Clinical and Population Studies

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. Senescent cells may persist in vivo long enough for pathological consequences to emerge before they undergo apoptosis and removal by phagocytes.

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, as well as impaired angiogenic properties. In complicated plaques, senescence of various cell types, including vascular smooth muscle cells and macrophages, was reported to favor both plaque rupture and atherothrombosis. Specifically, the accumulation of aged cells in advanced atherosclerosis as the result of high cell turnover and impaired phagocytic clearance was shown to elicit prominent tissue inflammation and matrix degradation, which result in a critical thinning of the fibrous cap 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 underresearch in women; most data originate from selected populations. The only community-based evaluation with a CVD priority was restricted to individuals 65 years and older. 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 range.
allows a relative T to S ratio to be calculated. Standard primers (tel1b: tel2b: 5'-GGCTTGCGTACCTCCCTACCCCTACCTA-CCCT-3') and cycling conditions were applied, whereas protocols were modified for control samples and data processing. To account for interplate measurement variations, standard DNA was located on each qPCR plate. In addition, a mathematical algorithm was implemented to correct for interassay differences in qPCR efficiency (supplemental Methods). As previously shown, 2 TM measurement by qPCR was highly reproducible (intra-assay and interassay coefficients of variation, 1.2% and 2.4%, respectively) and closely correlated with Southern blot–based mean telomere restriction fragments (TRF) assessment (supplemental Figure I). A brief comparison of both methods is given in the supplemental data.

Assessment of Atherosclerosis

Carotid atherosclerosis was assessed in 1995, 2000, and 2005 using a standardized ultrasonographic protocol involving scans of the internal (bulbous and distal segments) and common carotid (proximal and distal segments) arteries of either side. Atherosclerotic lesions were identified in B mode (10 MHz) based on wall thickening, wall surface irregularity, and plaque ulceration. The maximum axial diameter of plaques was measured on the near and far walls at each of 8 vessel segments. Follow-up evaluations allowed differentiation of 2 stages of atherosclerosis progression. (1) Early atherogenesis was defined by the presence of atherosclerotic lesions in segments previously free of atherosclerosis or the enlargement of nonstenotic lesions by a relative increase in the plaque diameter exceeding twice the measurement error of the method; it reflects conventional atherosclerosis processes. (2) Advanced atherogenesis was assumed when the plaque growth criterion was met and carotid stenosis of greater than 40% emerged or progressed; it reflects complicated atherosclerosis featured by plaque rupture and atherothrombosis. The 2 progression categories were highly reproducible (κ coefficients >0.8, n=100). The current analysis uses data from 1995 and 2000 because a 10-year focus would result in a substantial decline in sample size and statistical power. In addition, intima-media thickness (IMT) was measured in plaque-free sections of both common carotid arteries using standard methods, and the mean maximum IMT was calculated.

Clinical Evaluation

The composite CVD end point, hereinafter termed “new-onset CVD,” encompassed cases of myocardial infarction, stroke, and vascular death during follow-up (1995–2005). Fatal and nonfatal myocardial infarctions were defined confirmed when World Health Organization criteria for definite disease status were met. Ischemic stroke and transient ischemic attacks were classified according to the criteria of the National Survey of Stroke. Symptomatic peripheral artery disease and stable angina were diagnosed by a positive response to the Rose Questionnaire and a vascular nature of complaints confirmed by standard diagnostic procedures (ankle-brachial pressure index or angiography and exercise ECG or coronary angiography). Revascularization procedures (angioplasty and surgery) were carefully recorded. Deaths from myocardial infarction, sudden cardiac deaths, rupture of aortic aneurysms, or ischemic stroke were treated as vascular mortality. A family history of myocardial infarction was classified as positive if at least 1 male first-degree relative younger than 50 years or at least 1 female first-degree relative younger than 55 years had experienced a myocardial infarction. Corresponding age limits for family history of stroke were ages of 55 and 60 years, respectively. The ascertainment of clinical events and procedures did not only rely on patient self-reports or hospital discharge codes, but on a meticulous review of medical records provided by general practitioners, death certificates, Bruneck Hospital files, and the extensive clinical and laboratory examinations performed as part of the study protocols. The major advantages of the Bruneck Study are that virtually all subjects living in the Bruneck area were referred to 1 and the same local hospital and that the network existing between the local hospital and general practitioners allowed retrieval of practically all medical information on persons living in the area.

Methods

Study Subjects and Examination

The Bruneck Study is a prospective population-based survey on the epidemiology, pathophysiology, and prevention of cardiovascular and cerebrovascular diseases. The study protocol was approved by the pertinent ethics committee, and all participants gave their written informed consent. In 1990, 1000 individuals aged 40 to 79 years were randomly chosen from the inhabitants of Bruneck in South Tyrol, Italy, based on an age- and sex-stratified strategy (125 persons per sex and decade). The population of Bruneck is exclusively white and of heterogeneous geographic origin, with sizeable segments of Austro-German or Italian background. Population mobility within the survey area was low (0.2% per year). Follow-up examinations were performed in 1995, 2000, and 2005, with participation rates exceeding 90%. Analyses for the current investigation focused on the follow-up period between August to September 1995 and September to October 2005. Of 826 individuals who participated in the 1995 follow-up investigation, a total of 800 blood samples (96.9%) were available for TL assessment. Risk factors were recorded by validated standard procedures, as outlined in the supplemental Methods section (available online at http://atvb.ahajournals.org).

Laboratory Methods and TL Measurement

Venous blood samples were taken after an overnight fast and 12-hour abstinence from smoking and were immediately frozen or processed. All laboratory parameters were quantified by standard methods. DNA extraction was performed with a commercially available kit (Invisorb Blood Universal Kit) following standard procedures. Leukocyte TL was measured in quadruplicate using a quantitative polymerase chain reaction (qPCR) technique developed by Cawthon; this technique compares signals from the telomere repeat copy number (T) to a single-copy gene 36B4 copy number (S) and allows a relative T to S ratio to be calculated. Standard primers (tel1b:
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.

**Results**

The distribution of the T to S ratio in the general community and its relation to a mean telomere restriction fragment...
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,
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 loge-transformed T to S ratio was not associated with IMT (β=−0.2 [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-
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 atherogenesis (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 atherogenesis but emerged as strongly and independently associated with advanced atherogenesis, 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

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

**Figure 2.** Multivariable odds ratios of early and advanced atherogenesis (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.
vascularity. 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, such as impairment of endothelial repair and vessel remodeling, unfavorable changes in gene expression and cell phenotype, upregulation of immunoattractants (intercellular adhesion molecule-1 and vascular cell adhesion molecule-1) and prothrombotic molecules (plasminogen activator inhibitor-1), and matrix degeneration 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. 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). 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). 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. 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.

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 Study or Cardiovascular Health Study) or associations in subgroups only (Framingham Offspring Study). A small study 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. 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. 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. (2) In the Bruneck cohort, TL significantly correlated with family history of CVD. In line with a previous report, 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 reproducibility 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. 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.

References


