.

Vanzelfsprekend begin ik met de drijvende kracht achter het telomeerproject: dr. P. van der Harst. Beste Pim, sinds ik jou ken heeft het woord ambitie een gezicht gekregen. Met enorme werklust en wetenschappelijke interesse ben je in dit project gestapt. Met onder andere dit proefschrift als resultaat. Er gaan er vast nog vele volgen, maar mijn 'groenheid' in onderzoeksland blijft je vast nog wel even bij. Zo werden we ruim 3 jaar terug op een zonnige lentedag verrast door het feit dat praktisch alle kranten en radiojournaals onderzoeksresultaten meldden die, ten onrechte, aan ons werden toegedicht. Vanzelfsprekend waren er naast dit 'akkefietje' nog wel meer tegenslagen en moeilijke momenten tijdens mijn promotietraject en juist op zulke momenten verraste je mij met wijze woorden en een hart onder de riem. Bedankt hiervoor.

Mijn promotores prof. dr. W.H. van Gilst en prof. dr. D.J. van Veldhuisen.

Beste Wiek, bedankt voor alle hulp bij mijn promotietraject. Jouw praktische aanpak en originele gedachtengangen hebben mij altijd weer op weg geholpen. Ook buiten het lab ben je altijd bereikbaar voor een goed gesprek, mits deze gepaard gaat met een goede dosis humor. Ik heb dit altijd met veel plezier geobserveerd. Bedankt voor de eindsprint waarin we het afronden van de promotie in goede banen hebben kunnen leiden.

Beste Dirk Jan, jouw schier onuitputtelijke bron van enthousiasme voor de wetenschap heeft de Groningse telo-trein al sinds het vertrek van brandstof voorzien. Wat ik vooral bewonder zijn je uiterst snelle analyses en manier van denken. Dit gecombineerd met een wetenschappelijk raffinement maken dat ons team altijd op de rails is gebleven en het eindstation zonder al te veel vertraging heeft bereikt.

Mijn tweede co-promotor dr. R.A. de Boer. Beste Rudolf, bedankt voor het meedenken achter de schermen en de directe begeleiding tijdens de perioden dat Pim in het buitenland werkte. Hierdoor heb jij geregeld de wekelijkse telo-meeting voorgezeten. Dat hierin ook gelachen werd was voor mij een nieuwe, niet onplezierige, ervaring. Je humoristische en daadkrachtige aanpak heb ik altijd gewaardeerd, bedankt hiervoor.

De leden van de beoordelingscommissie, prof. dr. M.P. van den Berg, prof. dr. G. Pasterkamp en prof. dr. A.H. Zwinderman wil ik bedanken voor het kritisch doorlezen van mijn proefschrift.

Liza Wong: nog een naam zonder wie dit boekje er niet had gelegen. Als telo-team werkten we 3,5 jaar lang praktisch altijd zij aan zij. Ik heb dit als zeer leerzaam én heel erg gezellig ervaren! Het blijft me een raadsel hoe Pim twee zo verschillende persoonlijkheden heeft gevonden voor dit project, maar gelukkig zaten we qua 'belangrijke dingen in het leven' veelal op één lijn. Uitvoerig hebben we menig onderwerp besproken. Dagen en soms nachten lang zaten we op het lab, en daar zat echt geen minuut van stilte bij! Dat ga ik zeker missen. Promoveren is niet altijd een eenvoudig proces, maar gelukkig wist ik dat ik altijd op jouw steun en medewerking kon rekenen. Je altijd oprechte en rationele uitspraken hielpen mij vaak om de dingen helder te (blijven) zien. Liza, nogmaals heel erg bedankt voor alle hulp en onze prettige samenwerking. Ik ben blij dat het telo-team op deze dag weer compleet is en je mij als paranimf wil bijstaan.

Mijn tweede paranimf Ridwan Maulana: het mooie van een huisgenoot die ook promovendus is, is dat je elkaars problemen (her)kent, begrijpt en soms zelfs kunt oplossen. Met name over statistiek hebben we veel gesproken, en naast boerenkool of soto ayam was de multilevel analyse dan ook geregeld onderdeel van ons avondmaal. Bedankt voor je ondersteuning op moeilijke momenten en je altijd vrolijke gezelschap. Ik ben blij en trots dat je mij vandaag bijstaat als paranimf.

Ook mijn collega's van het Poort- en later Triadegebouw wil ik hier bedanken. Ik heb het altijd erg met jullie naar mijn zin gehad. Zeker wanneer de gesprekken zich wat verbreedden tijdens het samenzijn buiten werktijd. In random volgorde: Marcelle, Ali, Hessel, Lieuwe, Sheba, Suzan, Bart, Rob, Kevin, Marthe, Ismaël, Jan-Pieter, Pieter, Pieter-Jan, Jasper, Wouter, Mirjam, Caroline, Lennaert, Karim, Daan, Youlan, Frank, Nicolas, Arjen, Matthijs, Renée, Chris en in het bijzonder mijn kamergenoten van de hart**falen**kamer: Willem-Peter: bedankt voor de gezellige momenten. Jij was altijd wel te porren voor een praatje of discussie over welk onderwerp dan ook. Ook buiten het werk, toen we met je 1 dag oude auto strandden met een lekke band bleef je daar de lol van inzien. Bedankt voor alle goede momenten op en buiten het werk. Anne: bedankt voor de altijd leuke en gezellige momenten op onze kamer. Tussen het werk door hebben we er heel wat afgelachen. Naast gevoel voor humor zaten we ook vaak op andere vlakken op één lijn. Je carrièreswitch

kwam dan ook niet echt als een verrassing. Ik hoop dan ook dat je 'in Afrika' je roeping vindt! Ook al waaieren we (lees: jullie!) over Nederland uit, toch hoop ik dat we onze kamerreünies kunnen voortzetten en ik verheug me dan ook al op onze trip naar/op het strand.

De collega's van de Cardio Research wil ik hier ook bedanken voor de altijd prettige samenwerking: Greetje, Trienke, Karin, Geert, Margriet, Carlien bedankt en in het bijzonder Peter: jij hebt mij de praktische uitvoering van klinische trials geleerd. Een nauwkeuriger leermeester moet nog geboren worden! Gelukkig ging dit altijd wel gepaard met een keur aan anekdotes en gezelligheid, bedankt hiervoor. Carolien: ik zal onze, al dan niet altijd even zinnige, gesprekken onder het genot van een straffe bak koffie missen. Ook erg bedankt voor het altijd waarderen van de (schitterende) koffiemokken die ik voor onze kamer aanschafte en het werven van je halve, hier niet bij naam te noemen, geboortedorp voor onze Telosophy studie!

Naast het Triadegebouw heb ik een groot deel van mijn promotietijd op het lab van de Experimentele Cardiologie doorgebracht. Tijdens, en vooral na, het behalen van de dagelijkse target van 1152 PCR reacties was er altijd wel wat te beleven. Hiervoor wil ik Hisko, Irma, Lili, Meimei, Bo, Mariusz, Anne-Margreet, Leonie, Michael, Reinout, Rik, Laura, en Hongjuan bedanken. Ook buiten het lab was het altijd gezellig, met als hoogtepunt het (zeil)weekend naar Terschelling en Hamburg waar we elkaar van een heel andere kant hebben leren kennen. Verder wil ik de volgende mensen van het lab natuurlijk ook bedanken voor alle hulp: Herman, Irene, Hassan, Carla, Marjan, Bibiche, Janny, Inge, Bianca, Silke, Linda, Martin en in het bijzonder Jacko: bedankt voor je begeleiding bij het sorteren en prepareren van de Prevend samples. Ik hoop dat je pipetten ondertussen weer schoon zijn. Annet: bedankt voor het opzetten van een keur aan experimenten met "zeldzame zell'n". Met veel geduld heb je me de labtechnieken geleerd, maar daarnaast hebben we al zwervend door het ziekenhuis ook een hoop lol gehad. Germaine: op dezelfde dag zijn we begonnen aan dit project als vreemde eenden in de bijt, op een nog op te richten lab. Je bent er in geslaagd de telomeer-PCR op te zetten en te optimaliseren tot een echte high throughput assay. Dit was ook wel nodig gezien de scheepsladingen DNA die vanuit de hele wereld ondertussen in ons lab zijn beland. Jouw expertise, wetenschappelijk denken, geduld en precisie waren hierin absoluut onontbeerlijk. Gelukkig had je tussendoor ook altijd tijd voor een praatje en goed advies én hebben we samen een hoop afgelachen om (slechte) woordgrapjes. Ik vind het dapper en verstandig dat je weer bent gaan studeren en weet zeker dat je een hele goede dokter zult worden!

Ook wil ik hier de mensen van de COACH-groep bedanken voor de goede en prettige samenwerking. In het bijzonder Tiny en Ivonne voor de input en hulp bij het opzetten van hoofdstuk 3.

De goede samenwerking met de mensen van de athero-express in Utrecht heeft geleid tot hoofdstuk 4. Wouter Peeters, Gerard Pasterkamp en Louise Catanzariti bedankt voor jullie snelle hulp bij het analyseren van de data en het opschrijven van de resultaten.

Dat er zo weinig longitudinale telomeerstudies zijn, komt waarschijnlijk door de lastige statistiek die hier mee gepaard gaat. Ik wil Roy Stewart hier bedanken voor het geduldig begeleiden van mijn eerste stapjes in de complexe wereld van multilevel analyse. Ook wil ik Koos Zwinderman bedanken voor de uitleg hierover. Als ik me niet vergis was jij het die zei dat je multilevel analyse na enige bestudering wel kunt gebruiken maar waarschijnlijk nooit helemaal kunt doorgronden. Deze stelling en je praktische tips gaven mij in de trein terug naar Groningen weer moed, en heeft uiteindelijk geleid tot hoofdstuk 5.

Beste Alma en Audrey: bedankt voor al jullie hulp tijdens mijn promotietraject. Vragen waar ik al dagen mee worstelde werden door jullie in een handomdraai opgelost. Heel erg bedankt voor al jullie hulp de afgelopen jaren.

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Naast collega's zijn er natuurlijk nog andere mensen die een promovendus (in)direct steunen. Ik wil hier dan ook mijn vrienden en kennissen bedanken voor de steun en afleiding. Mayke, Bert, Ferdinand en Esther bedankt voor het vullen van mijn zondagavonden met mooie verhalen en nutteloze feitjes tijdens het pubquizen. Ik wil hier ook de beide Tjeerd S-en bedanken voor menig telomeergefilosofeer, hulp bij layout én het meedenken over de stellingen (waarvan slechts enkelen publicabel bleken).
Lieve Betta, bedankt voor je altijd opbeurende gemekker. Je zult altijd mijn favoriete lievelingsgeit blijven.

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The Road goes ever on and on
Out from the door where it began.
Now far ahead the Road has gone,
Let others follow it who can!
Let them a journey new begin,
But I at last with weary feet
Will turn towards the lighted inn,
My evening-rest and sleep to meet.

[J.R.R. Tolkien, Lord of the Rings]