Telomere length as cardiovascular aging biomarker: the long road to the clinic

Brief title: Telomere length and cardiovascular aging

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Abstract

Telomeres shorten with age, the major risk factor for atherosclerotic cardiovascular disease (aCVD). The observation of shorter telomeres in aCVD patients thus suggested that critical telomere shortening may contribute to premature biological aging and aCVD. Telomere length (TL) is therefore often suggested as a causal aCVD risk factor, a proposal supported by recent Mendelian randomization studies; however, epidemiological research has shown disappointingly low effect sizes. It therefore remains uncertain whether telomere shortening is a cause of aCVD or merely a consequence. We argue that elucidating the mechanistic foundation of these findings is essential for any possible translation of telomere biology to the clinic. Here, we critically evaluate evidence for causality in animal models and human studies and review popular hypotheses and discuss their clinical implications. We identify four key questions that any successful mechanistic theory should address, and we discuss how atherosclerosis-associated local telomere attrition may provide the answers.

Condensed abstract: Replicative senescence caused by critical telomere shortening is an antitumor mechanism that may also promote premature biological aging. Observation of shorter telomeres in atherosclerosis patients therefore led to suggestions that telomere length is a causal cardiovascular risk factor. Though causality is also supported by recent genomics studies, epidemiological research consistently showed disappointingly low effect sizes. We argue that elucidating the mechanistic foundation of these findings is crucial to translating telomere biology to the clinic. Here, we critically review popular hypotheses, discuss their clinical implications, and propose four key standing questions to help evaluate mechanistic theories.

Keywords: atherosclerosis, biological aging, telomerase, epidemiology

Abbreviations
aCVD – atherosclerotic cardiovascular disease
DKC – dyskeratosis congenita
GWAS – genome-wide association study
ht Q-FISH - high-throughput quantitative fluorescence *in situ* hybridization
qPCR – quantitative polymerase chain reaction
SNP – single nucleotide polymorphism
TERC - telomerase RNA component
TERT – telomerase reverse transcriptase
TL – telomere length
TRF – telomere restriction fragment

Introduction

While postulating the theory of DNA replication-associated telomere attrition in 1971, Alexey Olovnikov dubbed telomeres "the heel of Achilles" of DNA, immediately recognizing their potential impact on aging (1). At the cellular level, his prediction was shown to be accurate about 20 years later: telomeres indeed shorten with age and cell division (2,3), explaining the limited proliferative lifespan of normal somatic cells (4). Unsurprisingly, the detection at the turn of the new millennium of telomere shortening in association with atherosclerosis (5) led many to propose a causal role in cardiovascular aging as well, especially when telomere length (TL) was also linked to lower cardiovascular survival (6).

As a result, TL received intense attention as putative risk factor for atherosclerotic cardiovascular disease (aCVD), especially given that TL can be considered a "mitotic clock" that chronicles an individual's biological age rather than the calendar age. More specifically, the inherent promise of TL is that it may pinpoint the timing of disease onset. A decade of research, however, demonstrated only a weak association of TL with aCVD. Moreover, animal studies indicated a complex relationship between telomerase activity, telomere attrition, and atherosclerosis development, casting doubt on any putative causal relationship. Nonetheless, the causality hypothesis was soon revived by genomics research identifying genetic determinants of TL that also predict cardiovascular risk (7,8).

Although causality is back at the center of mechanistic theories regarding telomeres and cardiovascular aging, most theories do not explain the low effect size in epidemiological studies, together with other counterintuitive findings. Mechanistic insight is, however, indispensable for establishing TL as a clinically relevant aCVD risk factor and for a meaningful interpretation of commercial TL tests. In this viewpoint article, we discuss historical and alternative hypotheses

and their potential impact on patient care. The main pieces of evidence for or against each hypothesis are summarized in **Table 1**. Moreover, we outline several concepts and anomalies that remain controversial or unresolved in the field, highlighting them as *Key Questions* (Table 2). Yet, first, we provide a brief introduction of telomere length biology.

Telomeres, telomerase and replicative senescence

Telomeres are the nucleoprotein complexes capping chromosome ends. In humans, they consist of a hexameric repetitive DNA sequence bound by a specific set of nucleoproteins, the shelterin complex (9) (**Figure 1**). The canonical telomere sequence is $(TTAGGG)_n$, and although variant repeats exist, their position in the telomere and mechanism of generation are only now beginning to be elucidated (10). In healthy humans, average TL ranges from 5 to 12 kilobase pairs. At the proximal end, the telomere is separated from the rest of the chromatin by the subtelomere, a dynamic patchwork of sequence blocks that are often conserved between chromosomes. At the distal end, the telomere terminates in a single-stranded G-rich overhang that recoils into the double-stranded telomere DNA, forming a complex D-loop-t-loop structure (Figure 1). This structure distinguishes telomeres from double-strand breaks and is lost upon critical telomere attrition, which thus induces p53-mediated replicative senescence (9).

As originally postulated by Olovnikov (1), telomere shortening is caused by the end-replication problem, which is the inability of DNA polymerase to replicate the very end of the lagging strand. However, another major source of telomere attrition is oxidative stress (11). *In vitro*, replicative senescence arises when somatic cells stop dividing upon reaching a donor-specified number of divisions, known as the Hayflick limit (4). This limit was attributed by Olovnikov to critical telomere shortening, which was verified by the observations that telomeres

indeed shorten *in vitro* (2) and that the Hayflick limit can be overcome by ectopic telomerase expression (12).

Telomerase is a ribonucleoprotein consisting of a reverse transcriptase (TERT) and its primer, the telomerase RNA component (TERC), which elongates individual telomeres, e.g. to extend TL for the next generation. Though expressed in normal germ cells and to a lesser extent in stem cells, telomerase shows very limited expression in most somatic tissues; exceptions include blood mononuclear cells in children (13) and specific cells of the cardiovascular system (14). On the other hand, tumor cells reactivate telomerase expression to bypass replicative senescence and gain immortality (15), although telomeres can also be lengthened by an alternative mechanism (16).

Further studies evaluating the impact of telomere biology *in vivo* finally led to the wide acceptance of TL as a "mitotic clock" and anticancer mechanism (16). A crucial piece of evidence was the observation that germline telomerase complex mutations in humans often lead to bone marrow failure diseases, such as dyskeratosis congenita (DKC), which features critically short telomeres and heterogeneous geriatric-like phenotypes including aberrant skin pigmentation, nail dystrophy, leukoplakia and premature graying (17). The increasing disease severity over generations, with progressively shorter telomeres, underscores the causal role of telomere biology in DKC and therefore its *in vivo* relevance (18). However, the fact that aCVD is not a common DKC feature despite critically short telomeres suggests a complicated relation between telomere attrition and aCVD (**Table 2**, *Key Question 1*).

Epidemiological TL studies of (cardiovascular) aging: tissues, methods, and early results

As critical telomere attrition was put forward as one of the causes of physiological aging (19), its putative role in aCVD was investigated, most notably in epidemiological studies. In

adults, TL and the telomere attrition rate are strongly correlated between the different tissues of an individual (20). Therefore, in humans, TL has typically been measured in blood leukocytes or mononuclear cells, a straightforward choice for measuring "systemic" TL in epidemiological studies. However, despite a clear correlation in TL when comparing different tissues from the same individual, leukocyte telomeres are shorter than those of other tissues, possibly as a consequence of the higher hematopoietic stem cell proliferation rates in early life (20,21).

There are three common methods for TL measurement in blood: (i) telomere restriction fragment (TRF) analysis, which relies on DNA digestion and Southern blot (2); (ii) qPCR of the telomeric repeat sequence (22); and (iii) high-throughput quantitative fluorescence *in situ* hybridization (HT Q-FISH) (23). These methods largely differ in high-throughput character and reproducibility, but also in what is intrinsically measured, as some methods also include subtelomeric fragments and possibly nonfunctional variant repeats in their estimates, or can only measure mean TL rather than the shortest telomere (23,24). Nevertheless, all three methods are able to detect large differences, as observed, for example, in DKC (17).

The first studies supporting the *in vivo* relevance of TL as a marker of biological age reported mean blood-cell telomere attrition rates of roughly 30bp/year in adults (3,25). More recent research shows that telomeres shorten much faster during early life, with attrition becoming more moderate only after about two decades (21). Other important TL determinants identified in humans include inheritance, leading to major interindividual TL differences, and sex, with longer telomeres in women versus age-matched men (25,26). Significantly shorter telomeres were also found to be associated with higher pulse pressure and pulse-wave velocity in men (26). Importantly, Samani and coworkers detected shorter telomeres in coronary artery disease patients (5), in line with subsequent findings showing an association between shorter

telomeres and higher cardiovascular-related mortality (6). Moreover, Minamino and coworkers identified short-telomere-mediated senescence in atherosclerotic lesions, providing mechanistic evidence supporting a causal link between telomere attrition and aCVD (27). As TL is largely inherited, these studies gave rise to a first causality hypothesis, that individuals inheriting shorter telomeres are predisposed to atherosclerosis and thus aCVD, making leukocyte TL an appealing candidate as a clinical risk factor for aCVD (**Figure 2A**).

This hypothesis implies that factors affecting TL *at birth*, such as sex and inheritance, will have major health implications later in life, and this spurred a search for additional TL determinants. Paternal age at conception was shown to positively correlate with offspring TL (28,29), most likely due to increasing sperm-cell TL in older fathers (30). Thus the first causality hypothesis leads to the counterintuitive suggestion that children of older fathers would have a lower cardiovascular risk. More recent studies focused on the impact of environmental factors during gestation, showing that TL of newborn infants is influenced by intrauterine effects such as exposure to maternal stress, smoking, folic acid, and pre-eclampsia (31). Placenta and cord-blood TL were also found to be inversely associated with pre-pregnancy maternal BMI (32). These findings suggest that TL may mediate the impact of *in utero* exposures on the development of aCVD later in life.

TL as cardiovascular risk factor: popular mechanistic hypotheses

The on-average shorter leukocyte TL in patients with clinically manifest aCVD pleads for shorter TL to be already present in early atherosclerotic lesions (Figure 2A). Though leukocyte telomeres are somewhat shorter than vascular tissue telomeres (Figure 2A) (20), the high correlation between both should make leukocyte TL an excellent clinical proxy. However, large-scale population studies in aCVD-free individuals did not identify shorter leukocyte telomeres in

subjects featuring pre/subclinical atherosclerosis compared to non-atherosclerotic controls, contrasting results for advanced atherosclerosis (33-35). Although additional studies demonstrated a clearly significant independent association of shorter telomeres with cardiovascular risk, e.g. (36,37), the effect size was typically very limited. Though this can to a certain extent be attributed to the effect of other risk factors, it contrasts the clear cut-offs observed with confirmed causality, such as DKC (**Table 2**, *Key Question 2*). Indeed, one might expect atherosclerosis, if it would be caused by inheritance of shorter telomeres, to be accompanied by DKC-like symptoms, given that TL in leukocytes is on average shorter than, for example, in muscle (**Table 2**, *Key Question 1*). These and more recent epidemiological studies demonstrated that inheriting shorter telomeres is unlikely to be a cause of atherosclerosis (38), and alternative hypotheses have been postulated.

For example, the finding that leukocyte TL is associated with oxidative stress and inflammation even in healthy individuals, e.g. (39), suggested that cardiovascular risk-related systemic oxidative stress and inflammation might accelerate telomere attrition, thereby mitigating the impact of the inherited component of TL. The attrition-rate hypothesis thus implies that variation in adult telomere attrition rates is more important than variation in TL inheritance (33). Moreover, given the systemic impact of oxidative stress and inflammation and the similar TL attrition rates in somatic tissues (20), the TL attrition rate in blood cells would reflect that in vascular tissue (**Figure 2B**). This theory implies that the telomere attrition rate could serve as a clinical aCVD biomarker. However, variation in adult leukocyte telomere attrition rate was found to be too low to substantially alter TL compared with inheritance (40). Since the variation is too small to significantly modulate the association between TL and clinical atherosclerosis, this result suggests a higher blood TL attrition rate to be rather an

epiphenomenon (**Figure 2C)**. More recently, the lack of association between leukocyte telomere attrition rate and atherosclerosis was also demonstrated directly in a smaller longitudinal study (41).

In situ telomeres and telomerase in cardiovascular disease

Before examining more recent epidemiological studies, it is important to note that many important contributions in the field of TL and atherosclerosis research come from *in situ* studies, for example, the identification of vascular endothelial cells with short telomeres and senescence-associated phenotypes in atherosclerotic lesions (27). Moreover, direct analysis of arteries revealed large differences between control participants and patients, as well as between disease-free and diseased regions within the same patient, affecting both vascular smooth muscle (42) and endothelial cells (43). In atherosclerotic plaques, both cell types also show clear evidence of telomere-based senescence (27,42). Although these were pivotal findings, further studies were required to discern cause from consequence.

This research often relied on mice, and entailed the creation of genetically-engineered model systems given the overall far longer telomeres in *Mus musculus* compared to humans. For example, late-generation telomerase (*TERC*) knock-out mice, which have critically short telomeres, were found to be protected from cancer while exhibiting an aging phenotype associated with p53 activation, suppression of mitochondrial master regulators, ventricular dilation, myocardial thinning, cardiac dysfunction, and sudden death (44,45). Although these findings illustrate a possible causal role for telomere attrition in heart failure, the situation for atherosclerosis appears to be more complex. For example, *TERT*-null mice exhibit premature hair graying and reduced lifespan and hematopoietic regenerative potential, but not atherosclerosis (46). Moreover, in humans, strong telomerase expression or activity was found in

atherosclerotic plaques, particularly in proliferating cells in early lesions (47), and in neointimal macrophages (48). Moreover, telomerase is activated during atherosclerosis development in low-density lipoprotein receptor-deficient mice (48), and atherosclerosis is significantly attenuated in late-generation mice deficient for both apolipoprotein E and TERC with critically short telomeres, possibly due in part to impaired proliferation of hematopoietic cells within atherosclerotic lesions (49).

Although mouse studies cannot be readily translated to humans, the combined data clearly demonstrate the importance of telomerase activation in atherosclerosis. Also, these results question the concept that longer telomeres always protect against aCVD (**Table 2**, *Key Question 3*). Moreover, these findings illustrate the need for separate investigation of not only disease onset and progression, but also the tissues involved (macrophages, vascular tissues).

(Inheritance of) shorter telomeres as a cause of aCVD: genomic evidence and caveats

Previous epidemiological and in *situ* results thus cast significant doubt on a causal role for shorter telomeres in aCVD; however, more recent genome-wide association studies (GWAS) added a new dimension to the puzzle. The main goal of the GWAS was to identify genetic variants explaining the large inherited component of TL (measured in leukocytes). This analysis identified a small set of loci, including clearly significant results for several *TERT* and *TERC* single nucleotide polymorphisms (SNPs), but all with very small R^2 values (<0.5%) (7,8). These results were initially perceived as disappointing given the large heritable component in TL variability, which is thus more likely due to direct, "epigenetic" inheritance of gamete telomeres in the zygote (50). However, the surprise was that alleles responsible for shorter telomeres were, as a combined genetic risk score, also associated with higher aCVD risk (7,8,51). Given that aCVD cannot alter the TL-determining genotypes, these "Mendelian randomization" results

indicate that telomeres play a causal role in aCVD. As discussed higher, such a causal effect most likely does not act through atherosclerosis onset. It may for example relate to suboptimal or defective repair of atherosclerotic lesions already present.

Although these results suggest that *inheritance* of shorter telomeres is a risk factor for aCVD, there are considerations that could undercut this conclusion. A first assumption of this interpretation would be that the identified alleles indeed determine the *inherited* component of TL; however, this is uncertain because the GWAS used adult leukocyte TL. Another assumption is the absence of pleiotropic effects: if the implicated GWAS loci have functions beyond determining TL, their lower expression or activity could lead to inheritance of shorter telomeres and aCVD independently. For example, the Wnt/β-catenin pathway and E2F1-based transcription have both been implicated in atherosclerosis and suggested to be modulated by telomerase independently of TL (52-54). Although the existence of telomere-independent telomerase activity has been disputed (55), pleiotropic and related effects cannot be readily discarded. For example, abundant evidence links TL to genetic variation in the RNA biogenesis factor *NAF1*, yet the most commonly studied variant has been repeatedly demonstrated to be associated with both longer telomeres and a *higher* aCVD risk (7,8,51,56). Moreover, one study (with almost 300,000 participants) reported a clearer aCVD risk association for "genetically determined TL" than for directly measured TL (8), an unanticipated finding given the very low power of the SNPs to explain TL (*Key Question 4*). Nevertheless, this finding could also reflect the more accurate determination of genotype than of leukocyte TL.

Shorter telomeres as a causal factor in aCVD: key questions, possible answers and guidelines

It is somewhat ironic that the field began with clear mechanistic hypotheses, and yet while causality is now accepted, the underlying mechanism remains elusive. Given the direct implications for clinical applicability (see Figure 2) besides established risk factors, we argue that mechanistic insight is crucial and should be more actively pursued. Therefore, throughout the manuscript we identified a set of *Key Questions*, often related to putative anomalies, that any mechanistic hypothesis should address (**Table 2**).

These key questions do not disprove a causal role for telomere biology in aCVD; however, they do question the consensus interpretation that *inheritance* of shorter telomeres is the primary risk factor. This idea was also challenged by very recent results from Benetos and colleagues, who evaluated both leukocyte and skeletal muscle TL in atherosclerosis (38). These authors used skeletal muscle, a minimally replicative tissue, as a proxy for the inherited component of TL. Intriguingly, they found an association with atherosclerosis for leukocyte TL, but not muscle/inherited TL. Moreover, the difference between leukocyte and muscle TL, previously demonstrated to particularly arise from faster leukocyte telomere attrition during early life (21), showed a stronger association with atherosclerosis than did leukocyte TL itself (38). The authors concluded that factors affecting blood cell telomere attrition during early life (rather than inheritance of shorter/longer telomeres) may modulate aCVD risk later in life (38). Hence, they suggest that senescence of bone marrow derived progenitor cells involved in vascular endothelial repair may be involved in aCVD (57).

Importantly, the observation by Benetos et al. that early life leukocyte TL attrition is an aCVD risk factor, whereas inheritance of shorter telomeres is not (38), requires reconsideration of the GWAS results. Indeed, given the use of adult leukocytes in the GWAS, it is not unlikely that the latter studies identified *genetic determinants of leukocyte TL attrition during early life*

and that exactly these variants are associated with aCVD risk (58,59). On the other hand, most of the *Key Questions* specifically challenge a causal involvement of leukocytes/bone marrow derived cells (Table 2). Yet, it should be noted that leukocytes not necessarily play a role in aCVD: the genetic determinants of (early life) leukocyte TL dynamics (including telomerase activity (13)) will likely influence the TL and telomerase activity observed *in situ* as well, given the consistently shorter telomeres observed in atherosclerotic plaques and the telomerase activation in atherosclerosis (see above). Likely drivers of accelerated telomere attrition in atherosclerotic lesions are thus local atherosclerosis-associated oxidative stress and inflammation, as well as low telomerase activity or similar counteracting mechanisms *in situ* (**Figure 2D**, right panel). This would hold until critical shortening leads to replicative senescence, defective repair of existing lesions, and concomitant aCVD (58).

This hypothesis addresses most of the *Key Questions* in **Table 2**. In particular, it eliminates a role for critical leukocyte telomere attrition and thus a link with DKC (*Key Question 1*). Moreover, it explains the low biomarker value of leukocyte TL in aCVD (*Key Questions 2* and *4*). Additionally, in this hypothesis, the suggested, counterintuitive atheroprotective effect of higher paternal age, which most likely determines the *inherited* component of TL, is no longer relevant. Nevertheless, animal studies indicate that more research is required to determine if telomerase and longer telomeres, e.g. of vascular tissue, always protect against aCVD (*Key Question 3*).

A first guideline for future research is indeed to further develop and study genetically-modified animals with cell-type-specific and/or conditional deletion or overexpression of telomerase and other telomere-related proteins. The use of such models may allow one to convincingly establish a causal link, for example by testing whether TL restoration or attrition in

specific cell types (e.g., specific blood and vascular cells) aggravates or inhibits aCVD. As second guideline, we recommend to focus on early-life telomere dynamics and the difference between muscle and blood TL in epidemiologic studies, which will lead to novel mechanistic insight regarding genetic effects and the exact tissues involved. As third guideline, we argue that more extensive human *in situ* research (vascular tissues) will help to this end, as clearer differences and possibly higher relevance largely compensate the more complex sample collection.

Conclusion

More than 15 years of epidemiological and animal-model research have established a robust link between TL and aCVD, but with very low effect sizes. Despite the small effect, this link appears to be more than an epiphenomenon, since genetic analyses indicate causality. These analyses still need to be reconciled with the rest of the literature and with the set of standing key questions presented here. Within this framework, we argue that local telomere attrition in atherosclerotic lesions provides an appropriate mechanistic theory, but that verification will require additional genetic and animal-model based experiments. Indeed, there is a pressing need to test our theories against the often-unexpected results of epidemiological and animal studies before we move to the clinic.

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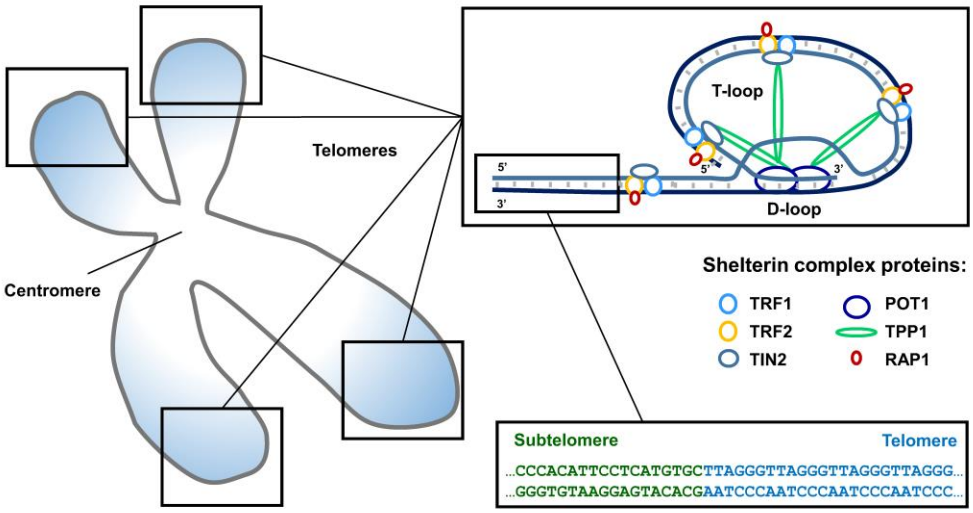

Figure 1: Telomeres at the ends of mammalian chromosomes. Telomeres consist of $(TTAGGG)_n$ tandem repeats with a single-stranded G-rich overhang that recoils into the double-stranded telomere, forming a D-loop, T-loop structure. The telomere structure is determined by the protein subunits of the shelterin complex.

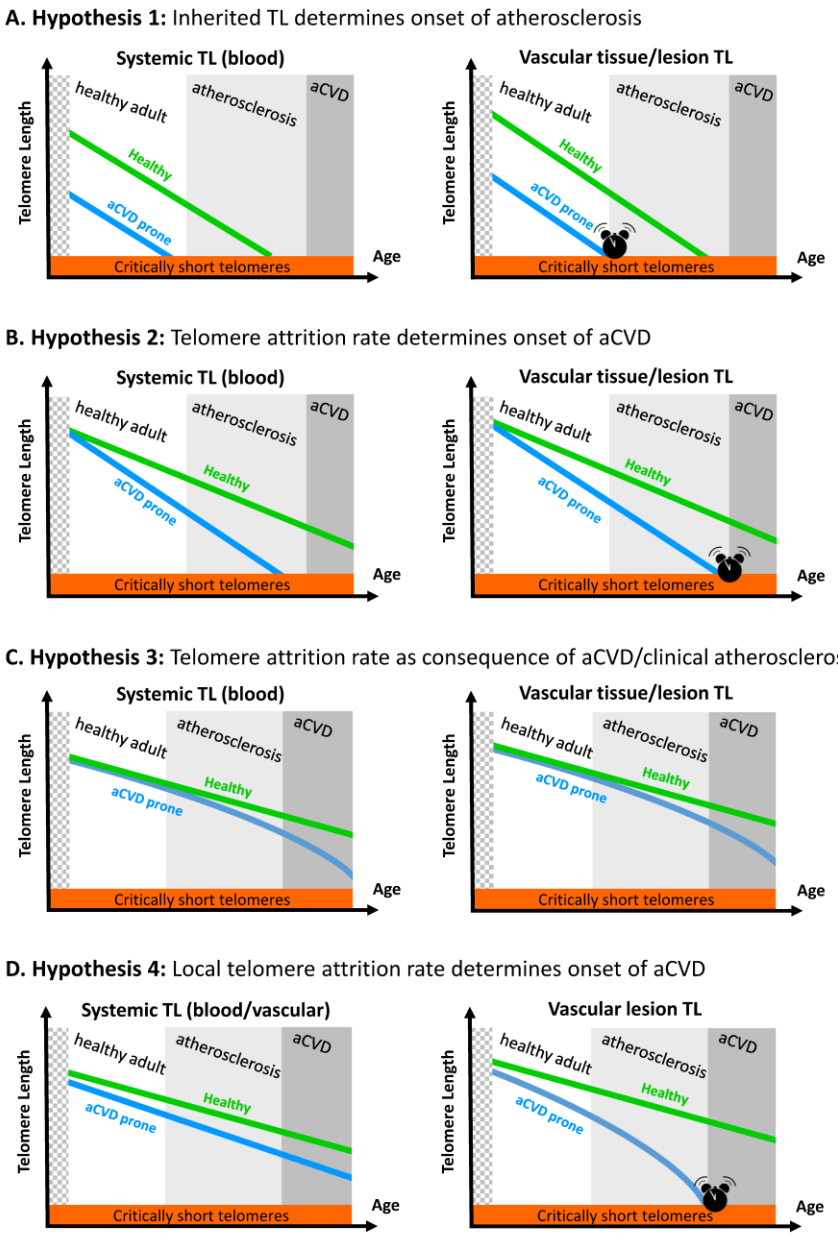

Figure 2 (Central illustration): Overview of prominent hypotheses regarding the role of telomere biology in aCVD, for both leukocyte/systemic TL (left panels) and vascular lesion TL (right panels). Note that leukocyte telomeres are typically shorter than vascular tissue telomeres (20). A. Hypothesis 1: Inheritance of shorter telomeres leads to atherosclerosis. B. Hypothesis 2: Accelerated telomere attrition leads to aCVD. C. Hypothesis 3: Telomere attrition is an epiphenomenon arising from atherosclerosis/aCVD-associated oxidative stress and inflammation. D. Hypothesis 4: The local telomere attrition rate in atherosclerotic lesions determines aCVD onset, but is not reflected in systemic TL (either in leukocytes or healthy vascular tissue). Alarm clock represents mitotic clock (reflecting critical telomere attrition); aCVD, atherosclerotic cardiovascular disease; TL, telomere length.

501 **Table 1 – summary of evidence for or against hypotheses on the role of TL in aCVD**

Evidence	H1	H2	H3	H4	Ref.
No close association between DKC and aCVD	✗	✗	✓	✓	(17)
Leukocyte TL is independently associated with aCVD	✓	✓		✓	(36,37)
Shorter leukocyte TL was observed in advanced, but not in pre/subclinical atherosclerosis	✗	✓	✓	✓	(33-35)
Leukocyte TL is associated with oxidative stress and inflammation, also in healthy subjects		✓	✓		(11,39)
Leukocyte TL attrition in time is limited compared to overall leukocyte TL variance, and not associated with aCVD		✗	✓		(40,41)
Alleles determining leukocyte TL predict aCVD risk	✓	✓	✗	✓	(7,8,51)
Clearer vascular than leukocyte TL differences with aCVD	✗	✗		✓	(27,42,43)
Telomerase knock-out mice do not develop aCVD	✗				(46,49)
Telomerase is active in atherosclerotic lesions				✓	(47,48)
(Proxy for) early life leukocyte telomere attrition rate rather than inherited/muscle TL is associated with aCVD	✗	✓			(38)

H1: inherited component of TL determines onset of atherosclerosis; H2: telomere attrition rate determines onset of aCVD; H3: telomere attrition rate is the consequence of aCVD; H4: local telomere attrition rate determines onset of aCVD. aCVD, atherosclerotic cardiovascular disease; DKC, dyskeratosis congenita; Ref.; references; and TL, telomere length. Check and X marks indicate supporting and contradicting evidence respectively, empty fields a non-straightforward relation.

Table 2 – Key questions to be addressed by mechanistic theories of TL-aCVD interaction

Key question	Ref.
1. Why are critically short telomeres in DKC not accompanied by aCVD and *vice versa*? *Since DKC patients with telomerase complex mutations inherit very short telomeres, one would expect aCVD to be a symptom of DKC if (inherited) short telomeres cause aCVD, especially if blood cells (featured by shorter telomeres than other tissues) play a causal role. Conversely, if inherited shorter telomeres lead to premature replicative senescence and hence aCVD, one would expect that leukocytes (with on average shorter telomeres) would encounter replicative senescence first, leading to DKC-like symptoms in addition to aCVD.*	(17,18)
2. Is causality compatible with the absence of a clear leukocyte TL threshold in aCVD? *Replicative senescence is initiated by telomere attrition below a critical threshold. The lack of a clear leukocyte TL threshold in aCVD therefore contradicts a role for inherited/leukocyte TL in aCVD.*	
3. Do longer telomeres always protect against atherosclerosis? *In animal models, shorter telomeres have been shown to protect against atherosclerosis, and telomerase activation was associated with atherosclerosis initiation.*	(47-49)
4. Why are the genetic determinants of (adult leukocyte) TL more strongly associated with aCVD than directly measured (adult leukocyte) TL? *In the only study providing both estimates (with 290,022 participants), the genetically determined TL (estimated from SNPs) was more significantly associated with ischemic heart disease risk than directly measured TL (note: TL in this study was measured by less precise qPCR method).*	(8)

aCVD indicates atherosclerotic cardiovascular disease; DKC, Dyskeratosis congenita; TL, telomere length; and Ref., references providing background information.