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Ageing, telomeres, and heart failure

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Ageing, Telomeres, and Heart Failure

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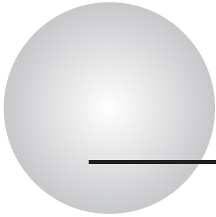

Introduction

CHRONIC HEART FAILURE

Chronic heart failure (CHF) is one of the most prevalent chronic diseases, affecting approximately 3% of the general population. It is even more frequently present in the elderly, with a prevalence of 10-20% in people aged above 70 years. The prevalence of CHF increased dramatically over the past decennia, due to a combination of increased ageing of the general population and increased survival due to improved medical care for CHF patients.[1]

Although treatment options for CHF have improved tremendously over the past decennia, it is still one of the major causes of morbidity and mortality in Western countries. CHF impairs quality of life[2] and has a 5-year survival rate as low as ~50%, which is worse than for most types of cancer.[3]

TELOMERES – THE BEGINNING

In 1908 Alexis Carrel, a surgeon who was awarded with the Nobel Prize for his work on vascular suture and organ transplantation, became interested in growing cells outside of the body. Het started cell culture experiments to investigate the life span of cells in vitro. He succeeded in keeping cell cultures from chicken embryo heart alive, and after 85 days of successfully culturing these cells he published an article in which he concluded that cells could be kept alive for a long time, if not indefinitely, outside the body.[4] His cell cultures seemed to really grow indefinitely, and that experiment was finally terminated after 34 years.[5]

The concept of infinite cell growth also became attractive to other investigators. When Leonard Hayflick and Paul Moorhead tried to repeat these experiments, they came to the contrary conclusion that cells could only enter cell cycle a fixed number of times, before going into "senescence", a state in which cells were not dead but had stopped dividing. They provided convincing evidence that withspoke the results and conclusions from Carrel's experiments. Nowadays, we acknowledge that Hayflick and Moorhead were right about the concept of limited cell division. This phenomenon is now widely known as the Hayflick limit.[6] Intriguingly, the truth behind the experiments of Alexis Carrel has never been revealed. Some scientists speculate that is it probable that the cell cultures were kept alive by the accidental or deliberate addition of fresh cells to the cultures daily.[7,8]

The investigations in cell cycle and the limiting factor in this process continued. One could say that the discovery of telomeres followed inevitably. In 1978, the first report of telomeres was made by Elizabeth Blackburn in the protozoan Tetrahymena.[9] Telomeres are the distal ends of chromosomes. They consist of repeats of nucleotide sequences. Due to erosion, the 'wear and tear' of our DNA, and incomplete DNA replication after every cell cycle telomeres shorten throughout lifetime. When telomeres become critically short, cells will eventually become senescent or dysfunctional.[10] Therefore, it is not surprising that short telomere length has been associated with organ dysfunction and disease.

TELOMERES AND CHF

Telomere length has been associated with cardiovascular disease and risk factors. Most evidence has been gathered on the association between short telomere length and atherosclerotic disease.[11-14] More recently, telomeres have also been

demonstrated to be associated with CHF, in both mouse models and patients.

Life style choices and other environmental factors can influence telomere length negatively.[15-17] However, telomere length has also shown to be largely determined by parental telomere length.[18,19] This gives rise to the intriguing question whether inherited short telomeres could be a factor in people that contributes to the (familial) predisposition for atherosclerotic disease and chronic heart failure, independently from life style choices.

AIMS OF THIS THESIS

Despite the clinical observations on the association between telomere length and CHF, the precise mechanism – if there is one - behind this association is still unclear. In addition, the evidence of a causal relationship between short telomeres and CHF is lacking. This thesis aimed at further exploring the associations between telomere length and CHF and risk factors. Subsequently, a mechanistical explanation for these associations has been sought.

The first part of this thesis focuses on the clinical associations between telomere length and CHF and risk factors. Chapter 1 provides an overview of the associations between telomere and chronic heart failure and associated factors. Chapter 2 and 3 describe the associations between anaemia and renal dysfunction – both frequent co-morbidities of CHF –and short telomere length. Chapter 4 reports a longitudinal study aimed at assessing associations between telomere length trajectory and risk factors for CHF. To date, only few longitudinal studies have been performed investigating the associations between telomere length and cardiovascular risk factors in time.

The second part of this thesis focuses on providing a possible mechanistical explanation for the relationship between heart failure and short telomere length. Chapter 5 describes a premature ageing mouse model and the use of this model in exploring the underlying mechanisms linking telomere shortening and cardiovascular disease. In Chapter 6, telomere length in cardiovascular progenitor cells – one of the possible underlying mechanisms linking telomere shortening and cardiovascular dysfunction - has been investigated in ischaemic heart failure patients, healthy controls, and their offspring. Chapter 7 summarises the recent discoveries on the role of telomere length in cardiovascular disease and discusses future perspectives in telomere length in the field of cardiovascular research. Chapter 8 is a summary of the other chapters of this thesis and discusses some fututre perspective of the role of telomere length in cardiovascular research and practice.

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1 Telomere biology in heart failure

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ABSTRACT

The incidence and prevalence of cardiovascular disease increases progressively with advancing age. Cardiovascular disease is a major cause of morbidity and mortality in Western Countries. In the near future, as the population ages, it is expected that the population prevalence of cardiovascular disease will increase dramatically, imposing a major social and economical burden on society. Not only is age closely related to the development and progression of cardiovascular disease, but genetic and environmental factors also play an important role. Recently, a chromosomal mechanism, telomere shortening, has been considered a driving force by which genetic and environmental factors jointly affect biological ageing, and possibly the risk for developing age-associated diseases. Telomeres are the extreme ends of chromosomes and shorten progressively during every cell cycle and therefore can be considered an indicator of biological age. In heart failure, telomere length is severely reduced. In the current review, we will discuss the emerging role of telomere biology in the pathophysiology of heart failure.

INTRODUCTION

The incidence of cardiovascular disease, including atherosclerosis and chronic heart failure, increases progressively with advancing age.[1] Cardiovascular disease represents one of the major causes of morbidity and mortality in Western Countries. In the near future, as the population ages, it is expected that the prevalence of patients with cardiovascular diseases will increase dramatically, imposing a major social and economical burden on society. Although medical and interventional therapies have greatly improved event free survival, the prognosis of chronic heart failure (CHF) remains poor[2-4] and the search for new strategies to improve outcome continues.[5] Many patients with disorders associated with cardiovascular ageing also have concomitant chronic disorders of other organ systems, including those of the kidneys and lungs.

Not only are age and concomitant diseases strongly related to the development and progression of CHF, but genetic and environmental factors also play important roles. Only recently, have telomeres been considered as a driving force by which genetic and environmental factors jointly affect biological age and pace of ageing, and consequently the risk of developing disorders related to ageing.[6]

In vitro, most somatic cells can undergo only a finite number of cell divisions before reaching senescence. This phenomenon was discovered in the sixties by the famous experiments of Leonard Hayflick.[7] This so-called “Hayflick limit” originates from progressive shortening of telomeres during each cell division. Therefore, telomeres are considered indicators or markers of biological age. The potential role of telomeres and telomerase in the development and progression of cardiovascular diseases is only just beginning to be recognised.[6]

In the current review, we discuss the potential role of telomeres and telomere maintenance in chronic heart failure and risk factors.

TELOMERES AND TELOMERASE

Structure and function

Chromosomal integrity is required for an organism to function and survive.[8] Several mechanisms contribute to chromosomal integrity. One essential mechanism is chromosomal capping by telomeres. However, the exact function and regulation of chromosomal capping by telomeres are only just beginning to be understood.[9,10] Telomeres are specialised functional deoxyribonucleic acid (DNA)–protein complexes which are located at both extreme ends of each chromosome. Telomeres are arranged in such a way that they can form loop structures (T- and D-loops), that act as a protective chromosomal “cap” (figure 1). The telomere sequence varies among species. In humans telomeres are composed of arrays of (TTAGGG)n up to 20 kilo-base pairs in length, terminating in a 3’ single stranded DNA overhang consisting of 100–400 nucleotides.[11,12]

The chromosomal caps formed by telomeres prevent the loss of genetic information and prevent the chromosomes from being recognised as double stranded DNA breaks by DNA damage signalling mechanisms. Telomere capping also prevents the detrimental end-to-end fusion and chromosomal degradation,

which leads to cellular senescence or even apoptosis.[9,10]

Using quantitative fluorescence in situ hybridization techniques, the length of telomeres on specific chromosomes has been studied. In humans, women tend to have longer telomeres than men. This difference has been attributed to potential telomerase upregulation by oestrogens.[13] For all chromosomes there is a linear correlation between length and age.[14] It has been suggested that the telomere length of chromosome 17p is shorter than the median telomere length.[15] However, 13p and 19p also have been identified as having the shortest telomeres.[14,16] In women, accelerated shortening of telomeres has been documented in the inactive X chromosome.[17] In males, there does not seem to be a difference in attrition rate between the Y and X chromosomes.[14] Nevertheless, it remains to be determined whether low average telomere length or specific chromosome arms are responsible for inducing senescence.

Unfortunately, telomeres are unstable structures. In each cell the holoenzyme DNA polymerase aims to replicate telomeres, just like any other chromosomal regions. The regular DNA polymerases fail to completely replicate the human telomeric DNA, a difficulty referred to as the "end-replication problem". Up to a few hundred base pairs of telomeric DNA are lost during each mammalian mitosis. This erosion of telomere length is a cumulative process and eventually the telomere will progress into a critical short, dysfunctional one, leading to cellular senescence or even apoptosis.[10,18-20] Since telomeres mark the number of cell divisions, they are regarded as a biological counter, and as such a marker of biological age.[18,21-24] However, telomere length per se is only one of the many variables which determine the potential of telomeres to form a protective structure.[8]

In contrast to chronological age, defined by date of birth, it may be possible to modify or influence biological ageing. Indeed, species as a whole are not ageing leading to extinction, but instead are reproducing for many millions of years. Circumventing the Hayflick limit by maintaining telomeric DNA length can be

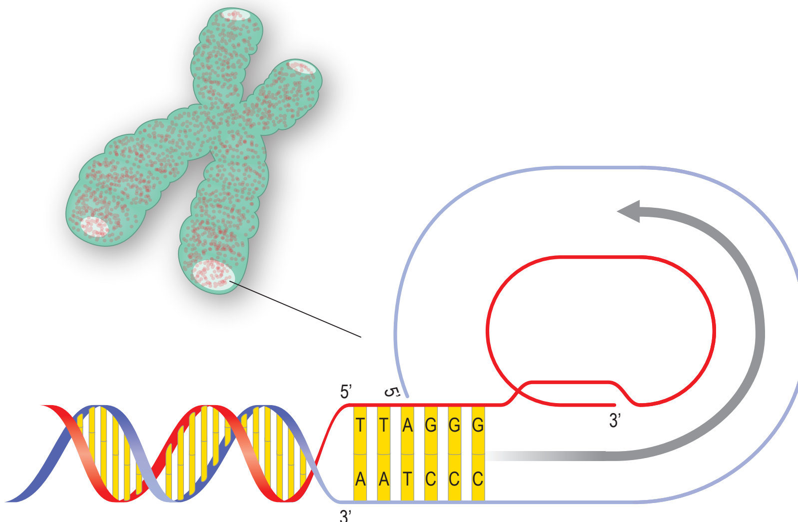

Figure 1. Simplified depiction of a telomere folded in T-loop formation. Telomeres consist of specific nucleotide repeats (TTAGGG and AATCCC) at both ends of each chromosome.

achieved by several mechanisms, including by the specialised ribonucleoprotein enzyme telomerase, which can add the specific TTAGGG repeats to the chromosomal ends.[25] Under physiological conditions, in humans, telomerase is only active in embryogenic stem cells, germline cells and some epithelial and lymphoid progenitor cells.[26,27] Human telomerase is composed of two essential protein components, human TElomerase Reverse Transcriptase (hTERT), and an RNA component, the human Telomerase RNA Component (hTRC).[28] Increased activity of telomerase has been linked to immortalisation of cells, uncontrollable growth and even malignancies.[18] Decreased activity of telomerase and extremely short telomeres have been linked to dyskeratosis congenita, a congenital, multi-system disorder, phenotypically characterized by mucocutaneous abnormalities, pulmonary aberrations, premature ageing, and early death due to bone marrow insufficiency.[29,30] Both the X-linked and autosomal dominant form of dyskeratosis congenita are associated with defects of telomerase and short telomeres. This strongly suggests that telomerase activity is required to maintain telomere length. In addition, short telomeres and absence of telomerase have been shown to result in premature ageing in humans.[29,30]

Telomere repeat binding factors and telomere function

Telomeres are unable to form the protective T- and D-loops without the assistance of several essential proteins. The single most important are the mammalian Telomere Repeat binding Factors (TRF) 1 and TRF2. TRF1 and TRF2 can bind directly to the telomeric DNA region and facilitate the formation of the protective loops (figure 1). In the absence of TRFs, telomeres lose these protective loop structures. Telomere length itself is an important determinant of the ability to form protective loops. However, in the presence of sufficiently high levels of TRF2, even short telomeres can form protective loop structures.[9] Nevertheless, an abundance of TRF2 proteins has also been related to increased telomere shortening in vitro.[9,31] Too much TRF2 seems to lead to compromised repair of oxidative telomeric damage, although it does not affect repair of genomic DNA.[31] A satisfactory explanation for these apparently contrary effects of TRF2 has yet to be provided.

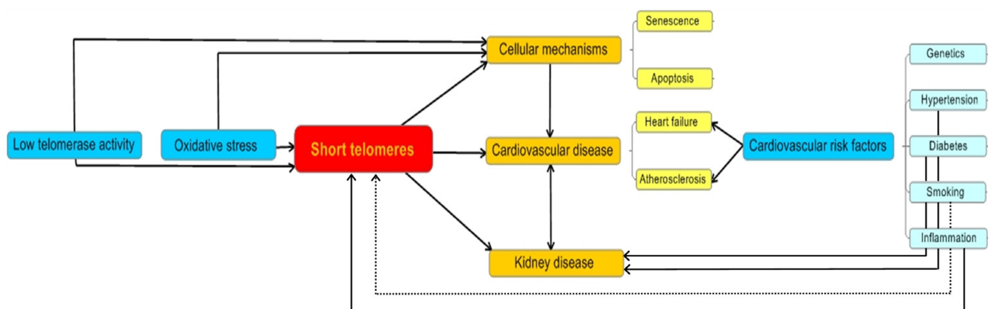

Figure 2. Relationships between telomeres and cellular processes, cardiovascular disease, and kidney disease. Dashed arrow: associative relationship. Continuous arrows: potential causal relationships.

Oxidative stress and telomere length
In addition to telomerase dysfunction, several other processes are associated with increased telomere attrition rate and short telomeres. Oxidative stress, independent of its origin, is considered the major cause of telomere erosion.[32] Cultured vascular smooth muscle cells and endothelial cells exposed to oxidative stress, exhibit increased shortening of telomeres and accelerated cellular senescence.[33] In addition, telomerase activity decreases in response to oxidative stress, which is thought to be a direct consequence of oxidative stress, rather than the result of premature senescence.[34] Smoking and obesity are well-known factors causing in vivo oxidative stress, and are both linked to decreased telomere length.[35] Alternatively, strategies to reduce oxidative stress (e.g. a state of hypoxia or by overexpression of anti-oxidant enzymes) can maintain telomere length and have been associated with increased telomerase activity.[36,37]

TELOMERES, TELOMERASE AND FACTORS LEADING TO CHRONIC HEART FAILURE

CHF is a complex, multi-causal, polygenic disorder of different aetiologies which is becoming increasingly prevalent as the population ages.[38,39] Hypertension, diabetes, and smoking are well-known risk factors for the progression of coronary artery disease and the development of CHF (figure 2), but have also been related to reduced telomere length. We will discuss risk factors for CHF and their relationship with telomere dynamics below.

Hypertension
Hypertension, leading to left ventricular hypertrophy and diastolic dysfunction, is an important early factor involved in the development of CHF. In addition to diastolic dysfunction, it leads to systolic dysfunction and ventricular dilatation.[40,41]

Hypertension has been shown to be associated with reduced telomere length in several, but not all, studies.[42-44] In hypertensive men, white blood cell (WBC) telomere length has been shown to be shorter compared to normotensive men, even after adjustment for chronological age.[43] In patients with type 1 diabetes, hypertensive subjects had shorter telomeres than non-hypertensive, but this difference failed to reach significance after additional adjustment for age.[44] Pulse pressure, the difference between diastolic and systolic blood pressure, which is known to increase with age, is regarded as an indicator of biological ageing of central arteries[45], and predicts cardiovascular mortality.[46] In men, telomere length in white blood cells (WBCs) negatively correlates with pulse pressure, independently from age.[21,45] The relationship between telomere length and pulse pressure in women is inconsistent.[21,45] The renin-angiotensin system is associated with outcome in patients with coronary heart disease.[47] Interestingly, in the Framingham Heart Study, shorter telomere length was also related to higher renin-to-aldosterone ratio, especially in participants with hypertension.[48] Interestingly, telomerase knockout mice with reduced telomere length also suffer from hypertension due to an increased expression of the endothelin-converting enzyme and consequently increased serum levels of the vasoconstrictor peptide endothelin-1.[49]

Diabetes mellitus

Diabetes mellitus is another important risk factor for the development of atherosclerosis and CHF. Diabetes can lead to cardiac dysfunction through several complementary mechanisms. Increased levels of non-esterified fatty acids in diabetes alter the activity of K-channels at the myocyte membrane, leading to decreased cardiac contractility.[50] In addition, the heart is less affected by insulin resistance compared to other organs. As insulin acts as a growth factor, stimulating cell growth through nuclear transcription pathways, hyperinsulinaemia can induce cardiac hypertrophy.[51] Furthermore, hyperglycaemia in diabetes results in increased production of deleterious advanced glycation end products (AGEs), which negatively affect cardiac contractility and ventricular filling pressures.[51]

Diabetes is also associated with reduced telomere length. Type 1 diabetes mellitus patients have shorter telomeres in WBCs compared to non-diabetic controls.[44] Potential confounders such as age, duration of diabetes, or albuminuria showed no independent correlation with telomere length. Type 2 diabetes mellitus has also been associated with shorter telomeres compared to healthy age- and sexmatched controls.[52] There may also be a difference between monocyte and lymphocyte telomere length. Monocyte telomere length has been demonstrated to be significantly shorter in type 2 diabetic patients, but no difference was observed for lymphocyte telomere length.[53] The reason for the differential expression of telomere length among subpopulations of peripheral WBCs is unclear. Interestingly, insulin resistance has also been reported to be associated with reduced telomere length.[43,54]

Cigarette smoking

Cigarette smoking is widely known to be related to the development of coronary heart disease (CHD)[55] and consequently the development of CHF. Smoking predisposes to several other atherosclerotic syndromes, including intermittent claudication, cerebrovascular disease, and glomerular sclerosis. Smoking decreases nitric oxide bioavailability and subsequently vasomotor function, and increases inflammation, leukocyte adhesion, and platelet activation.[55] Smoking slightly increases the number of circulating leukocytes, thereby possibly promoting systemic inflammation.

Smoking has also repeatedly been associated with reduced telomere length. In women, the mean telomere length of WBCs is shorter in smokers than in non-smokers in a dose-dependent manner.[35] Furthermore, a dose-dependent negative relationship between pack years and telomere length in peripheral lymphocytes was found in both patients with chronic obstructive pulmonary disease and controls with normal lung function.[56] Also, among bladder cancer patients and controls, telomere length of WBCs was gradually decreased with increasing number of pack years smoked.[57] However, not all studies have consistently observed a negative relationship between smoking and telomere length, possibly due to confounding factors.[23,58]

Atherosclerosis
In addition to the association between telomeres and the risk factors for atherosclerosis, a relationship between telomeres and atherosclerotic disease itself has also been observed.[23,59-62] WBC telomere length in patients who had a myocardial infarction before the age of 50 years was shorter than in healthy controls, independently from other atherosclerotic risk factors.[23] These findings have been confirmed in other cohorts of patients with coronary disease.[59,62] Not only are WBC telomeres shorter, but telomeres of coronary artery endothelial cells of atherosclerotic plaques are also shorter compared to non-atherosclerotic segments and healthy controls.[60]

Telomere length also predicts future coronary heart disease. In a study, involving 383 subjects, shorter WBC telomere length was associated with an approximately three times higher risk of myocardial infarction.[23] This finding was recently confirmed in a larger study involving 1542 subjects, which demonstrated that subjects with a WBC telomere length of the lowest or middle tertile were at increased risk of developing CHD compared to individuals with the longest telomeres.[63] In addition, the absolute benefit of pravastatin treatment was greatest in patients with the shortest telomeres.[63]

Whether short telomeres are causally involved in the pathogenesis of hypertension, diabetes, or atherosclerosis requires further evaluation. The alternative explanation, that telomere length marks the cumulative life burden of leukocyte turnover or inflammation, cannot be denied.

Valvular heart disease
Valvular heart diseases, such as aortic valve stenosis, can also lead to the development of CHF due to prolonged increased cardiac strain, which causes left ventricle hypertrophy and eventually dilatation and compromised cardiac pump function. Aortic valve abnormalities can be congenital or degenerative, and age-related.[64] Recently, degenerative aortic stenosis has also been associated with decreased leukocyte telomere length independently from possible confounding factors. This may be due to a telomere-dependent decrease in regenerative capacity associated with ageing.[64]

Iatrogenic
Many anti-cancer drugs cause cardiotoxicity or introduce a risk of delayed cardiovascular events. Although direct effects on cardiomyocytes are likely, chemotherapy also induces permanent telomere shortening in blood and bone marrow and possibly in other cells.[65] Currently, several new strategies in the oncology field are aimed at inhibiting telomerase to slow down cell proliferation. Monitoring of the patients included in studies of these new strategies for cardiovascular side effects, is therefore important.

TELOMERES, TELOMERASE, AND CHRONIC HEART FAILURE

CHF is characterised by increased myocyte apoptosis.[66,67] Several studies in animal models have provided important pathophysiological insights into the role

of telomeres and telomerase in cardiac failure and myocyte apoptosis.[67-70] Later generations of telomerase knockout (Terc$^{-/-}$) mice show progressively shortened telomeres. Telomere shortening in these mice is associated with attenuated myocyte proliferation, increased apoptosis and cardiac myocyte hypertrophy. Eventually, left ventricular failure and pathological cardiac remodelling, mimicking the end stage dilated cardiomyopathy of humans, develops in these mice with critically short telomeres.[69] In vitro experiments with cultured rat myocytes have demonstrated that downregulation of TRF2 leads to telomere attrition, activation of the pro-apoptotic protein Chk2, and eventually apoptosis. Conversely, upregulation of TRF2 can protect myocytes from premature apoptosis.[67] Mechanical myocyte stress, comparable to cardiac strain in hypertension, also shortens telomeres and induces Chk2 related apoptosis. In this in vitro model, forced hTERT expression could reverse telomere attrition and related apoptosis.[67]

Recently, it has been suggested that cardiac cells are not simply a homogenous population of post-mitotic cells. Instead, the myocardium consists of a heterogeneous population of myocytes from different biological age categories, myocytes appear to age prematurely under pathophysiological conditions. Experiments in mice have demonstrated that the heart is constantly repopulating the myocyte compartment to replace old, functionally impaired myocytes with younger ones.[71] The old, senescent, poorly contracting myocytes were found to have severely shortened telomeres, while the young and more efficient cells had longer telomeres.[71] A possible source of myocytes for repopulation of the myocardium could be the pool of cardiac progenitor cells (CPCs). It has been reported that CPCs have stem cell like potential, increase in number after myocardial infarction[72], and can migrate to damaged regions of the myocardium and generate young myocytes.[73] In human failing hearts, telomeres are shorter compared to healthy, age-matched controls. This suggests that telomere shortening in the heart does not necessarily accompany normal ageing. A possible explanation for the increased number of dysfunctional, prematurely aged myocytes could be the shortened telomeres observed in failing hearts.

In animals with forced TERT expression, telomerase activity is increased. This prevents telomere erosion and results in increased myocyte density, either by hyperplasia or decreased apoptosis.[70] Myocytes with forced TERT expression also exhibit increased incorporation of mitosis markers compared to control in the first weeks after birth. This suggests a delay of cell cycle exit, and thus replicative abilities of myocytes, under the influence of telomerase activity.[70] Another histological hallmark of heart failure is a decreased capillary density.[74] Endothelial progenitor cells are an important source of vascular repair and maintenance.[75] Short telomeres significantly reduce the angiogenic potential of progenitor cells.[76] Over-expression of TERT, resulting in an increase of telomerase activity and maintenance of telomere length, prolongs the life span and proliferation potential of cultured vascular smooth muscle cells.[36]

As mentioned previously, atherosclerosis — a risk factor for heart failure — is associated with shorter telomeres. However, recent studies in humans have also suggested an important role for telomeres in the pathophysiology of non-ischaemic CHF. Endomyocardial biopsies from 19 elderly patients with dilated

cardiomyopathy were compared with biopsies from 7 subjects of comparable age but without cardiomyopathy. Myocytes from the aged diseased hearts showed significant telomeric shortening, cellular senescence, and cell death.[66] Using confocal microscopy, a 39% reduction in average telomere length in CHF patients compared to healthy controls was observed.[66] These preliminary findings were recently substantiated in a large cohort of 620 CHF patients compared to 183 age- and sexmatched controls (figure 3).[62] Telomeres were shown to be related to the severity of heart failure as they were shorter in patients with higher New York Heart Association (NYHA) class. Ischaemic aetiology was an additional factor associated with shorter telomeres in patients with CHF. Even the number of atherosclerotic manifestations was associated with shorter telomeres (table 1).[62] In patients with CHF, telomere length was shorter in those with renal dysfunction than in those without.[77] There are suggestions that telomere length is associated with reduced ejection fraction in the elderly (table 1).[78]

Notwithstanding all these promising associations, we have to be careful when drawing conclusions regarding cause or effect. The exact mechanism explaining the relationship between reduced telomere length and CHF remains to be elucidated. Although data from experimental models strongly suggests a causal role, evidence beyond associations in humans is now required. The major limitation of almost all human data is its cross-sectional nature, or the lack of telomere length follow-up. Interestingly, not much is known about telomere length in specific subpopulations of circulating WBCs. Most researchers have determined telomere length in easily accessible circulating WBCs. However, these might not be the most relevant cells to consider in CHF.

THERAPEUTIC OPPORTUNITIES

If short telomeres and decreased telomerase activity indeed play a role in pathogenesis of cardiovascular disease, this provides opportunities for intervention. Telomere length in easily obtainable WBCs might provide an early marker of increased cardiovascular risk and could therefore be used to identify patients who would benefit most from early primary preventive treatment strategies.[23,42,63] For example, TRF2 in endothelial progenitor cells can be increased by statin therapy in vitro.[79] These statin treated EPCs also have lower levels of Chk2.[79] Telomere modifying strategies might be useful in stem cell transplantation or for intracoronary infusion of cells after myocardial infarction. Gene therapy is another conceivable approach. For example, specific over-expression of telomerase or TRF2 could contribute to the stability of telomeres, which in turn could contribute to better function of cells directly involved in angiogenesis. Forced hTERT expression in mice has been shown to lead to ventricular hypertrophy without compromising ventricular function and increased tolerance to ischaemia.[70] However, as we discussed earlier, increased telomerase activity can also lead to immortalisation of cells, which could possibly result in the growth of malignancies. As with all treatments, the balance between therapeutic benefit and harmful side effects must be found.

Besides pharmacological and gene therapy, behavioural changes could also

reduce telomere attrition rate. Smoking cessation and increased physical activity also have potential as effective interventions, and may be exceptionally effective in patients with short telomeres.

Table 1. Literature overview of the relationship between telomeres and heart failure

Study subjects	Main findings	Ref.
Suggestive for causal relationship between telomeres and heart failure		
Dogs	TERT upregulation, indicating increased telomerase activity, was found shortly after induced heart failure. Possibly, telomerase activity increases in newly developed heart failure to preserve telomere length and rescue cellular function, and declines as heart failure progresses.	76
Mice and mouse myocytes	Counteracting telomere shortening by TERT overexpression has beneficial effects on cardiac morphology, decreases infarct size in vivo, and rescues myocytes from apoptosis after hypoxia in vivo and in vitro.	78
Mice	Ablation of telomerase, and subsequently short telomeres, lead to myocyte apoptosis, decreased cardiomyocyte number, abnormal myocardial fibre morphology, decreased cardiac myocyte proliferation, myocyte hypertrophy, decreased +dP/dt, decreased –dP/dt, decreased LV developed pressure, elevated LV end-diastolic pressure, development dilated cardiomyopathy, left ventricular failure, and death due to heart failure.	77
Mice	Cardiac strain induces telomere shortening and myocyte apoptosis in the myocardium. Over-expression of TERT has cardioprotective effects.	75
Rats	Functionally competent cardiac progenitor cells, which are thought to be able to regenerate damaged or aged myocardium, had longer telomeres than the aged cardiac progenitor cells.	81
Associative relationship between telomeres and heart failure		
Human, myocardium	Shorter telomere length was measured in myocardium of hearts with dilated cardiomyopathy, compared to healthy controls.	75
Human, myocardium	Myocytes from hearts diseased from ageing cardiomyopathy had approximately 40% shorter telomeres compared to aged control hearts.	74
Human, myocardium	In chronic ischemic heart failure myocardial telomerase activity was increased compared to normal hearts, but less increased than in hearts with acute myocardial infarction. In chronic ischemic heart failure more cardiac progenitor cells with short telomere length were found compared to acute myocardial infarction.	80
Human, leukocytes	Severity of heart failure, expressed in NYHA class, is correlated to shorter telomere length. Telomere length was shorter in case of ischaemic aetiology of heart failure.	69
Human, mononuclear cells	Ventricular ejection fraction was correlated to shorter telomere length in a population of age above 85 years.	85

CONCLUSIONS AND PERSPECTIVES

Telomere length and telomerase have recently been shown to be associated with cardiovascular disease and its risk factors. Critically short telomeres, changes in telomere-binding proteins, and decreased telomerase activity have all been implicated in the activation of cellular damage pathways, and eventually cellular dysfunction, senescence and apoptosis. It remains to be elucidated whether WBC telomere shortening, which is frequently observed in CHD and CHF is a cause or a consequence of the disease. Future experimental and epidemiological studies to determine telomere length in relation to cardiac function will contribute to our understanding of the role of telomeres in cardiovascular disease and might open up new avenues for risk stratification and interventions.

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2 Anaemia is associated with shorter leukocyte telomere length in patients with chronic heart failure

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ABSTRACT

Anaemia is highly prevalent and associated with poor prognosis in patients with chronic heart failure (CHF). Reduced erythroid proliferation capacity of haematopoietic progenitor cells is associated with reduced telomere length, a marker of cellular ageing. We hypothesize that short telomere length contributes to the susceptibility to develop anaemia in patients with CHF.

We studied 875 CHF patients, of whom 254 (29%) fulfilled the WHO criteria of anaemia. Telomere length in DNA from peripheral leukocytes was measured with real-time quantitative polymerase chain reaction.

Age, gender, and baseline differences adjusted telomere length was correlated with haemoglobin levels (partial r=.130; P =0.011). One standard deviation shorter telomere length was associated with an increased risk of having anaemia [odds ratio (OR), 1.31; 95% confidence interval (CI), 1.12–1.53; P=0.001]. This observation was not affected by adjustment for potential confounders (OR, 1.38; 95% CI, 1.05–1.81; P = 0.021 after adjustment for age, gender, erythropoietin levels, renal function, left ventricular ejection fraction, age of CHF onset, blood pressure, history of stroke, diabetes, and B-type natriuretic peptide levels).

Shorter telomere length increases the odds of having anaemia in CHF patients. This finding supports the hypothesis that cellular ageing in CHF contributes to the susceptibility to develop anaemia.

INTRODUCTION

Anaemia is common and associated with a poor prognosis in patients with chronic heart failure (CHF).[1, 2] Several factors in CHF contribute to the development of anaemia, including iron deficiency, renal failure, haemodilution, the use of angiotensinconverting enzyme-inhibitors, and bone marrow dysfunction.[3-5]

Bone marrow cells derived from patients with CHF have decreased in vitro colony-forming activity.[6] In addition, reduced functionality of bone marrow cells has been demonstrated in patients with coronary artery disease. This phenomenon is associated with shorter telomere length, a marker for cellular ageing.[7]

The cause of the bone marrow dysfunction in patients with cardiovascular disease has not been elucidated, but has been suggested to originate from accelerated cellular ageing.[7] If shorter telomere length in the haematopoietic bone marrow stem cells impairs function and proliferative capacity, this might indeed lead to decreased erythropoiesis and consequently anaemia. Telomere length of bone marrow cells is closely reflected by that of the easily accessible circulating leukocytes.[7] Therefore, we explored the potential association between reduced leukocyte telomere length and the presence of anaemia in patients with CHF.

METHODS

This study was a substudy of the Coordinating study evaluating Outcomes of Advising and Counseling in Heart Failure (COACH) of which the main findings have been published.[8-10] In brief, patients were eligible when 18 years of age or older, and had typical signs and symptoms of heart failure and evidence of structural heart disease. COACH enrolled both patients with impaired and preserved ejection fraction. In total, 875 (86%) of the 1023 patients from the COACH study participated in this substudy. The non-participating patients (n = 148) were excluded based on missing DNA samples (n = 133) and missing haemoglobin (Hb) values (n = 15). The Medical Ethics Committee of each participating centre approved both the main COACH study and this DNA substudy.

According to the World Health Organization criteria, anaemia was defined as Hb levels <13.0 g/dL for men and <12.0 g/dL for women.

Storage conditions of blood and DNA and DNA isolation

Venous blood samples were collected into tubes containing EDTA and stored immediately at -80˚C. It has been shown that long-term storage of whole blood samples at this temperature does not influence yield or quality of leukocyte DNA.[11] DNA was isolated using the Qiagen QIAmp® 96 DNA Blood kit (Catalog number 51162, Qiagen, subsidiary Benelux B.V. Venlo, The Netherlands; disposables Greiner Bio-One B.V.). Briefly, 200 mL of lysis buffer was added to a mixture of protease and 200 mL of blood, and incubated for 10 min at 56˚C. Subsequently, 200 mL of ethanol was added to precipitate DNA, and DNA was bound to the silica-gel membrane of the QIAmp 96-well plate. Two washing steps followed, and finally DNA was eluted in 200 mL of elution buffer containing 10 mM Tris–Cl and 0.5 mM EDTA, pH 9.0.

DNA was stored at -80˚C after isolation and thawed at 4˚C before the

quantitative polymerase chain reaction (qPCR) measurements on telomere length were performed. All qPCR measurements were completed within 4 weeks. Storage of DNA at 4˚C for this short time period does not influence DNA quality.[11, 12]

Laboratory measurements
Serum creatinine and urea levels were measured with a colorimetric assay (Roche/Hitachi Modular P analyzer). Haemoglobin levels were measured on the automated haematology analyser (Sysmex XE-2100). B-type natriuretic peptide (BNP) was measured using a fluorescence immunoassay kit (Triage®, Biosite Incorporated, San Diego, CA, USA). Erythropoietin (EPO) levels were determined with the Immulite 2500 Erythropoietin assay.

Telomere length
Mean telomere length was measured by qPCR in leukocytes, as described previously.[13, 14] Telomere length is expressed as T/S ratio, with 'T' being the telomere repeat copy number and 'S' the single gene copy number; in our study, this was the 36B4-gene. The qPCR thermal cycling profile was the same for both T and S, and began with a 95˚C incubation period for 15 min. Forty cycles of 15 s at 95˚C and 1 min at 58˚C followed. The specificity of all T and S amplifications was determined by melting curve analysis. All measurements were performed in triplicate on separate qPCR plates, but in the same well positions. T and S reactions for each sample were also done in the same well position to eliminate position-dependent effects on the measurements. The mean ± standard deviation (SD) coefficient of variation between three measurements was 7 ± 5% for the T assay and 6 ± 4% for the S assay.

Left ventricular and renal function
Left ventricular ejection fraction (LVEF) was determined mainly by echocardiography. A small subset of LVEF data were obtained by MUGA scan or gated SPECT.

At enrolment, renal function of all patients was estimated [estimated glomerular filtration rate (eGFR)] using the simplified modification of diet in renal diseases equation [186.3 x (serum creatinine/88.4)$^{-1.154}$ x age$^{-0.203}$, in women multiplied by 0.742].[15]

Statistical analysis
Telomere length ratio and BNP levels were natural log transformed because of the skewed distributions. The log-transformed values were used in all further analyses on telomere length and BNP.

Baseline characteristics were compared between anaemic and nonanaemic patients by independent sample t-test or χ^2 test as appropriate. We used standard logistic and linear regression analysis for anaemia and Hb levels, respectively. The relationship between Hb levels and leukocyte telomere length cannot be assumed to be linear, and therefore, we also modelled it as a fractional polynomial function [a local mean (0 degree) polynomial smooth at 95% CI level based on the Epanechnikov kernel function evaluated at 50 points]. Two-sided P-values of < 0.05 were considered statistically significant. Statistical analyses were performed with

the use of STATA version 10.0 for Windows software (StataCorp LP, College Station, TX, USA).

RESULTS

Baseline characteristics according to the presence or absence of anaemia are presented in table 1. The study population consisted of 61% males. Median age was 73 years, median eGFR 53 mL/min/1.73m^2, median LVEF 30%, and most patients were in NYHA class II and III. Patients with anaemia were older, had a lower eGFR, longer history of heart failure, higher LVEF, higher BNP levels, lower diastolic blood pressure, and a more frequent history of diabetes mellitus and stroke (table 1).

Table 1. Baseline characteristics

Patient characteristics	Anaemic (N=254)	Non-anaemic (N=621)	Total (N=875)	P
Hemoglobin (g/dL)	11.4 [10.5-12.1]	14.3 [13.4-15.2]	13.5 [12.2-14.8]	
Telomere length (T/S ratio)	0.65 [0.55-0.81]	0.71 [0.60-0.87]	0.69 [0.59-0.86]	<0.001
Age (years)	76 [68-80]	73 [62-78]	73 [64-79]	<0.001
Male (n (%))	161 (63)	374 (60)	535 (61)	0.38
eGFR (mL/min/1.73m^2)	45 [32-61]	57 [44-71]	53 [40-68]	<0.001
Creatinine (μmol/L)	135 [101-172]	108 [89-136]	113 [90-144]	<0.001
Erythropoietin level (mIU/mL)	20.7 [16.3-25.1]	13.7 [11.7-15.8]	15.8 [13.8-17.7]	<0.001
Age of onset CHF (years)	72 [64-79]	70 [60-77]	71 [61-77]	0.009
Body mass index (kg/m^2)	26 [23-30]	26 [24-30]	26 [24-30]	0.14
Left ventricular ejection fraction (%)	33 [25-45]	30 [22-42]	30 [23-44]	0.04
NYHA class (n (%))				0.87
II	125 (50)	313 (51)	438 (51)	
III	115 (46)	282 (46)	397 (46)	
IV	9 (4)	18 (3)	27 (3)	
BNP (pg/mL)	697 [341-1140]	430 [185-836]	493 [222-952]	<0.001
Heart rate (beats/min)	72 [64-82]	72 [64-80]	72 [64-80]	0.60
Blood pressure (mmHg)				
Systolic	115 [100-130]	115 [104-130]	115 [101-130]	0.37
Diastolic	65 [60-71]	70 [60-80]	70 [60-76]	<0.001
Medical history (n (%))				
Myocardial infarction	119 (47)	250 (40)	369 (42)	0.07
Hypertension	106 (42)	261 (42)	367 (42)	0.94
Diabetes mellitus	90 (35)	156 (25)	246 (28)	0.002
Atrial fibrillation/flutter	113 (44)	275 (44)	388 (44)	0.96
Stroke	37 (15)	51 (8)	88 (10)	0.005
Previous admission (n (%))	97 (38)	176 (28)	273 (31)	0.004

Data is presented as "median [interquartile range]" or "number (%)". The body-mass index is the weight in kilograms divided by the square of the height in meters. eGFR – estimated glomerular filtration rate, NYHA - New York Heart Association functional class, CHF – Chronic Heart Failure, LVEF – left ventricular ejection fraction, BNP – B-type natriuretic peptide

Chronic heart failure was mainly due to ischaemic heart disease (n = 369, 42.2%). Other causes of CHF were dilated cardiomyopathy (n = 182, 20.8%), chronic hypertension (n = 128, 14.6%), valvular heart disease (n = 81, 9.3%), and hypertrophic and restrictive cardiomyopathy (n = 25, 2.9%).

Telomere length in the anaemic group was significantly shorter than that in the non-anaemic group ($P < 0.001$) (figure 1). In univariate logistic regression analysis, anaemia was predicted by telomere length (table 2). This remained significant after adjustment for age and gender, and also after additional adjustment for other baseline imbalances (table 2).

To give an indication of the significance of telomere length in our prediction model, we compared the effect of telomere length to that of renal function in predicting anaemia. In the multivariate model, the effect of 1 SD change in telomere length was approximately as large as a 35 mL/min/1.73m^2 decrease in eGFR, a well-known independent risk factor for anaemia.

Supplementary linear regression analyses were performed to explore the continuous relationship between Hb levels and telomere length (figure 2). Shorter telomere length was associated with lower Hb levels (correlation coefficient = 0.119, $P < 0.001$). This remained significant after adjustment for age and gender [standardised coefficient β (Std β) = 0.095, P = 0.004], and after additionally adjusting for EPO level, eGFR, LVEF, stroke, diabetes mellitus, age of onset CHF, diastolic blood pressure, and BNP (Std β = 0.130, P = 0.011).

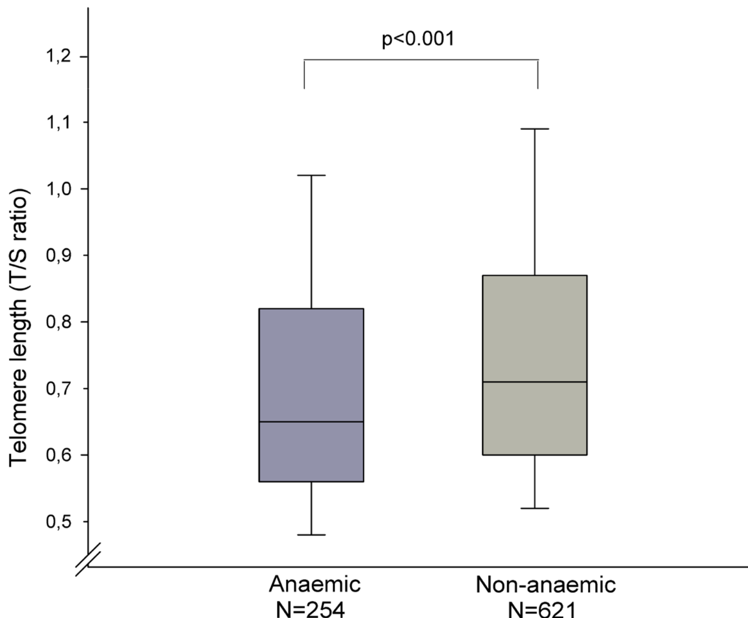

Figure 1. Telomere length in the anaemic vs non-anaemic group. Telomere length is expressed as Telomere/Single copy gene (T/S) ratio. The boxes represent median T/S ratio with 25th and 75th percentiles. Telomere length of anaemic patients with chronic heart failure is significantly shorter than non-anaemic patients.

Table 2. Odds ratio for anaemia in univariate and multivariate logistic regression models.

		Odds ratio	95% confidence interval for the Odds ratio	p
Model 1	Telomere length (SD)	1.31	1.12 – 1.53	0.001
Model 2	Telomere length (SD)	1.25	1.07 – 1.46	0.005
	Age (10 years)	1.23	1.07 – 1.42	0.004
	Male gender	1.21	0.89 – 1.65	0.222
Model 3	Telomere length (SD)	1.38	1.05 – 1.81	0.021
	Age (10 years)	1.24	0.57 – 2.68	0.585
	Male gender	1.88	1.07 – 3.30	0.029
	EPO level (SD)	1.49	1.14 – 1.95	0.004
	eGFR (10mL/min/1.73m^2)	1.11	0.97 – 1.26	0.138
	LVEF (10%)	1.09	0.89 – 1.33	0.424
	History of stroke	2.65	1.21 – 5.77	0.014
	History of diabetes mellitus	1.42	0.81 – 2.51	0.223
	Age of onset CHF (10 years)	1.19	0.58 – 2.42	0.634
	DBP (10 mmHg)	1.27	1.01 – 1.59	0.040
	BNP (100 pg/mL)	1.03	0.99 – 1.06	0.134

NS = not statistical significant, SD = standard deviation, eGFR = estimated Glomerular Filtration Rate, LVEF = left ventricular ejection fraction, CHF = chronic heart failure, DBP = diastolic blood pressure, BNP = B-type Natriuretic Peptide

Model 1: relationship between telomere length and anaemia
Model 2: relationship between telomere length and anaemia, adjusted for age and gender
Model 3: relationship between telomere length and anaemia, adjusted for age, gender, and all baseline differences
The Odds ratio reflects the risk of having anaemia corresponding to a standard deviation decrease of telomere length, expressed as Telomere/Single copy gene (T/S) ratio, a 10-years increase of age, being a male, a standard deviation increase of EPO level, a 10mL/min/1.73m^2 decrease of eGFR, a 10% increase of LVEF, a history of stroke, a history of diabetes mellitus, a 10 year increase of age of onset of CHF, a 10mmHg decrease of diastolic blood pressure, and a 100pg increase of serum BNP level

Since EPO is a major stimulating factor of erythroid proliferation of the bone marrow, we also performed a linear regression analysis of EPO on Hb levels. Haemoglobin level was indeed associated with EPO level univariately (correlation coefficient = -0.302; P < 0.001). This persisted after adjustment for age and gender (Std β = -0.281, P < 0.001), and after adjustment for telomere length, eGFR, LVEF, stroke, diabetes mellitus, age at onset of CHF, diastolic blood pressure, and BNP (Std β = -0.236, P <0.001). We did not find a relationship between telomere length and EPO level (correlation coefficient = -0.058; P = 0.177).

DISCUSSION

In the present study, we found that shorter telomere length is associated with an increased prevalence of anaemia in patients with CHF, whereas EPO level did not show such an association. One SD shorter telomere length was associated with the risk of anaemia comparable to a decrease in eGFR of 35 mL/min/1.73m^2.

Anaemia is a frequent co-morbidity in patients with CHF and is related to poor prognosis.[1] Anaemic CHF patients have a nearly doubled risk of death compared with non-anaemic patients and for each g/dL lower Hb level, the annual risk of mortality increases by 4.6%.[2] On the other hand, polycythaemic CHF patients

with Hb levels > 15 g/dL are liable to worse survival as well.[16] This indicates that strict regulation of Hb level in CHF can improve survival, and should be a point of attention in clinical practice. Unfortunately, anaemia in CHF is often multifactorial of origin or due to chronic disease, and is not readily amenable to therapy.[17, 18]

There are several lines of evidence suggesting that shorter telomere length causes bone marrow dysfunction and possibly plays a contributory role in the development of anaemia.[19-21] Dyskeratosis congenita and Fanconi's anaemia are classical examples of diseases characterized by shorter telomere length.[19, 20, 22] Indeed, bone marrow failure and anaemia are the hallmark features of both diseases. Anaemia in patients with Fanconi's anaemia was even more severe when annual telomere shortening rate was higher.[21] In apparent healthy subjects, telomere length has also been independently associated with decreased red blood cell count, but not with other haematopoietic cell counts, such as leukocytes and thrombocytes.[23] This is in line with the finding that the telomere length of haematopoietic progenitor cells is indicative of their erythroid proliferation capacity in vitro.[24] Erythroid progeny of the haematopoietic progenitor cells with shorter telomeres showed a significantly lower number of population doublings compared with haematopoietic progenitor cells with longer telomeres.[24] Interestingly, telomere length did not affect proliferation of other haematopoietic cell lines.[24]

Taken together, these data show that short telomere length is associated with the impairment of erythroid proliferation. This could be an additional causal factor for the high prevalence of anaemia in CHF patients, besides the established risk factors. In our study, the anaemic group was on average 3 years older than the non-anaemic group. We observed an association of age with telomere length, with a 0.0038 ± 0.0007 decrease in the T/S ratio per year increase in age. In multivariable adjustment, we confirmed that the slight difference in age between the anaemic and non-anaemic groups does not explain the association between telomere length and the risk of anaemia.

The cause of telomere shortening in CHF is still unclear. Short telomeres could be causally related to the development and progression of CHF, but could also be a consequence of CHF-associated factors, such as a history of hypertension, increased oxidative stress due to activation of the renin–angiotensin system[25, 26], or prolonged systemic hypoperfusion due to ventricular pump failure.[27] In-depth research into the precise effects of telomere attrition on CHF and risk factors is needed to draw more definite conclusions about the nature of this relationship.

Telomere length could also possibly serve as a new biomarker for severity of CHF and a predictor of anaemia in CHF. Of course, large prospective studies on progression of CHF and incidence of anaemia are mandatory to determine the clinical applicability of telomere length as a novel biomarker.

Over the past few years, it has become common to use qPCR to determine telomere length. Several studies have compared the results of qPCR with the previously frequently used telomere restriction fragment (TRF) analysis by Southern blotting. The correlation coefficient between T/S ratio and TRF estimated that telomere length is high (r between 0.65 and 0.82).[13, 28, 29] Thus, using qPCR for measuring telomere length is reliable and, in addition, has several practical advantages compared with TRF analysis.

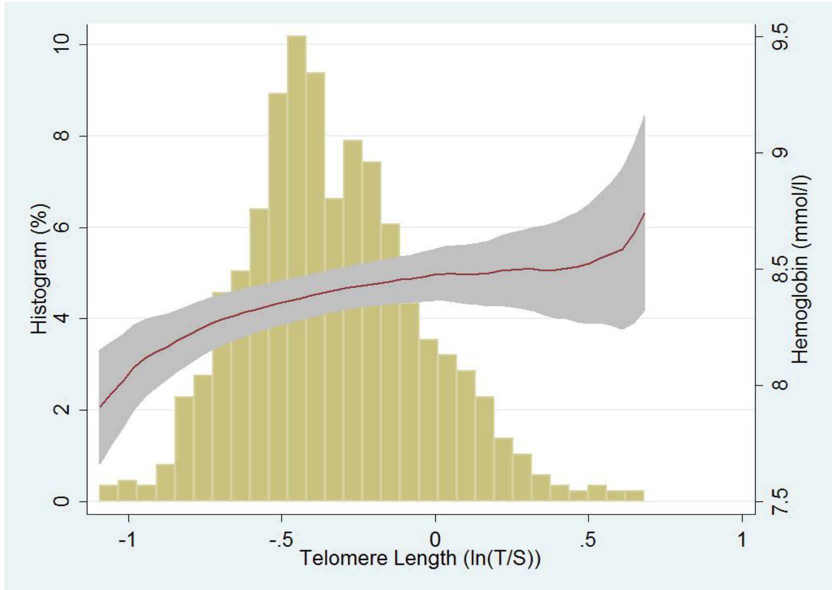

Figure 2. Haemoglobin levels histogram and association with telomere length. The bars of the histogram represent haemoglobin levels (left Y-axis: percentage of subjects per bar). Black line represents the squared relationship between continuous haemoglobin levels and telomere length (right Y-axis: haemoglobin levels in mmol/L; X-axis: telomere length in ln(Telomere/Single copy gene (T/S) ratio). The shaded area indicates the 95% confidence limits as estimated by the fractional polynomial function. Longer telomere length correlates with higher haemoglobin levels.

A limitation of our study might be the measurement of telomere length in circulating leukocytes, and not in bone marrow cells, which are more difficult to obtain. However, it has recently been shown that telomere length of circulating leukocytes strongly correlates with telomere length of bone marrow cells ($r = 0.75$, $P < 0.001$).[7] Secondly, our study was performed in a crosssectional manner and therefore conclusions about the character of the observed association—causal or only associative—cannot be drawn.

In conclusion, we demonstrate that shorter telomere length is associated with an increased risk for the presence of anaemia in CHF patients, even when adjusted for age, renal function, and other potential confounders. The effect of one standard deviation shorter telomere length is comparable to a 35 mL/min/1.73m^2 decrease in eGFR. These findings support the hypothesis that short telomere length contributes to the susceptibility to develop anaemia in patients with CHF. Further research on telomeres and their role in CHF is warranted, as a better understanding of telomere biology might reveal whether telomere shortening is cause or consequence in the pathophysiology of CHF, and this provides us with new therapeutic potentials for CHF.

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3 Renal dysfunction is associated with shorter telomere length in heart failure

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Clin Res Cardiol 2009;98(10):629-34

ABSTRACT

Renal dysfunction is a frequent comorbidity associated with high mortality in patients with chronic heart failure (CHF). The intrinsic biological age might affect the ability of the kidney to cope with the challenging environment caused by CHF. We explored the association between leukocyte telomere length, a marker for biological age, and renal function in patients with CHF.

Telomere length was determined by a real-time quantitative polymerase chain reaction in 866 CHF patients. Renal function was estimated with the simplified Modification of Diet in Renal Disease (MDRD) equation.

The median age was 74 [inter-quartile range 64-79] years, 61% male, left ventricular ejection fraction of 30 [23-44]%, and the estimated glomerular filtration rate (eGFR) was 53 [40-68] mL/min/1.73m2. Telomere length was associated with renal function (correlation coefficient 0.123, $p<0.001$). This relationship remained significant after adjustment for age, gender, age of CHF onset (standardised-Beta 0.091, $p=0.007$). Also additionally adjusting for the severity of CHF and baseline differences did not change our findings.

The association between shorter leukocyte telomere length and reduced renal function in heart failure suggests that intrinsic biological ageing affects the ability of the kidney to cope with the systemic changes evoked by heart failure.

INTRODUCTION

Chronic heart failure (CHF) is an age-associated disease with a high prevalence and incidence in Western Society.[1, 2] Risk factors associated with increased mortality in patients with CHF include hypotension, anaemia, increased BNP levels, activation of the renin-angiotensin system, and decreased renal function.[3-10] The precise nature of renal dysfunction in CHF patients remains to be elucidated. It has been suggested that the decreased cardiac output, increased inflammation and oxidative stress may challenge the function and integrity of the kidney in patients with CHF.[11, 12] At some point, a glomerulus may be irreversibly damaged, leading to "nephron dropout" and accumulating into a progressive decline of renal function. Recently, we provided preliminary data suggesting a possible association between shorter telomere length and reduced renal function in a retrospective study.[13] We hypothesized that a more advanced intrinsic biological age, reflected by telomere length, increases the susceptibility of the kidney to lose function in the challenged physiological environment evoked by CHF.

Telomeres are considered indicators of biological age and are heritable structures located at the extreme ends of chromosomes. Telomeres consist of specific nucleotide repeats, in humans TTAGGG.[14-16] In conjunction with several telomere-binding proteins, telomeres protect chromosomes from recognition and degradation by DNA damage signalling pathways.[17] When telomeres become critically short, they lose their protective function and cells become genetically instable, causing senescence or apoptosis.[14] Telomeres are incompletely replicated by DNA polymerase, causing cumulative attrition of length after each cell division and marking replicative history.[16] Additional telomere attrition can be caused by damaging external factors (e.g., oxidative stress, activation of the renin-angiotensin system).[18, 19] The aim of our study is to explore whether systemic leukocyte telomere length is associated with renal function in patients with CHF.

METHODS

This study was a sub-study of the Coordinating study evaluating Outcomes of Advising and Counseling in Heart Failure (COACH) of which the main findings have been published.[20, 21] The COACH study assessed the value of additional support by a specialised heart failure nurse in the treatment of CHF. Eligible patients were aged 18 years or older, had typical signs and symptoms, and evidence for structural heart disease confirmed by cardiovascular imaging. Patients did not necessarily have to have impaired left ventricular ejection fraction. At inclusion, patients were stable and on oral heart failure medication. In total, 157 (15%) of the 1023 patients who participated in the COACH were not included in this sub-study, mainly because of no available DNA (N=133) or missing serum creatinine values (N=18). This study has been approved by the local Medical Ethics Committee. All patients gave written informed consent.

Renal function and Telomere Length
Glomerular filtration rate (eGFR) was estimated at enrolment with the simplified Modification of Diet in Renal Diseases (MDRD) equation [186.3 x (serum creatinine/88.4)$^{-1.154}$ x age$^{-0.203}$, in women multiplied by 0.742], which is one of the most precise and accurate formulas for calculating GFR.[22] A venous blood sample was taken from the patients during the first outpatient visit and DNA isolated from it according to standard protocols (Qiagen, subsidiary Benelux B.V. Venlo, The Netherlands; QIAmp 96 DNA Blood kit, catalogno. 51162). Mean leukocyte telomere length was measured by quantitative polymerase chain reaction (qPCR) in leukocytes, as previously described in detail.[23] Telomere length is expressed as T/S ratio, which is the relative ratio of telomere repeat copy number “T” to a single-gene copy number “S” (36B4). All samples were assayed in triplicates on separate PCR plates, but in same well positions. The mean±SD coefficient of variation was 7±5% for the T-assay, and 6±4% for S assay.

Statistical analysis
Telomere length ratio was natural log transformed to obtain a normal distribution. Baseline characteristics were compared among quartiles of eGFR by one-way analysis of variance, Kruskis-Wallis test, or Chi square when appropriate. Pearson correlation coefficient was used to assess the association between leukocyte telomere length and renal function. Standard linear regression techniques were used to adjust for age and gender in a second model and additionally for age of CHF onset in a third model. This third basic model was used to subsequently adjust for baseline differences. Because renal function cannot be assumed to be linearly related to leukocyte telomere length, it was also modelled as a fractional polynomial function. A two-sided P-value of <0.05 was considered to indicate statistical significance. All statistical analyses were performed with use of STATA version 10.0 for Windows software (StataCorp LP, College Station, TX, USA).

RESULTS

Baseline characteristics according to quartiles of eGFR are presented in table 1. The study population consisted of 61% men, median age was 74 years, median left ventricular ejection fraction (LVEF) was 30%, with most patients in NYHA class II and III (together 97%). Patients with decreased renal function were less likely to be men, and more likely to be older of age, to have higher NYHA class, hypertension, diabetes, atrial fibrillation or flutter, lower hemoglobin levels, and a previous admission for CHF (table 1).

Estimated GFR decreased with age at a yearly rate of 0.70±0.058 mL/min/1.73m^2 (p<0.001). Telomere length ratio decreased steadily at a mean rate of 0.0035±0.00064 per year of increase of age (p<0.001).

Telomere length was 0.719 (interquartile range 0.609-0.881) in the quartile with the highest eGFR, 0.710 (0.604-0.855) in quartile 2, 0.673 (0.582-0.834) in quartile 3, and 0.667 (0.571-0.825) in the quartile with the lowest eGFR (p=0.031). When leukocyte telomere length was modelled as a continuous predictor, renal function decreased gradually with shorter telomere length. Pearson correlation coefficient

Table 1. Baseline characteristics

Patient characteristics	Quartiles of estimated GFR (eGFR)				Total	P
	1 (N= 216)	2 (N= 217)	3 (N= 216)	4 (N= 217)	N= 866	
eGFR (mL/min/1.73m^2)	79 [73-88]	61 [57-65]	46 [43-49]	31 [26-36]	53 [40-68]	
Creatinine (μmol/L)	83 [71-91]	104 [90-113]	131 [113-141]	174 [153-205]	113 [91-144]	<0.001
Telomere length (T/S ratio)	0.72 [0.61-0.88]	0.71 [0.60-0.85]	0.67 [0.58-0.83]	0.67 [0.57-0.82]	0.69 [0.59-0.85]	0.031
Natural log T/S ratio	-0.37 ±0.28	-0.34 ±0.28	-0.32 ±0.27	-0.31 ±0.28	-0.34 ±0.28	0.031
Age	66 [57-74]	73 [64-79]	75 [67-81]	78 [71-81]	74 [64-79]	<0.001
Male Gender, n (%)	148 (69)	140 (65)	134 (62)	107 (49)	529 (61)	<0.001
NYHA class, n (%)						0.001
II	135 (63)	112 (53)	99 (46)	87 (41)	433 (51)	
III	78 (36)	93 (44)	109 (51)	115 (54)	395 (46)	
IV	3 (1)	7 (3)	6 (3)	10 (5)	26 (3)	
Age of onset CHF– yr	64 [54-73]	71 [62-76]	71 [63-78]	74 [68-79]	71 [61-78]	0.001
LVEF (%)	30 [22-40]	30 [21-44]	30 [23-45]	33 [25-43]	30 [23-44]	0.44
Body Mass Index (kg/m^2)	26.0 [23.5-29.4]	26.3 [23.9-29.7]	26.2 [23.7-29.7]	26.1 [23.0-29.4]	26.1 23.5-29.6]	0.71
Blood pressure (mmHg)						
Systolic blood pressure	110	120	115	120	115	0.002
	[100-125]	[105-130]	[105-130]	[100-137]	[101-130]	<0.001
Diastolic blood pressure	65 [60-76]	70 [60-80]	65 [60-70]	65 [60-75]	69 [60-75]	
Heart rate (beats/min)	76 [66-86]	72 [66-80]	72 [64-80]	72 [64-80]	72 [64-82]	0.03
Medical history, n (%)						
Diabetes	52 (24)	52 (24)	60 (28)	81 (37)	245 (28)	0.005
Hypertension	81 (38)	77 (35)	93 (43)	113 (52)	364 (42)	0.002
Myocardial infarction	77 (36)	85 (39)	97 (45)	103 (47)	362 (42)	0.05
Atrial fibrillation/flutter	76 (44)	91 (42)	107 (50)	110 (51)	384 (44)	0.003
Stroke	18 (8)	17 (8)	26 (12)	26 (12)	87 (10)	0.29
Laboratory measurements						
NT-pro-BNP (pg/mL)	2027 [1259-4242]	1983 [1130-3624]	3016 [1202-4742]	4572 [1506-10664]	2530 [1259-5548]	<0.001
Hemoglobin (mmol/L)	8.7 [8.0-9.3]	8.8 [7.9-9.3]	8.3 [7.6-9.1]	7.8 [7.1-8.6]	8.4 [7.6-9.2]	<0.001
Previous admission, n (%)	48 (22)	56 (26)	69 (32)	102 (47)	275 (32)	<0.001
Current medication, n (%)						
RAS-inhibitors	189 (88)	189 (87)	182 (84)	155 (71)	715 (83)	<0.001
Beta-blockers	145 (67)	149 (69)	141 (65)	135 (62)	570 (66)	0.52
Diuretics	205 (95)	212 (98)	205 (95)	206 (95)	828 (96)	0.39
Digoxin	77 (36)	63 (29)	74 (34)	52 (24)	266 (31)	0.034
Statins	78 (36)	91 (42)	85 (39)	79 (36)	333 (38)	0.56

Normally distributed data is presented as means ± SD, skewed distributed data as median [interquartile range]. The body-mass index is the weight in kilograms divided by the square of the height in meters. eGFR – estimated glomerular filtration rate, NYHA - New York Heart Association functional class, CHF – Chronic Heart Failure, LVEF – left ventricular ejection fraction, NT-pro-BNP - N-terminal pro-B-type natriuretic peptide, RAS-inhibitors - renin-angiotensin-system inhibitors (angiotensin-converting enzyme inhibitor and/or angiotensin-receptor blocker). Diuretics include loop diuretics, thiazides, and aldosterone antagonists.

for the association between telomere length and eGFR was 0.123 ($p<0.001$). The relationship between renal function and telomere length remained significant after adjustment for gender and age (standardised beta 0.090; table 2). In the third basic model we also adjusted for the age of CHF onset (figure 1). Our findings did not change after additionally adjusting for baseline differences (diabetes, hypertension, history of myocardial infarction, NYHA class, systolic blood pressure, diastolic blood pressure, heart rate, atrial fibrillation, NT-pro-BNP, hemoglobin levels, use of renin-angiotensin system inhibitors, and digoxin; table 2).

DISCUSSION

A frequent co-morbidity factor and powerful predictor of mortality in CHF is decreased renal function.[4, 6, 7] The main finding of this study is that reduced leukocyte telomere length, as a marker for advanced intrinsic biological age, is associated with decreased renal function in patients with CHF. This observation remained significant after adjustment for several confounders, including age, age of CHF onset, and severity of CHF.

Telomere length is associated with CHF. We recently demonstrated telomere length to be shorter in 620 patients with CHF compared to healthy controls.[23] This was also observed by others.[24] In addition, levels of TRF2 – one of the telomere-stabilizing proteins – in the myocardium of heart failure patients was found to be down-regulated by approximately 50% compared to healthy controls.[24] Interestingly, Werner et al. found that physical exercise in mice up-regulated TRF2, and protected

Table 2. Univariate and adjusted standardised beta for association between renal function and telomere length

	Std-Beta	**95%CI**	**P-value**
Model 1	0.123	0.057-0.189	<0.001
Model 2	0.090	0.023-0.157	0.008
Model 3	0.091	0.024-0.158	0.007
Model 3			
+ Diabetes	0.090	0.023-0.157	0.008
+ Hypertension	0.091	0.024-0.159	0.008
+ Previous myocardial infarction	0.092	0.024-0.158	0.007
+ NYHA Class	0.085	0.018-0.153	0.013
+ Systolic blood pressure	0.088	0.021-0.155	0.010
+ Diastolic blood pressure	0.090	0.023-0.157	0.009
+ Heart rate	0.090	0.023-0.157	0.009
+ Atrial fibrillation/flutter	0.091	0.024-0.157	0.008
+ NT-pro-BNP	0.103	0.011-0.194	0.028
+ Hemoglobin	0.100	0.010-0.187	0.029
+ RAS-inhibitors	0.074	0.007-0.142	0.031
+ Digoxin	0.094	0.027-0.161	0.006

Model 1: univariate. Model 2: adjusted for age and gender. Model 3; adjusted for age, age of heart failure onset, and gender. NYHA - New York Heart Association functional class, NT-pro-BNP - N-terminal pro-B-type natriuretic peptide, RAS-inhibitors - renin-angiotensin-system inhibitors (angiotensin-converting enzyme and/or angiotensin-receptor blocker)

the myocardium from doxorubicin-induced apoptosis.[25] Thus, telomere biology is not only associated with CHF, but seems to be a modifiable factor in heart failure. Possibly, telomeres are a new therapeutic target in heart failure.

A retrospective analysis of the cohort of 620 CHF patients suggested a potential association between telomere length and renal function.[13] Obviously, retrospective analysis is susceptible to type-1 errors. The current prospective study, however, provides important independent confirmation of these preliminary findings. Reduced renal function might be associated with shorter telomere length in patients with CHF for several reasons. First, the processes in biological ageing and renal senescence associated with renal function decline include a decreased ability of aged nephrons to cope with diseased states. CHF elicits systemic changes, including decreased cardiac output, inflammation, oxidative stress, and activation of the renin-angiotensin system.[9, 11] Nephrons with shorter telomeres might be less resistant to these challenges and more likely to enter a senescence state, become dysfunctional or even apoptotic. The phenotype of human renal senescence has indeed been described previously as the loss of mass and function, including a loss of glomerular filtration rate.[26] Second, leukocytes telomeres mark replicative history and therefore might mark the cumulative inflammatory burden a patient has been exposed to.[27] Inflammation is a major causal factor of vasculo- and glomerulopathy and consequently might cause a decrease in renal function. Finally, other factors associated with biological ageing (e.g., accumulation of advanced glycation endproducts) might cause renal dysfunction and coincide with shorter telomere length.[28]

The cross-sectional nature of our study does not allow drawing definite conclusions concerning the nature of the observed association. Although we used multiple statistical adjustments, we cannot exclude possible confounding factors that may have obscured the observed relationship.

In conclusion, decreased renal function was associated with reduced leukocyte telomere length in patients with CHF. This observation support the hypothesis that increased intrinsic biological age affects the kidney in its ability to cope with the systemic changes evoked by CHF and might explain, at least in part, why renal function is closely related to mortality in patients with CHF.

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4 Telomere loss due to smoking and obesity

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ABSTRACT

Human telomeres attrition with age and short telomeres are associated with several age-associated diseases. There is considerable inter-individual variation in the rate of telomere attrition. Determinants of telomere attrition have not been fully elucidated.

To identify determinants of telomere attrition, we measured a total of 17,796 telomere length samples by multiplex monochrome real time quantitative polymerase chain reaction in all DNA available samples from 8,074 subjects collected on three occasions (at baseline, ~4.3 years, and ~6.6 years of follow-up). Multilevel growth models were created to identify factors influencing telomere dynamics.

We observed an attrition rate of 0.57 ± 0.08 relative telomere lengths units (RTLU) per year. Telomere attrition rate was increased in older subjects, with a larger increase in males compared to females. The major environmental factors determining telomere attrition rate were smoking (-0.67 ± 0.16 RTLU per year (yr) smoking; $P<0.0001$) and multiple components of the obesity related metabolic syndrome (waist-hip ratio: -2.66 ± 0.99 RTLU per 1.0 ratio per yr; $P=0.007$, glucose levels: -0.76 ± 0.38; RTLU per mmol/L/yr $P=0.045$ and HDL-cholesterol levels: +0.91 ± 0.24 RTLU per mmol/L/yr $P< 0.001$).

Smoking and the metabolic syndrome accelerate telomere attrition in humans. These modifiable factors may contribute to their adverse impact on risk of numerous age-associated diseases.

INTRODUCTION

Telomeres are the terminal ends of linear chromosomes, consisting of large numbers of tandem repeats of a simple DNA sequence (TTAGGG in humans), that are essential structures involved in maintenance of chromosomal stability and cell cycle control. In humans, the length of telomeres has been considered to mark the inter-individual variation of biological ageing for several observations: (1.) in vitro, somatic cells 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, and (3.) in vitro, the amount of telomere loss during each cellular division varies and is affected by environmental factors. When the mean telomere length reaches a critical value to produce inadequate telomeric end protection a DNA damage signal is generated which causes cell growth arrest or cell death. As such, telomere erosion is an important tumor-protection mechanism. However, a considerable number of studies have now shown an adverse association between shorter telomere length and other ageing-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] Several cross-sectional studies have shown an association between telomere length and demographic variables as well as life-style cardiovascular risk factors. However, such studies cannot establish whether such factors affect telomere attrition. This requires longitudinal studies with serial measurements of telomere length in the same subjects. Therefore we studied telomere length dynamics in a large population based cohort with longitudinal follow-up to identify the factors associated with temporal changes in telomere length.

METHODS

Study population

This analysis was performed in the framework of the Prevention of Renal and Vascular End Stage Disease study (PREVEND; www.prevend.org). 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. 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 measurement

To avoid any impact of variation in DNA extraction method on telomere length measurement, all samples analysed in the current study were (re)extracted

uniformly using the same DNA extraction kit (QIamp 96 DNA blood kit, catalog # 51162, Qiagen, Venlo, The Netherlands) from non-defrosted full-blood samples according to the instructions of the manufacturer. DNA samples from different collection moments were mixed and randomly extracted to neutralise potential batch-effects. Samples with a DNA concentration > 70 µg/mL (determined by the nanodrop method) were diluted to reach a final concentration of 20 – 60 µg/mL. Detailed information on the telomere length measurement is provided in the supplement. In brief, mean leukocyte telomere length was measured using a novel multiplex monochrome real time quantitative polymerase chain reaction technique.[9] This technique allows carrying out the telomere specific amplification and the reference gene amplification in a single reaction well with quantification measurements at different temperatures.[9] Samples were compared to a calibration curve which was run 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 (kindly 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 (RTLU). All samples were measured in triplicate and the average of the three runs was used to provide the mean RTLU for each individual.

We adhered to arbitrary categorisation of telomere trajectories previously reported (1.) RTL shortening, when >10% decrease of RTL was present at T3 compared to baseline; (2.) 10% or less change of RTL at T3 compared to baseline, and (3.) RTL elongation for > 10% increase in RTL at T3 compared to baseline.[10, 11]

Other measurements and definitions

All participants completed a questionnaire on demographic profile, cardiovascular disease history, medication use and smoking habits. Smoking was categorised 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 mmHg or diastolic blood pressure (DBP) of ≥ 90 mmHg or the use of antihypertensive medication. Blood pressures were defined as normal when SBP < 120 mmHg and DBP < 80 mmHg. 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/m^2. Subjects were categorised as non-obese when BMI was below 25 kg/m^2. All subjects in between were categorised 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. Hypercholesterolaemia 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]

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 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.

RESULTS

Baseline characteristics

Baseline characteristics of the subjects studied are presented in table A. The median age at baseline was 48 [range 28 to 75] years with 50.1 % females and 94.9% Caucasian. At baseline (T1) telomere length could be successfully determined in 8,074 subjects (94% of available DNA samples). At baseline, shorter telomere length was associated with higher age, male gender, higher blood pressure, hypercholesterolaemia, diabetes, higher waist-hip ratio and body mass index, and cigarette smoking (tables 1 and 2).

Telomere dynamics

In addition to the baseline measurements (T1), we obtained 9,230 follow-up measurements from 2 moments of follow-up; 3,572 for the second time point (T2, on average at 4.3 years after T1) and 5,457 for the third time point (T3, which was on average 6.6 years after T1). Using the unconditional growth model we observed an average telomere attrition rate of -0.57±0.078 RTL per year in the longitudinal analysis, which is similar to the attrition rate derived from the cross-sectional analysis (-0.47 (-0.52 to -0.42)) (table 1). However, inspection of the data showed

Table 1: baseline values and association with baseline telomere length

	Baseline-value (n=8074)	B (+ 95% C.I.)	Standar-dized B	p-value*
Age (years)	48 [39 – 60]	-0.47 (-0.52 to -0.42)	-0.205	<0.001
Male Gender (%)	4027 (49.9)	-2.51 (-1.26 to -3.76)		<0.001
Systolic blood pressure (mm Hg)	126 [114 – 141]	-0.030 (-0.066 to 0.006)	-0.021	0.098
Diastolic blood pressure (mm Hg)	73 [67 – 80]	-0.029 (-0.102 to 0.043)	-0.010	0.425
eGFR (mL/min.x 1.73m^2)	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
Body Mass Index (kg/m^2)	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
C-reactive protein (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-Cholesterol (mmol/L)	1.27 [1.03 – 1.56]	6.28 (3.99 to 8.57)	0.0647	<0.001
Cholesterol-HDL ratio	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		-2.20 (-3.37 to -1.03)		<0.001
<6	447 (16.4)			
6-20	1786 (65.3)			
> 20	499 (18.3)			

Data is presented as "median [interquartile range]" or "number (percentage)". eGFR; estimated glomerular filtration rate, HDL; high-density lipoprotein, p-value after adjustment for age and gender.

considerable variation among individual telomere trajectories. In 44.1% of subjects RTL shortening occurred (defined as >10% decrease of RTLU), 21.9 % had a stable RTL trajectory (defined 10% or less change of RTLU) and in 34.0 % of subjects, we observed RTL elongation (defined as >10% increase of RTLU) after 6.6 years. Increasing age and male gender were again 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 per year for females and 0.470±0.209 per year 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 details the modelling of age, gender, ethnicity and baseline RTL for our primary model providing the conditions in which further covariates were evaluated.

Table 2 : baseline RTL for subgroups

	N (%)	Mean RTL (95% CI)	Median T/S (IQR)	P-value *	Adjusted P-value †
Gender				<0.001	<0.001
Female	4047 (50.1)	3.75 (2.85 to 4.64)	1.026 (0.844 – 1.251)		
Male	4027 (49.9)	0.16 (-0.74 to 1.06)	0.982 (0.8179 – 1.201)		
Race					
Caucasian	7664 (94.9)	1.68 (1.03 to 2.33)	0.999 (0.829 – 1.221)		
Negroid	77 (1.0)	12.46 (5.86 to 19.06)	1.111 (0.911 – 1.435)	0.01	0.016 ‡
Asian	167 (2.1)	5.86 (1.06 to 10.66)	1.019 (0.850 – 1.302)	0.06	0.333 ‡
Others	166 (2.1)	5.89 (1.22 to 10.56)	1.072 (0.844 – 1.276)	0.07	0.289 ‡
Hypertension				<0.001	0.013
Yes	2577 (31.9)	-3.57 (-4.67 to -2.47)	0.949 (0.785 – 1.140)		
Intermediate	3241 (40.2)	3.68 (2.69 to 4.66)	1.026 (0.843 – 1.244)		
No	2256 (27.9)	5.81 (4.59 to 7.03)	1.042 (0.866 – 1.288)		
Diabetes				<0.001	0.024
Yes	203 (2.5)	-8.00 (-11.78 to -4.22)	0.915 (0.758 – 1.107)		
No	7871 (97.5)	2.22 (1.57 to 2.86)	1.004 (0.833 – 1.230)		
Obesity				<0.001	0.001
Yes	1259 (15.8)	-2.28 (-3.83 to -0.72)	0.963 (0.800 - 1.159)		
Intermediate	3255 (40.7)	0.39 (-0.61 to 1.40)	0.984 (0.814 - 1.211)		
No	3473 (43.5)	4.85 (3.88 to 5.82)	1.035 (0.858 - 1.265)		
Hypercholes-terolaemia				<0.001	0.005
Yes	1682 (20.9)	-3.01 (-4.36 to -1.66)	0.956 (0.798 – 1.159)		
Intermediate	3713 (45.8)	1.58 (0.63 to 2.53)	0.996 (0.824 – 1.224)		
No	2679 (33.3)	5.73 (4.64 – 6.82)	1.048 (0.865 – 1.275)		
Smoking				<0.001	<0.001
Yes	2742 (34.1)	-0.44 (-1.53 to 0.65)	0.979 (0.812 – 1.198)		
Stopped	2929 (36.4)	1.78 (0.72 to 2.84)	0.996 (0.828 – 1.218)		
No	2373 (29.5)	4.93 (3.78 to 6.09)	1.041 (0.856 – 1.266)		

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

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: In the longitudinal analyses the binary trait of hypertension 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, hypercholesterolaemia or elevated total and LDL-cholesterol level were not associated with increased telomere shortening. An increase of cholesterol/HDL ratio and triglycerides 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).

Smoking*:* Smokers had a higher telomere attrition rate, than subjects who never smoked or who stopped smoking before the baseline measurement (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 (table 3). The distribution of fat, as measured by waist-hip-ratio, was also associated with telomere attrition rate (table 3, figure 1).

Multi factorial 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 full model included baseline RTL, age, gender, age x gender, smoking, waist-hip ratio, glucose and HDL-cholesterol. In this 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.

DISCUSSION

Here we describe the first comprehensive evaluation of the determinants of telomere attrition in humans. Few studies have evaluated longitudinal telomere length dynamics and all previous longitudinal studies were insufficiently powered (up to 662 subjects[19]) to adequately address the relationships of cardiovascular risk factors and longitudinal changes of telomere length. Previous small longitudinal studies have reported associations of waist hip ratio,[10] HDL cholesterol,[19] and smoking[20] but were unable to replicate each others findings. In the present evaluation 8,074 subjects were studied on multiple occasions (17,796 total samples), providing the first convincing data demonstrating that blood pressure, body composition, smoking habits and glucose- and lipid metabolism are all associated with telomere length attrition rate over time, independent of age, gender and baseline telomere length. In addition, we also observed a significant gender effect, as was suggested previously.[20]

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]
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 support earlier studies suggesting more dynamic changes, although the overall average length is likely to

Table 3: Estimates of annual telomere attrition rate

Model	Variables included	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 (RTLU)	-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 × male gender	-0.021 (-0.041 to -0.001)	0.043
Hemodynamics factors	Systolic blood pressure (10 mm Hg)	-0.074 (-0.143 to -0.004)	0.038
	Diastolic blood pressure (10 mm Hg)	-0.150 (-0.286 to -0.014)	0.031
Metabolic factors	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 (kg/m^2)	-0.035 (-0.063 to -0.007)	0.013
	Waist-hip ratio	-4.065 (-5.870 to -2.259)	<0.001
	HDL-cholesterol (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
Full model	Time (years)	-0.255 (-0.526 to 0.015)	<0.001
	Baseline telomere length (RTLU)	-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

decrease in time. This observation supports the notion of the previous suggested homeostatic process of telomere length. 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.

Nevertheless, considering some of the potential 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. One of the limitations of our study is the large number of missing values in T2. Besides a natural lost to follow-up, the major reason for missing values is incomplete collection of whole blood for DNA isolation at T2 as it was initially thought not to be relevant to re-collect DNA samples. 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. The little variation in ethnicity could also be considered a limitation. Although we observed ethnicity-specific differences in baseline RTL, we did not

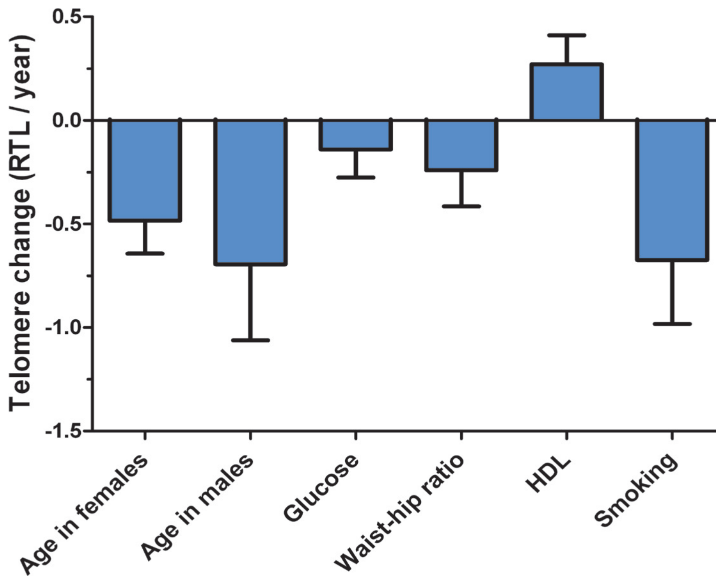

Figure 1: effect size on RTL change in the multifactorial model
The effects of the variables present in the full model on RTL change. The 0-line represents the basic attrition rate for non-smoking subjects when all other variables are at the mean. The boxes represent the additional telomere attrition rate for an increase of respectively: 10 years for age, one standard deviation for 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.

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. Nevertheless, these collection differences and duration of storage differences are factors that are unavoidable factors of a longitudinal design and more likely will affect our quantitative estimates rather than our qualitative conclusions.

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. One could hypothesise that modifying smoking behaviour and obesity would lead to a slower pace of biological ageing, 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.

In conclusion, we here present the largest study measuring telomere length to date and provide evidence for a longitudinal association of accelerated telomere attrition due to smoking, higher waist hip ratio, low HDL-cholesterol levels and high glucose levels. This notion support the hypothesis that smoking and the metabolic syndrome increase biological ageing pace.

SOURCES OF FUNDING

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CONFLICTS OF INTEREST

We declare that we have no conflict of interest.

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Supplement Material

Telomere measurements

All laboratory work and PCR data analysis 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 triplicate 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.[2,3] 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 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 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 in every 384-well plate. Good linearity was observed across this range (R2 = 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).[4] The S albumin primers were albu: **CGGCGGCGGGCGGCGCGGGCTGGGCGG**AAATGCTGCACAGAATCCTTG albd: **GCCCGGCCCGCCGCGCCCGTCCCGCCG**GAAAAGCATGGTCGCCT-GTT. The predicted product size is 106 bp. Bold 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).[5] 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.[6] 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 (r2=0.99, P<0.0001, inter-run CV 3.9%)[7,8] (figure S1). 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

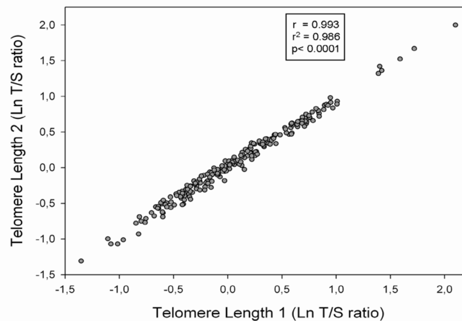

Figure S1. Correlation between telomere lengths of 216 subjects measured on two different occasions.

improve readability each telomere length was multiplied by 100.

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.[9,10]

All participants completed a questionnaire on demographic profile, cardiovascular disease history, medication use and smoking habits. Smoking was categorised 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 categorised as non-obese when BMI was below 25 kg/m2. All subjects in between were categorised 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. Hypercholesterolaemia 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.[11]

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 full 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.

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 full 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 ratio	0.96	1	0.327
DBP	0.94	1	0.332
Gender x age, gender	6.74	2	0.034
Full model	3.85	6	0.797

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

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5 Telomere biology in cardiovascular disease: the TERC$^{-/-}$ mouse as a model for heart failure and ageing

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ABSTRACT

Atherosclerosis and heart failure are major causes of morbidity and mortality in Western countries. Recent studies are suggesting involvement of telomere biology in the development and progression of age-associated conditions, including hypertension, atherosclerosis, and heart failure. Whether any of these reported associations are based on causal relationships remains to be elucidated. The construction of telomerase deficient ($TERC^{-/-}$) mice might provide a potential instrumental model to study the involvement of telomere biology in cardiovascular disease. Here, we review the current available information from all studies performed in $TERC^{-/-}$ mice providing information on the cardiovascular phenotypic characteristics. Although this mouse model has proven its value in the understanding of the role of telomere biology in cancer, stem cell, and basic telomere research, only few studies were specifically designed to answer cardiovascular related questions. The $TERC^{-/-}$ mice provide exciting opportunities to expand our knowledge of telomere biology in cardiovascular disease and the potential identification of novel targets of treatment.

INTRODUCTION

Cardiovascular disease is the leading cause of morbidity and mortality in Western countries.[1] Although experimental and epidemiological studies have identified many factors involved in pathogenesis of atherosclerosis and heart failure, our understanding is still incomplete. Recently, telomere biology has entered the cardiovascular research field as a potential factor involved in the initiation and progression of atherosclerosis and heart failure.[2-5] Decreased telomere length has been associated with heart failure and atherosclerosis in human cross-sectional studies. However, there is no convincing human evidence implicating telomeres as a cause of atherosclerosis or heart failure. The construction of genetically modified mice with short telomeres provides an opportunity to increase our knowledge on the nature of the relationship between changes in telomere biology and cardiovascular disease. This mouse model has advanced our understanding of the role of telomeres in cancer, but there are only a few specific studies focuses on cardiovascular disease. Here, we will review all available information on cardiovascular phenotypic characteristics of the telomerase deficient mice. We expect that future experiments in these mice might help to fill in important deficiencies in our knowledge on the role of telomeres in cardiovascular disease.

TELOMERE BIOLOGY

Structure and function of telomeres

Telomeres are the distal ends of chromosomes – present in all eukaryotes – and are made up of tandem repeats of specific DNA sequences (TTAGGG in vertebrates). The length of telomeres varies among and within species, but take up to approximately 15 kilobases in humans and 40 to 80 kilobases in mice.[6] Telomeric DNA terminates in a 3'-single stranded overhang, which is well-protected in a three-dimensional structure, the so-called T-loop.[5] Little is known about the dynamics of the T-loop formation, but it is clear that telomeric proteins are involved in this process. Telomeres associate with several proteins to form a telomere-protein complex, called the shelterin complex.[7,8] Thus far, six telomeric proteins have been identified – the best-known proteins probably being Telomere Binding Factor 1 and 2 (TRF1 and TRF2). Other telomeric proteins are Protection of Telomeres 1 (POT1), Ras-associated Protein 1 (Rap1), tripeptidylpeptidase 1 (TPP1), and TRF1- and -TRF2-interacting Nuclear Protein 2 (TIN2).[8,9] As far as known today, these proteins do not have any function elsewhere in the cellular machinery.[8]

Cellular signalling in response to telomere dysfunction

The essential function of telomeres is protecting the chromosomal ends from being recognised as double stranded DNA breaks. The protective mechanisms of telomeres are generally attributed to the unique features of the T-loop, as it hides the final open end of the DNA strand. If telomeres become dysfunctional or critically short, they will lose their protective properties and several DNA damage signalling mechanisms will be activated.

In mammalian cells, DNA breaks will be marked by DNA damage foci

following phosphorylation of the histone H2AX sites by the protein kinases ATM (ataxia-telangiectasia mutated) and ATR (ataxia-telangiectasia- and Rad3-related).[10] ATM and ATR will also activate the DNA checkpoint kinases Chk1 and Chk2 (which have an important role in cell cycle control). Chk1 and Chk2, in turn, will activate the anti-apoptotic protein p53 [11], leading to the expression of p21, a cyclin-dependent kinase inhibitor. P21 expression is linked to cellular senescence (figure 1). Activation of the checkpoint kinases can also lead to senescence, through inhibition of Cdc25 (cell division cycle 25). Next to the induction of senescence through p21, p53 can also initiate the apoptosis pathway.[7,8] A far less well understood mechanism through which telomere dysfunction causes senescence is the induction of p16. In senescent human and mouse cardiomyocytes, telomere reduction was related to upregulation of p16.[12,13,14] p16 blocks the cyclin dependent kinases CDK4 and CDK6. As a consequence, the protein RB (retinoblastoma protein) remains in its active, hypophosphorylated form. Active RB inhibits cell cycle progression and induces senescence (figure 1).[7,15]

In yeasts, the exonuclease 1 (EXO1) is shown to be involved in telomere-associated cellular damage responses when telomeres become critically short.[16,17] Recently, a role for EXO1 in these damage responses has been confirmed in mice.[18] The role of EXO1 is discussed in more detail below.

Cellular damage response initiated by telomere signalling can also involve DNA break repair systems. If telomeres are critically short or the shelterin complex is disturbed, the telomeric end will be recognised as DNA break in need of repair. In

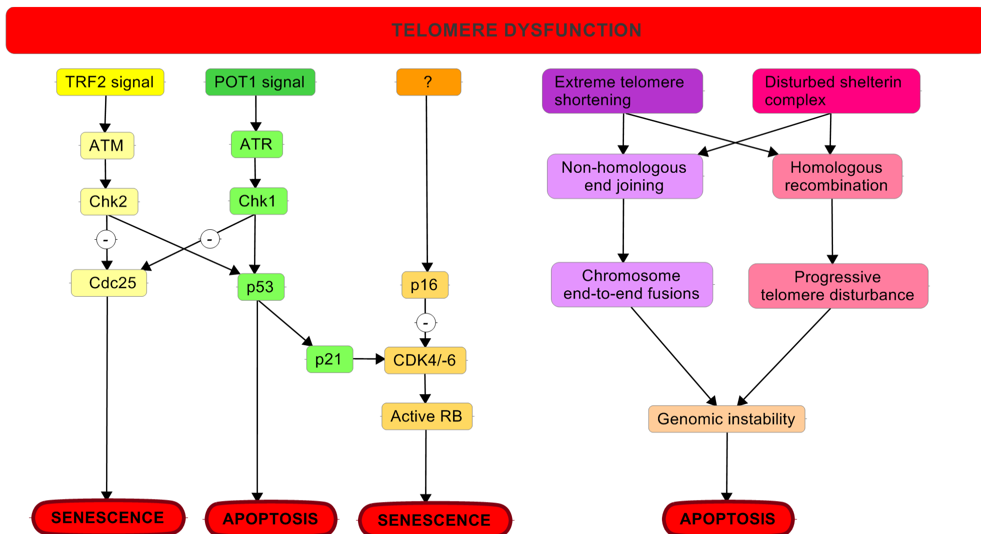

Figure 1. Schematic overview of some cellular signalling pathways in response to telomere dysfunction.

POT1: Protection of Telomeres 1; ATR: ataxia-telangiectasia mutated; TRF2: Telomere Binding Factor 2; ATR: ataxia-telangiectasia- and Rad3-related ; Cdc25: cell division cycle 25; Cdk4/-6: cyclin dependent kinases 4 and 6; RB: retinoblastoma protein.
For details see text.[7-11,15]

response, two DNA repair mechanisms will come into action; 1.) non-homologous end joining (NHEJ) and 2.) homologous recombination (HR). NHEJ of telomeres results in chromosomal end-to-end fusion and causes further telomere damage and dysfunction. Both NHEJ and HR eventually will lead to cellular dysfunction, genomic instability and apoptosis (figure 1). More details on NHEJ and HR at telomeric ends are extensively reviewed elsewhere.[8]

Acquired telomere erosion

After every cell cycle, telomeres lose a number of telomeric base pairs. This phenomenon is also known as the "end-replication problem". As a consequence, telomeres mark replicative history and are therefore considered a marker of chronological ageing.[12,19,20] Telomere length of human peripheral white blood cells has indeed consistently been associated with age.[21-24] Besides replicative stress, several external stressors have also been associated with telomere shortening. Most evidence exists for the telomere eroding effects of UV radiation and oxidative stress.[25,26] Telomere length at any time is a resultant of length provided at birth, replicative and environmental stresses. Telomere attrition is variable and might be different during life time, but has been estimated to be on average approximately 30 base pairs per year.[27]

Structure and function of telomerase

Telomeres can be elongated by the ribonucleoprotein enzyme telomerase, which adds TTAGGG repeats to the 3' end of DNA strands (figure 2). Telomerase consists of two core compounds, Telomerase Reverse Transcriptase (TERT) and Telomerase RNA Component (TERC), which serves as a template for addition of telomeric repeats to DNA strands.[28] Under physiological circumstances, telomerase expression is undetectable in most human cells, with the major exceptions being embryogenic stem cells, germline cells, and certain epithelial and lymphoid progenitor cells.[29,30]

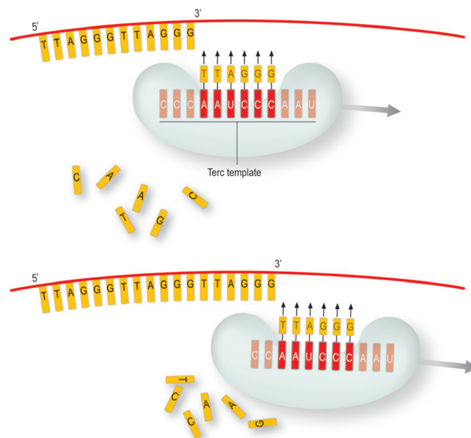

Figure 2. Elongation of a telomere by telomerase.

Telomerase can elongate telomeres. Telomerase carries an RNA template that is used as a template to add telomeric nucleotide repeats, in human TTAGGG, to the 3'-ends of telomeres.

TELOMERE BIOLOGY AND THE CARDIOVASCULAR PHENOTYPE IN HUMANS

Telomere length has repetitively been linked to cardiovascular disease states. One possible explanation for the association between telomere dysfunction and cardiovascular disease is that short telomeres lead to cellular dysfunction (e.g. diminished proliferative capacity) and increased senescence and apoptosis. Implications of diminished proliferative capacity for the cardiovascular system might include the exhaustion of the progenitor pool with repair capacity.[31] As a result, neovascularization in ischemic heart disease and vascular regeneration in atherosclerotic disease could be limited. In addition, cellular stability is essential for maintenance of organ function, especially in scarcely regenerating tissue, such as the myocardium. Increased cellular vulnerability or actual increased senescence and apoptosis due to telomere dysfunction can promote heart failure. Alternatively, the association between telomere dysfunction and cardiovascular disease may be that the disease itself causes the telomere to dysfunction or shorten. So although the precise mechanisms connecting telomere biology to the different cardiovascular phenotypes remain to be defined, convincing lines of evidence are supporting a role for telomeres in cardiovascular (patho)physiology, and below we briefly summarize these data.

Telomeres and cardiovascular risk factors

Reduced telomere length is associated with the presence of cardiovascular risk factors.[4] For smoking, the evidence is most convincing.[32,33,34] Reduced telomere length is also associated with insulin resistance and obesity[24,27,32], type 1 and type 2 diabetes[35,36], hypertension[37], activation of the renin-angiotensin-aldosterone system[38], and renal failure.[39]

Telomeres and atherosclerosis

Short telomeres have been implicated in vascular senescence and atherosclerosis.[40] Endothelial cells derived from atherosclerotic plaques have shorter telomeres compared to endothelial cells derived from non-atherosclerotic areas of the same individual.[41,42] Patients with atherosclerosis also have shorter telomeres in their leukocytes compared to healthy, age-matched controls.[41,43,44]

Telomeres and heart failure

Evidence is accumulating involving telomere biology in the development of heart failure. In endomyocardial biopsies from patients with dilated cardiomyopathies more cells were senescent and telomeres were shorter compared to age-matched controls.[13,45] Interfering with either function or expression of TRF2, one of the proteins that associate with telomeres, triggers telomere erosion and apoptosis in cardiomyocytes.[45] As myocardial tissue is difficult to obtain, human studies have evaluated telomere length in circulating leukocytes. Telomere length of leukocytes is also shorter in patients with heart failure compared to healthy controls.[43] Moreover, shorter telomeres have recently been found in bone marrow cells of patients with

atherosclerotic disease.[46] Shorter telomere length in the bone marrow led to the speculation that it might affect the function of endothelial progenitor or other repair cells.[31]

The suggestion of the existence of cardiac progenitor cells (CPCs) as a repair source of the adult heart has raised the possibility that telomeres are important for the function of these cells.[47,48,49] Telomere shortening is indeed observed and associated with aged and senescent CPCs.[49]

Unfortunately, despite significant clinical, in vivo and in vitro associations between cardiovascular disease phenotypes and telomere biology, definite proof for a causal role of telomeres in the development of atherosclerosis or heart failure has not been generated.

THE TELOMERASE DEFICIENT MICE

Telomerase deficient mouse models

The notion that mouse and human tumors express elevated levels of telomerase activity, while normal adjacent tissue lacks measurable activity, led to the development of models to study telomere biology. In 1997, dr. Maria A. Blasco reported the successful generation of telomerase deficient mice by knocking out the RNA template of telomerase (TERC$^{-/-}$ mice).[50] This mouse model was established to serve as a tool to study tumor formation and cell viability in the absence of telomerase. Besides the RNA template, another essential component of telomerase is the telomerase reverse transcriptase (TERT). Not surprisingly, TERT$^{-/-}$ mice have also been constructed.

Although originally generated to serve research related to oncology, the telomerase deficient mice have provided valuable information for ageing-associated diseases, including those related to cardiovascular disease.

General phenotype of the telomerase deficient mouse

The telomeres of the TERC$^{-/-}$ mice shorten at a rate of approximately 5 kilobasepairs in every subsequent generation.[50] Therefore, experiments using TERC$^{-/-}$ mice commonly involve several generations (G) of these mouse compared to wild types. Telomere length in G3 TERC$^{-/-}$ mice decreases as much as 50% during ageing from one to 16 months.[51] TERC$^{-/-}$ mice of later generations show increasingly severe structural abnormalities on cellular level, including undetectable short telomeres, aneuploidy, and chromosomal end-to-end fusions.[50] In general, animals with phenotypical features of ageing have shorter telomeres than age-matched controls from the same generation lacking a clear ageing phenotype. Later generations TERC$^{-/-}$ mice show a decreasing life span (table 1). Autopsy of late generation spontaneously diseased animals could not identify a clear cause of death, comparable to natural death in humans.[51] Later generation and aged TERC$^{-/-}$ mice show many more defects than earlier generation and younger TERC$^{-/-}$ mice. These defects include reduced body size and weight, hair graying and loss, infertility and testicular atrophy, spleen atrophy, signs of immunosenescence, and bone marrow proliferative defects.[51,52] Wound healing is also impaired in aged TERC$^{-/-}$ animals.[51] Reduced angiogenic potential of these mice has been suggested as an explanation

for this observation. Below we will focus in more detail on the knowledge of the angiogenic potential in these mice.

Although some interesting studies have been performed on TERT$^{-/-}$ mice, these mice are substantially less well-studied compared to TERC$^{-/-}$ mice. The construction of the TERT$^{-/-}$ mice has first been reported in 1999.[53] Both the TERT$^{-/-}$ and the TERC$^{-/-}$ mice do not show significant phenotypic abnormalities at early age in the first generation. Unfortunately, information on the general phenotype of the TERT$^{-/-}$ mice of later generations is sparse and data on fertility of these mice are reported differently by different groups, possibly due to different backgrounds of the mice. Unchanged litter size in G1 and G2 TERT$^{-/-}$ mice (progeny from a chimeric and a wild type C57Bl/6 mouse) has been reported by some[53], while others experienced dramatically reduced litter size in G2 TERT$^{-/-}$ mice on a pure C56Bl/6 background.[54] Indisputable is the fact that later generation TERT$^{-/-}$ and TERC$^{-/-}$ mice both have considerable shorter telomeres than their wild type litter mates.[54,55,56]

In general, homozygous TERT and TERC deficient mice display a similar phenotype. However, one remarkable difference between heterozygous TERT and TERC deficient mice has been observed. In contrast to TERC$^{+/-}$ mice, TERT$^{+/-}$ mice do not have detectable defects in telomere maintenance and elongation. In both heterozygous mice the mRNA levels of their knocked out gene are approximately 30-50% of that of wild-type mice. The differences are therefore unlikely to be attributable to differences in target gene expression. Presumably, both TERC and TERT are essential for telomere maintenance and elongation, but in contrast to TERT, gene copy number and transcriptional regulation of TERC are limiting for telomerase activity.[56,57]

Hypertension

Several cross-sectional human studies associate blood pressure parameters with telomere length of circulating leukocytes.[37,58] Only one study is available evaluating blood pressure in telomerase deficient mice.[59] In conscious mice, tail sphygmomanometry revealed that TERC$^{-/-}$ mice from G1 showed higher systolic blood pressures compared to wild type. In G3 mice, both systolic and diastolic blood pressure were increased compared to wild type and G1 mice. Studying anaesthesized G3 and wild-type mice with invasive haemodynamic studies did not show functional modifications in the nitric oxide system or the responsiveness to angiotensin II. Thus, the differences in blood pressure do not seem to be attributable to these two systems. Interestingly, the response to endothelin – a powerful vasoconstrictor – was diminished in G3. In concordance, treatment with bosentan, an endothelin receptor antagonist, resulted in a more pronounced drop of blood pressure in G3 mice compared to wild type. Furthermore, plasma and urine endothelin levels were gradually and significantly increased in G1 and G3 mice. To further explore the role of endothelin in the observed differences in blood pressure between TERC$^{-/-}$ and wild type mice, mRNA expression of endothelin converting enzyme (ECE) was measured. TERC$^{-/-}$ mice showed higher mRNA expression of ECE-1 and specific inhibition of ECE only reduced blood pressure in TERC$^{-/-}$ mice. In vitro experiments with transfection of deletion mutants of the ECE-1 promotor suggested that the AP-1 binding sequence of the ECE-1 promotor is involved, so

that transcriptional control of ECE-1 may be disrupted in $TERC^{-/-}$ mice.[59] Whether long term treatment with endothelin receptor blockers is beneficial in $TERC^{-/-}$ mice remains to be determined. In addition, these functional data obtained in the $TERC^{-/-}$ mice have not been translated back to human studies, meaning we do not know yet whether ECE activity is also involved in the association between hypertension and telomere length in humans.

Atherosclerosis

The association between reduced telomere length in leukocytes with the development and presence of atherosclerotic manifestations in humans is demonstrated by several independent groups.[41,44,60,61] As mice in general are resistant to the development of atherosclerosis[62], many groups are using the apolipoprotein E (ApoE)-deficient mice, which do develop atherosclerosis when exposed to a high fat diet.[63] Interestingly, $ApoE^{-/-}$ mice were inter-crossed with $TERC^{-/-}$ mice to study the effects of telomeres on the development of atherosclerosis.[64] Fourth generations of $TERC^{-/-}ApoE^{-/-}$ and $TERC^{+/+}ApoE^{-/-}$ mice were challenged with a high cholesterol high fat diet. Remarkably, generation four (G4) $TERC^{-/-}ApoE^{-/-}$ mice developed less atherosclerotic lesions compared to G4 $TERC^{+/+}ApoE^{-/-}$ mice (table 2). In addition, the atherosclerotic plaques of G4 $TERC^{-/-}ApoE^{-/-}$ mice were morphologically in a less advanced stage of atherosclerosis, compared to G4 $TERC^{+/+}ApoE^{-/-}$. This suggests that the absence of telomerase activity is protective for atherosclerotic disease. These observations could not be attributed to differences in serum cholesterol levels.[64] It was also observed that the proliferative capacity of macrophages and lymphocytes was decreased in G4 $TERC^{-/-}ApoE^{-/-}$ mice compared to G4 $TERC^{+/+}ApoE^{-/-}$, suggesting reduced inflammatory capacity. This might explain the differences in atherosclerotic manifestations in this experimental model, since progression of atherosclerosis is partly dependent of functional immunocompetent cells.[64]

Experiments in these mice providing more definite proof, e.g., by a bone-marrow switch with immune-competent cells or endothelial specific $TERC^{-/-}$, have not been reported up to date.

Angiogenesis

Evidence for involvement of telomerase in angiogenesis has been provided by adenovirus-mediated transfer of TERT in the rat hind limb ischemia, which enhances capillary density in the ischemic tissue.[65] Also, $TERC^{-/-}$ mice have been studied using both matrigel implants and murine melanoma grafts.[66] In an in vivo matrigel assay, late generation $TERC^{-/-}$ mice showed diminished angiogenic potential compared to wild type mice. Early generation $TERC^{-/-}$ mice, who have normal telomere length, did not have impairment of angiogenesis as assessed by the in vivo Matrigel assay.[66] This suggests that short telomere length limits the angiogenic potential, and not the absence of functional telomerase itself. Also an in vivo angiogenesis model using murine melanoma cells showed decreased tumor formation efficiency and growth rate in later generations $TERC^{-/-}$ mice. Microvessel density in tumor cryosections was stained with an anti-CD31 antibody – an endothelial cell marker – and it was shown that the microvessel density of G5 $TERC^{-/-}$ tumors was only half of the wild-type and G2 $TERC^{-/-}$ tumors.[66]

Cardiac myocytes and ventricular failure

The effects of telomerase deficiency on cardiac myocyte size, number, proliferative potential and myocyte apoptosis has been studied in combination with cardiac function in G2 and G5 TERC$^{-/-}$ mice of the original mixed background and compared to wild type mice. The progressive decrease of telomere length in cardiomyocytes of successive generations of TERC$^{-/-}$ mice was associated with an increase of p53 expression.[67] G5 TERC$^{-/-}$ mice suffer from severe left ventricular failure, characterized by increased end diastolic left ventricular pressure, decreased maximally developed left ventricular pressure and disturbed contractility and relaxation of the left ventricle. These mice also showed anatomical changes of the heart, similar to dilated cardiomyopathy in human, together with decreased total number of myocytes and increase of myocyte hypertrophy. In addition, apoptosis of myocytes was an approximate 40% greater in G5 TERC$^{-/-}$ mice, compared to wild type and G2 TERC$^{-/-}$ mice.[67] In G2 TERC$^{-/-}$ mice, only a slight decrease in left ventricular pressure compared to wild type mice was observed.[67] These data suggest that late generation of TERC$^{-/-}$ mice spontaneously develop pathological cardiac remodeling and severe ventricular dysfunction. Another study showed that exercise increased TRF2 expression and prevented doxorubicin-induced cardiac apoptosis in wild type mice, but not in TERT$^{-/-}$ mice. This suggests that, in absence of telomerase, upregulation of telomere-stabilizing proteins is challenged and cardiac apoptosis is more severe.[68]

In conclusion, telomerase deficient mice provide a model to study the efficacy of telomerase based therapies for heart failure. However, it should be taken into account that the majority of patients who develop heart failure have coronary artery disease.

Stem cell biology and tissue regeneration

A key process in tissue and organ homeostasis is the mobilization of stem cells for maintenance and repair. Evidence is supporting a role for bone marrow derived cells in the maintenance and regeneration the endothelium.[69] More controversial is whether the heart is also harbouring progenitor cells in adult life.[48,49] The TERC$^{-/-}$ mice have provided us with more insights in the role of telomerase and telomere length in several well-characterized stem cell subtypes, including haematopoietic, epidermal, and neural stem cells.

Haematopoietic progenitor cells from G1 TERC$^{-/-}$ mice have a normal capacity to grow and differentiate in vitro. Mature haematopoietic organ structure and function seem to be well compensated in TERC$^{-/-}$ deficient mice, as no changes in peripheral blood count and profile were observed through successive generations and mature immunocytes show normal responses to mitogenic or infectious stimuli.[70] However, in vitro haematopoietic colony-forming unit (CFU) assays revealed that later generation TERC$^{-/-}$ mice have a significant decrease in total number of CFU-granulocyte-monocyte, CFU-granulocyte, -erythrocyte, -monocyte, -megakaryocyte, and decreased high-proliferative-potential colony forming cell colonies.[70] In addition, serial and competitive transplantations of TERC$^{-/-}$ bone marrow stem cells showed reduced long-term repopulating capacity compared to wild type cells.[71,72] This indicates that long-term renewal of haematopoietic stem

Table 1. Characteristics of different generations of TERC$^{-/-}$ mice

	WT	G1	G2	G3	G4	G5	G6
Lifespan	Normal	Normal	Normal/ Reduced #	Normal/ Reduced #	Normal/ Reduced #	Normal	Reduced
Ageing phenotype (hair graying, alopecia)	Normal	-	-	Accelerated	-	-	Accelerated
Body weight	Normal	Normal	Normal	Normal/ 20% reduction# §	20% reduction# §	15% reduction	20-25% reduction *
Blood pressure	Normal	Hypertensive #	-	Hypertensive #	-	-	-
Left ventricular function	Normal	-	Slightly impaired	-	-	Severely impaired	-
Myocyte size	Normal	-	Increased	-	-	Severely increased	-
Angiogenic potential	Normal	-	Normal	Decreased	Decreased#	Decreased#	Decreased
Wound healing	Normal	Normal	-	Impaired	-	-	Impaired

Observed in mice with C57BL6 genetic background, all other observations were in mice with originally mixed background
§ Observed in animals > 4 months of age
* Observed in animals > 6 months of age

cells is compromised upon telomere loss.

In different generations of TERC$^{-/-}$ inbred mouse the epidermal stem cell number has been compared.[73] In G1 and even more pronounced in G3 TERC$^{-/-}$ mice, greater numbers of epidermal stem cells were present in the bulge area of the hair follicle. Interestingly, the epidermal stem cells in TERC$^{-/-}$ mice showed a defect in their mobilization. Coincidentally, the proliferation index in different compartments was lower than that of wild-type follicles. In addition, in vitro culture of keratinocytes from G1 and G3 TERC$^{-/-}$ mice formed fewer and smaller colonies than those of wild type.[73] Thus, epidermal stem cells in TERC$^{-/-}$ mice are less functional than in wild type, and the increased numbers of epidermal stem cells in the TERC$^{-/-}$ mice are possibly due to accumulation in the follicles, as the epidermal stem cells have impaired capacity to mobilize.

Although bone marrow and stem cells are considered important in the pathogenesis and possible treatment of cardiovascular disease, including atherosclerosis and heart failure, studies focuses on the role of telomere biology are lacking. In this regard also, the TERC$^{-/-}$ model provides a good model to study the efficacy of stem cell-based therapies for heart failure.

COUNTERACTING THE EFFECT OF TELOMERE DYSFUNCTION

As telomere biology is linked to cardiovascular pathology, targeting it provides new and promising therapeutic avenues to be explored. Here, we will discuss data investigating several possibilities of counteracting the deteriorating effects of dysfunctional telomeres.

Exonuclease-1 deletion

One of the proteins that mediate response to telomere dysfunction is exonuclease 1 (EXO1).[18] It has been shown that EXO1 deletion prolongs the lifespan of G3 TERC$^{-/-}$ EXO1$^{-/-}$ mice, compared to G3 TERC$^{-/-}$EXO1$^{+/+}$ mice. EXO1 deletion preserves organ function of the intestinal tract and the haematopoietic system[18], both organ systems that are normally affected in the TERC$^{-/-}$ mice.[50] Remarkably, this beneficial effect is present in 12-15, but not 24 months old G3 TERC$^{-/-}$EXO1$^{-/-}$ mice, suggesting that in old mice EXO1-independent mechanisms are responsible for the disturbed organ homeostasis. The beneficial effect of EXO1-deletion is not caused by preservation of telomere length. Telomere length in the G3 TERC$^{-/-}$EXO1$^{-/-}$ mice is comparable to G3 TERC$^{-/-}$ EXO1$^{+/+}$ mice. The precise mechanisms of the beneficial effects of EXO1-deletion on mice with dysfunctional telomeres remain to be elucidated.

Telomerase upregulation

Under physiological circumstances, telomerase activity is absent or undetectable in the adult myocardium. Overexpression of TERT rescues telomerase activity and preserves telomere length in the adult mouse myocardium and induces cardiomyocyte hypertrophy, without fibrosis or impaired cardiac function. In addition, infarct size after experimental myocardial infarction was substantially reduced in TERT transgenic mice compared to wild type mice.[74]

Table 2. Mouse models of telomerase manipulation and the cardiovascular phenotype

Model	**Animals (genetic background)**	**Cardiovascular phenotype**	**Ref. no.**
Telomerase deficiency	Mouse, TERC$^{-/-}$ (C57BL6 background)	Hypertension	58
		Decreased angiogenic potential (>generation 3)	65
		Inflammation: decreased Lymphocyte (T and B) proliferative response	69
	Mouse, TERC$^{-/-}$ (mixed background)	Myocyte hypertrophy, left ventricular failure and dilatation	66
		Decreased angiogenic potential (>generation 3)	65
	Mouse, TERT$^{-/-}$ (mixed background)	Inhibited upregulation of telomere-stabilizing protein TRF2 following exercise. No protection against doxorubicin-induced cardiomyopathy.	67
	Mouse, TERC$^{-/-}$ ApoE$^{-/-}$	Less atherosclerosis, decreased proliferative capacity of inmunocompetent cells	63
	Mouse, TERC$^{-/-}$ Atm$^{-/-}$	Depletion of haematopoietic progenitor cells	74
	Mouse, TERC$^{-/-}$ Wrn$^{-/-}$	Development of diabetes mellitus type 2	75
TERT depletion	Rat, transfection of double negative TERT	Abrogation of angiogenesis	64
TERT over-expression	Mouse, transfection of cardiac specific TERT	Myocyte hypertrophy, left ventricle hypertrophy without diminished function, increased cardiac ischemia tolerance in myocardial infarction	73

CONCLUSIONS AND FUTURE PERSPECTIVES

Evidence in humans for an association between telomere length and cardiovascular related phenotypes, including atherosclerosis and heart failure, is rapidly accumulating.[2,3,4,43] Understanding of the nature of these associations might be paramount in our understanding of the aetiology and pathogenesis of these diseases. Telomere biology already offers therapeutic targets in the treatment of cancer, but whether it can also provide novel therapeutic targets in coronary artery disease, heart failure, or optimalization of stem cells treatment remains to be discovered. For a rapid increment of our knowledge on the involvement of telomere biology in cardiovascular disease, the TERC$^{-/-}$ mice might turn out to be instrumental. Although this mouse model has proven its value in oncology, stem cell, and basic telomere research, up to date it is only sparsely used in the cardiovascular field. Studies with the TERC$^{-/-}$ mice might teach us valuable lessons on the involvement of telomere biology in age-associated hypertension, atherosclerosis, angiogenesis, and cardiac remodeling after myocardial infarction, as well as efficacy of telomerase and stem cell based therapies.

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CONFLICT OF INTEREST

None.

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6 Telomere length of circulating leukocyte subpopulations and buccal cells in patients with ischaemic heart failure and their offspring

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ABSTRACT

We aimed to find support for the hypothesis that telomere length (TL) is causally involved in the pathogenesis of ischemic heart failure (IHF). We measured TL in IHF patients and their high-risk offspring and determined whether mean leukocyte TL reflects TL in CD34+ progenitor. We additionally measured TL of offspring of patients and controls to examine heritability throughout different cell types.

TL was measured by qPCR in overall leukocytes, CD34+ progenitor cells, mononuclear cells (MNCs), and buccal cells in 27 IHF patients, 24 healthy controls and 60 offspring. TL in IHF patients was shorter than healthy controls in leukocytes (p=0.002), but not in CD34+ cells (p=0.39), MNCs (p=0.31) or buccal cells (p=0.19). Offspring of IHF patients had shorter TL in leukocytes than offspring of healthy subjects (p=0.04) but not in other cell types. Controls and offspring showed a good within person correlation between leukocytes and CD34+ cells (*r* 0.562; p=0.004 and *r* 0.602; p=0.001, respectively). In IHF patients and offspring the correlation among cell types was blunted. Finally, we found strong correlations between parent and offspring TL in all four cell types.

Reduced leukocyte TL in offspring of IHF subjects suggests a potential causal link of TL in ischemic heart disease. However, this causality is unlikely to originate from exhaustion of TL in CD34+ progenitor or MNC cells as their lengths are not well captured by overall leukocyte TL. Additionally, we found strong correlations between parent and offspring TL in all examined cell types, suggesting high heritability of TL among cell types.

INTRODUCTION

Telomeres are the terminal ends of the DNA strands, and shorten during life because of incomplete DNA replication after cell cycling or damaging environmental factors. Cells with critically short telomeres become dysfunctional, and can eventually even go into apoptosis.[1,2] Recently, telomere biology has been implicated in ageing-associated cardiovascular diseases. Most data has been generated on establishing the association between short mean overall leukocyte telomere length (TL) and ischemic heart disease (IHD).[3-6] In addition, it has been suggested that presumably healthy offspring of patients with ischemic heart disease already have shorter TL compared to healthy offspring of controls.[7] An open question remains whether telomere length is causally involved in the development of heart disease, and if so, what the underlying mechanism is. Short overall mean leukocyte telomere length has been viewed as a reflection of short telomere length in other cells, possibly of vascular progenitor cells, and thereby providing a link to an impaired vascular repair mechanism potentially causing ischemic heart disease.

To further dissect the association of ischemic heart disease with mean overall leukocyte TL we need to establish whether mean overall leukocyte TL is a reflection of TL in different cell types or whether it is more or less specific for leukocytes. Of particular interest in this regard are the CD34 positive (CD34+) cells as it is thought that these cells might be cardiovascular progenitor cells and play a role in cardiovascular repair.[8-10] Short TL in CD34+ cells might provide a mechanism for the association with IHD as their cellular dysfunction might impair cardiovascular repair. Furthermore, mean leukocyte telomere length has not been compared to non-circulating non-vascular cells and it is unknown whether leukocytes might merely be a reflection of overall TL of the whole body.[11]

We have investigated telomere length in circulating leukocytes, CD34+ cells, mononuclear cells, and the non-systemic non-circulating buccal cells in patients with ischemic heart failure (IHF) – which is the most extreme phenotype of IHD – and compared them to healthy, age-matched controls. Since occurrence of IHD is highly familial and telomere length is an inheritable trait[12-14], we also aimed to determine whether telomere length in the different cell types is shorter in offspring of IHF patients compared to offspring of healthy controls.

METHODS

Ethics statement

The study was approved by the local Medical Ethical Committee for human research of the University Medical Center Groningen (UMCG) and adheres to the Declaration of Helsinki. All participants were aged 18 or older and provided written informed consent for participation in this study.

Study design

The Telosophy study is an observational, prospective case-control study. Groups consisted of 1) patients with stable ischemic heart failure (IHF) and 2) age and gender matched healthy controls. In addition, of both groups we included offspring;

(3) offspring of IHF patients, and (4) offspring of healthy controls.

The inclusion criteria for the IHF patients were: presence of coronary artery disease (previous myocardial infarction and/or coronary revascularisation), left ventricular ejection fraction (LVEF) ≤ 40%, having heart failure for at least 6 months and being stable on optimal medication for heart failure for at least 4 weeks prior to the study visit, and having at least one healthy biological child that was willing to participate. Exclusion criteria were having had an ischemic cardiac event in the past 12 months and having severe cardiac valvular disease. Inclusion criteria for controls were being age- and gender- matched to the IHF patients, and having at least one healthy biological child who was willing to participate. Exclusion criteria for healthy controls were having known atherosclerotic disease or heart failure, a family history of premature cardiovascular disease, and having a partner (who is the biological parent of the participating offspring) with known cardiovascular disease. In addition, main exclusion criteria for all participants were having systemic inflammatory diseases, haematopoietic diseases, severe renal or liver disease, uncontrolled hypothyroidism, premature ageing syndromes, or malignancies, which could all influence telomere length.

Study visit

IHF patients were recruited from the outpatient clinic of the Universitair Medical Center Groningen in Groningen, the Netherlands. Healthy controls were recruited with local advertisements or screened at the pre-operative outpatient clinic for planned selective non-cardiovascular surgery (small plastic surgery, ophtalmic surgery or minor orthopaedic procedures).

Subjects underwent 1 study visit to assess medical and family history and life style factors and to undergo a physical exam. Venous blood samples (60 mL in collected in tubes precoated with EDTA and 2.5 mL in the PAXgene Blood RNA tube, catalogno. 762174, Qiagen, Venlo, The Netherlands) and a buccal mucosa cells (four buccal swabs (isohelix, SK-4)) samples were taken.

Isolation of CD34+ cells

Peripheral blood mononuclear cells were obtained from venous whole blood after density gradient centrifugation (Ficoll Paque Plus, catalogno. 17-1440-02, GE Healthcare Europe GmbH, Diegem, Belgium). The mononuclear cell (MNC) fraction was incubated with magnetic beads conjugated to anti-CD34-antibody (CD34 Microbead Kit, catalogno. 130-046-702, Miltenyi Biotec, Bergisch Gladbach, Germany). Subsequently, CD34+ cells were obtained from the MNC fraction by magnetic bead cell selection (MidiMACS separator, Miltenyi Biotec, Bergisch Gladbach, Germany). Flow cytometric analysis of mononuclear cells showed that incubation with anti-CD34-antibody and magnetic beads increased the CD34+ cell isolation from 0.39% to 58.81% (data not shown).

Telomere length measurement

DNA isolation of CD34+ cells, MNCs, and buccal swabs was performed according to manufacturer's protocol (NucleoSpin Tissue kit, catalogno. 740952, Macherey-Nagel/Bioké, Leiden, The Netherlands). Relative telomere length (TL) was assessed

by monochrome multiplex quantitative PCR method, previously described in detail[15,16], and expressed as the ratio of telomere (T) to reference copies (S), further called T/S ratio. Samples of IHF patients, controls, and both offspring groups were randomly assigned to the plates and plate positions, with each plate containing samples of all four groups. All samples were run in triplicate. Samples with a coefficient of variation larger than 0.10 were run again. No samples had a coefficient of variation larger than 0.10 after the second run.

Data were characterised as outliers when >4 times standard deviation and were excluded from all statistical analyses (N=1).

Statistical analysis

Skewed variables were natural log transformed to acquire normal distribution. Differences in means between the groups were tested with Student's T-test or Chi square test. Pearson (or Spearman) and linear regression techniques were used to asses associations between variables of interest. Multivariate linear regression analysis was used to make adjustments. All statistical analyses were performed in SPSS version 16.0 (SPSS inc. Chicago, Illinois). A two-sided p-value of <0.05 was interpreted to indicate statistical significance.

RESULTS

Baseline characteristics

Baseline characteristics per group are presented in table 1. All IHF patients had experienced previous myocardial infarction. On average, IHF patients were aged 69 years, had a mean LVEF of 27%, 85% was male, and were well treated.

Offspring of IHF patients had a higher BMI and tended to have more frequently hypercholesterolaemia compared to offspring of controls.

Telomere length in leukocyte subpopulations in patients, controls, and their offspring

In concordance with previous finding[17,18] we observed shorter leukocyte TL in IHF patients compared to controls (figure 1). We also observed shorter leukocyte TL in offspring of IHF patients compared to offspring of healthy controls (figure 1). One of the aims of this study was determining whether telomere length of CD34+ cells is different in IHF patients compared to healthy controls. We did not find a difference in TL between IHF patients and controls in CD34+ cells (mean TL ± standard deviation 0.56 ± 0.17 and 0.60 ± 0.14, respectively, p=0.39). Also the TL of MNCs was similar (0.46 ± 0.12 and 0.49 ± 0.10, respectively, p=0.31) as were TL of buccal cells (0.97 ± 0.27 and 1.04 ± 0.19, respectively, p=0.19). In offspring, TL observed in CD34+ cells (0.66 ± 0.16 and 0.71 ± 0.16; p=0.22), MNCs (0.62 ± 0.17 and 0.62 ± 0.17; p=0.96), and buccal cells (1.17 ± 0.42 and 1.21 ± 0.25; p=0.47) were comparable.

These results clearly indicate that there is no significant difference in CD34+ cell TL between IHF patients and controls. CD34+ cell dysfunction as a result of short TL is therefore an improbable player in the pathogenesis of IHF.

Table 1. Baseline characteristics

Patient characteristics	I: CHF patients N=27	II: Healthy controls N=24	P-value	III: Offspring of CHF patients N=29	IV: Offspring of healthy controls N=25	P-value
Age (years)	69 ± 6.9	66 ± 6.6	0.14	40± 6.8	38 ± 6.0	0.28
Male (n (%))	23 (85)	20 (83)	0.86	17 (59)	9 (36)	0.10
Age of onset CHF (years)	60 ± 8.5	-	-	-	-	-
Body mass index (kg/m^2)	28.0 ± 5.0	25.3 ± 3.0	0.04	27.2 ± 4.5	24.0 ± 2.4	0.002
Left ventricular ejection fraction (%)	27 ± 8.2	-	-	-	-	
NYHA class (n (%))			-			-
II	12	-		-	-	
III	15	-		-	-	
IV	0	-		-	-	
CRP (mg/L)	0.0 ± 0.0	0.0 ± 0.0	0.38	0.0 ± 0.0	0.0 ± 0.0	0.55
NTproBNP (pg/mL)	665 [364-1524]	52 [35-63]	<0.001	24 [11-41]	19 [8-45]	0.97
Heart rate (beats/min)	66 ± 8	67 ± 9	0.76	69 ± 13	71 ± 10	0.47
Blood pressure (mmHg)						
Systolic	114 ± 15	133 ± 18	<0.001	123 ± 18	121 ± 17	0.64
Diastolic	68 ± 10	80 ± 11	<0.001	76 ± 11	77 ± 13	0.71
Medical history (n (%))						
Myocardial infarction	27 (100)	0 (0)	<0.001	0 (0)	0 (0)	1.00
Hypertension	4 (15)	5 (21)	0.57	2 (7)	1 (4)	0.64
Diabetes mellitus	8 (30)	1 (4)	0.02	1 (3)	0 (0)	0.35
Atrial fibrillation/flutter	11 (37)	0 (0)	<0.001	1 (3)	0 (0)	0.35
Stroke	3 (11)	0 (0)	0.09	0 (0)	0 (0)	1.00
Hypercholesterolaemia	13 (48)	0 (0)	<0.001	4 (14)	0 (0)	0.05
Medication						
Beta-blocker	26 (96)	3 (13)	<0.001	1 (3)	0 (0)	0.35
ACE inhibitor and/or	27 (100)	3 (13)	<0.001	1 (3)	1 (4)	0.92
AII antagonist	7 (26)	0 (0)	0.007	0 (0)	0 (0)	1.00
Aldosterone antagonist	22 (81)	1 (4)	<0.001	2 (7)	1 (4)	0.64
Diuretic	24 (89)	1 (4)	<0.001	3 (10)	0 (0)	0.10
Statin	26 (96)	0 (0)	<0.001	0 (0)	0 (0)	1.00
Anticoagulant						

Data is presented as mean ± standard deviation or number (%).
CHF – chronic heart failure; MNC – mononuclear cells; CD34+ – CD34-positive cells; The body-mass index is the weight in kilograms divided by the square of the height in meters; NYHA – New York Heart Association functional class; CRP – C-reactive protein; NTproBNP – N-terminal proB-type Natriuretic Peptide; ACE – Angiotensin Converting Enzyme; AII – Angiotensin II.

Strong correlation between leukocyte and CD34+ in controls and their offspring, but not IHF patients and their offspring

Another aim of this study was to investigate whether within one individual the more frequently reported leukocyte TL mirrors TL of the cells of our interest, the CD34+ cells. Therefore, we examined the correlation between TL of leukocytes and CD34+ cells. Interestingly, we found strong correlations between TL of leukocytes and CD34+ cells in controls and their offspring but not in IHF patients or their offspring (table 2).

Strong correlations between parent and offspring TL in all cell types
We found a positive association of TL between parent and offspring in leukocytes (correlation coefficient *r* 0.440, p=0.002). We also observed this association in CD34+ cells (*r* 0.401, p=0.004), MNCs (*r* 0.526, p<0.001),), and in buccal cells (*r* 0.391, p=0.005) (figure 2). These associations remained significant after correction for age of parent and offspring (for leukocytes *r* 0.448, p=0.004, for MNCs *r* 0.547, p<0.001; for CD34+ cells *r* 0.397, p=0.004; for buccal cells *r* 0.368, p=0.010). We could not validate earlier findings suggesting an association of paternal age at time of birth with offspring telomere length.[13]

DISCUSSION

Shorter mean leukocyte TL is a remarkable and consistent finding in subjects with ischemic heart disease, but the reason is not known. Nevertheless, short overall mean leukocyte telomere length has been viewed as a reflection of short telomere length in other cells, possibly of vascular progenitor cells, and thereby providing a link to an impaired vascular repair mechanism potentially causing ischemic heart disease. We indeed observed a good correlation between overall mean leukocyte telomere length and CD34+, MNCs and buccal cells in healthy subjects and also in their offspring. However, these high intra-individual correlations were lost in subjects with IHF and their offspring. The major difference in telomere length between IHF patients and controls was observed in the overall leukocyte pool, not specifically in CD34+, MNCs or buccal cells as a source of non-blood derived cells. We confirmed earlier findings, suggesting shorter leukocyte telomere length in offspring of patients with coronary artery disease versus offspring of healthy controls.[7] Finally, we confirmed the strong associations between parent and offspring TL in all four cell types we examined.

The comparable TL of CD34+ cells in cases and controls and both offspring groups are consistent with earlier findings[19] and strongly suggest that telomere shortening of CD34+ cells is not a major player in the pathophysiology of IHD. If there is any role of TL in this process – the consistent finding of shorter leukocyte TL in IHD patients does support this allegation – it would rather involve other leukocyte populations. It is known that leukocyte function changes with ageing. In the elderly, specific immune responses might be diminished, but many other functions are unchanged or even augmented compared to young persons.[20] A state of low-grade chronic inflammation has been recognised in elderly, which makes them prone to chronic inflammatory diseases, including atherosclerosis.[20] Macrophages, and to a lesser extent other leukocytes, catalyse the process of atherosclerosis by eliciting an enhanced systemic inflammatory response, possibly through increased oxidative stress.[20-22] Interestingly, it has been demonstrated that macrophages with short telomeres are more susceptible to damage from oxidative stress, and in addition have higher intracellular concentrations of oxidative stress molecules[23], which are suggested to be a driving force in the development and progression of atherosclerosis.[21] These data strongly suggest that ageing – chronologically or biologically – of leukocytes can possibly augment their unbeneficial contribution to IHD.

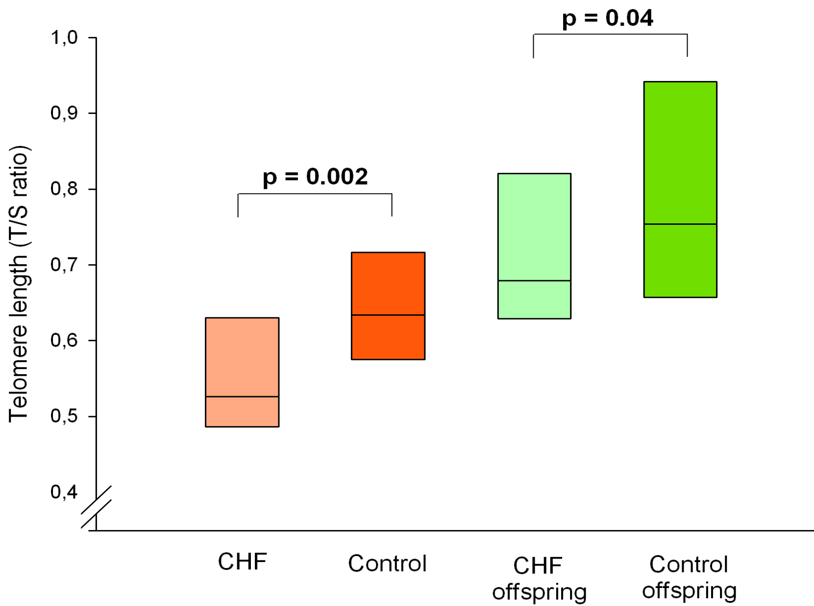

Figure 1. Mean leukocyte telomere length of all groups.

A secondary focus of our study was the heredity of TL traits among cell populations. Heritability of mean leukocyte telomere length has been demonstrated previously.[12,14,24] We now add to this the heritability patterns in TL derived from different cell fractions. A tissue specific TL regulation has been suggested, since TLs differ between different types of tissue.[11] In addition, external influences on TL are acknowledged. For instance, it has been shown that vascular endothelial cells that endure more hemodynamic sheer stress have shorter telomeres than endothelial cells in low pressure arteries.[25] Also, oxidative stress is a well-known factor that causes telomere shortening.[26] Our results indicate that despite external influences and tissue specific TL regulation, TL is a highly inheritable trait throughout different cell types.

Table 2. Correlation coefficients between Cd34+ cells and other cell types within patients, controls, and offspring

	Correlation coefficient *r*	P-value
CHF patients	0.313	0.137
Healthy controls	0.562	0.004
Offspring of CHF patients	0.210	0.324
Offspring of healthy controls	0.602	0.001

Strengths and limitations

Our study is strengthened by the fact that we not only examined TL of IHF patients and controls, but also of their offspring. Our finding that TL in healthy offspring of IHF patients is shorter than in healthy offspring of controls supports the hypothesis that, next to the genetic predisposition to coronary artery disease deriving from specific genes, TL might also be a factor contributing to familial predisposition to IHD. Furthermore, we separated leukocyte cell fractions to determine differential expression of TL. This is significant, since knowledge on which specific cell types contribute to the shorter mean leukocyte TL is necessary in order to identify a potential mechanism underlying the association between TL and IHD. To our knowledge, our study is the first study that investigated both heritability features and differential expression of TL in leukocyte subpopulations. Also, our study was a prospective study, meaning all measures were taken in advance to preserve blood and tissue samples taken from participants, which contributed to high accuracy of experimental procedures and laboratory measurements.

We also have to acknowledge some limitations. The separation of white blood cells was limited to MNCs and CD34+. Because of potential functional relevance,

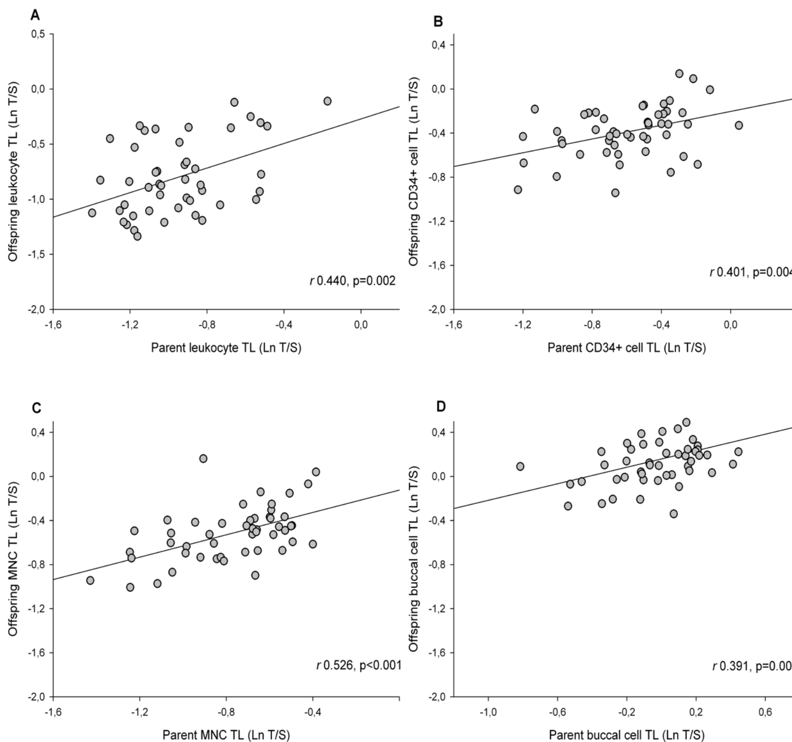

Figure 2. Correlations between parent and offspring telomere length in leukocytes (A), CD34+ cells (B), mononuclear cells (C), and buccal cells (D)

our main interest was the CD34+ cell population. However, we demonstrated that the CD34+ cell population is not the cell population that drives the difference in leukocyte TL between IHF patients and controls. This difference must thus lie in other leukocyte cell populations that we did not separately analyse in this study.

Conclusions

In conclusion, we found that TL is shorter in leukocytes of IHF patients and their healthy offspring compared to healthy controls and their healthy offspring. This is supporting evidence for a causal role of TL in familial predisposition to ischemic heart disease. The fact that we did not find a difference in TL of CD34+ cells between IHF patients and healthy controls, suggests that these cells are not involved in the potential mechanism linking short TL and ischemic heart disease. Furthermore, we found good correlations between parent and offspring TL in all cell types we examined, indicating a strong inheritance pattern of TL. Evidence for a causal role of TL in ischemic heart disease will be helpful in finding new therapeutic targets.

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7 Ageing, telomeres, and heart failure

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ABSTRACT

During normal ageing, the heart undergoes functional, morphological and cellular changes. Although ageing per se does not lead to the expression of heart failure, it is likely that age-associated changes lower the threshold for the manifestation of signs and symptoms of heart failure. In patients, the susceptibility, age of onset and pace of progression of heart failure are highly variable. The presence of conventional risk factors cannot completely explain this variability. Accumulation of DNA damage and telomere attrition results in an increase in cellular senescence and apoptosis, resulting in a decrease in the number and function of cells, contributing to the overall tissue and organ dysfunction. Biological ageing, characterized by reduced telomere length, provides an explanation for the highly interindividual variable threshold to express the clinical syndrome of heart failure at some stage during life. In this review, we will elaborate on the current knowledge of ageing of the heart, telomere biology and its potential role in the development of heart failure.

INTRODUCTION

Congestive heart failure (CHF) is a highly prevalent condition affecting more than 15 million patients in Europe alone.[1] The incidence and prevalence of CHF increases steeply with age and is likely to rise in the next decade due to the increasing average age of the population.[2] The prognosis of CHF is poor with a 4-year survival of only~50%.[1] Advanced age is one of the major risk factors for the development of CHF. Nevertheless, the susceptibility, age of onset and pace of progression are highly variable. The presence of conventional risk factors cannot completely explain this variability. The process of biological ageing affects most cells, organisms and species. Although ageing itself does not need to lead to CHF, it is likely that age-associated changes lower the threshold for the expression of this syndrome. Significant changes in diastolic function, hypertension, atherosclerosis, valve calcifications, senile cardiac amyloid depositions all are associated with ageing and an increased risk of the clinical signs and symptoms of CHF.[3] The molecular mechanisms involved allow time for accumulated damage to occur and include free radicals, advance glycation endproducts, apoptosis and senescence. Accumulation of DNA damage and telomere attrition can result in an increase in senescent cells in tissue and organs and can subsequently result in decreased function, providing an explanation for the lower threshold to express the clinical manifestation of heart failure. Here, we will briefly overview the current knowledge of the cardiac changes associated with ageing. One aspect of ageing in relation to CHF is of particular interest to us, telomere biology. Telomeres consist of an evolutionary conserved repetitive nucleotide sequence and are located at the terminal ends of the chromosomes.[4,5] The process of telomere erosion has been put forward as an intracellular counting mechanism that runs parallel with the ageing process and the susceptibility to develop CHF.

FUNCTIONAL CHANGES OF THE AGEING HEART

At the functional level, there are no age-related changes in cardiac output, end-diastolic or end-systolic volumes or ejection fraction at rest in healthy subjects.[6] However, the myocardial stiffness increases with advancing age, resulting in an elevated left ventricular end-diastolic pressure at rest and with exertion.[7] Early diastolic filling is decreased in elderly, a phenomenon seen on echocardiography as a change of the early to late filling velocity (E/A ratio).[8] Important age-associated changes are particularly noticeable during exercise. Although the stroke-volume increase during exercise of the young and old is comparable, the old tend to augment stroke volume during exercise more through cardiac dilatation with an increase in end-diastolic volume, whereas the young rely more on increase in the ejection fraction with no cardiac dilatation.[9] During exercise, the older heart rate has a lesser increase in frequency and blood pressure has a greater increase.[9] The difference in heart rate response is associated with a decrease in response to catecholamines and axonal degeneration of sympathetic neurons innervating the atria.[10,11] In addition, the number of pacemaker cells in the sinoatrial node diminishes significantly, from around 50% in youth to less than 30% in elderly.[12] One of the major vascular changes

during ageing is the decrease in aortic distensibility and decrease in the 'Windkessel function' explaining the frequently observed isolated systolic hypertension in elderly.[7]

MORPHOLOGICAL AND CELLULAR CHANGES OF THE HEART

The mature myocardium constitutes of cardiomyocytes and supporting connective tissue.[13] Healthy cardiac tissue is roughly composed of 20–25% cardiomyocytes, although this number varies among different species.[13] During ageing, most components of the myocardium undergo structural changes. The change in morphology is characterized by a loss of myocytes number, with a subsequent hypertrophy of remaining viable myocytes.[14] The remaining myocytes frequently contain multiple nuclei (polyploidy). The mechanism responsible for loss of myocytes is likely to be apoptosis, or programmed cell death. In parallel, there is an increase in collagen content, fibrosis and depositions of 'senile' cardiac amyloid and lipofuscin.[15-17] Ageing cardiomyocytes display prolonged contraction and relaxation caused by changes in calcium homeostasis.[18] Prolonged contraction with decreased force of the myofilaments is associated with down-regulation of genes that encode proteins that are involved in contractile activity, such as troponin and myosin forms in the aged heart.[19,20] Age-related changes of the mitochondria include a tendency to become larger but less efficient, with a decreased ATP production per cell.[21]

CARDIOMYOCYTE TURNOVER DURING AGEING

The paradigm that all cardiomyocytes are terminally differentiated has been challenged. Recent experiments using human left ventricular myocardial cells and carbon-dating techniques have established that DNA of cardiomyocytes continues to be synthesized many years after birth, indicating that cells in the human heart do renew well into adulthood.[22] Nevertheless, cardiomyocyte DNA synthesis decreases with age. Mathematical modelling predicts a ~1% cardiomyocyte renewal rate at the age of 25 and 0.45% at the age of 75. Considering this turnover rate, at the age of 50 years, 55% of the cardiomyocytes remain from the time around birth. There was no significant difference observed in ^{14}C integration of DNA synthesis in cardiomyocytes from subjects with cardiac pathology. Interestingly, the ^{14}C data also indicated a substantially higher renewal rate for non-cardiomyocytes, with a median annual turnover of 18%. Unfortunately, data derived from ^{14}C integration do not allow the identification of the source of new cardiomyocytes. These could be derived either from cardiomyocyte duplication or from some sort of progenitor pool.[22]

Advances in mouse genetic engineering allow cell tracking using 'fate-mapping' approach. Cells expressing the cardiomyocyte-specific alpha-myosin can be genetically labeled by an inducible recombination technique leading to permanent GFP expression of only cells that expressed alpha-myosin at time of induction.[23] If at a later stage, stem cells (which initially do not express alphamyosin) contribute to cardiomyocyte renewal or regeneration, the percentage of GFP-positive myocytes will decrease. These experiments indicated that stem cells do not replace adult

mouse cardiomyocytes during at least 1 year of ageing. However, in the setting of myocardial infarction or pressure-overloaded hearts, a significant decrease in percentage of GFP-positive myocytes was observed, suggesting that precursor cells participate in the formation of new cardiomyocytes after injury.[23] The bone marrow is a pool for multiple types of progenitor cells, which are believed to contribute to cardiovascular repair.[24] Other possible sources of stem cells that can be stimulated to cardiogenic differentiation are located in adipose tissue or in the bone marrow.[24]

One of the main determinants of functionality of bone marrow progenitor cells is age. A study in young and old bone marrow-derived endothelial progenitor cells (EPCs) showed that endothelial progenitor cells from 3-month-old donor mice augmented angiogenic capacity in the recipient 18-month-old mice, whereas EPCs from 18-month-old donor mice showed no effect.[25] Furthermore, young mesenchymal stem cells (MSCs) showed higher angiogenic response to anoxia than old MSCs in vitro, and were more resistant to apoptotic stimuli.[26] This was supported by a study in a myocardial infarction (MI) model. Infarct size of rats with experimental MI was attenuated after intramyocardial injections of young MSCs, whereas injection of old MSCs did not lead to any changes. In addition, cardiac function was preserved after MI in all rats that received MSCs—young, old or a mixture—but the beneficial effect on cardiac function was the largest in rats that received only young MSCs.[26] Age does not only determine functionality of progenitor cells but is also possibly associated with the number of progenitor cells. [27-29]

TELOMERE BIOLOGY

Telomeres are specialised DNA structures made up of tandem repeats (TTAGGG in humans) located at the end of chromosomes.[4,5] Telomeres have a critical function as they serve as protective caps, preventing the chromosomal ends to be accidently recognised as DNA double strands by the DNA damage–repair system, and activation of the p53 or p16INK4a pathway, which eventually leads to senescence or apoptosis. The G-rich strand of the telomere, in conjunction with specialised proteins, form a so-called telomere loop (T-loop) concealing the terminal ends of the DNA strands (figure 1). These specialised proteins include telomeric repeat binding factor 1 (TRF1) and 2 (TRF2), which can both bind directly to double-stranded telomere DNA. Other telomere-associated proteins include the protein protection of telomeres 1 (POT1), binding directly to single-stranded telomere DNA, and repressor activator protein 1 (Rap1), TPP1 and TRF1-interacting nuclear factor 2 (TIN2) (figure 2). Telomeres lose 30–150 base pairs during each cell division (also known as the end replication problem) due to the inability of DNA polymerase to fully replicate the 3′ end of the DNA strand. Additional erosion occurs in presence of damaging environmental factors, e.g., oxidative stress.[30] Because telomere length marks the cumulative replicative history and cumulative exposure to environmental factors, it is strongly associated to date of birth (chronological) age and is even considered a marker of biological/cellular ageing. When the telomere reaches a critical short length, the cell will no longer divide and can become dysfunctional or senescent. On average, cells are estimated to reach senescence after ~50 population

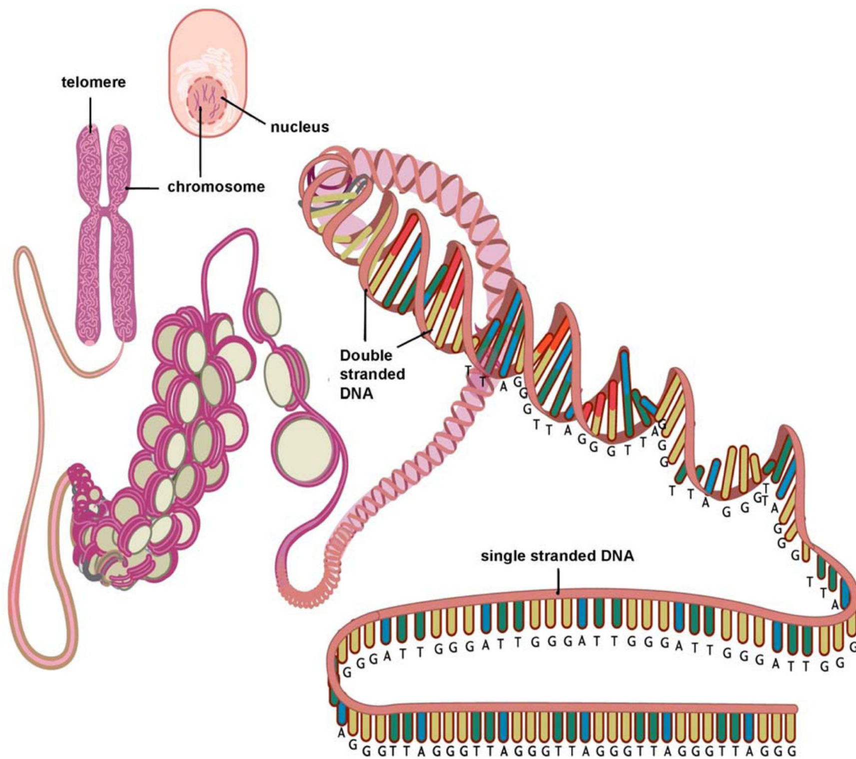

Figure 1. Simplified scheme depicting the structure of the telomere and its location on the chromosome and in the cell.
Reproduced with permission.[68]

doublings. Not only telomere length per se but also disruption of the associated telomere-binding proteins responsible for the three-dimensional loop structure can induce chromosomal instability, senescence or apoptosis.

The function of the ribonucleoprotein enzyme telomerase is the elongation of the telomere sequence by addition of nucleotides to their ends. In most mature cells, telomerase activity is nearly undetectable, with exceptions including embryogenic stem cells, germline cells, malignantly transformed cells and some epithelial and lymphoid progenitor cells. Telomerase consists of two main components, telomerase RNA component (TERC) and telomerase reverse transcriptase (TERT). A third component (dyskerin) serves to stabilize the complex (figure 3). A less well-understood mechanism through which telomeres can be elongated has been named 'alternative lengthening of telomeres' (ALT). This mechanism is thought to be dependent on the cellular homologous recombination machinery, one of the systems for the reparation of DNA double-strand breaks. The ALT pathway is not an alternative to telomerase in cells that lack telomerase activity. Instead, the ALT pathway acts concurrently to telomerase.[31]

Recently, the paradigm that telomeres are transcriptional silent has been

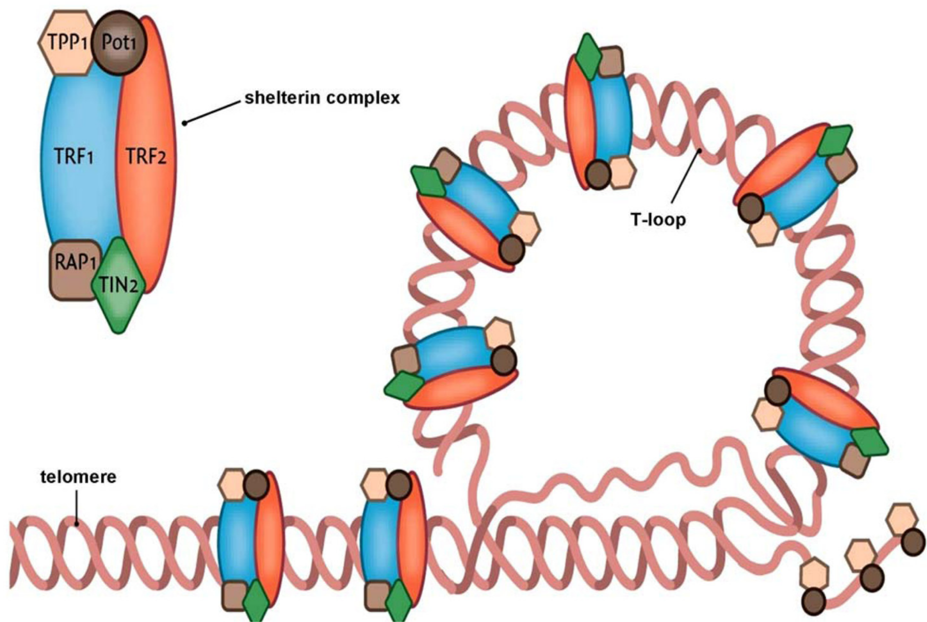

Figure 2. Simplified scheme depiction the terminal end of the telomere concealing the terminal single-stranded part with help of the shelterin complex.
Reproduced with permission.[68]

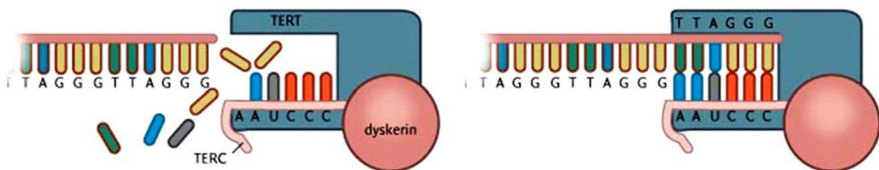

Figure 3. Schematic overview of telomerase. Active telomerase is composed from 2 RNA-complexes (TERC; only 1 depicted) and 2 telomere-reverse-transcriptase (TERC; 1 depicted) stabilized by dyskerin.
Reproduced with permission.[68]

broken. Telomeric repeat-containing RNA (TERRA) is an in length heterogeneous non-coding RNA forming an integral component of the telomeric structure.[32,33] As the sequence of TERRA is complementary to TERC, it might be involved in the regulation of telomerase activity or regulation of the DNA damage response at short telomeres. However, the exact function of TERRA remains to be discovered.

Telomere length is highly variable among individuals of the same age. Already at birth, remarkable differences in telomere length can be detected. Several studies have suggested that telomere length can be predicted by the telomere length of

the parents. Heritability of telomere length has been estimated to be as high as 82%.[34] The effect of paternal telomere length is more obvious than that of maternal telomere length. The strength of the association with paternal telomere length is in addition larger for father–son than for father–daughter relationships.[35] Genome-wide scans have mapped loci associated with telomere length on chromosome 12[36], 14[37], 18[38] and 3 near TERC (component of the enzyme telomerase).[39]

Several environmental factors are also associated with telomere length and possible telomere attrition rate. Most important are oxidative stress[30] and factors related to oxidative stress such as smoking[40] and UV radiation.[41]

TELOMERES AND HEART FAILURE

Telomere length has been related to factors predisposing to CHF, including hypertension[42], diabetes mellitus[43], premature myocardial infarction[44] and activation of the renin-angiotensin-aldosterone system (RAAS).[45] The effect of RAAS inhibition, however, is not clear, since presumably all patients that suffer from CHF are taking RAAS-inhibiting medication, leaving little or no CHF patients without RAAS-inhibiting mediation to serve as valid controls. Of course, the effect of RAAS inhibition could very well be investigated in an experimental heart failure model, but to date no data on this matter have been reported.

Not surprisingly, patients with CHF also have shorter telomeres compared to healthy age- and gender-balanced controls.[46] Telomere length has even been associated with the severity of CHF symptoms and outcome.[46,47] In addition, worse renal function (a powerful predictor of outcome in CHF and not directly related to vascular function[48-50]) has been associated with telomere length in subjects with CHF.[51,52] It is tempting to speculate this is also due to higher senescence in the kidney and consequently nephron dropout. In apparent healthy elderly, one standard deviation of shorter leukocyte telomere length was associated with 5% reduced left ventricular ejection fraction and telomere length alone accounted for 12% of the observed variability.[53] Telomere length is usually measured in leukocytes but has also been evaluated in cardiac tissue from patients with CHF. Patients with dilated heart failure have ~25% shorter cardiac telomeres compared to healthy controls.[54] In aged diseased hearts characterized by mild hypertrophy and biventricular failure, shorter average telomere length was found.[55] Interestingly, cardiomyocytes with severely shortened telomeres (<2.5 kbp) were positive for p16INK4a, a marker for cellular senescence. The fraction of p16INK4a-positive cardiomyocytes was much larger in aged diseased heart compared to non-diseased controls. These findings are consistent with the idea that short telomere length is associated with increased levels of cellular senescence facilitating—if not causing—heart failure.[55]

The most prominent question concerning the association between telomere length and heart failure is the causality of this association. Does short telomere length directly contribute to the development and progression of heart failure, or does short telomere length itself or the underlying cause of heart failure lead to accelerated telomere shortening? An attractive explanation could be that telomere dysfunction is a common pathway through which risk factors act and increase cardiomyocyte senescence and dysfunction. The diminished regenerative capacity

might also include exhaustion of the progenitor pool with repair capacity.[56-58] Evidence for a causal role has been provided by telomerase knockout mice. Fifth-generation telomerase knockout mice have severely reduced telomere length and suffer from severe left ventricular failure, characterized by increased end-diastolic left ventricular pressure, decreased maximally developed left ventricular pressure and disturbed relaxation and contractility very similar to that observed in human dilated cardiomyopathies.[59] On the other hand, stabilizing telomeres by over-expression of TRF2 prevents doxorubicin-induced cardiac apoptosis in wild-type mice, but not in telomerase-deficient mice.[60] However, convincing evidence in humans for a causal role is lacking. Shorter telomeres could be a consequence or an epiphenomenon rather than a cause of CHF. Oxidative stress or increased inflammatory status, both related to outcome[61], could provide a true basis of the development of CHF and at the same time explain reduced telomere length.

CONCLUSIONS AND FUTURE PERSPECTIVES

Recent clinical CHF trials, for example with statins[62], have not fulfilled their promises in improving prognosis of CHF.[63,64] Therapeutic strategies to improve myocardial function and outcome in CHF are urgently needed, and new medicines are rapidly being introduced.[65-67] Telomere biology might be involved in the biology of ageing and age-associated pathology. Telomeres are connected to the basic biology of ageing and trigger cellular senescence. It still needs to be established whether telomere biology is causally involved in the development of CHF in humans. Large, prospective, longitudinal studies are needed to provide us with more in-depth insights into the nature of the association between telomere length and CHF. These studies can clarify whether short telomere length predicts the development of CHF—which possibly supports a causal role—or telomere length merely shortens after manifestation of CHF. Of course, strongest evidence for a causal role would be delivered by interventional studies that demonstrate the cardiac effects of actively altered telomere length. At this time, however, this approach encounters not only several practical obstacles, but also moral objections, since our awareness of all the consequences of telomere length manipulation is surely incomplete. Nevertheless, answering the question on causality unambiguously in the near future is essential, as it will allow the development of novel strategies in the treatment and prevention of CHF, for example by beneficially modifying stem cells currently used in experimental trials.

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8 Summary and future perspectives

Chronic heart failure (CHF) is a condition affecting millions of people, especially elderly, worldwide. Large scale investigations have contributed to the discovery of several risk factors for CHF, but the precise mechanisms of CHF have not fully been elucidated.
Telomeres are the distal ends of our chromosomes. They consist of repeats of specific nucleotide sequences, enabling them to fold in to a special structure called T-loop. Due to this T-loop formation, the distal ends of our DNA strands are hidden and importantly protected from damaging external influences, like oxidative stress or UV radiation.

In natural course of life, telomeres shorten gradually due to incomplete DNA replication and erosion. Cells with critically short telomeres have shown to become dysfunctional, senescent or even go into apoptosis. It is therefore not surprising that short telomere length can lead to organ dysfunction.
Approximately a decade ago, telomere length has been introduced as a possible new player in the pathophysiology of CHF. Clinical associations between short telomere length and CHF have been reported repetitively. However, suggested explanations for this association were only scarcely supported by scientific data. In this thesis, we investigated the association between short telomere length and CHF.

The first part of this thesis focuses on the clinical associations between telomere length and CHF.

Chapter 1 provides an overview of the literature on the association between telomere length and CHF or risk factors. Short telomere length has repetitively been associated with atherosclerosis and myocardial infarction. Furthermore, it also has been associated with classic cardiovascular risk factors, such as hypertension, diabetes, and cigarette smoking.

Chapter 2 and 3 show that telomere length is associated with co-morbidities of CHF in patients. Chapter 2 reports the association of short telomere length with prevalence of anaemia, another frequently present co-morbidity of CHF. The question rises whether this is a causal relationship or not. Unfortunately, the cross-sectional approach of the study does not allow drawing any conclusions on causality. However, other studies have shown that short telomere length in the bone marrow impairs haematopoietic potential of bone marrow cells, and that leukocyte telomere length reflects bone marrow telomere length very well. Although this is indirect evidence, these data do suggest that the anaemia in CHF could be the result of a diminished proliferative potential of haematopoietic bone marrow cells with short telomeres. Chapter 3 describes the inverse association on telomere length and renal function, indicated by estimated glomerular filtration rate, in patients with CHF. There are several possible explanations for this association. Renal dysfunction causes increased oxidative stress. Oxidative stress is one of the recognised factors that induces telomere shortening. Telomere length would then serve as a systemic marker for damage originating from decreased renal function. Another possible example could however also be that short telomere length is a causal factor for development of renal dysfunction in CHF patients, assuming that leukocyte telomere length reflects telomere length of other cell types. Cells with short telomeres are possible more vulnerable for damaging environmental factors. Short

telomere in renal cells would then result in a cellular vulnerability in the kidney, and pose an increased risk on a CHF patient to develop renal failure. Chapter 4 describes the large, longitudinal study of telomere length in association with cardiovascular risk factors in time in approximately 8000 subjects without a cardiovascular history of disease. This is the first clinical study of this magnitude with telomere length measured at multiple time points. This study showed that telomere attrition rate was associated with the well-known cardiovascular risk factors smoking, obesity, and glucose levels. High HDL-levels are associated with a decreased telomere attrition rate. Based on these study results, one could hypothesize that the pace of biological ageing can be influenced by change in life style patterns, which is also in line with epidemiological evidence.

The second part of the thesis aimed at the possible mechanisms underlying the observed associations between short telomere length and CHF.

Chapter 5 describes the telomerase deficient mouse model as a model for cardiovascular research. Telomerase deficient mice were originally generated for cancer research, as telomerase activity is often a key factor in the process of carcinogenesis. However, it also serves as an excellent model to explore the possible mechanisms connecting telomeres and cardiovascular disease. For fundamental research, non-human models are indispensable since highly controlled environments or genetic resemblance is often required to elucidate the precise influence of a factor of interest. In chapter 6, we investigated whether circulating cardiovascular progenitor cells would have shorter telomere length in CHF patients than healthy controls. We confirmed that leukocyte telomere length is shorter in CHF patients than in healthy controls, and demonstrated that this could also be observed in the healthy offspring of both groups. We also showed that telomere length was not different between CHF patients and healthy controls in circulating cardiovascular progenitor cells, making it very unlikely that these progenitor cells are involved in the role of telomere biology in CHF.

Finally, chapter 7 describes the ageing cardiovascular system and the role of telomere length in this ageing process, and it provides an overview of recent scientific findings on telomere length in relation to cardiovascular disease.

Future perspectives.

The main question regarding the role of telomere length in CHF remains the nature – causal or not – of this relationship. Most evidence for this association comes from observational clinical studies. These studies have predominantly used leukocyte telomere length, presumably because of easy access (venous blood puncture) to leukocyte DNA.[1-7] It is however not established whether short leukocyte telomere length reflects overall short telomere length, including short telomeres in the myocardium. In addition, we and others have shown that leukocyte telomere length within one individual can fluctuate in time. It would be interesting to see whether the association between telomere length and CHF extents to myocardial telomere length. This would give us a clue whether the potential mechanisms linking short telomere length and CHF is to be sought on cellular level within the myocardium or more systemic influences affecting both leukocyte telomere length and cardiac

function. However, as mentioned before, obtaining large numbers of human cardiac tissue samples is difficult. Therefore, the telomerase knock out mouse model could be of possible value. It would be interesting to see whether late generation telomerase knock out mice (with short telomeres) would develop heart failure after –for example- experimental myocardial infarction more rapid or severe compared to early generation mice with longer telomeres. It would also be interesting to see how both generation mice would respond to classic heart failure medication. Possibly, this could contribute to a more tailor-made medical treatment for patients with CHF.

One could wonder what the clinical applicability of telomere length could be. This depends substantially on whether telomere length is causal to CHF. If causality can be established, one could think of telomere-stabilizing or even elongating therapeutic strategies. It has for example been suggested that statin treatment stabilises telomere length[8], and that patients with coronary artery disease with shortest telomere length benefit most from statin treatment.[2] Theoretically, it is possible to transplant cells with upregulated telomerase, and consequently longer lifespan, to enforce the compromised heart. However, repeated studies have pointed out difficulties with harbouring of circulating cells in cardiac tissue.[9] Another problem is the viability of these transplanted cells. This opportunity therefore sounds potentially promising, but has many obstacles to overcome and the beneficial effects are to be established.

In another scenario, telomere length could serve as a biomarker for CHF. For diagnostic purposes it will probably not be useful, because of the large interindividual variation of telomere length. However, it is potentially useful in monitoring progression of CHF, since it has been suggested that a gradual inverse relationship exists between telomere length and severity of disease in CHF patients.[3] Before this can be applied in clinical practice, this gradual effect must be investigated more extensively.

To conclude, in this thesis we have confirmed the inverse association between telomere length and CHF and associated factors. We have established factors associated with telomere length in time. Also, we have ruled out one possible mechanism underlying the role of telomere length in CHF. Despite our efforts, the question on the nature of the association between CHF and telomere length still remains. To elucidate the precise role of telomere length in the pathophysiology of CHF and its potential role in therapeutic or diagnostic strategies, more mechanistical as well as longitudinal observational studies are needed.

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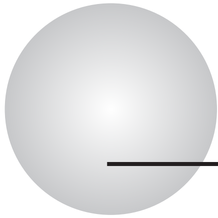

Samenvatting en toekomstperspectieven

Chronisch hartfalen (CHF) is een aandoening die wereldwijd miljoenen patiënten treft. Hoewel veelvuldig onderzoek tot de ontdekking van verschillende risicofactoren voor CHF heeft geleid, zijn de precieze mechanismen van het ontstaan en de progressie van CHF nog niet geheel duidelijk.

Ongeveer een decennium geleden werd voor het eerst geopperd dat telomeerlengte mogelijk een rol speelt in de pathofysiologie van CHF. Telomeren zijn de uiteinden van chromosomen. Door de speciale structuur waarin telomeren "opgevouwen" zijn, beschermen ze het uiteinde van het DNA waaruit chromosomen zijn opgebouwd. Ernstige telomeerverkorting kan leiden tot celdisfunctie of zelfs apoptose, geprogrammeerde celdood, wat schadelijk voor het organisme kan zijn. Dit beschermingsmechanisme is echter niet feilloos en er vindt tijdens het leven door verschillende mechanismen toch verkorting plaats van telomeren.

Uit onderzoeken is gebleken dat patiënten met CHF kortere telomeren hebben dan hun gezonde leeftijdsgenoten. Korte telomeerlengte lijkt dus samen te hangen met CHF. Belangrijk is de vraag of telomeerlengte verkorting vooraf ging aan het ontstaan van CHF of dat het het gevolg is van CHF, of zelfs helemaal niets met elkaar te maken hebben maar slechts "toevallig" tegelijkertijd worden geobserveerd in CHF patiënten. Indien telomeerverkorting vooraf gaat aan CHF, betekent dit dat telomeerlengte mogelijk een factor is die bijdraagt aan het ontstaan van CHF en daarbij mogelijk ook een ingang is voor een nieuwe behandeling.

Hoewel verschillende onderzoekers een associatie tussen korte telomeerlengte en CHF hebben geconstateerd, is er weinig gespeculeerd over mogelijke verklaringen voor deze associatie en is er nog minder wetenschappelijk bewijs dat deze mogelijke verklaringen ondersteunt. In dit proefschrift hebben wij de associatie tussen korte telomeerlengte en CHF onderzocht.

Het eerste gedeelte van dit proefschrift richt zich op de klinische associaties tussen telomeerlengte en CHF.

Hoofdstuk 1 geeft een overzicht van de literatuur over de associatie tussen telomeerlengte en CHF of risicofactoren voor CHF. Korte telomeerlengte is herhaaldelijk in verband gebracht met atherosclerose (in de volksmond "vaatverkalking") en hartinfarcten. Daarnaast is telomeerlengte ook geassocieerd met klassieke risicofactoren voor hart- en vaatziekten, zoals hoge bloeddruk, suikerziekte en roken.

Hoofdstukken 2 en 3 laten zien dat telomeerlengte ook geassocieerd is met nevenziekten die vaak optreden bij CHF patiënten. Hoofdstuk 2 beschrijft de associatie tussen korte telomeerlengte en het voorkomen van anemie (bloedarmoede), wat ook een vaak voorkomende nevenziekte van CHF is. De vraag rijst of het hier gaat om een causaal verband. Helaas laat de opzet van dit onderzoek niet toe dat we op basis van dit onderzoek hier conclusies over trekken. Andere onderzoeken hebben echter laten zien dat telomeerlengte van witte bloedcellen sterk overeenkomt met telomeerlengte van beenmergcellen en dat korte telomeerlengte in het beenmerg – waar rode bloedcellen worden aangemaakt – de bloedvormende capaciteit van het beenmerg vermindert. Dit is natuurlijk indirect bewijs, maar het wekt wel de suggestie dat bloedarmoede in CHF patiënten zou kunnen voortkomen uit verminderde functie van het beenmerg met korte telomeren. In hoofdstuk 3 is

te lezen dat er een omgekeerde relatie bestaat tussen telomeerlengte en geschatte nierfunctie in CHF patiënten. Er zijn verschillende denkbare verklaringen hiervoor. Slechte nierfunctie veroorzaakt verhoogde oxidatieve stress. Oxidatieve stress is een bekende factor die telomeerlengteverkorting veroorzaakt. In dit scenario zou telomeerlengte dus een marker zijn voor schade aan het lichaam dat veroorzaakt wordt door nierfalen. In een ander scenario zou telomeerlengte ook een causale factor kunnen zijn voor het ontstaan van nierfalen in CHF patiënten, als we ervan uit gaan dat telomeerlengte van witte bloedcellen (die wij hebben gemeten) een afspiegeling is van telomeerlengte van andere lichaamscellen. Cellen met korte telomeren zijn kwetsbaarder voor schadelijke externe factoren. Als de nierencellen korte telomeren hebben, zouden de nieren kwetsbaarder zijn en sneller last functieverlies kunnen krijgen. Hoofdstuk 4 beschrijft een groot, longitudinaal onderzoek naar de associatie tussen telomeerlengte en cardiovasculaire risicofactoren in een populatie van ongeveer 8000 mensen met albuminurie (eiwit in de urine). Dit is het eerste klinische onderzoek van deze omvang met telomeerlengte gemeten op meerdere tijdstippen. We hebben laten zien dat snelheid van telomeerverkorting geassocieerd is met bekende risicofactoren voor hart- en vaatziekten, zoals roken, overgewicht en hoog bloedsuikergehalte. Daarentegen was een hoog bloed-HDL gehalte (het "goede cholesterol") geassocieerd met langzamere telomeerverkorting. Op basis van deze onderzoeksresultaten zou je kunnen speculeren over de invloed van leefstijl en –gewoontes op telomeerlengteverkorting. Door een gezondere leefstijl aan te houden kan wellicht telomeerlengteverkorting worden vertraagd.

Het tweede gedeelte van dit proefschrift richt zich op de mogelijke mechanismen die ten grondslag liggen aan de geobserveerde klinische associaties tussen korte telomeerlengte en CHF.

In hoofdstuk 5 wordt het telomerase-deficiënte muismodel beschreven als model voor cardiovasculair onderzoek. Telomerase-deficiënte muizen werder oorspronkelijk gebruikt voor onderzoek naar kanker, omdat telomerase-activiteit vaak een rol speelt bij het ontstaan van kanker. Dit muismodel kan echter ook uitstekend worden gebruikt om eventuele onderliggende mechanismen van de relatie tussen telomeerlengte en hart- en vaatziekten te onderzoeken. Om mechanistisch onderzoek te doen zijn niet-menselijke onderzoeksmodellen onontbeerlijk omdat vele omgevingsfactoren (zoals bijvoorbeeld woonomgeving en dieet) controleerbaar moeten zijn en bovendien de genetische achtergrond van de onderzochte organismen nagenoeg gelijk moet zijn. Het is niet moeilijk voor te stellen dat dit bij mensen praktisch onhaalbaar is en we daarom diermodellen nodig hebben.

In hoofdstuk 6 hebben we telomeerlengte onderzocht in leukocyten en circulerende cardiovasculaire voorlopercellen (cellen die schade aan het hart- en vaatstelstel zouden kunnen reparere) en vroegen ons af of deze cellen een kortere telomeerlengte hebben bij CHF patiënten dan bij gezonde controles. We hebben bevestigd dat dit het geval is in leukocyten. Daarnaast hebben we laten zien dat dit zelfs het geval is bij de (nog) gezonde kinderen van beide groepen. Het is bekend dat kinderen van ouders met hart- en vaatziekten een hoger risico hebben op het ontwikkelen van hart- en vaatziekten. Dat telomeren al korter zijn bij de gezonde

kinderen van CHF patiënten vergeleken met gezonde kinderen van gezonde patiënten zou impliceren dat telomeerlengteverkorting vooraf gaat aan het ontstaan van CHF. Daarnaast hebben we laten zien dat telomeerlengte in de voorlopercellen niet verschillend is in de groepen, wat een rol voor deze cellen in de relatie tussen telomeren en CHF erg klein maakt.

Tenslotte hebben we in hoofdstuk 7 de veroudering van hart en vaten en de rol van telomeren in dit verouderingsproces en hart- en vaatziekten beschreven.

Toekomstperspectieven.

De centrale vraag met betrekking tot de associatie tussen telomeren en CHF blijft of het een causaal verband betreft of niet. De meeste wetenschappelijke bewijzen voor deze associatie zijn voortgekomen uit observationele klinische onderzoeken. Deze onderzoeken hebben voornamelijk naar telomeerlengte in witte bloedcellen bekeken, omdat deze gemakkelijk te verkrijgen zijn met een "bloedprik".[1-7] Het is echter nog niet onomstotelijk vastgesteld dat telomeerlengte in witte bloedcellen telomeerlengte in de rest van het lichaam en met name het hart weerspiegelt. Dit is belangrijk om te weten, omdat we dan weten of de link tussen korte telomeerlengte en CHF mogelijk in het hartweefsel zit of dat we het moeten zoeken in overkoepelende systemische factoren die invloed hebben op zowel telomeerlengte als hartfunctie. Hartweefsel van mensen verzamelen is echter zeer moeilijk en het is dus de vraag wanneer er overtuigend bewijs uit grootschalige onderzoeken komt dat telomeerlengte in het hart wel of niet wordt weerspiegeld door die van witte bloedcellen.

Zoals eerder gezegd kan het telomerase-deficiënte muismodel uitkomst bieden. Het zou interessant zijn om te onderzoeken of late generaties van deze muizen – die korte telomeren hebben – sneller of ernstiger hartfalen krijgen dan gezonden wild type muizen na een hartinfarct en wat het effect van medicatie tegen hartfalen is in beide groepen.

Een andere vraag is wat de klinische toepasbaarheid van telomeerlengte is. Dit wordt in grote mate bepaald door de aard van de relatie tussen telomeerlengte en CHF (causaal of niet). Als deze relatie causaal blijkt te zijn, zouden telomeerstabiliserende of zelfs –verlengende strategieën denkbaar kunnen zijn. Statines zouden bijvoorbeeld telomeerlengte stabiliseren.[8] In theorie is het ook mogelijk om cellen met kunstmatig verlengde telomeren te transplanteren om het verzwakte hart te ondersteunen of herstellen. Hier komen echter serieuze problemen bij kijken. Ten eerste blijkt het in de praktijk erg lastig om de "losse" cellen verweven te krijgen in hartweefsel.[9] Daarnaast lijken deze cellen verminderd levensvatbaar. Transplantatie van deze cellen klinkt wellicht veelbelovend, maar er moeten nog veel obstakels in de praktijk worden overwonnen en de effectiviteit moet daarna nog worden aangetoond.

Telomeerlengte zou ook kunnen dienen als marker van CHF. Om CHF te diagnostiseren zou het waarschijnlijk niet toepasbaar zijn, omdat telomeerlengte erg kan verschillen tussen verschillende mensen. Mogelijk kan het wel ingezet worden om verslechtering van CHF te monitoren. Patiënten met ernstig CHF lijken namelijk kortere telomeren te hebben dan patiënten met minder ernstig CHF.[3] Dit zou eerst beter onderzocht moeten worden voordat we het bij patiënten kunnen toepassen.

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Concluderend hebben we in dit proefschrift bevestigd dat er een omgekeerde relatie bestaat tussen telomeerlengte en CHF en CHF-gerelateerde factoren. Ook hebben we laten zien dat deze relatie in de tijd blijft bestaan. Daarnaast hebben we aangetoond dat de cardiovasculaire voorlopercellen geen rol spelen in het eventuele onderliggende mechanisme van de relatie tussen telomeerlengte en CHF. Ondanks onze pogingen deze relatie grondig te onderzoeken, blijft de vraag bestaan of het gaat om een causaal verband of niet. Om de precieze rol van telomeren in CHF en eventuele klinische toepasbaarheid van deze kennis te begrijpen, is er zowel meer mechanistisch als longitudinaal onderzoek nodig.

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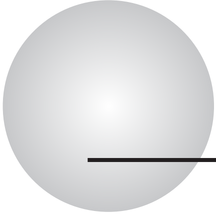

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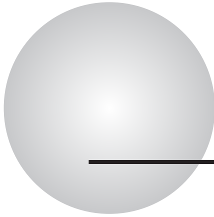

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