Arteriosclerosis, Thrombosis, and Vascular Biology

BRIEF REVIEW

Telomerase as a Therapeutic Target in Cardiovascular Disease

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ABSTRACT: Shortened telomeres have been linked to numerous chronic diseases, most importantly coronary artery disease, but the underlying mechanisms remain ill defined. Loss-of-function mutations and deletions in telomerase both accelerate telomere shortening but do not necessarily lead to a clinical phenotype associated with atherosclerosis, questioning the causal role of telomere length in cardiac pathology. The differential extranuclear functions of the 2 main components of telomerase, telomerase reverse transcriptase and telomerase RNA component, offer important clues about the complex relationship between telomere length and cardiovascular pathology. In this review, we critically discuss relevant preclinical models, genetic disorders, and clinical studies to elucidate the impact of telomerase in cardiovascular disease and its potential role as a therapeutic target. We suggest that the antioxidative function of mitochondrial telomerase reverse transcriptase might be atheroprotective, making it a potential target for clinical trials.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: aging ■ atherosclerosis ■ cardiovascular diseases ■ oxidative stress ■ T-lymphocytes ■ telomerase ■ telomere

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In the first half of the 20th century, Hermann J. Muller and McClintock[1] already described the instability of broken chromosomes in the fruit fly and maize. However, structural changes at the end of the chromosomes were not detected. It was therefore concluded that those terminal regions must have a special function in protecting the chromosomes. Muller distinguished these unique regions from the remainder of the chromosomes and termed them telomeres, combining the Greek words for end (telo) and segment (meros). Forty years later, Szostak and Blackburn[2] demonstrated tandem hexanucleotide repeat sequences at the end of yeast chromosomes and concluded that those sequences are the functional telomeres. Today, it is well known that telomeres in mammals are composed of tandem repeats of the hexanucleotide sequence TTAGGG, which adopt a higher order structure and interact with a number of DNA-binding proteins.[3–5] Subsequently, Elizabeth Blackburn and her graduate student Carol Greider discovered that a cellular ribonucleoprotein complex that they called telomerase exists in Tetrahymena.[6] Shortly thereafter, the enzyme telomerase was also detected in eukaryotes.[7] The enzyme telomerase consists of the catalytic subunit telomerase reverse transcriptase (TERT) and the noncoding telomerase RNA component (TERC), which interact with multiple telomerase-associated proteins, like the TRF1 and TRF2 (telomeric repeat binding factors 1 and 2). TERC serves as the template for TERT-dependent telomere elongation. Telomeres, the caps of the end of the chromosomes, protect them from end-to-end fusion. Telomeres undergo shortening during each cell division due to the end replication problem, a process that is counteracted by the enzyme telomerase.[8] For long time, it was believed that telomerase is only expressed in tumor cells, stem cells, and highly proliferating cells. However, it has become evident that telomerase is also detectable in differentiated, nondividing, or low proliferating cells of the cardiovascular system.[9,10]

CANONICAL TELOMERASE FUNCTION: TELOMERE LENGTH

Telomere Length Across Species

When compared with other species, humans have relatively short telomere length (TL) ranging from 5 to 15 kb in

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Nonstandard Abbreviations and Acronyms

CHD	coronary heart disease
iNOS	induction of nitric oxide synthase
LTL	leukocyte telomere length
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
ROS	reactive oxygen species
TA	telomerase activity
TACTIC	Telomerase Activator to Reverse Immunosenescence in Acute Coronary Syndrome
TERC	telomerase RNA component
TERT	telomerase reverse transcriptase
TL	telomere length
TRAP	telomeric repeat amplification protocol
TRF1	telomeric repeat binding factor 1
TRF2	telomeric repeat binding factor 2

newborns, and yet they have much longer life spans than, for example, laboratory mice, which can have at birth TLs of ≈50 kb.[11] Recent studies on telomere biology of different species helped to resolve this apparent paradox and hence contributed important implications for understanding human aging and disease. By comparing TL in >60 different species, Gomes et al[12] concluded that the life span of a species cannot be predicted from TL at birth, and that there was a trend for short-lived species to have longer telomeres. In accordance with these findings, Whittemore et al[13] recently measured in parallel the TL in 9 species of birds and mammals and found that the telomere shortening rate, but not the initial TL alone, is a strong predictor of species life span. Accordingly, long-lived species, like humans and elephants, have a much lower telomere shortening rate (≈70–150 bp per year) than short-lived mice, which shorten telomeres at a rate of ≈7000 bp per year. These comparative studies suggest that critical telomere shortening and the induction of cellular senescence is a general determinant of species life span.

TL Across Human Tissues and Cell Types

Most studies to date have used unfractionated whole blood samples as a DNA source for TL measurements. Thus, our current understanding of the role of this parameter in cardiovascular disease is mainly based on measurements in total blood leukocytes. Less numerous investigations have looked into TL distribution among different human tissues. For example, Daniali et al[14] found significant differences in TL in circulating leukocytes, muscle, skin, and fat from the same individuals, yet TL strongly correlated between tissues. Furthermore, we could show similar rates of age-dependent TL attrition. More recently,

Highlights

- Loss of function mutations of telomerase reverse transcriptase, the catalytic subunit of telomerase, accelerate telomere shortening and limit T-lymphocyte proliferation but also impair the noncanonical role of telomerase in mitochondria.
- The atheroprotective role of mitochondrial telomerase reverse transcriptase stems from its antioxidative function and possibly from its role in vascular cell regeneration.
- Targeting telomerase and specifically the noncanonical functions of telomerase reverse transcriptase could prove beneficial in the treatment of cardiovascular aging and disease.

Demanelis and colleagues presented a unique data set comprising comparative measurements of relative TL in over 20 distinct tissue types from 952 donors from the Genotype-Tissue Expression (GTEx) Project.[15] We could confirm that TL is generally positively correlated across human tissue types and that leukocyte TL reflects tissue-specific TL for many somatic tissues, including coronary vessels. In addition, evidence was provided on how the cell type composition significantly affects TL measurement in human tissues, including blood leukocytes. For example, enrichment with granulocytes correlated with higher TL, and higher proportions of CD8+ T lymphocytes inversely correlated with total leukocyte TL (LTL). These results are in accordance with previous studies by us and others, demonstrating that TL in lymphocytes, and specifically in memory T cells, decreases significantly faster and is shorter than TL in granulocytes (Figure 1).[16,17] When comparing TL across different subpopulations of leukocytes in patients with coronary heart disease (CHD), we further detected the longest telomeres in myeloid cells, B lymphocytes, and naïve T cells, whereas differentiated memory CD8 T cells displayed the shortest TL.[18] We have specifically demonstrated that TL in myeloid leukocyte subsets, including progenitors, monocytes, and neutrophils, was 500 bp shorter in patients with CHD when compared with healthy individuals, whereas TL in differentiated memory CD8 T cells was already 1000 bp shorter, mainly as a result of cytomegalovirus seropositivity.[19] Based on these results, we postulated that TL shortening occurs faster in specific populations of circulating lymphocytes in patients with CHD compared with healthy controls, suggesting that TL measurements in defined cell subsets (eg, using Flow-FISH method, see below) might provide a more reliable marker of cardiovascular aging.

Quantification of TL

Several techniques for TL quantification have been developed and refined over the past decades. Whereas

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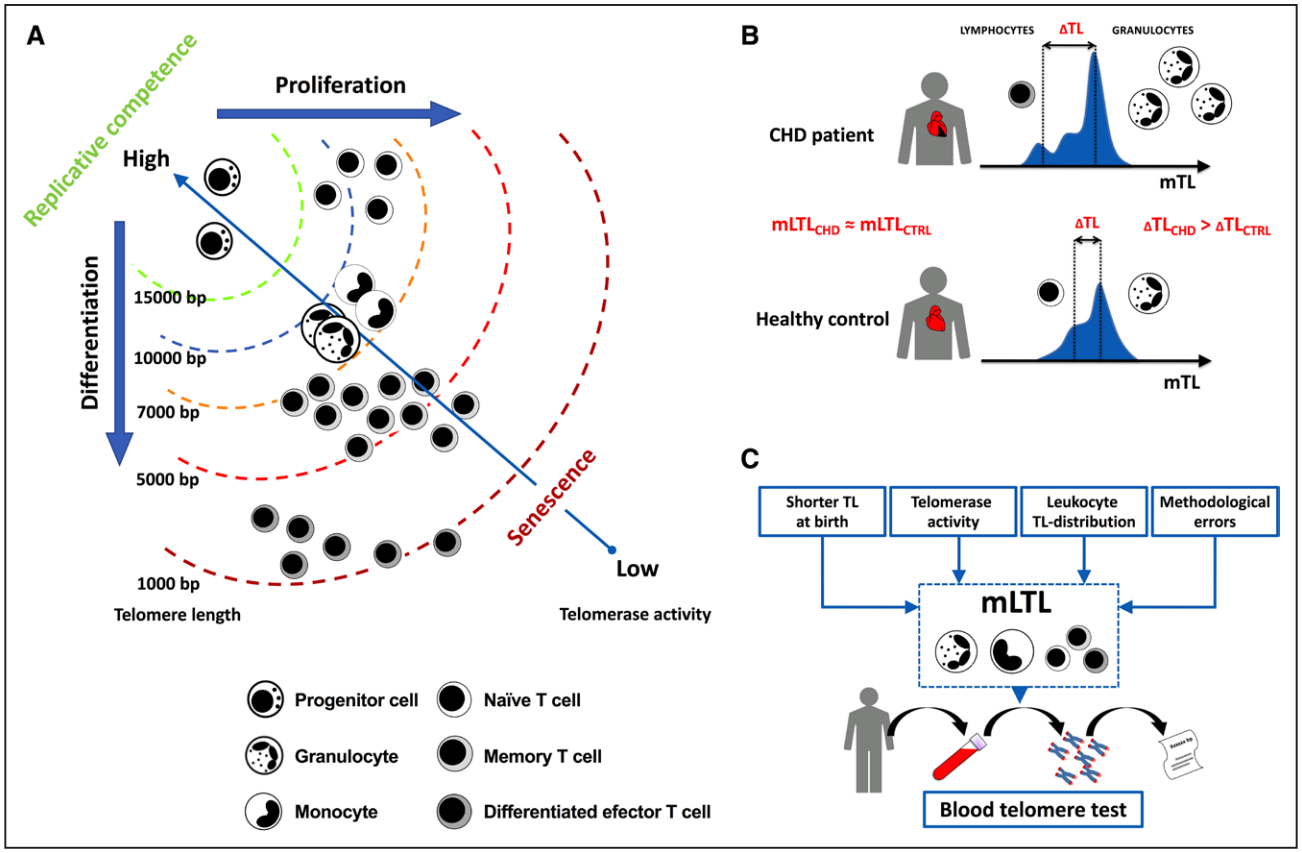

Figure 1. Telomere length in human blood cells–a reliable marker of cardiovascular disease?
A, Schematic of the telomeric landscape of human leukocyte subsets showing the relationships between telomerase activity and telomere length in different subsets of lymphocytic and myeloid cells. The originating cells with high telomerase activity, longest telomeres and maintained replicative capacity (eg, or naïve T cells) undergo rounds of replication and differentiation process throughout life span, marked by gradual telomere shortening and finally replicative and functional senescence (immunosenescence). **B**, Composition of leukocytes varies significantly among individuals and due to infection or comorbidity, even in the same individual from day to day. For example, coronary heart disease (CHD)-related systemic inflammation often results in peripheral granulocytosis. Expanded myeloid cells with relatively longer telomeres may mask the progressive telomere shortening in the highly diluted cells such as senescent T lymphocytes. In consequence, the measured mean leukocyte telomere length (mLTL) could seem very similar between patients with CHD and healthy controls. Our proposed estimation of the telomere gap between granulocytes and lymphocytes (ΔTL) using flow–FISH techniques can provide a granulocytosis-independent TL measure by unmasking telomere attrition in lymphocyte subsets.[18] **C**, In addition to variations in leukocyte distribution, mLTL might be influenced by multiple factors including genetic and environmental confounders, variable telomerase activity, methodological errors (comparability of techniques used for telomere length (TL) estimation in different test setups). These confounders should be taken into account when considering TL as an ideal biomarker for cardiovascular aging.

DNA-blotting-based (Southern blot) terminal restriction fragment analysis (TRF) remains a gold standard for TL measurement,[20,21] further technical advances have evolved, including quantitative fluorescence in situ hybridization (Q-FISH and Flow-FISH),[22,23] PCR-based methods (quantitative PCR and single TL analysis, STELA),[24–26] and dot blot analysis.[27] Technical aspects of the available TL quantification methods, including their advantages and limitations, as well as their applicability for clinical studies, are reviewed in more detail elsewhere.[23,24,28–30]

Assessment of Telomerase Activity

Telomeric repeat amplification protocol (TRAP), including its numerous modifications, is the most common method employed for the quantification of telomerase activity (TA).[31] The TRAP assay is a polymerase chain reaction (PCR)-based method that involves 3 basic steps: (1) extension of an oligonucleotide by telomerase-mediated addition of telomeric DNA repeats, (2) subsequent PCR amplification of the extension products, and (3) detection of telomerase products. Quantitative analysis of PCR products by real-time PCR offers higher precision, reproducibility, and applicability for high-throughput formats, as compared with traditional polyacrylamide gel electrophoresis (standard TRAP). More recently, the development of a droplet digital PCR version of the TRAP assay has enabled accurate quantitative assessment of TA in single cells.[32] However, several established challenges with either approach exist, which contribute to the reason that TA is not used clinically or even in most research studies. TA is difficult to standardize with a normal assay,

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since it does not return absolute values, thus requiring control cells as a standard. Other technical issues lie within the effect of PCR inhibitors such as heparin, changing composition of peripheral blood leukocytes, variability in counting cell input or generally the effect of cell cryopreservation on TA variability. Finally, it should be pointed out that TA is measured following lysis of complete cells, which might not represent the situation in vivo where enzymatic components of telomerase could be located in separate compartments of the cell.

LTL AND CARDIOVASCULAR DISEASE

TL in Heart Failure

Telomeres are shorter in leukocytes from patients with severe heart failure compared with healthy controls (Figure 2).[33] A very recent study shows that cardiomyocyte's telomeres are shorter in failing hearts compared with nonfailing donors, and hypertrophic hearts with reduced ejection fraction exhibit the shortest telomeres.[34] In contrast to circulating blood cells, no difference in cardiomyocyte's TL is evident with age. However, telomere attrition was present in both young and older patients with cardiac hypertrophy. We have recently demonstrated that accumulation of telomere-associated DNA damage induced by mitochondrial dysfunction and increased oxidative stress during aging promotes senescence of cardiomyocytes.[35] This alternative mechanism of telomere damage signaling acts through the classical p16/p21 pathways and provides a means by which a largely postmitotic tissue, which limited regenerative potential can accumulate senescence in the absence of proliferation and replicative telomere shortening.[36,37] Intriguingly, cardiomyocyte senescence is associated with expression of a proinflammatory and profibrotic senescence-associated secretory phenotype.[35] Together, the accumulation of senescent cells and the senescence-associated secretory phenotype promote maladaptive myocardial remodeling termed senescence-induced remodeling. Remarkably, pharmacological and pharmacogenetic clearance of senescent cells both attenuate cardiac hypertrophy, reduce the expression of profibrotic chemokines, reduce fibrosis, promote cardiomyocyte regeneration, and improve cardiac function.[35,38] Similar beneficial effects of clearance of senescent cells within the heart have been described by others.[39,40] It is possible therefore that oxidative stress both induces and is a result of myocardial senescence via a local increase in reactive oxygen species production locally by senescent cells and systemically via senescence-associated secretory phenotype-mediated inflammation. Further studies are warranted to assess whether this systemic inflammation could contribute to telomere shortening in immune cells via an increase in proliferation together with oxidative stress–mediated inhibition of telomerase.

TL and CHD

In his seminal pilot study in 2003, Cawthon et al[41] demonstrated that short LTL is predictive of higher mortality in over 60-year-old subjects, increasing the risk

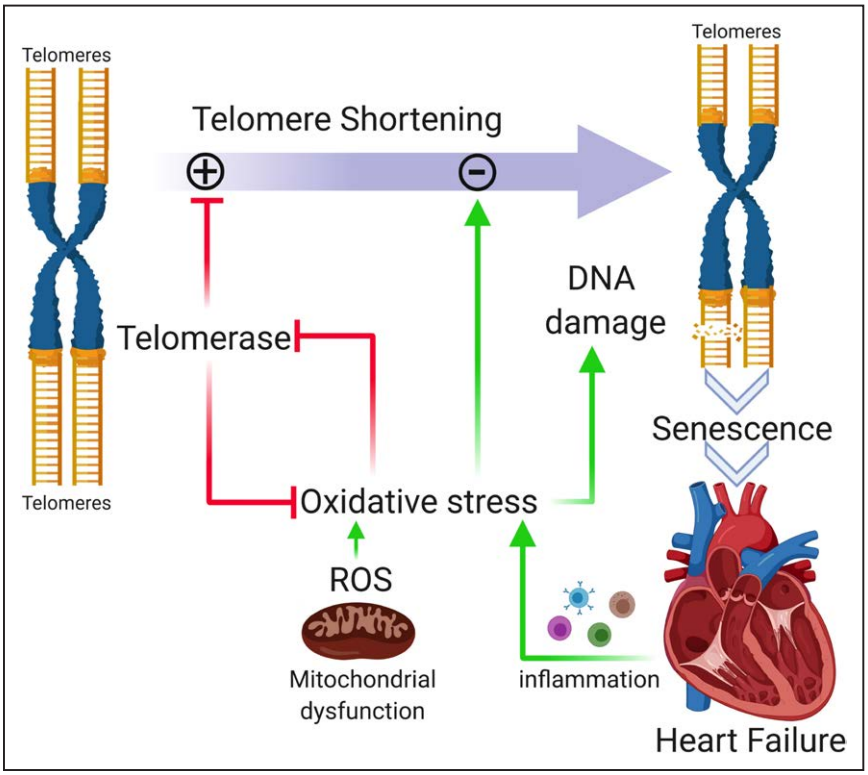

Figure 2. Interactions between oxidative stress and telomerase drive senescence-induced remodeling and heart failure. Oxidative stress from multiple sources, including mitochondrial dysfunction and inflammation, drives cellular senescence through multiple mechanisms. Oxidative stress directly accelerates telomere shortening via 8-oxodG formation but also inhibits telomerase transcriptional activity harming telomere homeostasis. Oxidative stress also leads to the formation of persistent telomere associated double-stranded DNA breaks, which induce cell senescence via activation of the DNA damage response. Telomerase reverse transcriptase (TERT) contributes to the control of mitochondrial reactive oxygen species (ROS) generation. Accordingly, mitochondrial TERT inhibition by oxidative stress results in increased ROS and thereby further increases intracellular oxidative stress. Telomere shortening and associated DNA damage contribute to senescence in multiple cell populations including cardiomyocytes. Cardiomyocyte senescence contributes to inflammation in the form of a senescence-associated secretory phenotype, which may promote maladaptive myocardial remodeling and systemic inflammation.

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of death from heart disease and infection by 3- and 8-fold, respectively. Several years later, Willeit et al[42] found an inverse relationship between LTL and cancer incidence as well as mortality. Inflammation is a common pathogenic factor in age-related diseases.[43] In the recent CANTOS trial, the inhibition of excess inflammation by canakinumab through targeting interleukin-1β innate immunity led to a reduction in both cardiovascular events and cancer but was associated with a higher incidence of fatal infection compared with the placebo arm.[44] Assuming a link between TL and inflammation, the CANTOS trial suggests that while chronic inflammation in age-related diseases probably leads to increased cell turnover, an association with shorter LTL is thus to be expected. In contrast, critically short TL in hematopoietic stem cells or progenitor cells that predisposes to fatal infections suggests a reverse causality. These data therefore do not clarify causes and consequences of short TL in the context of cardiovascular disease and cancer.

Multiple observational studies have shown that short LTL is associated with ischemic heart disease.[45–47] Likewise, cardiovascular risk factors such as hypertension, stress, smoking, or obesity have been associated with shorter telomeres.[48] The WOSCOPS study was the first larger trial to investigate TL in cardiovascular disease. It demonstrated in a subset of 1500 patients that shorter LTL was associated with a 2-fold higher risk of developing coronary artery disease (lower TL tertile versus higher TL tertile).[49] In addition, patients with the shortest TL benefited most from pravastatin treatment, without actually showing that TL responded to this beneficial treatment. The question arising from all these studies is about causality—whether short TL is in fact accelerating atherosclerosis or causing CHD. In our opinion, there is no evidence to date for this hypothesis. Indeed, the cross-sectional studies Asklepios[48] and PESA[50] found that LTL is not a significant independent determinant of subclinical atherosclerosis in middle-aged human populations. It is conceivable though that chronic inflammation and oxidative stress, which are both instrumental in the pathogenesis of atherosclerosis, exert different effects on telomerase, thus accelerating TL shortening in leukocyte populations. In addition, while an association between shorter telomeres and CHD has been proven for different leukocyte compartments, there is currently no evidence showing disease-related accelerated TL shortening in the human coronary circulation. Beyer et al,[9] for example, have shown no telomere shortening in either left ventricular tissue or microvessels when comparing healthy subjects to patients with coronary artery disease, while highlighting the noncanonical the role of telomerase in endothelial surveillance and coronary pathology (as described in more detail in the following sections).

TL and Cardiac Arrhythmias

LTL shortening as well as a high load of shorter telomeres have been shown to trigger cellular senescence, eventually indicating structural changes in cardiac myocytes during aging. Interestingly, one study in patients with implantable defibrillators found a high load-of-short telomeres and TA to be predictive for ventricular arrhythmias in patients with ischemic heart disease,[51] and far superior than relying on the deterioration of left ventricular function alone. These results are in contrast with the lack of evidence for a link between telomerase and atrial arrhythmias,[52,53] suggesting a link between senescence and left ventricular changes.

Meta-Analysis of TL Studies

Haycock et al[54] performed a meta-analysis of 24 studies with LTL measurements in a total of 43 725 participants and 8400 patients with clinically relevant cardiovascular disease. The authors found an inverse relationship between LTL and the risk of CHD, independent of conventional risk factors. The study revealed that the risk of being in the shortest versus the longest TL tertile, the relative risk for CHD was 1.54 (95% CI, 1.30–1.83). An association with cerebrovascular disease appeared less certain. Of note, the authors did not use per patient data for individual assessment of dose-response associations, making it difficult to establish TL as a biomarker for the prediction of CHD.

Genome-Wide Association Study With Telomerase Mutations

Although the association between short LTL and CHD can now be regarded as proven, there is no evidence for a causal relationship. Mendelian randomization studies are a valuable method to control for reverse causation and confounding in epidemiological studies.[55,56] It uses gene variation, for example, loss-of-function mutations, to examine their effect on disease without conducting a randomized trial. Scheller Madrid et al[57] performed a large Mendelian randomization study in 290 000 participants by looking at 3 single nucleotide polymorphisms associated with shorter TL (67 bp per allele), namely in the genes *TERT*, *TERC*, and *OBFC1* (a component of the CST complex, which binds to single-stranded DNA and is required to protect telomeres from DNA degradation and probably a more general role in DNA metabolism at nontelomeric sites). The authors then calculated the relative risk to suffer from ischemic heart disease and found significant risks for *TERT* (1.04 [95% CI, 1.02–1.06]) and *OBFC1* (1.05 [95% CI, 1.03–1.08]). Interestingly, *TERC* mutations were associated with shorter TL but not ischemic heart disease (1.01 [95% CI, 0.99–1.03]). In contrast to the protein-coding *TERT* and *OBFC1* genes,

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TERC codes an RNA template sequence that allows nuclear telomerase to copy the telomere strand. In our opinion, this large study offers an alternative interpretation in that (1) shorter telomeres are clearly a downstream readout of decreased TA and (2) shorter TL by itself is not necessarily pathogenic for a proinflammatory condition such as atherosclerosis. In fact, it is more likely (given the higher risk of infection-related death with short LTL) that short TL in white blood cells is rather protective for vascular inflammation (see below, Telomerase Mutations/Deletions in Murine Studies).

Telomere Hypothesis: TL in Leukocyte Subsets Suggests Systemic Influence on Different Blood Compartments

We found in our own studies that TL was 500 bp shorter in patients with CHD and previous myocardial infarction when compared with age-matched healthy controls.[19] Using flow cytometric sorting strategies, we managed to compare TL in 13 different leukocyte populations from patients and controls. In patients with CHD, we found the same loss of 500 bp in almost all myeloid and lymphoid cell subpopulations. CD8 T-lymphocytes presented the only exception, being biased by a high proportion of patients with Cytomegalovirus seropositivity, thus leading to excessive turnover and TL shortening in the CD8 compartment. We concluded from these data that identical TL shortening occurs in different blood compartments (bone marrow–derived myeloid cells as well as thymic progenitor cells), strongly suggesting that LTL shortening is caused by systemic factors (eg, cell turnover or DNA damage through inflammation or oxidative stress, as present in age-related diseases), rather than LTL being the cause of chronic diseases itself.

GENETIC DISORDERS WITH TELOMERASE DYSFUNCTION

To understand the clinical impact of telomerase dysfunction or reduced TA, it is useful to revisit genetic disorders with loss-of-function mutations in genes that either code for telomerase or lead to shorter telomeres. Dyskeratosis congenita is a rare inherited disorder that manifests itself at first with skin alterations, nail dystrophy, premature hair graying, and skin pigmentation. The disease is caused by mutations that affect telomere maintenance, hence all patients with dyskeratosis congenita have short telomeres.[58] Depending on the gene mutation and inheritance pattern, dyskeratosis congenita can lead to bone marrow failure in mid-life, accompanied by anemia and thrombopenia. Importantly, despite the presence of *TERT* or *TERC* mutations and shorter telomeres in patients with dyskeratosis congenita, atherosclerosis is not a clinical symptom, arguing strongly against a primary cause of TL shortening in atherosclerosis.

Hutchison-Gilford progeria syndrome is a so-called laminopathy, involving defects in the cell nucleus envelope secondary to mutations in the gene *LMNA*, coding for lamin A and lamin C proteins.[59] Lamins are filaments that support basic functions of the cell nucleus, such as chromatin organization, DNA replication, DNA repair, and cell-cycle progression.[60] Although there is no direct link to dysfunctional telomerase, patients exhibit short telomeres compared with age-matched controls because of accelerated shortening in the replication process.[61] Telomere dysfunction then results in genomic instability, activation of DNA damage responses, mitochondrial dysfunction, and stem cell exhaustion, which have all been implicated in the aging process.[62] Clinically, cellular senescence in patients with Hutchison-Gilford progeria syndrome culminates into an accelerated aging phenotype with a median life span of 14.6 years.[63] While cardiovascular disease is the dominant cause of death, this is not because of a typical atherosclerosis process caused by conventional risk factors but rather down to the direct adverse effect of progerin on endothelial cells, vascular smooth muscle cells, and possibly leukocytes and platelets as well, all in the absence of classical risk factors.[59,64]

TELOMERASE MUTATIONS/DELETIONS IN MURINE STUDIES

TERC Mutations

The first knockout mouse model for telomerase was established in 1997 for TERC[65] and in 2004 for TERT.[66] Chiang et al[66] found that both TERC and TERT are essential for telomere maintenance and elongation but that gene copy number and transcriptional regulation of TERC, but not TERT, are limiting for TA under the in vivo conditions analyzed. Given that the rate of telomere shortening even in TERC$^{-/-}$ mice is only about 5 kB during one generation, late generation null mice are needed to investigate the impact of critical telomere shortening. While first generation TERT$^{-/-}$ and TERC$^{-/-}$ mice have no obvious phenotype, late generation TERC$^{-/-}$ mice do show signs of aging, including hair graying, alopecia, loss of fertility, impaired wound healing, immunosenescence-related diseases, heart failure, various tissue atrophies, and reduced tissue regeneration (specially in highly proliferative tissues and cell types).

The progressive shortening of telomeres in later generation TERC$^{-/-}$ mice leads to induction of DNA damage response signaling, such as p53.[67] G5 TERC$^{-/-}$ mice suffer from myocyte loss and hypertrophy, leading to severe left ventricular failure, a phenotype reminiscent of that in patients with dilated cardiomyopathy.[67]

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Angiotensin II receptor blockers and angiotensin-converting enzyme inhibitors both have demonstrated induction of telomerase dependent protective effects in arterial vessels, by direct and indirect effects on vascular smooth muscle cells and endothelium.[68–70] The upregulation of endothelin-1 in TERC-deficient mice could indeed explain a link between telomerase and hypertension.[69]

Telomere shortening is considered detrimental in the setting of the vascular wall. However, late generation (G4) double knockout *Apoe*$^{-/-}$ *Terc*$^{-/-}$ mice with short telomeres develop less atherosclerosis than their littermate controls with intact *Terc* (*Apoe*$^{-/-}$ *Terc*$^{+/+}$), despite identical serum cholesterol level, presumably because the presence of critically short telomeres impairs the proliferative capacity of lymphocytes and myeloid cells (monocytes/macrophages; Figure 3).[72] This atheroprotective role of telomere shortening in *Apoe*-null mice is consistent with the notion that excessive proliferation of neointimal macrophages contributes to the development of proinflammatory atherosclerotic plaques. However, it is important to take into account that endothelial cell and smooth muscle cell dysfunction also plays a major role in atherosclerosis. For example, aged human atherosclerotic lesions accumulate senescent endothelial cells, perhaps in part due to progressive telomere attrition.[73,74] Interestingly, however, TERT expression and activity has been detected in atherosclerotic human coronary arteries, particularly in proliferating cells in early lesions.[75] Abundant TERT expression has been also reported in neointimal human macrophages, and atherosclerosis development in low-density lipoprotein receptor-deficient mice is associated with telomerase activation.[76] These studies illustrate the complex relationship between telomerase/telomere attrition and atherosclerosis development. Future studies using inducible and cell type-specific telomerase inactivation/overexpression are thus warranted to address the effects of altering telomere dynamics in different stages of atherosclerosis and should also examine the role of other telomere-associated proteins that regulate TL and structure.

TERT Mutations

Similar to late generation TERC$^{-/-}$ mice, G4 TERT$^{-/-}$ mice show progressively shorter telomeres and

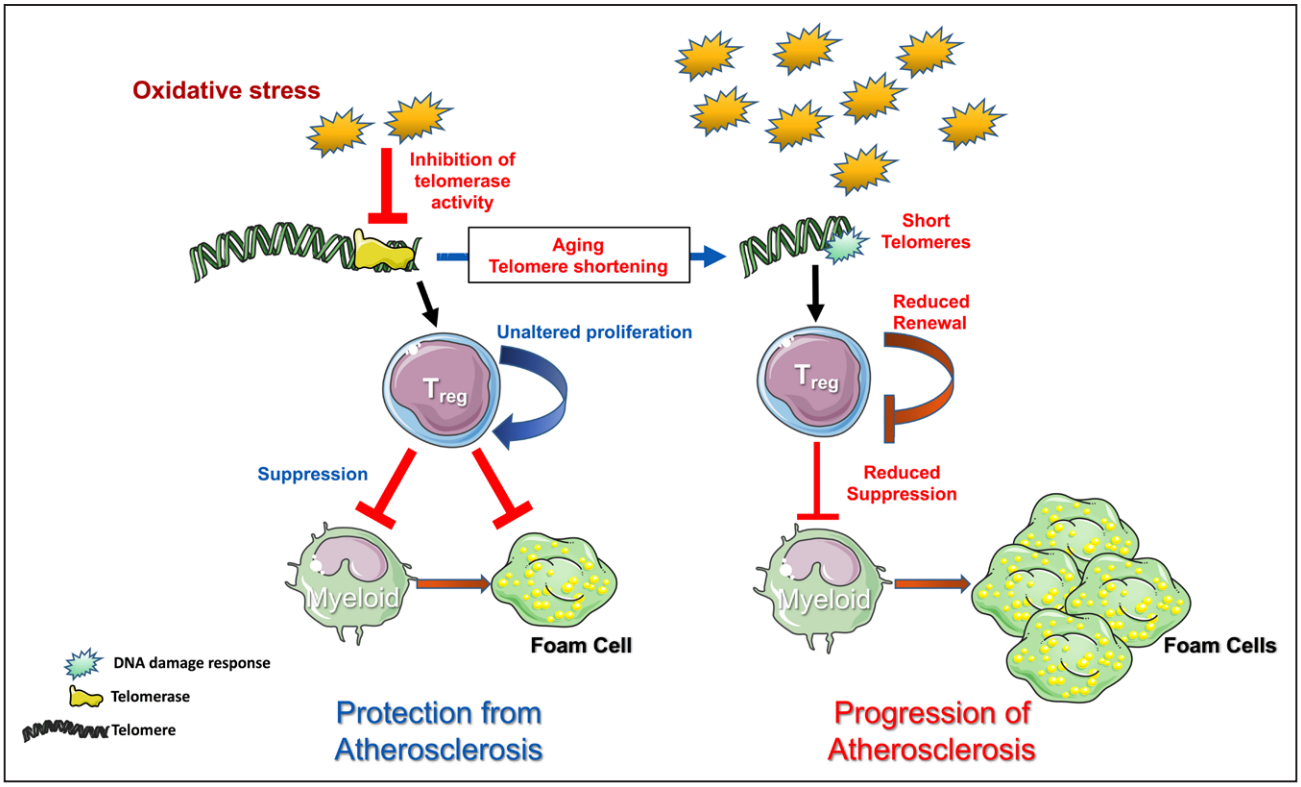

Figure 3. Contribution of telomerase to the development of atherosclerosis.
Telomerase consists of a catalytic subunit (TERT) and the RNA template (TERC), and is well described as an antiaging factor. Atherosclerosis is an age-related disease characterized by systemic oxidative stress and low-grade inflammation. TERT, but not TERC, has been shown to protect against mitochondrial-derived reactive oxygen species and mitochondrial DNA damage and is a likely cause of telomere independent contributions to the development of cardiovascular disease. We have recently investigated the effects of telomere length and telomerase activity in different subpopulations of leukocytes that could contribute to the development of atherosclerosis.[105] We could show that first, oxidative stress–mediated reduction in CD4$^+$ T-cell proliferation is TERT dependent. Second, telomerase function was not required for the ability of T-regulatory cells (T_{regs}) to protect against atherosclerosis; however, telomere length is critical for T_{reg} function. These results suggest that atherosclerosis development is affected by both increase in oxidative stress and acceleration of telomere attrition in T_{regs} via nuclear actions of telomerase.

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age-associated pathologies. Re-expression of TERT in those mice reverted some age-associated degenerative phenotypes in multiple organs including testis, spleen, and intestine.[77] Thus, one could assume that testing a TERT gene therapy setup in mice could be beneficial also in the context of cardiovascular diseases. This was tested using an adenovirus associated virus 9 for the expression of TERT in cardiomyocytes. This virus is nonintegrative and replication incompetent and thereby provides a relatively genome-safe, organ-specific TERT activation, which should counteract the ability of TERT to induce tumors. Injection of TERT-adenovirus associated virus 9 after acute myocardial infarction in mice resulted in improved ventricular function, reduced scar size, and increased survival.[77] This study by Jaskelioff et al showed an elongation of telomeres and an increase in the proliferation capacity of cardiomyocytes in the heart after TERT application.[77] However, this did not exclude telomere independent effects in the context of myocardial infarction, this will be further eluded to in the Section Noncanonical and Nonnuclear Effects of Telomerase. Nevertheless, TERT gene therapy could be a promising candidate; however, first more research efforts are required to understand the implication for cardiovascular diseases but also for the potential side effects occurring.

Werner et al[78] convincingly demonstrated that treatment of nondiabetic mice with pioglitazone, an antidiabetic drug and peroxisome proliferator-activated receptor agonist, increased TA in the aortic wall. As a functional consequence of telomerase induction, they find that monocytes from pioglitazone-treated TERT wild-type mice are more resistant to oxidative stress–induced apoptosis than those from TERT null mice. These results suggest that vascular rejuvenation could potentially be achieved by targeting telomerase in the vessel wall.

NONCANONICAL AND NONNUCLEAR EFFECTS OF TELOMERASE

Besides the pivotal role of TERT in ensuring maintenance of telomere homeostasis, evidence has accumulated demonstrating nontelomeric functions of the enzyme. TERT was shown to be involved in gene expression[79,80] and apoptosis protection independent of changes in TL.[78,81,82] The cardiovascular beneficial effects of physical exercise required TERT, because in first generation TERT-deficient animals, which still have normal TL despite the lack of TA, physical exercise did not protect against doxorubicin cardiotoxicity and vascular apoptosis.[82,83] Likewise, TERT is a key factor in regulating flow-mediated dilation in the mouse and human microcirculation.[9,84]

Remarkably, in addition to its conventional nuclear localization, TERT has also been detected in mitochondria in different mouse and human cell types (Figure 4).[85–87] TERT was shown to improve the mitochondrial membrane potential and complex I activity of the respiratory chain, reduce mitochondrial reactive oxygen species, interact with mitochondrial DNA and, thereby, protect mitochondrial DNA against damage, and inhibit apoptosis.[85,86] From data provided by the Santos and the Haendeler group that TERT can interact with mitochondrial DNA one could assume that mitochondrial TERT may interacts with RNAs within mitochondria. This is also further supported that the catalytic activity of TERT is required within mitochondria.[85,86] However, it remains unclear whether mitochondrial or nuclear TERT or both are responsible for these protective effects. Moreover, it is difficult to differentiate between overlapping functions of TERT in the nucleus and the mitochondria, because all published studies were done either in TERT-deficient cells and mice or after expressing TERT variants artificially targeted to specific cellular organelles while maintaining expression of the endogenous protein. The Haendeler and the Altschmied laboratories have now set out to provide tools to solve this problem by creating mice ubiquitously expressing low levels of nuclear or mitochondrial TERT. After backcrossing these animals onto a TERT-deficient background, the offspring will contain TERT only in one of the 2 subcellular compartments throughout the body and therefore allow the dissection between nuclear and mitochondrial functions of TERT in several disease models.

Another potential link of noncanonical TERT functions and autophagy could be assumed from the existing literature. In general, autophagy is a recycling process of damaged intracellular components, which has been investigated in cells of the cardiovascular system (for review, see study by Yan and Finkel[88]). Under physiological conditions, autophagy is a beneficial process to maintain cellular homeostasis. However, excessive levels of autophagy lead to apoptosis induction. Overexpression of TERT inhibits the mTORC1 (mammalian target of Rapamycin complex 1) in several tumor cell lines leading to increased autophagy in those cell lines.[89] Moreover, caloric restriction enhanced TA, autophagy, and diastolic dysfunction in the hearts of diabetic rats.[90] In a recent publication, Hughes et al[71] demonstrate that inhibition of autophagy switches the mechanism of microvascular flow-mediated dilation from nitric oxide to H_2O_2 in non-CAD, while activation of autophagy in coronary artery disease switches the mechanism of dilation from H_2O_2 to nitric oxide. Hence, telomerase seems to signal through autophagy to determine the mechanism of microvascular dilation in health and disease.

While LTL has been exhaustively studied in clinical studies involving patients with either existing or developing CHD,[33,45,46,49,91–93] TA has only been investigated in peripheral blood mononuclear cells (PBMCs) in a few small cohorts. Low TA in 64 healthy women was associated with exaggerated autonomic reactivity to acute

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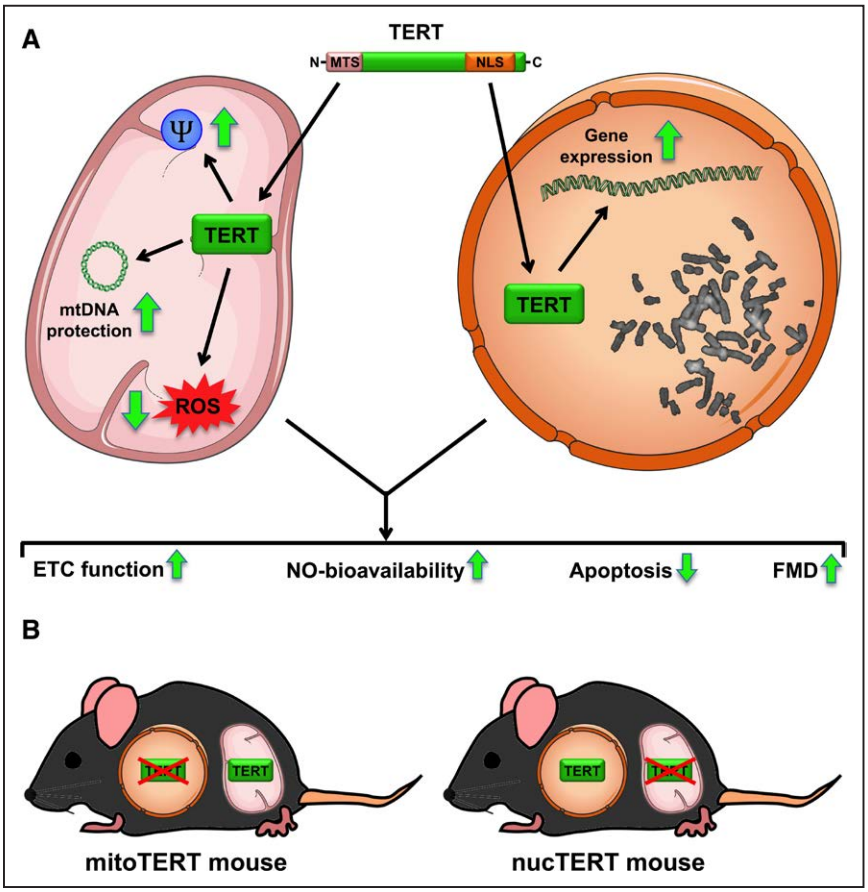

Figure 4. Noncanonical functions of telomerase reverse transcriptase.
A, Telomerase Reverse Transcriptase (TERT) contains an N-terminal mitochondrial targeting sequence (MTS) and a nuclear localization signal (NLS) in its C terminus that drives its mitochondrial and nuclear localization. Within the mitochondria, TERT improves mitochondrial membrane potential (Y), protects the integrity of mitochondrial DNA (mtDNA), and reduces the levels of reactive oxygen species (ROS). In addition to its telomeric functions, nuclear TERT has an impact on additional cellular functions (eg, gene expression). These noncanonical TERT functions improve the functionality of the mitochondrial electron transport chain (ETC), increase NO-bioavailability, reduce apoptosis in different cell types of the cardiovascular system, and improve flow-mediated dilation (FMD). However, the relative contribution of nuclear and mitochondrial TERT to these processes cannot be assessed with the experimental models currently available. **B**, New mouse models to unequivocally distinguish between nuclear and mitochondrial functions of TERT in vivo. The mitoTERT mouse ubiquitously expresses low levels of TERT exclusively imported into the mitochondria, while the nucTERT mouse expresses TERT exclusively imported into the nucleus. Therefore, the respective other cellular compartment is devoid of TERT allowing to characterize compartment-specific functions. Single elements for figures were taken from Servier Medical Art (https://smart.servier.com/), which is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

mental stress, elevated nocturnal epinephrine, and poor lipid profile.[94] Comprehensive lifestyle changes in 24 men with low-risk prostate cancer significantly increased leukocyte telomerase.[95] In contrast, TA in neutrophils from coronary plaques was elevated in one study from patients with unstable angina.[96] Inhibition of TA in vascular smooth muscle cells completely abrogates the proliferative response following vascular injury in a mouse model.[97] Taken together, the potential effect of telomerase will strongly depend on the underlying pathology as well as the cell type. It remains unknown how telomerase is regulated in PBMCs and specifically in lymphocytes from patients with CHD and/or acute myocardial infarction.

While cellular energy production occurs in mitochondria, a sufficient function and number of mitochondria per cell are required for the homeostasis of cells and tissue. Mitochondrial damage, and specifically mitochondrial mutations such as the large 4977 kb mitochondrial DNA (mtDNA4977) deletion, occurs in many tissues during aging, especially in the substantia nigra and the left ventricle.[98] The group from Andreassi has shown in 770 patients with coronary artery disease that short LTL as well as a high percentage of the mtDNA^{4977} deletion are predictors of mortality.[99]

Telomerase maintains TL in stem and progenitor cells allowing them to escape senescence.[100] Remarkably, human TERT has also been implicated in controlling stem cell function independently of its ability to elongate telomeres.[101] TERT is a transcriptional co-activator in Wnt signalling,[80] has a role in siRNA generation,[102] and regulates mitochondrial function.[103] There is also

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accumulating evidence that TERT contributes to somatic cell physiology. Previously, we have demonstrated that pharmacologically protective doses of statins induce TA and TERT-dependent proliferation in T-lymphocytes.[104] Conversely, oxidative stress, in the form of hyperoxia, suppresses both TA and TERT expression in T-cells and attenuates their proliferative activity.[105] Beyer et al have demonstrated additional noncanonical TERT functions. Human arterioles incubated with telomerase-inhibitor show a change in arteries mediator of flow-mediated dilation from the physiological dilator nitric oxide (NO) to pathological H_2O_2,[9] an effect that occurred independently of telomerase transcriptional activity and was suggested to be due to TERT-mediated inhibition of mitochondrial reactive oxygen species production. In line with this hypothesis, telomerase activation switched flow-mediated dilation back from H_2O_2 to NO.

Even in the context of cellular senescence, TERT can function independently of its transcriptional activity. Martin Bennett's group demonstrated that vascular smooth muscle cell senescence in atherosclerotic plaques was associated with telomere attrition, which occurs as a result of increased oxidative stress.[106] In vivo telomerase expression was capable of delaying vascular smooth muscle cell senescence without modifying TL.[106] We and our collaborators have also published multiple studies indicating an interaction between telomerase and oxidative stress both investigating the effect of oxidative stress on telomerase as well as demonstrating the telomere-independent protective effect of telomerase on oxidative stress.[86,87,107–110]

Collectively, these studies suggest that TERT could potentially protect leukocytes from oxidative stress without affecting TL, thus highlighting the importance of better understanding the noncanonical activities of TERT in atherosclerosis.

CLINICAL TRIALS AND TELOMERASE

Statins

While statins have clearly demonstrated beneficial cardiovascular outcome secondary to its cholesterol lowering mechanism,[111] the existence of further pleiotropic effects that might contribute to this remain controversial. In a randomized, placebo-controlled study, 100 hypercholesterolemic patients received 20 mg of atorvastatin daily or placebo for 12 months. TA was significantly increased in isolated PBMCs in the atorvastatin group but did not change in the placebo arm.[112] In line with these results, we have shown that pharmacologically relevant concentrations of atorvastatin increased TA by 6-fold in human and mouse PBMCs and CD4 T-lymphocytes, translating into a moderate increase in proliferation of T lymphocytes.[104] The proliferative effect of atorvastatin was ablated in the absence of the catalytic subunit TERT.

Another mechanistic link between telomerase, statins, and cardiovascular outcome comes from a substudy of the in the West of Scotland Primary Prevention Study.[49] In this first randomized case-control designed study, Brouilette et al[49] demonstrated the correlation between TL and the risk of developing coronary artery disease. TL was measured in white blood cell DNA from 1500 men with elevated plasma LDL cholesterol level. The study showed that individuals with shorter TL have about a 2-fold increased risk of developing coronary artery disease in the 5 years from the start of treatment. Interestingly, pravastatin completely attenuated this telomere-attributed risk. While this study serves as an excellent example of how patients with shorter telomeres are at increased risk and therefore benefit more from statin treatment, it does not prove causality. It might have been very well that statins simply reduced inflammation and activated mitochondrial TERT, thereby reducing reactive oxygen species.

Exercise

Like statins, the antiaging and protective effect of exercise against cardiovascular mortality, all-cause mortality and CAD is beyond doubt (for review, see study by Schuler et al[113]). Work by the research group of Ulrich Laufs and Christian Werner has elegantly demonstrated the impact of exercise on TL and cellular senescence in circulating leukocytes, which also reflected the changes in TL observed in cells in the arterial wall.[82,83] More recently, these authors have shown that 6 months of endurance exercise, but not resistance training, induces TA in PBMCs, which was paralleled by a small increase in LTL and iNOS (induction of nitric oxide synthase).[114]

Telomerase Activator TA-65 in Acute Coronary Syndromes Trial

The small-molecule telomerase activator cycloastrogenol (CAG) was discovered in an empirical cell-based telomerase activation screen using crude extracts and semi-purified fractions of Astragalus roots. TA-65MD is a purified and encapsulated form of CAG with increased bioavailability (T.A. Sciences, New York). TA-65MD has been shown to induce telomerase in different tissues in mice,[115,116] and in human lymphocytes and epithelial cells.[117] By increasing *TERT* mRNA transcription, TA-65MD leads to telomerase-dependent elongation of short telomeres and rescue of associated DNA damage. We hypothesize that telomerase deficiency in atherosclerosis leads to accelerated immune aging with telomere shortening in peripheral blood leukocytes, increased oxidative stress and inflammation, and finally impaired microvascular endothelial function, all of which contribute to the pathology of CHD. Activating telomerase with TA-65MD might lead to reduced oxidative stress, improved endothelial function and decreased telomere shortening

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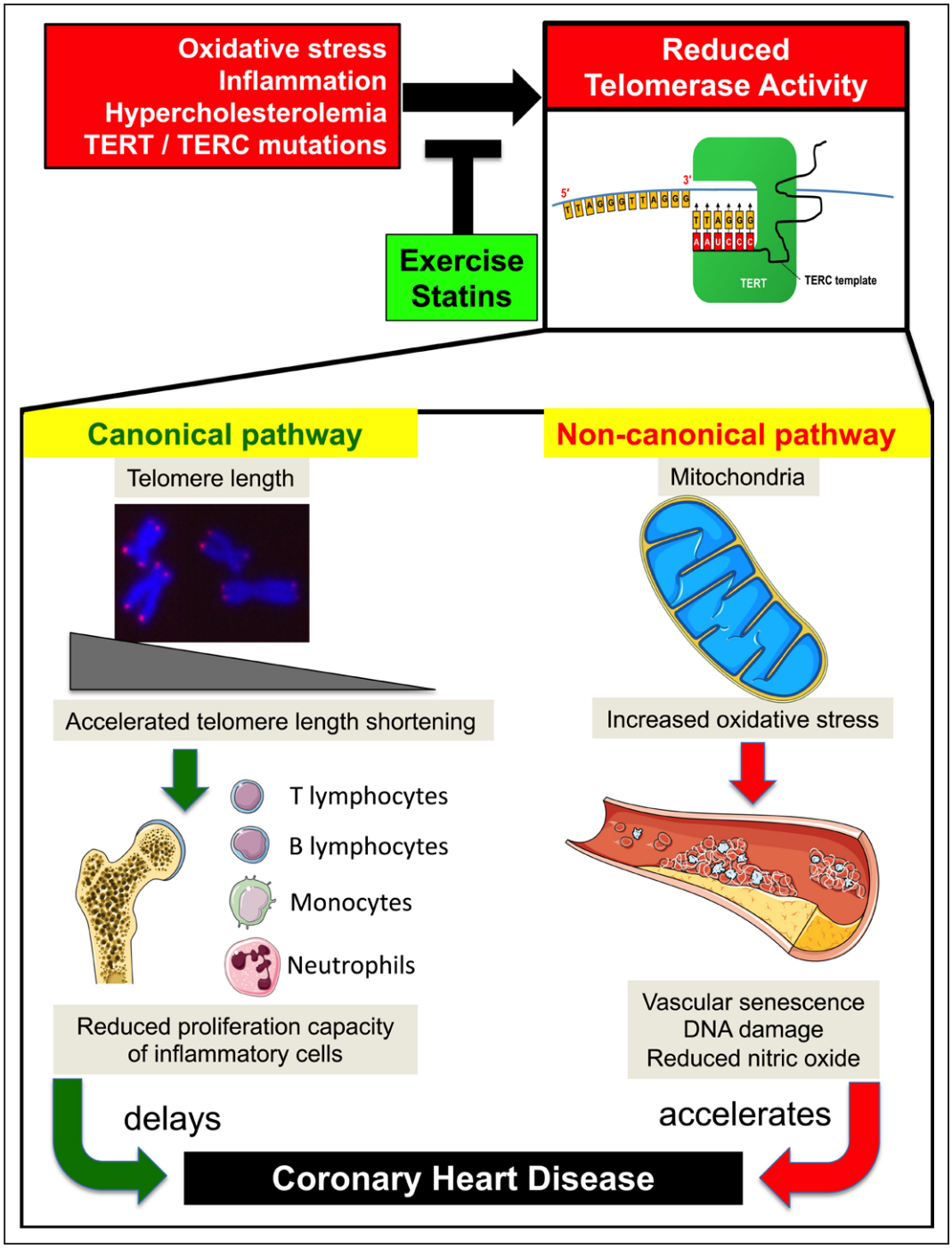

Figure 5. The Yin and Yang of telomerase in coronary heart disease.
Cardiovascular risk factors and mutations in telomerase RNA component (TERC) or TERT reduce telomerase activity (TA) in peripheral blood mononuclear cells, while exercise and statins lead to its induction. While reduced TA affects the canonical pathway to some degree, causing accelerated telomere shortening, other consequences (noncanonical functions) include less vascular nitric oxide, increased oxidative stress, enhanced DNA damage and senescence, contributing to atherosclerosis. Complete lack of telomerase such as in dyskeratosis congenita (DKC) leads to premature bone marrow failure and heart failure, but without atherosclerosis.

in patients with CHD. Our TACTIC trial (Telomerase Activator to Reverse Immunosenescence in Acute Coronary Syndrome), a double-blind, phase II, pilot randomized controlled trial[118] will investigate whether activation of telomerase by 12-month treatment of acute coronary syndrome patients with TA-65 reduces the percentage of proinflammatory CD8 T-lymphocytes, increases TL in leukocyte subsets, and improves endothelial function. If successful, a larger study with clinical outcome should follow.

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CONCLUSIONS

The effects of telomerase in cardiovascular medicine and biology are perceived as its canonical effect on TL. It is accepted now that patients with shorter TL, typically measured in circulating peripheral blood leukocytes, have a higher risk to develop heart disease while this TL associated, enhanced risk can be neutralized by treatment with statins. Patients with already established cardiovascular disease, or even those with risk factors alone, demonstrate shorter LTL as well. Hence, for many years, we have been led to think that the link between shorter telomeres and cardiovascular risk implies a causal relationship. Interestingly, while this could to be the case for heart failure, it is not for atherosclerotic disease (Figure 5). In fact, preclinical studies in mice point to the opposite—short telomeres prevent atheroma progression in mice. With the advent of large-scale genome-wide association studies, it seems clear that while mutations in telomerase can lead to shorter TL, this is not always associated with increased risk for CHD. Loss of function mutations of TERT, the catalytic subunit of telomerase, also impair its noncanonical role in mitochondria, diminishing the antioxidative function and possibly regenerative potential of vascular cells as well as T-lymphocytes. Given that statin therapy as well as endurance exercise both enhance TA, TERT and telomerase have emerged as a novel target to treat cardiovascular aging and its related inflammatory signaling. Telomerase also confers an antisenescent effect and given that senolytics have demonstrated beneficial recovering following myocardial infarction, this could be another preferable mechanism to combat cardiovascular aging. The use of telomerase activators in cardiovascular disease has been hampered by the fear of cancer induction, which, as far as we know, requires telomerase reactivation typically secondary to introduction to mutations in its promoter, rather than enhancement of preexisting TA. This is reflected by strong evidence that higher levels of physical activity are linked to enhanced TA and lower risk of several types of cancer. In conclusion, targeting telomerase and specifically the noncanonical functions of TERT could prove beneficial in the treatment of cardiovascular aging and disease.

ARTICLE INFORMATION

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Disclosures

None.

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