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Cellular Aging Reflected by Leukocyte Telomere Length Predicts Advanced Atherosclerosis and Cardiovascular Disease Risk

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Objective—To determine the association between leukocyte telomere length (TL) and atherosclerosis and its clinical sequelae stroke and myocardial infarction.

Methods and Results—Within the scope of the prospective population-based Bruneck Study, leukocyte TL was measured by quantitative polymerase chain reaction in 800 women and men aged 45 to 84 years (in 1995). The manifestation of cardiovascular disease (CVD) (1995–2005) and the progression of atherosclerosis (1995–2000) were carefully assessed. The TL was shorter in men than in women (age-adjusted mean [95% CI], 1.41 [1.33 to 1.49] versus 1.55 [1.47 to 1.62]; $P=0.02$) and inversely correlated to age ($r=-0.22$, $P<0.001$) and family history of CVD ($P=0.03$). Participants with CVD events during follow-up ($n=88$) had significantly shorter telomeres (age- and sex-adjusted mean [95% CI], 1.25 [1.08 to 1.42] versus 1.51 [1.45 to 1.57]; $P<0.001$). In multivariable Cox models, baseline TL emerged as a significant and independent risk predictor for the composite CVD end point and its individual components (myocardial infarction and stroke); however, this was not the case for de novo stable angina and intermittent claudication. Subjects in the top and bottom TL tertile group differed in their CVD risk by a factor of 2.72 (95% CI, 1.41 to 5.28), which is the risk ratio attributable to a 13.9-year difference in chronological age. Remarkably, in our atherosclerosis progression model, TL was strongly associated with advanced, but not early, atherogenesis. All findings were consistent in women and men.

Conclusion—Our findings indicate a differential role of telomere shortening in the various stages of atherosclerosis, with preferential involvement in advanced vessel pathology and acute vascular syndromes. (*Arterioscler Thromb Vasc Biol.* 2010;30:1649-1656.)

Key Words: cell ■ senescence ■ telomere myocardial infarction ■ stroke ■ atherosclerosis

Telomeres are nucleoprotein complexes composed of TTAGGG repeats at the extreme ends of chromosomes implicit in the maintenance of chromosomal integrity. Telomeres progressively shorten with each cell cycle and reflect replicative history at the cellular level. Excessive cell replication and telomere attrition lead to cell senescence, featured by cell cycle arrest, derogated cell viability, and changes in gene expression.^{1,2} Senescent cells may persist in vivo long enough for pathological consequences to emerge before they undergo apoptosis and removal by phagocytes.^{3,4}

A series of stimulating experimental investigations has suggested a tight interplay between cell senescence and atherosclerosis. In vitro studies of aged endothelial cells have revealed multiple proatherogenic changes in cell phenotype and yielded evidence of a decreased repair and vascular remodeling capacity,⁵ as well as impaired angiogenic properties.⁶ In complicated plaques, senescence of various cell

types, including vascular smooth muscle cells and macrophages, was reported to favor both plaque rupture⁷ and atherothrombosis.⁸ Specifically, the accumulation of aged cells in advanced atherosclerosis as the result of high cell turnover and impaired phagocytic clearance⁴ was shown to elicit prominent tissue inflammation and matrix degradation, which result in a critical thinning of the fibrous cap⁷ and potentially translate into excess cardiovascular disease (CVD) risk.

Although experimental evidence in support of a role of cell senescence and short telomere length (TL) in CVD is compelling, epidemiological support is sparse, with substantial under-research in women; most data originate from selected populations.^{9–11} The only community-based evaluation with a CVD priority was restricted to individuals 65 years and older.¹² To our knowledge, the current study is the second to investigate the putative association between leukocyte TL and CVD in a population-based cohort and the first to include a broad age

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Table 1. Baseline Characteristics in the Entire Study Population and Separately in Subjects With and Without CVD Events During Follow-Up (1995–2005)*

Characteristic	Total (N=800)	New-Onset CVD		P Value†
		No (n=712)	Yes (n=88)	
Demographic variables				
Age, y	62.7±11.1	61.8±10.8	70.0±10.5	<0.001
Male sex, %	49.4	47.6	63.6	0.005
Social status, %				
Low	60.0	58.0	76.1	0.004
Middle	22.5	24.0	11.4	
High	17.5	18.0	12.5	
Telomere length				
Relative T to S ratio (qPCR)	1.48±0.80	1.52±0.81	1.13±0.52	<0.001
TL, kB (SB)‡	8.04±1.09	8.10±1.11	7.60±0.71	<0.001
Intima-media thickness (via carotid ultrasonographic findings), mm	0.94±0.25	0.92±0.24	1.08±0.30	<0.001
Lifestyle and vascular risk factors				
Smoking, %	20.0	20.1	19.3	0.86
Total smoking, pack-years	12.2±16.6	11.6±16.0	17.4±20.5	0.01
Alcohol consumption, g/d	23.5±31.2	23.1±30.9	26.1±33.5	0.40
Physical activity (sport) index	2.4±0.9	2.4±0.9	2.0±0.8	<0.001
Body mass index§	25.7±3.9	25.6±3.8	26.3±4.5	0.17
Diabetes mellitus, %	11.0	10.1	18.8	0.02
Fasting glucose, mmol/L	5.7±1.3	5.6±1.3	6.1±1.6	0.009
Hypertension, %	67.9	67.6	70.5	0.58
Blood pressure, mm Hg				
Systolic	148.2±20.7	147.5±20.4	154.2±22.7	0.009
Diastolic	87.1±9.2	87.0±9.1	87.6±9.8	0.58
Lipoprotein(a), μmol/L¶	0.42 (0.16–1.34)	0.39 (0.16–1.30)	0.63 (0.19–1.75)	0.08
HDL cholesterol, mmol/L	1.5±0.4	1.5±0.4	1.5±0.5	0.14
LDL cholesterol, mmol/L	3.8±1.0	3.7±1.0	3.9±1.2	0.11
Triglycerides, mmol/L¶	1.3 (0.9–1.8)	1.2 (0.9–1.8)	1.4 (1.0–2.0)	0.01
High-sensitivity CRP, nmol/L¶	1.6 (0.9–3.1)	1.5 (0.8–3.0)	2.1 (1.2–4.1)	0.006
Ferritin, pmol/L	300.0±374.9	285.9±340.6	414.2±572.8	0.04
Fibrinogen, μmol/L	8.5±2.2	8.4±2.1	9.2±2.8	0.002
Creatinine, μmol/L	83.6±16.6	83.0±16.3	88.4±18.2	0.01
Ischemic stroke, myocardial infarction, stable de novo angina, PAD, and revascularizations (ie, previous CVD), %	6.9	4.9	22.7	<0.001

CRP indicates C-reactive protein; CVD, cerebrovascular disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PAD, peripheral artery disease; qPCR, quantitative polymerase chain reaction; S, single-copy gene *36B4* copy; SB, Southern blot; T, telomere repeat copy; TL, telomere length.

*Data are given as mean±SD unless otherwise indicated.

†Values are for the difference in variable levels between subjects with and without new-onset CVD, calculated with the χ^2 test, the Fisher exact test, or an independent-samples *t* test. Skewed variables were \log_e transformed for the *t* test.

‡Transformation of the relative T to S ratio (qPCR) to TL was performed with the following formula: TL (kB)=(6.016+1.365×relative T to S ratio). The supplemental Methods provides further explanation (available online at <http://atvb.ahajournals.org>).

§Calculated as weight in kilograms divided by height in meters squared.

¶Values with a markedly skewed distribution are given as median (interquartile range).

||When the analysis was adjusted for statins (overrepresented in subjects with new-onset CVD), the difference in LDL level achieved significance ($P=0.04$).

dures yielded similar results. $P<0.05$ (2 sided) was considered significant.

Results

The distribution of the T to S ratio in the general community and its relation to a mean telomere restriction fragment

(Southern blot) is illustrated in supplemental Figure I. Baseline demographics, lifestyle characteristics, vascular risk factors, and laboratory parameters in the study population (N=800) are demonstrated in Table 1. Data are presented separately for subjects who did and did not experience CVD

Table 2. Association Between Telomere Length and Composite and Individual CVD End Points in the Bruneck Study (1995–2005)*

Model	Incidence Rate per 1000 PY†	HR (95% CI)‡					P Value	
		In Tertile Groups of TL			Per 1-SD Decrease in log _e (Relative T to S Ratio)			
		High	Middle	Low	0.5	1.0		1.5
Composite CVD end point (n=88)*								
No adjustment	12.6	1.00	3.06 (1.58–5.94)	4.71 (2.49–8.93)				<0.001
Adjustment for age, sex, and previous CVD		1.00	2.33 (1.19–4.56)	3.18 (1.66–6.08)				<0.001
Multivariable adjustment 1§		1.00	2.27 (1.16–4.43)	2.88 (1.50–5.54)				<0.001
Multivariable adjustment 2¶		1.00	2.23 (1.14–4.38)	2.72 (1.41–5.28)				0.001
Extended composite CVD end points								
Multivariable Cox model								
1 (n=104)**	15.1	1.00	2.11 (1.17–3.80)	2.25 (1.25–4.05)				0.02
2 (n=113)¶¶	16.5	1.00	1.61 (0.94–2.75)	1.67 (0.98–2.84)				0.08
3 (n=129)#	19.2	1.00	1.53 (0.94–2.51)	1.53 (0.93–2.49)				0.08
Individual disease end points								
Myocardial infarction (n=43)	6.0	1.00	2.60 (0.94–7.22)	3.58 (1.32–9.70)				0.04
Stroke (n=46)	6.5	1.00	2.05 (0.84–4.99)	2.24 (0.93–5.42)				0.02
Peripheral artery disease (n=15)	2.1	1.00	0.51 (0.12–2.06)	0.53 (0.14–2.06)				0.71
Stabile de novo angina (n=33)	4.6	1.00	1.16 (0.46–2.93)	0.76 (0.29–2.02)				0.85
Vascular death (n=45)	6.3	1.00	1.93 (0.68–5.49)	3.04 (1.13–8.19)				0.03

CVD indicates cardiovascular disease; HR, hazard ratio; PY, person-years.

*N=800. The composite CVD end points included stroke, myocardial infarction, and vascular death; and the extended composite end points included stroke, myocardial infarction, vascular death, and any revascularization procedure.

†Rates were calculated per 1000 PY of follow-up.

‡The HRs were derived from Cox models and calculated for TL tertile groups and separately for a 1-SD decrease in the log_e-transformed T to S ratio.

§Multivariable analysis built with a forward stepwise selection procedure. The following variables were selected for inclusion: age; sex; ferritin, high-sensitivity C-reactive protein, and lipoprotein(a) levels; and previous CVD.

¶Multivariable Cox models were adjusted for the following: age; sex; previous CVD; hypertension; pack-years of smoking; ferritin, high-sensitivity C-reactive protein, lipoprotein(a), and low- and high-density lipoprotein cholesterol levels; physical activity; diabetes mellitus; and alcohol consumption. For individual disease end points, adjustment was performed for prior manifestation of the disease of interest rather than for prior CVD.

¶¶This model also included symptomatic peripheral artery disease (intermittent claudication).

#This model also included stable de novo angina.

**This extended composite end point included stroke, myocardial infarction, vascular death, and any revascularization procedure.

during follow-up. Participants with CVD events during follow-up had significantly shorter telomeres (age- and sex-adjusted mean [95% CI], 1.25 [1.08 to 1.42] versus 1.51 [1.45 to 1.57]; $P<0.001$), were older, more likely to be men, physically inactive or diabetic, and had higher levels of systolic blood pressure, fibrinogen, high-sensitivity C-reactive protein, triglycerides, low-density lipoprotein cholesterol (after adjustment for statin therapy), and creatinine.

TL and Cardiovascular Risk Factors

The TL was shorter in men than in women (age-adjusted mean [95% CI], 1.41 [1.33 to 1.49] versus 1.55 [1.47 to 1.62]; $P=0.02$) and inversely correlated to age ($r=-0.22$, $P<0.001$).²¹ In age- and sex-adjusted analysis, TL was associated with high-density lipoprotein cholesterol ($r_p=0.09$, $P=0.01$) and apolipoprotein AI ($r_p=0.08$, $P=0.04$) and inversely associated with diabetes (age- and sex-adjusted mean [95% CI], 1.30 [1.13 to 1.48] versus 1.50 [1.44 to 1.56] in nondiabetics; $P=0.004$), ferritin ($r_p=-0.07$, $P=0.048$),

and high-sensitivity C-reactive protein ($P=0.05$). Remarkably, a correlation was found between TL and family history of CVD. The age- and sex-adjusted mean (95% CI) values of TL were as follows: 1.49 (1.44 to 1.55) in subjects with a negative family history ($n=735$), 1.36 (1.14 to 1.58) in subjects with a positive history for stroke or myocardial infarction in first-degree relatives ($n=49$), and 1.25 (0.86 to 1.63) in subjects with a positive history for stroke and myocardial infarction ($n=16$) ($P=0.03$ for trend).

TL and Cardiovascular Events

During follow-up, 88 study participants experienced a myocardial infarction, stroke, or vascular death (composite CVD end point; 12 cases in high, 32 in middle and 44 in low TL tertile group). As outlined in Table 2, baseline TL emerged as a highly significant and independent predictor of new-onset CVD. Each 1-SD decrease in log_e-transformed relative T to S ratio was associated with a 46% (95% CI, 16%–84%) greater risk for the composite CVD end point (multivariable model,

Table 3. Association Between Telomere Length and Composite, CVD End Points in Various Subgroups in the Bruneck Study (1995–2005)*

Multivariable Model	Events	Incidence Rate per 1000 PY†	HR (95% CI) per 1-SD Decrease in log _e (Relative T to S Ratio)‡	0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0
Sex				
Male (n=395)	56	17.0	1.5 (1.1–2.1)	
Female (n=405)	32	8.6	1.4 (1.0–2.1)	
BMI§				
<25 (n=366)	39	12.2	1.9 (1.3–2.7)	
≥25 (n=434)	49	12.9	1.3 (0.9–1.8)	
Medication				
No (n=531)	33	6.7	1.5 (1.0–2.1)	
Yes (n=269)	55	26.5	1.5 (1.1–2.1)	
Smoking status				
Nonsmoker (n=445)	45	11.4	1.5 (1.1–2.0)	
Current or ex-smoker (n=355)	43	14.1	1.5 (1.0–2.2)	
Age, y				
45–54 (n=230)	8	3.5	3.5 (1.5–8.4)	
55–64 (n=217)	20	9.8	1.4 (1.0–2.2)	
65–74 (n=204)	27	15.6	1.9 (1.2–3.0)	
75–84 (n=149)	33	34.2	0.9 (0.6–1.5)	
Type of CVD event				
Primary (n=745)	68	10.2	1.5 (1.2–2.0)	
Secondary (n=55)	20	54.6	1.7 (0.8–3.7)	

BMI indicates body mass index; CVD, cardiovascular disease; HR, hazard ratio; PY, person-years.
 *N=800. The composite CVD end point included stroke, myocardial infarction, and vascular death.
 †Rates were calculated per 1000 PY of follow-up.
 ‡The HRs were derived from Cox models and calculated for a 1-SD decrease in the log_e-transformed T to S ratio.
 §Calculated as weight in kilograms divided by height in meters squared.

P=0.001). The association also applies to myocardial infarction (hazard ratio [95% CI], 1.41 [1.02 to 1.96]), stroke (1.49 [1.08 to 2.07]), and vascular death (1.46 [1.04 to 2.05]), but not to stable de novo angina or intermittent claudication (Table 2). Accordingly, the strength of the relationship declined when considering angina and peripheral artery disease in an extended composite CVD end point (Table 2).

The link between TL and CVD was further strengthened by a number of sensitivity analyses. (1) The association between TL and CVD was consistent in various subgroups, including women and men and subjects with and without prior CVD (primary and secondary events) (Table 3). However, the association between TL and CVD disappeared after the age of 75 years (*P*=0.01 for the effect modification by age). (2) To further characterize the association between TL and CVD, hazard ratios were calculated for tertile groups of TL (Table 2). Subjects in the top and bottom tertile groups of TL differed in their CVD risk by a factor of 2.72 (95% CI, 1.41 to 5.28), which equals the risk ratio attributable to a 13.9-year difference in chronological age. Cumulative hazard curves for the composite CVD end point (vascular death, myocardial infarction, and stroke) are shown in Figure 1. (3) Findings remained robust when further enhancing the level of adjustment and checking the multivariable analysis for all variables in Table 1 and for regular medication (data not shown).

TL and Carotid Atherosclerosis

We tested the relationship between TL and carotid atherosclerosis. In both age- and sex-adjusted and multivariable regression analysis, the log_e-transformed T to S ratio was not associated with IMT (β =−.02 [*P*=0.24] and β =−0.009 [*P*=0.50], respectively). To eliminate the effects of chronological age and to avoid overadjustment, subjects of the 3 tertile groups of TL were closely matched for age and sex (supplemental data). In the matched groups (n=183 per group), the IMT levels were similar (high, middle, and low tertile groups of TL: 0.92, 0.96, and 0.92 mm, respectively; *P*=0.23). In the follow-up between 1995 and 2000, 294 (47.2%) of 623 study participants experienced early atherosclerosis (the occurrence of new plaques and the nonstenotic progression of preexisting lesions); 61 (18.9%) of 322 participants with preexistent atherosclerosis showed a stenotic transformation (advanced atherogenesis). Short telomeres were significantly associated with advanced, but not with early, atherogenesis (Figure 2). Details are depicted in the supplemental Table. These findings apply equally to women and men and emerged as independent of other risk factors. There was also no evidence for differential effects in other subgroups. The matching approach mentioned yielded a significant increase in the probability of advanced atherogen-

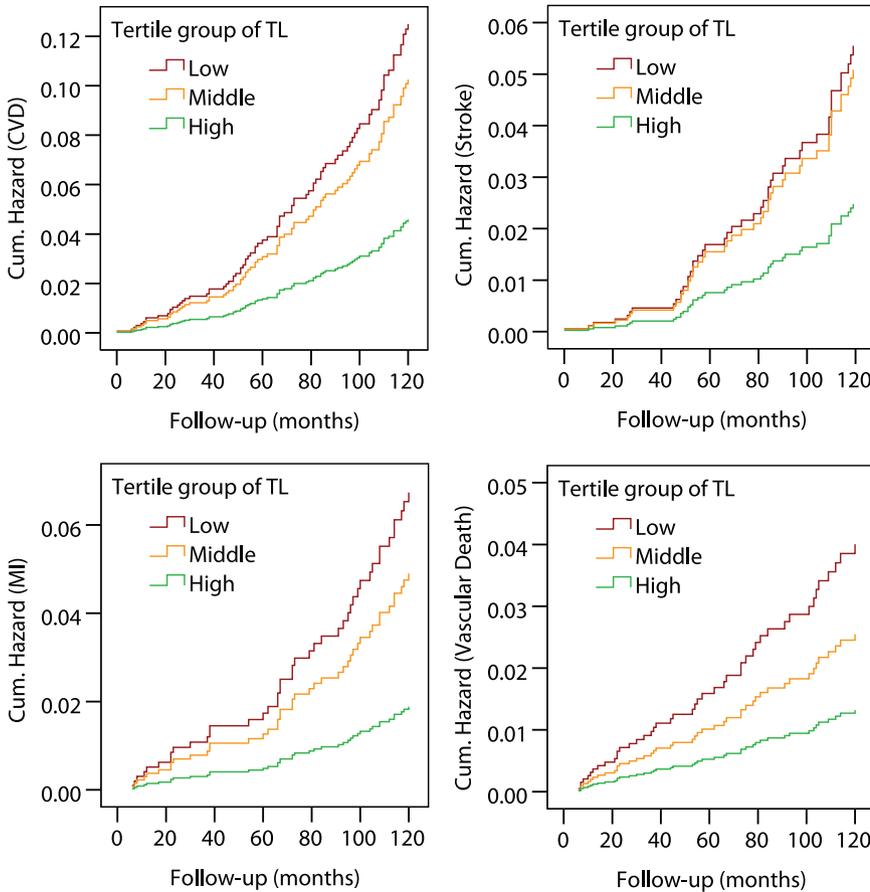


Figure 1. Cumulative hazard curves for CVD (n=88), vascular death (n=45), myocardial infarction (MI) (n=43), and stroke (n=46) manifesting between 1995 and 2005.

esis across TL tertile groups matched for age and sex (high, middle, and low tertile groups of TL: 8.9%, 21.5%, and 28.6%; $P_{\text{trend}}=0.001$) but not for early atherosclerosis (50.0%, 46.9%, and 45.7%; $P_{\text{trend}}=0.45$).

Discussion

The current prospective study of unselected subjects observed for 10 years unraveled a highly significant association between short leukocyte TL and CVD risk, independently of chronological age, sex, and standard risk factors. This finding

extends to myocardial infarction and ischemic stroke commonly originating from unstable plaques, but not to stable angina or intermittent claudication (Table 1). In our person-based progression model of carotid atherosclerosis, short TL was unrelated to early atherosclerosis but emerged as strongly and independently associated with advanced atherosclerosis, characterized by plaque fissuring/rupture and atherothrombosis (Figure 2).¹⁵⁻¹⁷

The mechanistic link between short TL and advanced atherosclerosis remains to be established. There is preliminary evidence that leukocyte TL adequately reflects TL in the

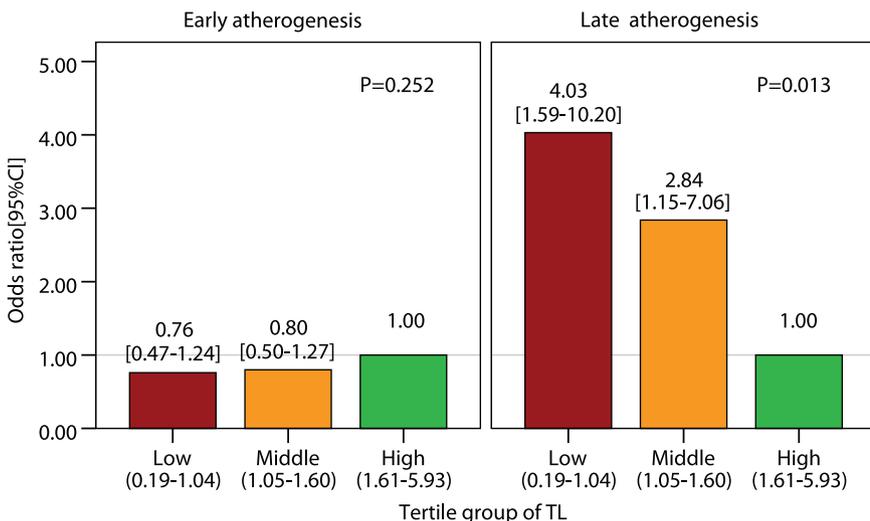


Figure 2. Multivariable odds ratios of early and advanced atherosclerosis (1995–2000) in TL tertile groups. The top tertile served as the reference category, and ranges of the T to S ratios in the 3 tertile groups are given in parentheses.

vasculature.²² Telomere shortening in key vascular cells, in turn, and the related phenomena of cellular aging and replicative cell senescence have been reported to evoke multiple proatherogenic consequences,¹ such as impairment of endothelial repair and vessel remodeling, unfavorable changes in gene expression and cell phenotype, upregulation of immunoattractants (intercellular adhesion molecule-1²³ and vascular cell adhesion molecule-1²⁴) and prothrombotic molecules (plasminogen activator inhibitor-1⁸), and matrix degeneration²⁵ with critical destabilization of plaques. In stable atherosclerosis, senescent cells are few, whereas in advanced complicated lesions, senescent cells accumulate as the result of high cell turnover, prominent oxidative and inflammatory stress, and impaired phagocytic clearance.^{4,26} Thus, the concept that telomere shortening preferentially affects advanced atherosclerosis is appealing, yet speculative, and awaits rigorous confirmation in future research. In particular, the adequacy of leukocyte TL as a surrogate of vascular TL has to be firmly established.

How do our findings reconcile with previous epidemiological studies? First, the bulk of studies with focus on cardiovascular end points revealed findings highly consistent with those of our analysis. In detail, a retrospective survey of 203 subjects with a premature myocardial infarction before the age of 50 years and 180 controls demonstrated significantly shorter telomeres among the subjects (difference, 299.7 ± 69.3 base pairs; $P < 0.001$).¹⁰ In a series of outpatients with stable coronary artery disease ($n = 780$), TL was associated with all-cause mortality after a mean follow-up of 4.4 years ($P = 0.02$).¹¹ As part of the West of Scotland Coronary Prevention Study, the TL in 484 men who developed coronary heart disease during a mean period of 4.9 years was compared with TL in 1058 age-matched controls. Individuals with shorter telomeres at recruitment faced a significantly higher risk of future coronary heart disease.⁹ As the only prospective population-based evaluation in the field, the Cardiovascular Health Study showed an inverse relationship between TL and CVD risk in a subgroup of 419 subjects.¹²

Second, most studies with a focus on vessel wall pathology measured IMT as a surrogate and precursor lesion of atherosclerosis and yielded no significant association with TL (Asklepios Study²⁷ and Cardiovascular Health Study¹²) or associations in subgroups only (Framingham Offspring Study²⁸). A small study²⁹ involving 163 hypertensive men directly visualized plaques in the carotid arteries and obtained a significant inverse association between TL and the presence of atherosclerosis; the large Asklepios Study failed to find such a relationship.²⁷ Investigations that monitor plaque development and/or differentiate between stages of atherosclerosis have not yet been published.

Two further aspects deserve brief consideration. (1) The association between TL and CVD risk did not extend to elderly subjects (those aged ≥ 75 years) (probability value for effect modification by age = 0.01) (Table 2). This is unlikely to be a chance finding because it is in perfect agreement with results from 3 previous studies.^{12,30,31} The breakdown of the association in aged individuals may reflect survival effects or the existence of a threshold, beneath which further telomere shortening does not confer additional risk.⁹ (2) In the Bruneck

cohort, TL significantly correlated with family history of CVD. In line with a previous report,³² this observation and the preferential association between TL and vascular risk in younger individuals suggest that the heritability of TL may contribute to the individual genetic susceptibility to CVD.

Our study has several strengths. (1) Robustness of findings: The associations obtained were internally consistent, valid for both women and men and of a magnitude considered biologically meaningful. (2) Study design: The Bruneck cohort is extremely well characterized with a near 100% follow-up and high-quality ascertainment of clinical endpoints. TL measurement was performed in quadruplicate, showed a high level of reproducibility²¹ and was corrected for qPCR efficiency. (3) Focus on atherosclerosis: We are able to differentiate between early and advanced stages of carotid atherosclerosis by applying a unique person-based atherosclerosis progression model.

Our study has shortcomings as well, including the limited number of outcome events and the fact that TL was measured in circulating blood leukocytes, an easily accessible source of DNA, rather than in atherosclerotic tissue per se. However, the validity of leukocyte TL as a surrogate for vascular TL was suggested by a recent study.²² Finally, the Bruneck Study cohort is entirely white, and findings should not be generalized to other ethnicities.

In conclusion, the Bruneck Study provides strong evidence for a link between TL and CVD. In concert with current experimental evidence, our findings lend support to the concept that replicative cell senescence as the result of telomere attrition contributes to the transition of stable to complicated plaques, thereby amplifying the risk of acute vascular syndromes.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Study subjects

The population of Bruneck is exclusively white and of heterogeneous geographic origin, with sizeable segments of the population being of either northern Italian or Austro-German descent. Population mobility within the Bruneck area is low, 0.2% per year at the time of the study. The official population register contains information obtained from the national census and is continuously updated regarding births, deaths, and changes of residence. The study population was recruited as a sex- and age-stratified random sample of all inhabitants aged 40 to 79 years: age groups were formed by decades and 125 women and 125 men from each age group were selected using a computer-based random number generator.

Clinical examination and history

All study participants underwent an ambitious examination program and completed a standardized questionnaire on medical history and various lifestyle factors, including current smoking, alcohol consumption, and physical activity. Diabetes was coded present according to the WHO definition (fasting blood glucose ≥ 126 mg/dl, two-hour glucose level ≥ 200 mg/dl after 75g oral glucose load, or pre-established diagnosis of diabetes). Hypertension was defined as a blood pressure $\geq 140/90$ mm Hg (mean of three independent readings obtained with a standard mercury sphygmomanometer after at least ten minutes of rest) or the use of anti-hypertensive drugs. Pack years of smoking in current smokers and ex-smokers were

defined as the average number of packs smoked daily multiplied by years of smoking. Body mass index was calculated as weight divided by height squared (kg/m^2). LDL cholesterol was computed from the Friedewald formula.¹ Physical activity was assessed with the Baecke score (sports index).² Average alcohol consumption was quantified in grams per day.³ Finally, socioeconomic status was assessed on a three-category scale (low, medium, high) based on information about occupational status and educational level of the person with the highest income in the household.

Efficiency correction of the quantitative PCR method

Differences in qPCR efficiency between samples were corrected with a mathematical algorithm developed by Pfaffl et al.^{4,5} In general, the qPCR method amplifies a defined DNA section and amplification kinetics are registered via a fluorescent signal. If the reaction is 100% efficient, the existing amplicons are copied completely and fluorescence doubles with every cycle. However, it has been shown that several factors, like phenol, ethanol, hemoglobin and heparin impede the qPCR⁶ and, thus, efficiency of 100% is a rare condition. For instance, if efficiency is only 50%, the measured signal will rise by 1.5 with every cycle. Considering the exponential increase, even slight differences in efficiency have a striking impact on qPCR.

The qPCR kinetics in the exponential phase of the PCR can be described as $F_n = F_0 \times (1 + Eff)^n$ with F_0 and F_n being the amount of fluorescence measured at the very beginning and after n PCR cycles, respectively, and Eff being the efficiency of the PCR reaction. By log-transforming the equation, an equation similar to the general linear equation $y = d + k \times x$ is formed: $\log F_n = \log F_0 + n \times \log(1 + Eff)$. On a log-transformed ordinate, this formula describes the linear regression line of data points in the exponential phase, with $\log F_0$ being the intercept and $\log(1 + Eff)$ the slope of the line (red line in Figure II).

In our study, we used the software LinRegPCR 7.2 published in March 2003 for efficiency computation.⁷ As depicted in Figure III, the program analyzes the log-linear part of the PCR by using linear regression and calculates intercept and slope. In detail, an iterative algorithm searches for lines consisting of at least four and no more than six data points with the highest R² value and a slope close to the maximum slope. Efficiency is determined by inserting the calculated slope in the following equation:

$$\text{slope} = \log(1 + \text{Eff}) \Leftrightarrow 10^{\text{slope}} = 1 + \text{Eff} \Leftrightarrow 10^{\text{slope}} - 1 = \text{Eff}$$

Calculation of T/S ratios

A control sample was added to each plate to account for inter-assay variability. The relative T/S ratio is described as the ratio of the telomere to a single copy gene (SCG) in one sample, set in relation to the same ratio in the control DNA. In keeping therewith, the formula for calculating the relative T/S ratios can be stated as:

$$F_n = F_0 \times (1 + \text{Eff})^n$$

$$\Rightarrow \text{Telomere} = F_{0\text{tel}} \times (1 + \text{Eff}_{\text{tel}})^{\text{Ct}(\text{tel})}$$

$$\Rightarrow \text{Single copy gene} = F_{0\text{SCG}} \times (1 + \text{Eff}_{\text{SCG}})^{\text{Ct}(\text{SCG})}$$

$$T/S \text{ Ratio (sample)} = \frac{(1 + \text{Eff}_{\text{tel, sample}})^{-\text{Ct}(\text{tel, sample})}}{(1 + \text{Eff}_{\text{SCG, sample}})^{-\text{Ct}(\text{SCG, sample})}}$$

$$T/S \text{ Ratio (control)} = \frac{(1 + \text{Eff}_{\text{tel, control}})^{-\text{Ct}(\text{tel, control})}}{(1 + \text{Eff}_{\text{SCG, control}})^{-\text{Ct}(\text{SCG, control})}}$$

$$\text{Relative T/S Ratio} = \frac{(1 + \text{Eff}_{\text{tel, sample}})^{-\text{Ct}(\text{tel, sample})}}{(1 + \text{Eff}_{\text{SCG, sample}})^{-\text{Ct}(\text{SCG, sample})}} / \frac{(1 + \text{Eff}_{\text{tel, control}})^{-\text{Ct}(\text{tel, control})}}{(1 + \text{Eff}_{\text{SCG, control}})^{-\text{Ct}(\text{SCG, control})}}$$

Southern Blot

5µg of genomic DNA were digested overnight with the restriction enzymes RsaI/Hinfi (Roche) and loaded onto a 0.5% agarose gel with 0.5µl marker (DIG-labeled Marker VII, Roche). After electrophoresis, gels were depurinated, denatured, and neutralized. Capillary transfer was performed overnight with 10X SSC using positively charged nylon membranes (Roche). Membranes were briefly rinsed with 2X SSC, cross-linked for 3min under UV light, washed for 30min with 2X SSC at 65°C and prehybridized with hybridization buffer (5X SSC, 1% blocking reagent by Roche, 0.02% SDS, 0.1% sarcosyl) for 4.5 hours at 65°C. Hybridization took place overnight at 65°C using 10pmol/ml DIG-labeled probe (CCCTAA)₃ (Microsynth) in hybridization buffer. Membranes were washed three times for 15 minutes at room temperature and 15 minutes at 65°C with 2X SSC/0.1% SDS. Detection of DIG-labeled probe and marker was performed using the DIG Luminescent Detection Kit for Nucleic Acids (Roche) according to the manufacturer's instructions. After 2 h of exposure, films (GE Healthcare) were developed and fixed (Kodak). Density measurements were performed with the Quantity One software by BioRad.

Comparison of quantitative PCR and Southern Blot

In our study, TL was measured by quantitative PCR for reasons as follows: (a) availability of a high-throughput method of assessment, (b) high accuracy and reproducibility and (c) requires much smaller amounts of DNA. However, Southern Blot was additionally performed in a random sample of our population (n=56) to validate TL assessment and to obtain an absolute value in base pairs. The correlation coefficient between T/S ratios (qPCR) and mean TRF (Southern Blot) was 0.63 (Online-only Data Supplement Figure I, Part B) and thus similar to correlation coefficients reported in previous comparable studies.⁸ In a subsample, Southern Blot was applied twice and dual measurement resulted in better correlation

($r=0.765$) or around 60% shared variance. Remaining disagreement between both methods is partly explained by the measurement error of both qPCR and Southern Blot. Moreover, Southern Blot does not provide the actual length of telomeres, but unlike qPCR includes the subtelomeric region as well.^{9,10}

Statistical analysis – matching procedure

To eliminate effects of chronological aging and avoid over-adjustment, key associations were replicated in TL tertile groups closely matched for age and sex. For each subject in the middle TL tertile group suitable subjects in the low and high tertile group identical in age (± 1 yr) and sex were identified to form matched triplets. In the case of several suitable matches all were numbered in ascending order and one was selected by means of a computer-based random number generator. Overall, 183 triples were generated; for 84 individuals in the middle tertile group no suitable matches could be identified.

SUPPLEMENTAL FIGURES

Figure I. Distribution of T/S ratio in the general community (A) and relationship between T/S ratio and mean telomere restriction fragment (TRF) assessed by Southern Blot in 56 DNA samples (B). The corresponding regression line with 95% mean prediction interval is plotted.

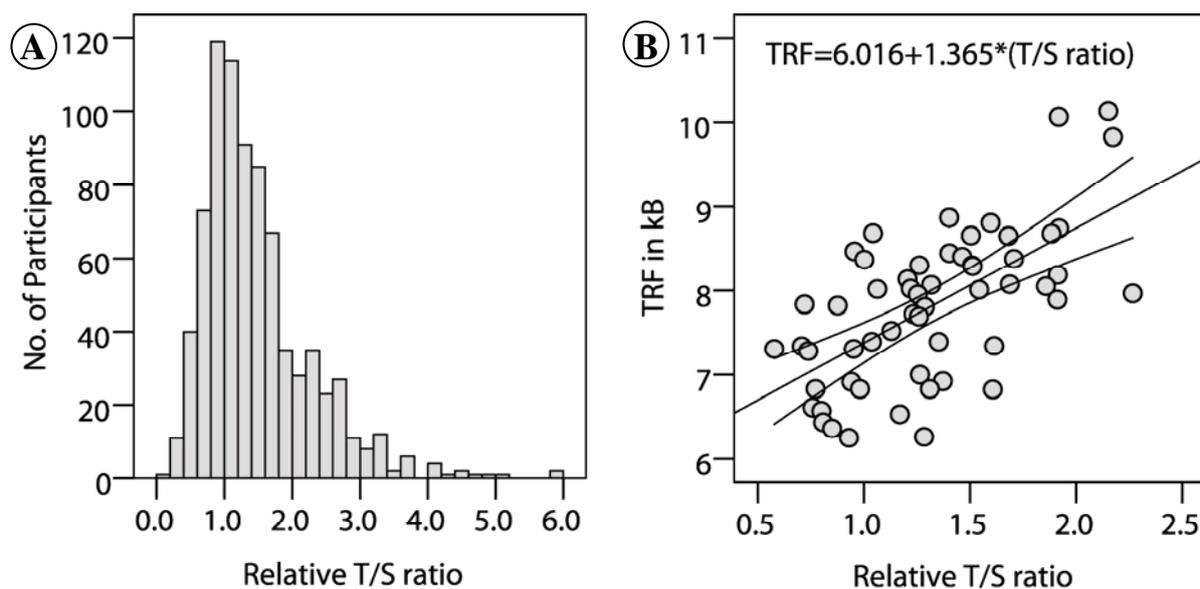


Figure II. Rise of fluorescence signal in a qPCR. The ordinate has a logarithmized scale.

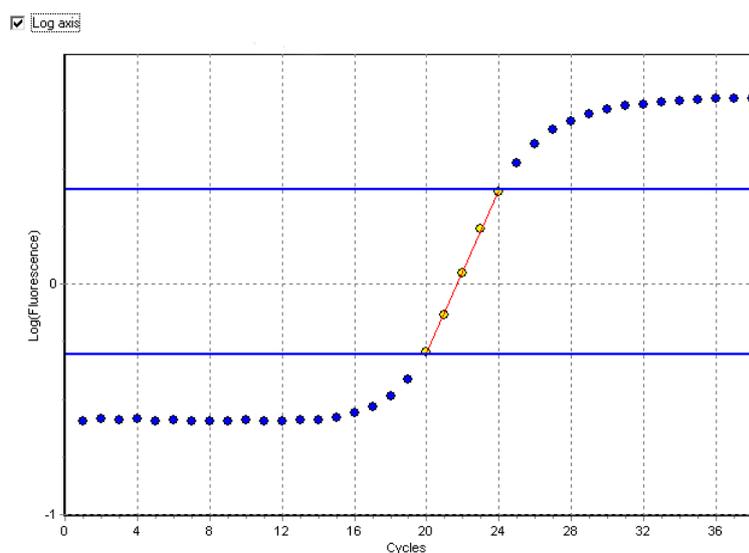
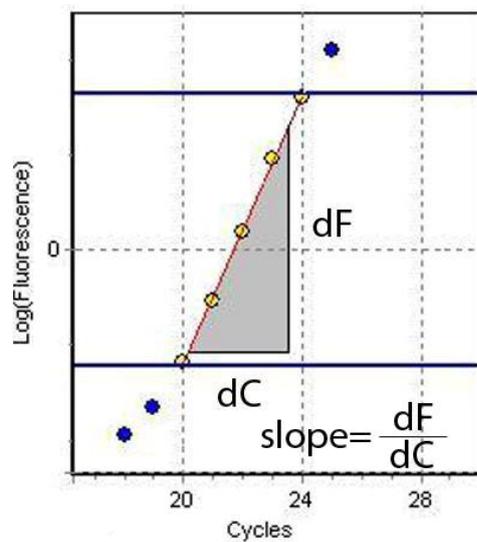


Figure III. Triangle for computation of the slope.



SUPPLEMENTAL TABLES

Table I. Association of TL with early and advanced atherogenesis in the Bruneck Study (1995–2000).

Multivariable model*	Odds ratios [95%CI] in TL tertile groups			P _{trend}
	High	Middle	Low	
Early atherogenesis*				
Entire population (294 out of 623)				
No adjustment	1.00	1.25[0.86–1.81]	1.17[0.80–1.73]	0.376
Adjustment for age, sex and AS	1.00	0.86[0.55–1.35]	0.82[0.51–1.30]	0.379
Multivariable model	1.00	0.80[0.50–1.27]	0.76[0.47–1.24]	0.252
Men (161 out of 301)				
No adjustment	1.00	1.35[0.79–2.30]	1.47[0.83–2.60]	0.178
Adjustment for age, sex and AS	1.00	0.96[0.51–1.77]	1.04[0.54–2.02]	0.924
Multivariable model	1.00	0.82[0.43–1.56]	0.96[0.49–1.91]	0.870
Women (133 out of 322)				
No adjustment	1.00	1.08[0.64–1.83]	0.92[0.54–1.59]	0.809
Adjustment for age, sex and AS	1.00	0.81[0.42–1.55]	0.66[0.34–1.29]	0.219
Multivariable model	1.00	0.75[0.37–1.51]	0.63[0.30–1.31]	0.208
Advanced atherogenesis*				
Entire population (61 out of 322)				
No adjustment	1.00	2.53[1.15–5.57]	3.93[1.79–8.65]	0.001
Adjustment for age, sex and stenosis	1.00	3.04[1.26–7.34]	4.59[1.89–11.14]	0.001
Multivariable model	1.00	2.84[1.15–7.06]	4.03[1.59–10.20]	0.003
Men (32 out of 177)				
No adjustment	1.00	4.07[1.11–14.88]	5.90[1.58–22.01]	0.007
Adjustment for age, sex and stenosis	1.00	5.61[1.27–24.89]	5.77[1.26–26.38]	0.034
Multivariable model	1.00	4.75[0.96–23.38]	4.98[0.93–26.59]	0.073
Women (29 out of 145)				
No adjustment	1.00	1.87[0.64–5.50]	3.16[1.13–8.79]	0.027
Adjustment for age, sex and stenosis	1.00	1.90[0.61–5.92]	3.83[1.29–11.43]	0.015
Multivariable model	1.00	1.80[0.54–5.95]	3.41[1.02–11.37]	0.046

* Odds ratios and confidence intervals were derived from logistic regression analyses and calculated for TL tertile groups. For adjustment in multivariable models see methods. 294 out of 623 (47.2%) study participants experienced early atherosclerosis (occurrence of new plaques and non-stenotic progression of preexisting lesions), while 61 out of 322 participants (18.9%) with pre-existent atherosclerosis showed a stenotic transformation (advanced atherogenesis).

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