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Telomere shortening over 6 years is associated with increased subclinical carotid vascular damage and worse cardiovascular prognosis in the general population

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Abstract. Baragetti A, Palmen J, Garlaschelli K, Grigore L, Pellegatta F, Tragni E, Catapano AL, Humphries SE, Norata GD, Talmud PJ (Bassini Hospital, Cinisello Balsamo, Milan; Università degli Studi di Milano, Milan, Italy; University College London, London, UK; Istituto di Ricerca e Cura a Carattere Scientifico (IRCCS) – Multimedica Hospital, Milan, Italy; and Barts and The London School of Medicine and Dentistry Queen's Mary University, London, UK). Telomere shortening over 6 years is associated with increased subclinical carotid vascular damage and worse cardiovascular prognosis in the general population. *J Intern Med* 2015; **277**:478–487.

Introduction. Leucocyte telomere length (LTL) is an important determinant of telomere function and cellular replicative capacity. The aim of the present study was to examine prospectively the associations between telomere shortening (TS) and both the progression of atherosclerosis and the incidence of cardiovascular events (CVEs).

Materials and methods. Leucocyte telomere length was measured by quantitative polymerase chain reaction to determine the ratio of telomere length to single-copy gene (T/S) in 768 subjects (462 female and 306 male) enrolled in a large general population survey [the Progressione della Lesione Intimale Carotidea (PLIC study)]. Common carotid artery intima–media thickness was determined at baseline and after 6 years of follow-up, and the associations between TS and the progression of atherosclerosis and incidence of CVEs were evaluated.

Results. Mean LTL was 1.25 ± 0.92 T/S (median 1.14) at baseline and 0.70 ± 0.37 T/S (median 0.70) after 6 years of follow-up. Median 6-year LTL change was −0.46 T/S [interquartile range (IQR) −0.57 to 1.06], equating to −0.078 T/S [IQR (−0.092 to 0.176)] per year. Of note, telomere lengthening occurred in 30.4% of subjects. After adjustment for classical cardiovascular disease (CVD) risk factors (age, gender, smoking, physical activity, alcohol consumption, systolic blood pressure, glucose levels, lipid profile and therapies), TS was associated with incident subclinical carotid vascular damage [hazard ratio (HR) 5.19, 95% confidence interval (CI) 1.20–22.4, $P = 0.028$]. Finally, subjects in whom LTL shortened over time showed an increased risk of incident CVE, compared to those in whom LTL lengthened (HR 1.69, CI 1.02–2.78, $P = 0.041$).

Conclusion. These data indicate that TS is associated with increased risk of subclinical carotid vascular damage and increased incidence of CVEs beyond CVD risk factors in the general population, whereas LTL lengthening is protective.

Keywords: ageing, cardiovascular disease, intima–media thickness, leucocytes telomere length, telomere shortening.

Introduction

During DNA replication, DNA polymerase moves in a 5′ to 3′ direction and this leads to a progressive loss of nucleotides at the 5′ end of chromosomes, because of the removal of an RNA primer template for the enzyme [1–4]. This effect is partially dampened by telomeres, which are short repeat nucleotide sequences at the extremities of DNA strands,

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protecting chromosome ends from physiological and pathological degradation and attrition [1–3]. The ends of telomeres consist of a 3′ single-strand overhang which is susceptible to single-strand breaks, particularly due to oxidative damage, because of their G-rich content. These breaks lead to additional telomere loss during replication [5, 6], and therefore, telomere length is indicative of the replicative capacity and cumulative genomic damage of somatic cells, reflecting biological ageing.

Telomerase is a holoenzyme, which comprises two conserved components: TERT (the core active component) and TERC (or TR; the essential RNA component); together these are responsible for maintaining telomere length [1]. Telomere length is genetically determined [7] and heritable [8, 9]. In a large genome-wide association study, seven key loci, including TERT and TERC, associated with mean telomere length were identified, and variation at these loci was associated with risk of coronary artery disease (CAD) [10]. As part of the ageing process, telomere shortening (TS) also induces a p53-dependent alteration in mitochondrial function [11] in slow turnover tissues such as the brain, pancreas or cardiovascular system. When a critical shortening is reached, the cell enters apoptosis [12].

In cross-sectional analyses, lower leucocyte telomere length (LTL) was associated with increased risk of CAD independently of classical cardiovascular disease (CVD) risk factors [13, 14]. Patients with monogenic and polygenic forms of coronary heart disease (CHD) also present with shorter telomeres [15]. However, to date, no prospective data are available on the impact of LTL change (ΔLTL) over time, with respect to the incidence of vascular damage and cardiovascular events (CVEs) in the general population.

The aim of this study was to investigate prospectively the association between ΔLTL, by evaluation of repeat LTL measurements from the same subjects from baseline to year 6 of the study, and both subclinical carotid vascular damage, as defined by carotid intima–media thickness (cIMT), and CVEs in subjects enrolled in a large survey of the general population [16–18].

Materials and methods

Study population

The Progressione della Lesione Intimale Carotidea (PLIC) study is a large survey of the general population [19, 20] followed at the Center for the Study of Atherosclerosis, Bassini Hospital (Cinisello Balsamo, Milan, Italy). Of the 2141 subjects recruited to the study, 768, (462 women and 306 men; all Caucasians) were selected by probability sampling for further examination; the impact of telomere length on cardiovascular damage, as assessed by cIMT, was investigated over 6 years of follow-up in this longitudinal observational study. Informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. Medical history and information about current therapies were obtained for all participants, blood pressure (mmHg) was measured, and body mass index (kg m^{-2}) and waist/hip ratio were calculated. Blood samples were collected for the determination of lipid profile, glucose level, leucocyte count, the presence of creatininaemia and levels of hepatic enzymes [alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyltransferase (γGT)]. CVD risk and the presence of the metabolic syndrome were assessed, as previously described [21, 22], following determination of the Progetto Cuore individual risk score [23]. International guidelines were followed for the diagnosis of diabetes [24] and hypertension [25]. Lifestyle, smoking and dietary habits were recorded, as previously reported [17, 26, 27].

Study outcomes

The main clinical outcome of the study was the incidence of subclinical carotid vascular damage [defined as the progression of cIMT in the common carotid artery (CCA-IMT)] greater than the 75th percentile of the median CCA-IMT of a Caucasian general population, according to international reference values [28]. During 6 years of follow-up, the incidence of CVEs was determined through outpatient records, diagnosis and hospital discharge registries. CVEs were defined as coronary, peripheral and cerebrovascular events, such as ischaemic heart disease (stable and unstable angina and silent and acute myocardial infarction), stroke, transient cerebral ischaemic attack and acute coronary syndromes; major surgery (i.e. percutaneous coronary angioplasty, arterial angioplasty, arterial bypass of the lower limbs and coronary bypass) was also included in this definition. To examine the associations between LTL and both clinical parameters and cardiovascular prognosis, subjects were divided into two groups with regard to telomere variation over the 6-year period: (i) 'LTL shorteners', that is, those in whom LTL shortened;

and (ii) 'LTL lengtheners', that is, those in whom LTL lengthened.

Biochemical measurements

Blood and urine samples were collected after an overnight fast. After centrifugation at 18 ***g*** for 12 min, samples were stored at −80 °C. Serum levels of cardiometabolic markers [total cholesterol, HDL cholesterol, triglycerides, apolipoprotein (apo) B, apoA-I and glucose] as well as hepatic enzymes [ALT, AST, γGT and creatine phosphokinase (CPK)] were measured, and creatinine and uric acid levels were determined colorimetrically using the Cobas Mira Plus analyser (Horiba; ABX, Montpellier, France). The LDL cholesterol fraction was calculated using the Friedewald formula. Total leucocyte and subfraction counts were determined [22].

Carotid IMT assessment and incident subclinical carotid vascular damage

Common carotid artery IMT was assessed through ultrasound scanning and measurement of IMT in the carotid arteries by a single expert sonographer, blinded to the subject's identity. An 8-MHz transducer (Biosound 2000 II sa, Indianapolis, IN, USA) with axial and lateral resolutions of 0.385 and 0.500 mm, respectively, was used [26]. CCA-IMT was determined in the lateral projection at five standardized points (5, 10, 20, 25 and 30 mm from the bulb dilatation) in both arteries and averaged to calculate the mean cIMT for each subject. In two scans performed by the same operator in 75 subjects, the mean difference in cIMT was 0.005 ± 0.002 mm and the coefficient of variation (CV) was 1.93%. The correlation between two scans was significant ($r = 0.96$; $P < 0.0001$). The presence of plaques was verified in the bulb, bifurcation and internal and external branches [20]. CCA-IMT was weighted for age, and subclinical carotid vascular damage was defined as thickening over 6 years greater than the 75th percentile of the median cIMT value for a general Caucasian population according to the international guidelines of the American Society of Echocardiography [28]. The annual CCA-IMT change was normally distributed (Figure S1).

Leucocyte telomere length assessment

Leucocyte DNA was extracted by the salting out method [29]. LTL was measured in 768 individuals representative of the PLIC cohort (Mann–Whitney nonparametric *U*-test; see below) using a quantitative polymerase chain reaction (PCR)-based method [30], as adapted in our previous studies [31] and further modified as outlined below. The relative telomere length was calculated as the ratio of telomere repeats to a single-copy gene (SCG) value (T/S ratio). For each sample, the quantity of telomere repeats and the quantity of SCG copies were determined in comparison with a single reference sample. Each sample was measured in triplicate, and those in the top and bottom 20% with regard to length were rerun to validate the results. In addition, four control samples (the same samples throughout), and a negative control, (water) were used for each run. All experiments were performed in triplicate using the Rotor-Gene 6000 analyser (Corbett Research Ltd, Cambridge, UK), and the raw data were processed using comparative quantification analysis (ROTOR-GENE 6000 software; Corbett Research Ltd). The specificity of all amplifications was determined by melting curve analysis. All analyses were processed blinded to sample status. For each control, we calculated the mean over all the runs and divided the T/S value by the mean for that control. The four control samples within each run were then averaged to obtain the correction factor for that run. The interassay CV was 7.3%.

Statistical analysis

SPSS v.19.0 for Windows (IBM Corporation, Chicago, IL, USA) was used for all statistical analysis of data. Shapiro–Wilk test was performed to verify the normal distribution of linear variables. For variables normally distributed, *t*-test was used for comparison and the mean ± standard deviation (SD) is presented; for variables non-normally distributed, Mann–Whitney nonparametric *U*-test was performed and median and interquartile range (IQR) is presented. For dichotomous variables, chi-squared test and relative risk (95% confidence interval) assessment were performed. Spearman correlation coefficients (ρ) are presented for univariate correlations between linear variables; standardized regression coefficients (β) are shown when multiple stepwise regression models (including all the covariates independently associated with the independent variable at univariate analysis) were used. Kaplan–Meier curves were used to estimate the cumulative risk of CVEs in subjects with shorter and longer LTL compared to baseline (visit 1); log-rank test was used to compare the two curves. Then, Cox regression models were used to

estimate the association between TS and the major clinical outcomes, weighting up the burden of the different clinical risk factors and for follow-up. Test of collinearity was performed to verify the presence of redundant variables. Receiver operating characteristic (ROC) curves were used for the estimation of the predictive value of annual ΔLTL for the studied outcomes. ROC comparison was performed using the Hanley–McNeil method [32], taking into account the correlation between the area under the curve (AUC) values. Forest plots were used to determine hazard with GraphPad prism 5® for Windows® (GraphPad Software® Inc., La Jolla, CA, USA). For all analysis, statistically relevant differences were considered for $P < 0.05$.

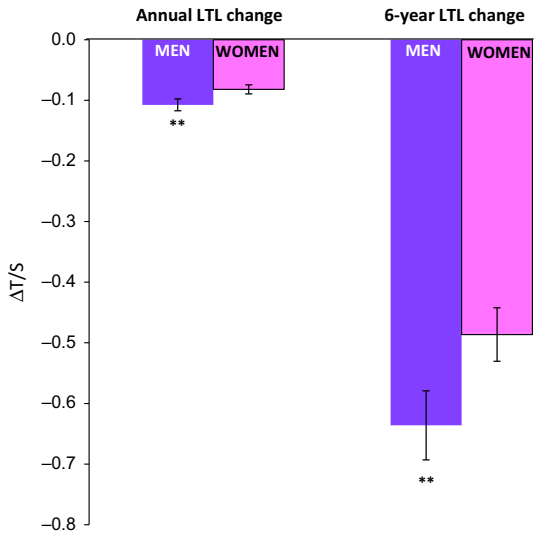

Fig. 1 *Leucocytes telomere length (LTL) change over the follow-up period. Pronounced annual and 6-year LTL changes were significantly higher in men (black bars) than in women (white bars) (**$P < 0.05$).*

Results

Leucocyte telomere length at randomization and ΔLTL after 6 years

The clinical and cardio-metabolic profile of 768 individuals from the PLIC study with repeat LTL measures is shown in Table S1. The selected sample was representative of the entire PLIC cohort (Mann–Whitney *U*-test and Kolmogorov–Smirnov nonparametric tests, data not shown). The median LTL of the population at visit 1 was 1.14 T/S (IQR 0.21–2.49). LTL at visit 1 was inversely correlated with age ($\rho = -0.119$, $P < 0.001$) (Figure S2) and was similar in women and men. After 6 years of follow-up, LTL was 0.70 (IQR 0.23–0.92) and median ΔLTL was −0.46 (IQR −0.57 to −1.06) and the median annual ΔLTL was −0.078 (IQR −0.092 to −0.176). In a regression model including age as an independent covariate, annual LTL shortening

Table 1 *Correlations between cardio-metabolic variables and both leucocyte telomere length (LTL) at baseline (visit 1) and LTL change in the selected 768 subjects*

	Telomere length at visit 1		LTL change (ΔT/S per year)	
	ρ	β	ρ	β
Age (years)	−0.138*	−0.057	0.021	−0.014
BMI (kg m^{-2})	0.014	−0.044	0.028	−0.022
Waist/hip ratio	0.094	0.003	0.086*	0.058
Glucose levels (mg dL^{-1})	0.050	−0.005	0.065	0.038
Systolic blood pressure (mmHg)	0.014	0.021	0.023	−0.026
Total cholesterol (mg dL^{-1})	−0.034	0.141	0.031	0.158
HDL cholesterol (mg dL^{-1})	−0.020	−0.156	−0.062	−0.125
LDL cholesterol (mg dL^{-1})	−0.034	0.129	0.036	0.145
Triglycerides (mg dL^{-1})	−0.019	−0.024	0.006	−0.057
apoA-I (mg dL^{-1})	−0.017	0.139	−0.036	−0.047
apoB (mg dL^{-1})	0.028	−0.180	0.024	−0.171

BMI, body mass index; apo, apolipoprotein; ΔT/S, change in ratio of telomere length to single-copy gene; ρ, univariate Spearman correlation coefficient; β, standardized regression coefficients, obtained from stepwise multiple linear regression models.

*$P < 0.05$. Test of collinearity was performed to evaluate the association between variables; no collinearity was found either between age and LTL at visit 1 or between age and LTL change (ΔT/S per year).

was greater in men than women (−0.107 ± 0.009 vs. −0.082 ± 0.007, $P = 0.039$; Fig. 1).

Leucocyte telomere length change and cardio-metabolic status

Next, we investigated the association between cardio-metabolic status and ΔLTL. Multiple linear stepwise regression analysis showed that age, but not other classical CVD risk factors, was independently associated with LTL (Table 1). Accordingly, annual ΔLTL was not independently associated with any classical CVD risk factor (Table 1). ΔLTL was increased in smokers; in addition to an increased ΔLTL in dyslipidaemic patients, LTL was longer (Table S2). LTL and ΔLTL values were similar in subjects with and without hypertension as well as in those with and without the metabolic syndrome (Table S2). Patients receiving antidiabetic, antihypertensive, hypolipidaemic or antithrombotic drugs showed similar ΔLTL, compared to untreated subjects (Figure S3).

ΔLTL is associated with increased subclinical carotid vascular damage

The association between ΔLTL and the incidence of subclinical carotid vascular damage was examined over the 6-year follow-up period. Both unadjusted and adjusted (age, gender, smoking, alcohol consumption, physical activity, blood pressure, glucose levels, lipid profile and therapies) higher annual ΔLTLs were independently associated with increased risk of incident subclinical carotid vascular damage [hazard ratio (HR) 5.19, (1.20–22.4) 95% CI, $P = 0.028$; Fig. 2 a]. Of note, this association was present in men [HR 8.72, (1.28–59.36) 95% CI, $P = 0.027$], but not in women (HR 0.69, 0.05–9.68, $P = 0.782$; Fig. 2 b,c). Compared to the model including only classical CVD risk factors, when annual ΔLTL was included, the ROC curve showed a significant increase in sensitivity and specificity [AUC 0.782, (0.731–0.834) 95% CI versus AUC 0.752, (0.698–0.807) 95% CI, $P = 0.041$; Fig. 3]. This further indicates that annual ΔLTL independently improves the predictive model for increased risk of subclinical carotid vascular damage. The findings were similar when overall ΔLTL was considered (data not shown).

Leucocyte telomere length lengthening is associated with reduced incidence of subclinical carotid vascular damage

About one-third (30.4%) of subjects were LTL lengtheners, maintaining a stable LTL or showing

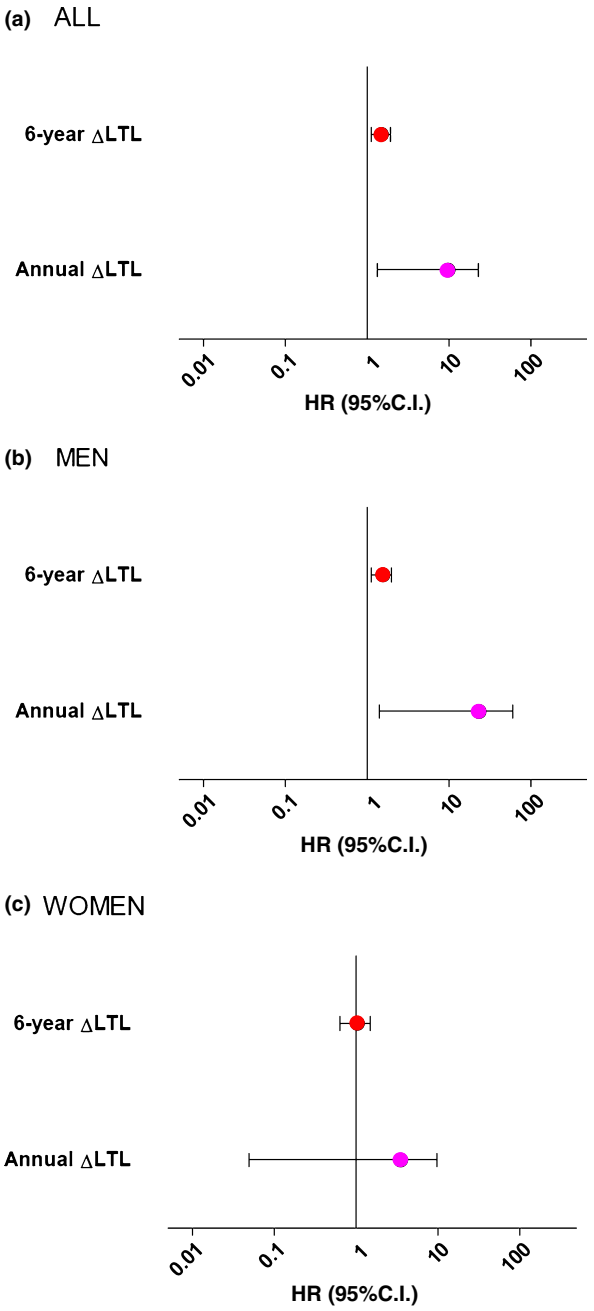

Fig. 2 *The risk of incident subclinical carotid vascular damage is shown for all subjects (a) and according to gender (b and c). The risk was calculated using a Cox regression model (adjusting for the follow-up period and for classical cardiovascular disease risk factors: age, gender, smoking, alcohol consumption, physical activity, blood pressure, levels of glucose, total cholesterol, HDL cholesterol and triglycerides and use of antihypertensive, antidiabetic and lipid-lowering drugs). Six-year change and annual change in leucocyte telomere length (ΔLTL) are shown. HR, hazard ratio; CI, confidence interval.*

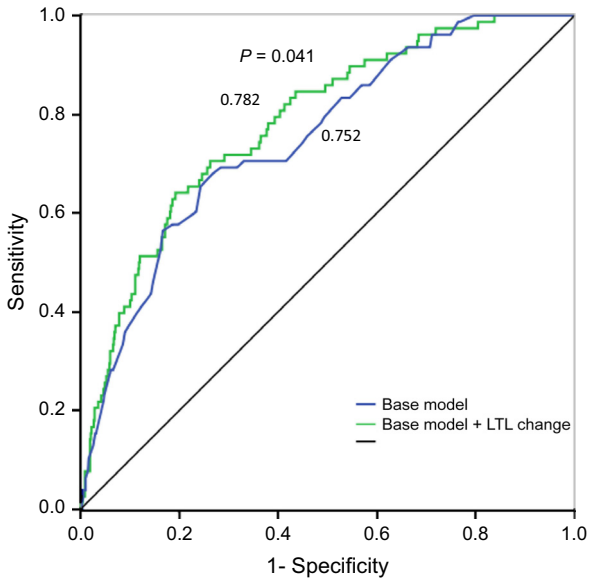

Fig. 3 *Receiver operating characteristic curves for the incidence of subclinical carotid vascular damage. Base model including classical cardiovascular disease risk factors (age, gender, smoking, alcohol consumption, physical activity, lipid profile, glucose levels and therapies) is shown (blue line). Inclusion in the model of change in leucocyte telomere length (LTL) together with the classical risk factors is shown (green line).*

telomere lengthening over 6 years of follow-up (Fig. 4). Compared to this group, the risk of incident subclinical carotid vascular damage was significantly higher amongst LTL shorteners (HR 1.12, 1.01–5.17, $P = 0.04$; Fig. 5 a). The increased risk remained statistically significant after adjustment for age, gender, smoking, alcohol consumption, physical activity, blood pressure, levels of glucose, total cholesterol, HDL cholesterol and triglycerides and use of antihypertensive, antidiabetic and lipid-lowering drugs (Fig. 5 a).

Leucocyte telomere length shortening is associated with increased cardiovascular morbidity

There was an increased incidence of CVEs amongst LTL shorteners compared to LTL lengtheners (HR 1.69, 1.02–2.78, $P = 0.04$ adjusted for age, gender and classical CVD risk factors; Fig. 5 b). CVE-free survival was significantly reduced in LTL shorteners during the 6-year follow-up period compared to LTL lengtheners (Fig. 6).

Discussion

Cross-sectional studies have shown an association between LTL and CVD [13, 14]. In a case–control study, age- and sex-adjusted mean terminal restriction fragment length, a measure of average telomere length, in leucocyte DNA of 203 patients with premature myocardial infarction (i.e. occurring below 50 years of age) was significantly shorter than that of 180 control subjects [13]. In the West of Scotland Primary Prevention Study, mean LTL at recruitment was a predictor of future

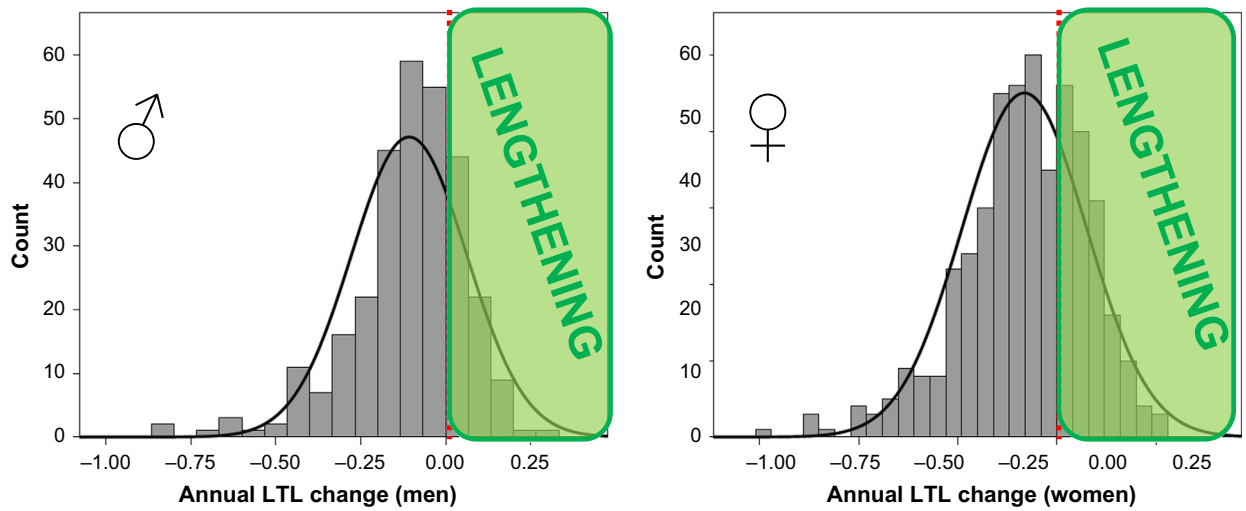

Fig. 4 *The 6-year change in leucocyte telomere length (LTL) according to gender. The green areas indicate the subjects in whom LTL lengthened over time.*

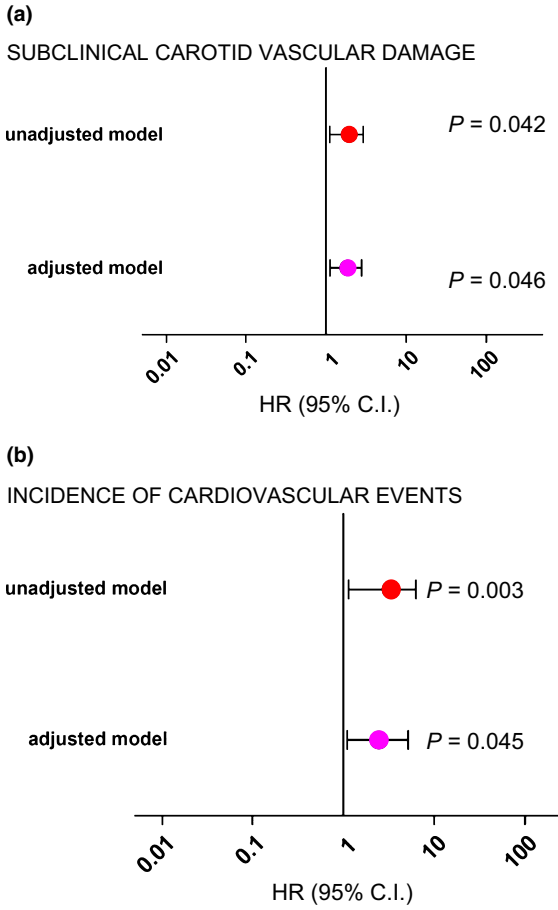

Fig. 5 *Risk (unadjusted and adjusted) of subclinical carotid vascular damage and of incidence of cardiovascular events during 6-years of follow-up in individuals in whom leucocyte telomere length (LTL) shortened compared to those with no change or LTL lengthening over time. Risks were calculated using a Cox regression model [adjusting only for the follow-up period, age and gender (unadjusted model) and further adjusting also for smoking, alcohol consumption, physical activity, blood pressure, levels of glucose, total cholesterol, HDL cholesterol and triglycerides and use of antihypertensive, antidiabetic and lipid-lowering drugs (adjusted model)]. HR, hazard ratio; CI, confidence interval.*

CHD events in middle-aged, high-risk men [14], whilst shorter LTL at baseline was associated with advanced vessel pathology and acute vascular syndromes in the Bruneck study [33]. Not all data, however, are consistent with the hypothesis that shorter LTL is associated with increased CVD risk, and LTL was associated with a modest or no increase in CHD risk in the general population in both the Canadian Nova Scotia Health Survey and

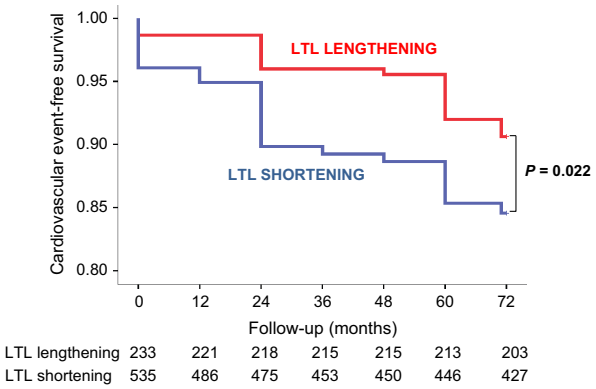

Fig. 6 *Kaplan–Meier event-free survival analysis for the incidence of cardiovascular events. Comparison between subjects with leucocyte telomere length (LTL) shortening and lengthening is shown (log-rank test).*

the Copenhagen General Population Study [34, 35]. These discrepancies could be the consequence of the single time-point analysis of LTL and support the need for longitudinal studies to provide estimations of telomere attrition rates and to further address the relation between telomere biology and CVD [36].

Here, we investigated, for the first time prospectively, the association between ΔLTL over 6 years and subclinical carotid vascular damage. LTL shortening was associated with increased incidence of subclinical carotid vascular damage in the general population. This observation in the total population was consistent with the result in men but not in women, in agreement with the finding that LTL is generally longer in women than in men [10, 37]. One possible explanation for this discrepancy is the ability of oestrogens to activate the telomerase promoter [38] and stimulate telomerase functionality through the phosphoinositol-3-kinase/Akt pathways [39] and favour p53-mediated DNA repair [40].

Another main finding of this study is that subjects in whom LTL remained unchanged, or lengthened over time, were protected from subclinical carotid vascular damage compared to LTL shorteners. Furthermore, a similar cardio-metabolic profile was observed in LTL lengtheners and shorteners. Telomerase acts preferentially on short telomeres *in vitro* and in mice [41–44]; similarly, humans with shorter LTL at enrolment more frequently presented with elongated LTL at follow-up [45–47], in agreement with the present findings (Figure S4).

Of note, it was previously shown that the induction of telomerase expression was able to rescue plaque vascular smooth muscle cell senescence, which was associated with accelerated oxidative stress-induced DNA damage and marked TS [48]. Here, we extend these findings, showing that LTL shortening is associated with subclinical carotid vascular damage.

We acknowledge the limitations of this study. First, LTL measurement is a highly sensitive technique; therefore, with an average CV of 7.3% (see Materials and methods), we cannot exclude the possibility that part of the variation in LTL might be related to the technique itself [49]. However, when the analysis was restricted to subjects within 1 SD (66%) of the overall ΔLTL distribution, increased LTL shortening remained associated with increased subclinical carotid vascular damage (data not shown). Secondly, we measured telomere length in leucocytes, and the findings from some studies have questioned the relevance of this measure in these cells with regard to vascular senescence. However, Wilson and colleagues found a strong correlation between LTL and telomere length in atherosclerotic plaques from patients with CAD [50].

In conclusion, we show for the first time an association between telomere length shortening and subclinical atherosclerosis in a large prospective study of CVD. By contrast, telomere lengthening is associated with reduced risk of CVD, independently of other classical CVD risk factors. Our data suggest the possibility that changes in LTL could act as a marker of vascular senescence. Further studies are warranted to address this issue.

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Conflict of interest statement

No conflict of interest to declared.

References

1 Armanios M, Blackburn EH. The telomere syndromes. *Nat Rev Genet* 2012; **13:** 693–704.
2 Blackburn EH. Telomeres and telomerase: the means to the end (Nobel lecture). *Angew Chem Int Ed Engl* 2010; **49:** 7405–21.
3 Blackburn EH, Greider CW, Szostak JW. Telomeres and telomerase: the path from maize, Tetrahymena and yeast to human cancer and aging. *Nat Med* 2006; **12:** 1133–8.
4 Armanios M. Telomeres and age-related disease: how telomere biology informs clinical paradigms. *J Clin Invest* 2013; **123:** 996–1002.
5 Petersen S, Saretzki G, von Zglinicki T. Preferential accumulation of single-stranded regions in telomeres of human fibroblasts. *Exp Cell Res* 1998; **239:** 152–60.
6 Serra V, Grune T, Sitte N, Saretzki G, von Zglinicki T. Telomere length as a marker of oxidative stress in primary human fibroblast cultures. *Ann N Y Acad Sci* 2000; **908:** 327–30.
7 Longhese MP, Anbalagan S, Martina M, Bonetti D. The role of shelterin in maintaining telomere integrity. *Front Biosci (Landmark Ed)* 2012; **17:** 1715–28.
8 Armanios M, Alder JK, Parry EM, Karim B, Strong MA, Greider CW. Short telomeres are sufficient to cause the degenerative defects associated with aging. *Am J Hum Genet* 2009; **85:** 823–32.
9 Goldman F, Bouarich R, Kulkarni S *et al.* The effect of TERC haploinsufficiency on the inheritance of telomere length. *Proc Natl Acad Sci USA* 2005; **102:** 17119–24.
10 Codd V, Nelson CP, Albrecht E *et al.* Identification of seven loci affecting mean telomere length and their association with disease. *Nat Genet* 2013; **45:** 422–7, 7e1–2.
11 Moslehi J, DePinho RA, Sahin E. Telomeres and mitochondria in the aging heart. *Circ Res* 2012; **110:** 1226–37.
12 Sahin E, DePinho RA. Axis of ageing: telomeres, p53 and mitochondria. *Nat Rev Mol Cell Biol* 2012; **13:** 397–404.
13 Brouilette S, Singh RK, Thompson JR, Goodall AH, Samani NJ. White cell telomere length and risk of premature myocardial infarction. *Arterioscler Thromb Vasc Biol* 2003; **23:** 842–6.
14 Brouilette SW, Moore JS, McMahon AD *et al.* Telomere length, risk of coronary heart disease, and statin treatment in the West of Scotland Primary Prevention Study: a nested case–control study. *Lancet* 2007; **369:** 107–14.
15 Maubaret CG, Salpea KD, Jain A *et al.* Telomeres are shorter in myocardial infarction patients compared to healthy subjects: correlation with environmental risk factors. *J Mol Med (Berl)* 2010; **88:** 785–94.
16 Norata GD, Garlaschelli K, Grigore L *et al.* Circulating soluble receptor for advanced glycation end products is inversely associated with body mass index and waist/hip ratio in the general population. *Nutr Metab Cardiovasc Dis* 2009; **19:** 129–34.
17 Norata GD, Garlaschelli K, Grigore L *et al.* Effects of PCSK9 variants on common carotid artery intima media thickness and relation to ApoE alleles. *Atherosclerosis* 2010; **208:** 177–82.
18 Predazzi IM, Norata GD, Vecchione L *et al.* Association between OLR1 K167N SNP and intima media thickness of the common carotid artery in the general population. *PLoS ONE* 2012; **7:** e31086.

19 Ammirati E, Cianflone D, Vecchio V *et al.* Effector memory T cells are associated with atherosclerosis in humans and animal models. *J Am Heart Assoc* 2012; **1:** 27–41.
20 Baragetti A, Knoflach M, Cuccovillo I *et al.* Pentraxin 3 (PTX3) plasma levels and carotid intima media thickness progression in the general population. *Nutr Metab Cardiovasc Dis* 2014; **24:** 518–23.
21 Norata GD, Ongari M, Garlaschelli K *et al.* Effect of the −420C/G variant of the resistin gene promoter on metabolic syndrome, obesity, myocardial infarction and kidney dysfunction. *J Intern Med* 2007; **262:** 104–12.
22 Baragetti A, Norata GD, Sarcina C *et al.* High density lipoprotein cholesterol levels are an independent predictor of the progression of chronic kidney disease. *J Intern Med* 2013; **274:** 252–62.
23 Giampaoli S, Palmieri L, Chiodini P *et al.* The global cardiovascular risk chart. *Ital Heart J Suppl* 2004; **5:** 177–85.
24 Basevi V, Di Mario S, Morciano C, Nonino F, Magrini N. Comment on: American Diabetes Association. Standards of medical care in diabetes–2011. *Diabetes Care* 2011; **34 (Suppl. 1):** S11–61. Diabetes Care 2011; 34: e53; author reply e4.
25 Mancia G, Fagard R, Narkiewicz K *et al.* 2013 ESH/ESC Guidelines for the management of arterial hypertension: the Task Force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *Eur Heart J* 2013; **34:** 2159–219.
26 Norata GD, Raselli S, Grigore L *et al.* Small dense LDL and VLDL predict common carotid artery IMT and elicit an inflammatory response in peripheral blood mononuclear and endothelial cells. *Atherosclerosis* 2009; **206:** 556–62.
27 Ammirati E, Bozzolo EP, Contri R *et al.* Cardiometabolic and immune factors associated with increased common carotid artery intima-media thickness and cardiovascular disease in patients with systemic lupus erythematosus. *Nutr Metab Cardiovasc Dis* 2014; **24:** 751–9.
28 Stein JH, Korcarz CE, Hurst RT *et al.* Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: a consensus statement from the American Society of Echocardiography Carotid Intima-Media Thickness Task Force. Endorsed by the Society for Vascular Medicine. *J Am Soc Echocardiogr* 2008; **21:** 93–111; quiz 89-90.
29 Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16:** 1215.
30 Cawthon RM. Telomere measurement by quantitative PCR. *Nucleic Acids Res* 2002; **30:** e47.
31 Salpea KD, Nicaud V, Tiret L, Talmud PJ, Humphries SE. The association of telomere length with paternal history of premature myocardial infarction in the European Atherosclerosis Research Study II. *J Mol Med (Berl)* 2008; **86:** 815–24.
32 Hanley JA, McNeil BJ. A method of comparing the areas under receiver operating characteristic curves derived from the same cases. *Radiology* 1983; **148:** 839–43.
33 Willeit P, Willeit J, Brandstatter A *et al.* Cellular aging reflected by leukocyte telomere length predicts advanced atherosclerosis and cardiovascular disease risk. *Arterioscler Thromb Vasc Biol* 2010; **30:** 1649–56.
34 Ye S, Shaffer JA, Kang MS *et al.* Relation between leukocyte telomere length and incident coronary heart disease events (from the 1995 Canadian Nova Scotia Health Survey). *Am J Cardiol* 2013; **111:** 962–7.
35 Weischer M, Bojesen SE, Cawthon RM, Freiberg JJ, Tybjaerg-Hansen A, Nordestgaard BG. Short telomere length, myocardial infarction, ischemic heart disease, and early death. *Arterioscler Thromb Vasc Biol* 2012; **32:** 822–9.
36 Nilsson PM, Tufvesson H, Leosdottir M, Melander O. Telomeres and cardiovascular disease risk: an update 2013. *Transl Res* 2013; **162:** 371–80.
37 Barrett EL, Richardson DS. Sex differences in telomeres and lifespan. *Aging Cell* 2011; **10:** 913–21.
38 Kyo S, Takakura M, Kanaya T *et al.* Estrogen activates telomerase. *Cancer Res* 1999; **59:** 5917–21.
39 Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* 2000; **407:** 538–41.
40 Sengupta S, Wasylyk B. Physiological and pathological consequences of the interactions of the p53 tumor suppressor with the glucocorticoid, androgen, and estrogen receptors. *Ann N Y Acad Sci* 2004; **1024:** 54–71.
41 Nordfjall K, Svenson U, Norrback KF, Adolfsson R, Lenner P, Roos G. The individual blood cell telomere attrition rate is telomere length dependent. *PLoS Genet* 2009; **5:** e1000375.
42 Farzaneh-Far R, Lin J, Epel E, Lapham K, Blackburn E, Whooley MA. Telomere length trajectory and its determinants in persons with coronary artery disease: longitudinal findings from the heart and soul study. *PLoS ONE* 2010; **5:** e8612.
43 Ehrlenbach S, Willeit P, Kiechl S *et al.* Influences on the reduction of relative telomere length over 10 years in the population-based Bruneck Study: introduction of a well-controlled high-throughput assay. *Int J Epidemiol* 2009; **38:** 1725–34.
44 Weischer M, Bojesen SE, Nordestgaard BG. Telomere shortening unrelated to smoking, body weight, physical activity, and alcohol intake: 4,576 general population individuals with repeat measurements 10 years apart. *PLoS Genet* 2014; **10:** e1004191.
45 Teixeira MT, Arneric M, Sperisen P, Lingner J. Telomere length homeostasis is achieved via a switch between telomerase-extendible and -nonextendible states. *Cell* 2004; **117:** 323–35.
46 Hemann MT, Strong MA, Hao LY, Greider CW. The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. *Cell* 2001; **107:** 67–77.
47 Bodnar AG, Ouellette M, Frolkis M *et al.* Extension of lifespan by introduction of telomerase into normal human cells. *Science* 1998; **279:** 349–52.
48 Matthews C, Gorenne I, Scott S *et al.* Vascular smooth muscle cells undergo telomere-based senescence in human atherosclerosis: effects of telomerase and oxidative stress. *Circ Res* 2006; **99:** 156–64.
49 Steenstrup T, Hjelmborg JV, Kark JD, Christensen K, Aviv A. The telomere lengthening conundrum–artifact or biology? *Nucleic Acids Res* 2013; **41:** e131.
50 Wilson WR, Herbert KE, Mistry Y *et al.* Blood leucocyte telomere DNA content predicts vascular telomere DNA content in humans with and without vascular disease. *Eur Heart J* 2008; **29:** 2689–94.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Distribution of the annual change in common carotid artery intima–media thickness (CCA-IMT) during 6 years of follow-up.

Figure S2. Correlation between age and leukocyte telomere length at baseline.

Figure S3. Effect of therapies on change in leukocyte telomere length (LTL)**.** Differences were compared with multiple stepwise linear regression models, including age, gender and cardiovascular disease risk factors (smoking, alcohol consumption, physical activity, blood pressure and levels of glucose, total cholesterol, HDL cholesterol and triglycerides). Six-year (left) and annual (right) LTL changes are shown. ΔT/S, change in ratio of telomere length to single copy gene. n.s., not significant.

Figure S4. Inverse correlation between leukocyte telomere length (LTL) at baseline (visit 1) and annual LTL change.

Table S1. Cardio-metabolic characteristics of the study population according to gender.

Table S2. Leukocyte telomere length (LTL) at baseline (visit 1) and change in LTL according to diagnosis of dyslipidaemia, hypertension, diabetes or the metabolic syndrome.

