Circulating Leukocyte and Carotid Atherosclerotic Plaque Telomere Length

Interrelation, Association With Plaque Characteristics, and Restenosis After Endarterectomy


Objective—Shorter leukocyte telomeres are associated with atherosclerosis and predict future heart disease. The goal of the present study was to determine whether leukocyte telomere length is related to atherosclerotic plaque telomere length and whether it is associated with plaque characteristics or recurrence of disease.

Methods and Results—Telomere length was measured by real-time quantitative polymerase chain reaction in atherosclerotic plaques and leukocytes in patients with carotid atherosclerosis undergoing carotid endarterectomy (n=684) and of leukocytes in age- and gender-balanced subjects without clinical atherosclerosis (n=780). Leukocyte telomere length was shorter in patients versus controls (0.99 [interquartile range (IQR): 0.79 to 1.26] versus 1.06 [0.80 to 1.39]; P=0.0007). Plaque telomeres were longer than leukocyte telomeres (1.42 [IQR: 1.21 to 1.77] versus 1.01 [IQR: 0.75 to 1.34]; P<1.00×10^{-5}) and independent of age. Leukocyte and plaque telomere length were only weakly correlated (correlation coefficient r^2=0.04, P=0.03). Patients, whose plaques showed marked macrophage infiltration and large lipid core, had longer plaque telomeres (1.61 [IQR: 1.32 to 2.04] versus 1.40 [IQR: 1.15 to 1.57]; P=0.006) and shorter leukocyte telomeres (0.88 [IQR: 0.75 to 1.20] versus 1.03 [IQR: 0.83 to 1.34]; P=0.02). Plaque telomere length was associated with restenosis 1 year after endarterectomy (OR 1.58±0.206; P=0.026 per SD decrease of plaque telomere length).

Conclusion—Leukocyte telomere length is associated with the presence of atherosclerotic carotid plaques but is not a proxy for local plaque telomere length. Plaque telomere length is related to plaque characteristics and development of restenosis following endarterectomy. (Arterioscler Thromb Vase Biol. 2011;31:1219-1225.)

Key Words: aging ■ atherosclerosis ■ restenosis ■ telomerase ■ telomere
studied the associations of telomere length with biochemical and histopathologic characteristics of disease. Previous research has shown that inflammatory, lipid-rich plaques on histopathologic examination were associated with a lower risk of restenosis, possibly because of remodeling and cell turnover induced by plaque remnants. We therefore also evaluated the relation between telomere length and recurrence of local disease, measured by carotid restenosis, after 1 year of follow-up.

**Methods**

**Design and Subjects**
We performed a case-control study to determine the association between circulating leukocyte telomere length and the presence of carotid artery stenosis. A detailed description is provided in the Supplemental Data, available online at http://atvb.ahajournals.org. In brief, subjects undergoing carotid endarterectomy (cases) were derived from the Athero-Express study (the Netherlands).

Age- and gender-balanced presumably healthy subjects (controls) were derived from the population based PREVEND study (the Netherlands). In cases, blood was drawn before surgery. In addition, plaques were harvested in cases during carotid endarterectomy, and plaque telomere length was compared intraindividually to that of circulating leukocytes.

Telomere length of both circulating leukocytes and plaques were studied in cases in relation to clinical, biochemical, and histopathologic characteristics of disease. Recurrence of disease and clinical outcome was evaluated by duplex follow-up at 1 year after endarterectomy to determine the presence of restenosis. Study protocols were approved by the appropriate institutional review boards and comply with the declaration of Helsinki. All subjects provided written informed consent.

**Atherosclerotic Plaque Characterization**
During surgery, atherosclerotic plaques were freshly harvested and divided into sections of 5 mm thickness. The segment with the greatest plaque burden was stained for histological examination with hematoxylin-eosin, CD-68 immunostain, α-actin, and picrosirius red. For details, see Supplemental Data.

**Telomere Length**
Telomere length was measured in triplicate using a real-time monochrome multiplex quantitative polymerase chain reaction method using a single-well strategy to measure telomere (T) relative to a single reference (S) signal based on the albumin gene. A single fluorescent DNA-intercalating dye (SYBR) was used to collect the T signals in early cycles, before S signal rises above baseline, and the S signals was collected at a temperature that fully melts the T product, sending its signal to baseline. The ratio of telomere and reference gene content (T/S ratio) is a relative measure of telomere length. All experimental DNA samples were assayed in triplicate. Seven concentrations of a reference DNA sample (standard) spanning a ∼12-fold range (5.2 to 60 ng) of DNA concentrations were prepared by serial dilution and analyzed in triplicate in every 384-well plate. Good linearity was observed across this range ($R^2=0.99$). Two wells received water as the no-template control, 2 wells were loaded with a human control sample, and 2 were loaded with DNA of a human leukemia cell line (1301) with extreme long telomeres (kindly provided by Dr Cesaro, L’Istituto Scientifico Tumori, Genova) as a positive/maximum control. For quality control, all samples were checked for concordance between triplicate values. The final coefficient of variation for the T amplicon was 1.52%, for the S amplicon it was 1.17%, and for T/S it was 3.3%. Reproducibility data were obtained for 216 subjects from PREVEND, and good agreement between T/S ratios, measured on different days, was observed ($r^2=0.99$, $P<0.0001$; interrun coefficient of variation, 3.9%). For details, see Supplemental Figure I and other supplemental material.

**Follow-Up**
The primary end point of the Athero-Express study was the occurrence of 50% or greater restenosis measured 1 year after intervention as determined by duplex ultrasound on the basis of the recommendations of the Society of Radiologists in Ultrasound. For details, see Supplemental Data.

**Statistical Methods**
Because of the skewed distribution, telomere length (T/S ratio) was log transformed. Controls were matched on age and gender with a propensity score matching algorithm (psmatch2) without other clinical, biochemical, or telomere length knowledge. Differences in patient characteristics between groups were tested by $t$ tests and the $\chi^2$ test when appropriate. A paired sample $t$ test was used to compare intraindividual plaque versus leukocyte telomere length. Pearson and standard linear regression techniques were used to associate telomere length with individual factors and to make adjustments. One primary clinical end point was defined: occurrence of ≥50% restenosis after 1 year. Logistic regression models were used to estimate the odds of experiencing the primary end point. Covariates that were introduced were univariately associated with restenosis (lipid core size and macrophage infiltration) or associated with plaque telomere length (diastolic blood pressure, diabetes). Analyses were performed using StataMP, version 10.1 (StataCorp) and SPSS, version 14.0 (SPSS Inc, Chicago, IL). A 2-sided probability value of $P<0.05$ was interpreted to indicate statistical significance.

An expanded description of the methods is provided in the Supplemental Data.

**Results**

**Population Characteristics**
The median age of patients with carotid atherosclerosis was 73 years (interquartile range [IQR]: 66 to 79), and for control subjects without clinical atherosclerosis, it was 72 years (IQR: 66 to 78). In both groups, 69% of subjects were male. Clinical and biochemical baseline characteristics of cases with carotid atherosclerosis (N=684) are presented in Table 1. For cases, 785 DNA samples were collected from atherosclerotic carotid plaques or leukocytes; from 101 subjects, both leukocyte and plaque samples were available. Clinical and biochemical characteristics for subgroups of cases are presented in Supplemental Table I. Detailed histological plaque characteristics are presented in Supplemental Table II.

**Circulating Leukocyte Telomere Length in Carotid Atherosclerosis and Controls**
Circulating leukocyte DNA was available from 390 cases. For each case, we selected 2 controls. A total of 780 presumably healthy age- and gender-balanced controls were included. Circulating leukocyte telomere length was considerably shorter in subjects with carotid atherosclerosis compared with controls (0.99 [IQR: 0.79 to 1.26] versus 1.06 [0.80 to 1.39]; $P=0.0007$ (Figure 1)). This difference was independent of age and gender. Leukocyte telomere length was associated with age ($\beta=-0.11$, $P=0.0002$). Addition of squared or cubed age terms was nonsignificant, indicating a linear relationship. In addition, adding an interaction term for age×case-control status was not significant ($P=0.216$), indicating a similar age-telomere length correlation in leukocytes for cases and controls.
Comparison of Leukocyte Telomere Length and Plaque Telomere Length

We observed a remarkably large difference between telomere lengths of circulating leukocytes compared with plaques for the total group (Figure 1). This did not change when we considered only the subgroup of which both telomere lengths were available (Supplemental Figure II). Median leukocyte telomere length was 1.01 (IQR: 0.75 to 1.34), and median plaque telomere length was 1.42 (IQR: 1.21 to 1.77), \( P = 0.007 \) (Figure 1). This difference was not explained by age and gender. To determine whether leukocyte telomere length is a reliable reflection of plaque tissue telomere length, we evaluated their pairwise correlation. We observed a positive, though weak, correlation (\( r = 0.210, P = 0.034 \)) (Figure 2). After we controlled for age and gender, the partial correlation coefficient remained similar (\( r = 0.215, P = 0.033 \)).

Telomere Length and Clinical and Biochemical Characteristics

Associations of leukocyte and plaque telomere length with clinical and biochemical characteristics for patients are presented in Table 1. In contrast with leukocyte telomere length, atherosclerotic plaque telomere length was not associated with age (Figure 3). Male patients tended to have shorter telomeres, although this did not reach statistical significance in leukocytes. Patients with diabetes had shorter plaque telomere length compared with nondiabetic patients (median T/S ratio: 1.32 [IQR: 1.10 to 1.48] versus 1.41 [IQR: 1.19 to 1.49], \( P = 0.002 \)).

![Figure 1](http://arch.ahajournals.org/)

**Figure 1.** Leukocyte and plaque telomere length in cases and controls. Shown are mean leukocyte and plaque telomere length in carotid artery stenosis cases and leukocyte telomere length in healthy controls. Whiskers represent 95% confidence intervals.

![Figure 2](http://arch.ahajournals.org/)

**Figure 2.** Correlation between leukocyte and plaque telomere length within the same subject, for 101 subjects for whom both measurements were available. Partial correlation coefficient controlling for age and gender = 0.215, \( P = 0.033 \).
Finally, plaque telomere length was associated with diastolic blood pressure. These associations persisted after adjustment for age and gender; the point estimate (95% confidence interval) was as follows: for male gender, −0.067 (−0.118 to −0.016), \( P = 0.01 \); for diabetes, −0.063 (−0.121 to −0.005), \( P = 0.032 \); and for diastolic blood pressure, 0.002 (0.001 to 0.004), \( P = 0.038 \).

Clinical presentation of the patient was not associated with leukocyte or plaque telomere length (see Supplemental Table III). Between asymptomatic and symptomatic patients, there was also no difference in leukocyte telomere length (median T/S ratio: 1.06 [IQR: 0.82 to 1.30] versus 0.98 [0.79 to 1.26], \( P = 0.52 \)) or plaque telomere length (1.39 [1.09 to 1.67] versus 1.38 [1.18 to 1.65], \( P = 0.26 \)).

**Telomere Length and Histopathologic Characteristics**

When analyzing plaque content, we noticed that plaque telomere length was shorter in restenosis-prone noninflammatory, fibrous plaques compared with the more restenosis resistant inflammatory atheromatous plaques: 1.40 (IQR: 1.15 to 1.57) versus 1.61 (IQR: 1.32 to 2.04), \( P = 0.006 \) (Table 2). However, telomere length in leukocytes of subjects with inflammatory atheromatous plaques were shorter compared with the noninflammatory fibrous plaques: 0.88 (IQR: 0.75 to 1.20) versus 1.03 (IQR: 0.83 to 1.34), \( P = 0.022 \) (Table 2).

Adjustment for age, gender, blood pressure, and diabetes did not change these findings (Figure 4). Plaque collagen or smooth muscle cell content was not associated with telomere length.

**Telomere Length and Incidence of Restenosis at 1 Year**

The incidence of restenosis of 50% or greater 1 year after carotid endarterectomy was evaluated with duplex ultrasound, and the overall restenosis incidence in our study was 17%. A plaque telomere length that was 1 SD shorter was associated with a 58% increased restenosis risk (\( P = 0.026 \); Table 3). This observation persisted after adjustment for age, gender, and other covariates associated with plaque telomere length (diabetes and diastolic blood pressure) or occurrence of restenosis (lipid core size and macrophage infiltration; \( P = 0.038 \), Table 3). Circulating leukocyte telomere length was not an independent predictor of carotid restenosis at 1 year (Supplemental Table IV).

### Table 2. Histopathological Plaque Characteristics and Telomere Length

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Leukocyte Telomere Length</th>
<th>Plaque Telomere Length</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Median T/S</td>
</tr>
<tr>
<td>Lipid inflammation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noninflammatory fibrous</td>
<td>330</td>
<td>1.03</td>
</tr>
<tr>
<td>Inflammatory atheromatous</td>
<td>61</td>
<td>0.88</td>
</tr>
<tr>
<td>Smooth muscle cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None/minor</td>
<td>115</td>
<td>0.96</td>
</tr>
<tr>
<td>Moderate/heavy</td>
<td>276</td>
<td>1.02</td>
</tr>
<tr>
<td>Collagen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None/minor</td>
<td>80</td>
<td>0.92</td>
</tr>
<tr>
<td>Moderate/heavy</td>
<td>311</td>
<td>1.02</td>
</tr>
</tbody>
</table>
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Discussion

In the current study, we observed that leukocyte telomere length in subjects with carotid atherosclerosis is shorter compared with age- and gender-balanced controls without clinical atherosclerosis. Previous research already reported no association between leukocyte telomere length and preclinical atherosclerosis, whereas the association between telomere length and clinically significant atherosclerosis is well established. We now confirm this association specifically in carotid atherosclerosis. In contrast to leukocyte telomere length, little is known about the potential differences in telomere length of cells originating from different tissues. We therefore measured telomere length in circulating leukocytes, as well as in atherosclerotic plaques, and made 4 important observations. First, we showed that telomeres in atherosclerotic plaques are considerably longer than those in circulating leukocytes. Second, in contrast to leukocyte telomere length, plaque telomere length was not associated with age. Third, although we did observe a statistically significant correlation between the 2 samples obtained from the same individual, the correlation was weak and explained less than 5% of the variance. Finally, we showed that leukocyte and plaque telomere length are differently related to atherosclerotic plaque characteristics.

In contrast to the study by Wilson et al., who reported an r ranging from 0.44 in subjects with asymptomatic abdominal aortic aneurysms to 0.68 in normal aortas, we found only a weak correlation between leukocyte and tissue telomere length. The difference is not likely to arise from telomere length measurement method, as both our study and that of Wilson et al. used quantitative polymerase chain reaction. Also, the age of the studied subjects was fairly similar. It is possible that the severity of local vascular disease might obscure the correlation with blood leukocyte telomere length. Wilson et al reported the strongest correlation in vessels from normal aortas of subjects who experienced intracerebral hemorrhage. The correlation in asymptomatic vascular disease was less strong. In our population, consisting of subjects with significant and symptomatic carotid atherosclerosis, the correlation observed was weak. In addition, our sample size was considerably larger that that of Wilson et al., who studied 32 vessel wall and blood leukocyte pairs.

The weak correlation that we observed suggests that telomere length in 1 cell type does not necessarily provide a good surrogate for the relative telomere length in other tissues. This implies that the extrapolation of telomere length associations observed in leukocytes to that of the atherosclerotic vessel merits careful consideration. There are several potential explanations for the absence of a strong association between leukocyte and plaque telomere length. We not only have to realize that both samples consist of a heterogeneous population of cells; we also need to appreciate the difference in replicative history of leukocytes compared with vessel wall derived cells. The replicative history might be very different as the cells circulating leukocytes originate from are thought to divide far more frequently than vascular cells. Also specifically in carotid atherosclerotic plaques, a very long turnover time has recently been reported. Furthermore, telomere length can also be modified by the telomere elongating enzyme telomerase. There is evidence that the activity of this enzyme differs between different types of tissue and in different situations. In healthy rat carotid arteries, telomerase activity is barely detectable. However, after balloon injury there is a 10-fold increase of telomerase activity. In patients with unstable angina, inflammatory cells present in the coronary artery plaque express considerably higher telomerase activity compared with circulating leukocytes. Local leukocyte telomerase reactivation in plaques is thought to prolong the lifespan of inflammatory cells, which might make it possible to maintain the inflammatory response. This phenomenon could be an explanation for the longer plaque telomeres we found in inflammatory lipid-rich plaques, as well as for the lack of correlation of plaque telomere length with age. These telomere length modifying factors and their interplay together could be held responsible for the seemingly paradoxical difference in telomere biology we observed between leukocyte and plaque tissue. Unfortunately, we did not have live cells available from the Athero-Express study to measure telomerase activity.

Recently, data from the Athero-Express study showed that the marked presence of inflammation and lipid-rich plaques

Table 3. Logistic Regression Analysis for 50% or Greater Restenosis (Primary End Point) for Plaque Telomere Length

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Univariate</th>
<th>P</th>
<th>Full Adjusted Model</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque telomere length, per SD shorter</td>
<td>1.58 (1.06 to 2.37)</td>
<td>0.026</td>
<td>1.65 (1.03 to 2.65)</td>
<td>0.038</td>
</tr>
<tr>
<td>Age, per 10 years</td>
<td>0.81 (0.62 to 1.06)</td>
<td>0.126</td>
<td>0.68 (0.41 to 1.11)</td>
<td>0.125</td>
</tr>
<tr>
<td>Male gender</td>
<td>1.22 (0.74 to 2.00)</td>
<td>0.433</td>
<td>0.99 (0.36 to 2.73)</td>
<td>0.978</td>
</tr>
<tr>
<td>Lipid core size*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% to 40%</td>
<td>0.60 (0.34 to 1.04)</td>
<td>0.070</td>
<td>0.30 (0.10 to 0.88)</td>
<td>0.028</td>
</tr>
<tr>
<td>&gt;40%</td>
<td>0.32 (0.17 to 0.62)</td>
<td>0.010</td>
<td>0.32 (0.09 to 1.13)</td>
<td>0.077</td>
</tr>
<tr>
<td>Macrophage infiltration</td>
<td>0.44 (0.27 to 0.70)</td>
<td>0.010</td>
<td>0.49 (0.19 to 1.27)</td>
<td>0.142</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>1.01 (0.99 to 1.03)</td>
<td>0.590</td>
<td>1.02 (0.98 to 1.06)</td>
<td>0.361</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0.93 (0.52 to 1.65)</td>
<td>0.810</td>
<td>1.36 (0.44 to 4.18)</td>
<td>0.597</td>
</tr>
</tbody>
</table>

*Compared with <10%.
reduced risk of restenosis during follow-up. With the current study, we add the observation that these plaque characteristics are also related to telomere length, but differently for leukocytes compared with plaque tissue. Circulating leukocyte telomere length was strongly associated with the presence of carotid atherosclerosis in the current study. However, among patients experiencing carotid atherosclerosis and undergoing endarterectomy, we did not find an association between leukocyte telomere length and restenosis at 1 year. This might indicate that the involvement of circulating leukocyte telomere length is more relevant early in the process of atherosclerosis and that local processes are more relevant in the restenosis process. In previous studies, it has been suggested that the plaque composition at baseline is related to the dynamic vascular remodeling process after the endarterectomy procedure. Inflammation and protease activity in the media and adventitia (the layers remaining after endarterectomy) might give rise to production of matrix metalloproteinases, which leads to thinning of the media and adventitia (the layers remaining after endarterectomy) might give rise to production of matrix metalloproteininas, which leads to thinning of the media and adventitia (the layers remaining after endarterectomy) might give rise to production of matrix metalloproteinases, which leads to thinning of the media and adventitia (the layers remaining after endarterectomy) might give rise to production of matrix metalloproteinases, which leads to thinning of the media and adventitia. This could be related to decreased media thinning and remodeling and therefore a higher risk of restenosis. These are all indicators that we should not use telomere length of circulating leukocytes as a proxy for that of the plaque, or more generally, target tissue of interest.

Telomere length of leukocytes was shorter in subjects with carotid atherosclerosis and associated with age. Interestingly, in cases with atherosclerosis, plaque telomere lengths were similar among different ages. It is tempting to speculate that a subject becomes a case at a certain threshold determined by his or her biological age as estimated by leukocyte telomere length and possibly unrelated to their date of birth.

There are some limitations to this study. Because our control cohort had a median age of 72 and its members were not specifically screened for presence or absence of carotid atherosclerosis, subclinical atherosclerosis is to be expected to be omnipresent. Consequently, the observed difference between cases and controls might be an underestimation of the true difference between cases and truly healthy subjects.

Also, the use of flow velocity in the definition of restenosis could be considered a limitation as these criteria are based on consensus not commonly accepted and might have their limitations in categorizing stenosis. Finally, we studied mean telomere length of DNA isolated from the overall leukocyte population and the overall cell population of the plaque. We cannot exclude the possibility that telomere length of the different cell populations within leukocytes or tissue is different. Plaque tissue harvested consisted mainly of intima, though traces of media cannot be excluded. A future major methodological challenge remains to analyze the individual component of the plaques (eg, with laser dissection techniques or magnetic beads), which will provide additional insights.

In conclusion, in contrast to what has previously been suggested, we have demonstrated that telomere length of circulating leukocytes does not provide a good surrogate for telomere length of the atherosclerotic plaque. Circulating leukocyte and atherosclerotic plaque telomere lengths are associated differently with plaque composition. Restenosis at 1 year appears to be associated with plaque telomere length. The current findings justify further research to determine differences among cells characterized in more detail.

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Disclosures
None.

References


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Circulating leukocyte and carotid atherosclerotic plaque telomere length; interrelation, association with plaque characteristics and restenosis after endarterectomy

J. Huzen, W. Peeters, et al.

Study Design and Population

To determine the association between circulating leukocyte telomere length and carotid artery stenosis we performed a case-control study. Cases were subjects undergoing carotid endarterectomy. Cases were derived from the *Athero-Express* study, of which the design has been reported in detail previously.\(^1\)\(^2\) The criteria to perform carotid endarterectomy were based on the recommendations by the Asymptomatic Carotid Atherosclerosis Study and Asymptomatic Carotid Surgery Trial studies for asymptomatic patients and the North American Symptomatic Carotid Endarterectomy Trial and European Carotid Surgery Trial studies for symptomatic patients.\(^2\) In brief, blood and carotid plaques of patients undergoing primary carotid artery endarterectomy were collected. After one year patients underwent duplex follow-up to determine target vessel patency. The primary endpoint of the study was the presence of restenosis which was defined as > 50% restenosis of the operated carotid artery after one year.

Presumably healthy subjects were derived from the population based *Prevention of REnal and Vascular ENd stage Disease* (PREVEND)\(^3\) study, an ongoing prospective study investigating the natural course of increased levels of urinary albumin excretion and its relation to renal and cardiovascular disease in a large cohort drawn from the general population. Details of this protocol have been described elsewhere.\(^3\)\(^4\) The PREVEND study includes a total of 8,592 subjects. To account for major differences in age and gender, we selected 2 age and gender balanced controls for each case using the psmatch2 algorithm\(^5\) (STATA).

Differences between telomere lengths of cells derived from circulating leukocytes were compared with cells derived from plaques in subjects undergoing carotid endarterectomy and of whom both samples were available (within subject comparison).
Finally, in cases telomere lengths of both circulating leukocytes and plaques were studied in relation to clinical, biochemical and histopathological characteristics.

Clinical outcome was evaluated by duplex follow-up at 1 year after endarterectomy to determine the presence of restenosis.

**Atherosclerotic Plaque Characterization**

During surgery, directly after excision, the atherosclerotic plaque specimens were taken to the laboratory. Plaques were divided in segments of 5-mm thickness along the longitudinal axis. The thickest plaque segment was fixed in formalin for immunohistochemical staining and remaining parts were freshly snap frozen to study protease activity and future protein and RNA expressions. The segment with the greatest plaque burden area was defined as the culprit lesion and used for histological examination.1 Semiquantitative scores were made for macrophage infiltration using CD-68, smooth muscle cell infiltration with alpha-actin, the amount of collagen with Picro-Sirius Red and the lipid core size with hematoxylin and eosin and Picro-Sirius Red stains. The scores for collagen and smooth muscle cells were composed as follows: 1: none or minor, 2: moderate or heavy staining. Inflammatory atheromatous plaques were categorized as follows: 1: no or minor macrophage infiltration and lipid core <40%, 2: moderate or heavy macrophage infiltration and lipid core >40%.

Histological observations were made blinded for clinical data and performed by 2 independent observers. The inter-rater and intra-rater reproducibility was assessed in 100 specimens. Briefly, 100 specimens were assessed by 2 independent observers and the ratings of both observers were compared with $\kappa$ statistics. To assess intra-observer reproducibility, the second observer reassessed the specimens 2 months afterwards with blinding for the previous assessments of the plaques. Both inter-observer and intra-observer reproducibility were found to be excellent ($\kappa = 0.6-0.9$).6

**Telomere length measurements**

Mean telomere length was measured with the recently modified QPCR protocol using a single well strategy to measure the telomere (T) and single reference (S) signal.7 All experimental DNA samples were assayed in triplicate.7,8 The ratio of telomere and reference gene content (T/S ratio) is a
relative measure of telomere length. PCR reactions were set up by aliquoting 8μL of master mix into each well reaction of a 384-well plate compatible with our Bio-Rad CFX384 real-time system on a C1000 thermal cycler, followed by addition of 2μl DNA (~20ng), for a final volume of 10μL per reaction. Seven concentrations of a reference DNA sample (standard) spanning a ~12-fold range (5.2 to 60 ng) of DNA concentrations were prepared by serial dilution and analyzed in triplicate in every 384-well plate. Good linearity was observed across this range (R² = 0.99). Two wells received water as the no template control (NTC), two wells were loaded with a human control sample and two with DNA of a human leukemia cell line (1301) with extreme long telomeres (kindly provided by dr. Cesaro, IST, Genova) as a positive/max control. The final concentrations of reagents in the PCR were 1U Titanium Taq DNA polymerase with the provided Titanium Taq PCR buffer, 0.75xSYBR Green I (Sigma), 0.2 mM of each dNTP, 1 mM DTT, 1M betaine, 900nM of each telomere primers (Telg and Telc), 900nM of each albumin (Albu and Albd). The primers were; telomere, telg, ACACTAAGGTTTGTTGTTTGGGTTTGGGTTTGGGTTAGTGT and tele, TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA, that generate a short, fixed-length product (for a further explanation and details see Cawthon 2009). The albumin primers were albu: CGGCAGCGGCGCGGCGCGGCGCGCTGGGGCGGaagtcgacagaatcttg albd: GCCCGGGCCCGCGCGCCGTCCCGC CGGCGGaagagca tgcgtgctt. The predicted product size is 106 bp. Capitalized bases of the albumin primers are non-template 5’ tag sequences that confer a high melting temperature on resulting PCR product (for a further explanation and details see Cawthon 2009). The thermal cycling profile was Stage 1: 15 min at 95°C; Stage 2: 2 cycles of 15 s at 94°C, 15 s at 49°C; Stage 3: 5 cycles of 15 s at 94°C, 15 s at 66°C; Stage 4: 32 cycles of 15 s at 94°C, 10 s at 60°C, 15 s at 72°C with signal acquisition, 10 s at 85°C, and 15 s at 89°C with signal acquisition. Stage 5; for QC a final dissociation stage was performed from 60°C to 95°C in steps of 0.05 s. At stage 4; the 72°C reads provide Ct values for the amplification of the telomere template (in early cycles when the S signal is still at baseline); the 89°C reads provided the Ct values for the amplification of the S template (at this temperature there is no signal from the telomere PCR product, because it is fully melted). For the reference DNA sample, each DNA concentration the Ct for albumin
occurred ~ 7.2 cycles later in cycling than the Ct for the telomere. The Bio-Rad CFX manager software was used to generate two standard curves for each plate as previously described. For quality control all samples were checked for concordance between triplicate values. The final coefficient of variation for the T amplicon was 1.52%, and for the S amplicon 1.17% and for T/S 3.3%. Reproducibility data was obtained for 216 subjects from PREVEND and good agreement between T/S ratios, measured on different days, was observed (r²=0.99, P<0.0001, inter-run CV 3.9%). See figure II.

**Follow-up**

Clinical outcome was evaluated by duplex follow-up at 1 year after endarterectomy to determine the presence of restenosis (defined as >50% restenosis). Patients underwent follow-up with duplex ultrasound (Philips Medical Systems, Eindhoven, the Netherlands). The definition of occurrence of 50% or greater restenosis was defined as a peak systolic velocity of at least 125 cm/s at the ipsilateral bifurcation. Duplex measurements were performed by investigators who were blinded for data regarding plaque phenotype and baseline characteristics.
Supplementary References


Table I. Clinical and biochemical baseline characteristics of cases divided for availability of DNA (plaque, circulating leukocytes, both).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Plaque only Telomere Group (n= 291)</th>
<th>Leukocyte only Telomere Group (n=292)</th>
<th>Combined Group (n= 101)</th>
<th>p-value (*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>73.0 (66.0 – 80.0)</td>
<td>73.5 (67.0 - 79.0)</td>
<td>73.0 (66.0 – 80.0)</td>
<td>0.846</td>
</tr>
<tr>
<td>Females (%)</td>
<td>30.6</td>
<td>31.5</td>
<td>31.7</td>
<td>0.440</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>22.8</td>
<td>24.1</td>
<td>19.8</td>
<td>0.390</td>
</tr>
<tr>
<td>Antihypertensives use (%)</td>
<td>91.1</td>
<td>90.1</td>
<td>89.2</td>
<td>0.420</td>
</tr>
<tr>
<td>Statins use (%)</td>
<td>79.7</td>
<td>72.7</td>
<td>73.3</td>
<td>0.022</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>27.0</td>
<td>29.7</td>
<td>23.0</td>
<td>0.272</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>154 (140 – 170)</td>
<td>155 (140 – 172)</td>
<td>160 (140 – 175)</td>
<td>0.176</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>83 (75 – 90)</td>
<td>80 (75 – 90)</td>
<td>80 (75 – 90)</td>
<td>0.160</td>
</tr>
<tr>
<td>Ln Hs-CRP (mg/dL) †</td>
<td>3.34 (1.68 – 5.96)</td>
<td>3.30 (1.34 – 7.73)</td>
<td>3.2 (1.31 - 5.60)</td>
<td>0.405</td>
</tr>
<tr>
<td>Hemoglobin (mmol/L)</td>
<td>8.7 (8.1 – 9.3)</td>
<td>8.7 (8.0 – 9.2)</td>
<td>8.8 (8.2 – 9.4)</td>
<td>0.660</td>
</tr>
<tr>
<td>Lipids (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- HDL cholesterol ‡</td>
<td>0.94 (0.72 – 1.36)</td>
<td>1.06 (0.88 – 1.33)</td>
<td>1.11 (0.92 - 1.33)</td>
<td>0.001</td>
</tr>
<tr>
<td>- LDL cholesterol §</td>
<td>2.37 (1.92 – 3.05)</td>
<td>2.70 (2.12 – 3.39)</td>
<td>2.53 (2.06 – 3.21)</td>
<td>0.432</td>
</tr>
<tr>
<td>- Triglycerides</td>
<td>1.23 (0.97 – 1.89)</td>
<td>1.53 (1.07 – 2.10)</td>
<td>1.14 (0.90 – 1.66)</td>
<td>0.871</td>
</tr>
</tbody>
</table>

(*) p-value for difference between leukocyte and plaque group. † High sensitivity C-reactive protein, ‡ High Density Lipoprotein cholesterol, § Low Density Lipoprotein cholesterol. Presented are the median (interquartile range) values or percentages (%).
**Table II.** Histopathological characterisation of cases

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Plaque only Group (n= 291)</th>
<th>Leukocyte only Group (n=292)</th>
<th>Combined Group (n= 101)</th>
<th>p-value (*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-inflammatory fibrous</td>
<td>78.4</td>
<td>84.4</td>
<td>94.4</td>
<td>0.156</td>
</tr>
<tr>
<td>Inflammatory atheromatous</td>
<td>21.6</td>
<td>15.6</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td>None or minor smooth muscle cells</td>
<td>30.0</td>
<td>28.1</td>
<td>31.0</td>
<td>0.286</td>
</tr>
<tr>
<td>Moderate or heavy smooth muscle cells</td>
<td>70.0</td>
<td>71.9</td>
<td>69.0</td>
<td></td>
</tr>
<tr>
<td>None or minor collagen</td>
<td>20.9</td>
<td>16.4</td>
<td>17.0</td>
<td>0.042</td>
</tr>
<tr>
<td>Moderate or heavy collagen</td>
<td>79.1</td>
<td>83.6</td>
<td>83.0</td>
<td></td>
</tr>
</tbody>
</table>

(*) p-value for difference between leukocyte and plaque group. Presented are the percentages.
Table III. Clinical presentation and telomere length

<table>
<thead>
<tr>
<th></th>
<th>Leukocyte</th>
<th></th>
<th>Plaque</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Median T/S</td>
<td>IQR</td>
<td>n</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>67</td>
<td>1.06</td>
<td>0.82 - 1.30</td>
<td>47</td>
</tr>
<tr>
<td>TIA</td>
<td>152</td>
<td>0.98</td>
<td>0.76 – 1.29</td>
<td>185</td>
</tr>
<tr>
<td>CVA</td>
<td>100</td>
<td>1.01</td>
<td>0.79 – 1.26</td>
<td>98</td>
</tr>
<tr>
<td>Amaurosis fugax</td>
<td>46</td>
<td>0.98</td>
<td>0.86 – 1.20</td>
<td>49</td>
</tr>
<tr>
<td>Other</td>
<td>27</td>
<td>0.91</td>
<td>0.79 - 1.25</td>
<td>14</td>
</tr>
</tbody>
</table>

TIA: transient ischemic attack; CVA: cerebrovascular event; IQR: inter quartile range.
P-values for differences among groups for leukocyte telomere length: p=0.93 and for plaque telomere length: p=0.23.
Table IV. logistic regression on 50% or greater restenosis (Primary endpoint) for circulating leukocyte telomere length

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Restenosis &gt; 50%</th>
<th>OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1 Normalised Leukocyte Telomere Length</td>
<td>1.020 (0.764-1.361)</td>
<td>0.894</td>
<td></td>
</tr>
</tbody>
</table>
**Figure I.** Mean Telomere length with 95% confidence intervals for carotid artery stenosis cases with both plaque and circulating leukocyte telomere length available.
Figure II. Correlation between telomere lengths of 216 subjects measured on two different occasions.