Blood leucocyte telomere DNA content predicts vascular telomere DNA content in humans with and without vascular disease


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Aims
Previous studies have suggested that reduced telomere length in circulating leucocytes in humans is associated with premature vascular disease and by implication, accelerated vascular ageing. Importantly, a link between telomere length in circulating leucocytes and the blood vessel wall has never been established. We, thus, investigated the relationship between vascular wall and circulating leucocyte telomere length in humans with and without overt vascular disease.

Methods and results
Aortic biopsies and paired blood leucocytes were obtained from 20 patients with asymptomatic abdominal aortic aneurysms (AAA), undergoing elective open repair, and 12 morphologically normal aortas from a group of cadaveric organ donors of similar mean age. Telomere content was compared by quantitative PCR and expressed as telomere:genomic DNA ratio.

The telomere:genomic DNA content was significantly reduced in wall biopsies of AAA vs. normal aorta, and this difference remained after adjusting for age and gender. There were strong correlations between leucocyte and vascular telomere content when the AAA and control groups were analysed either separately or grouped irrespective of the presence of vascular disease ($r = 0.62, P < 0.001$).

Conclusion
The findings demonstrate that leucocyte DNA content is predictive of vascular telomere content and is an accurate surrogate for human vascular age.

Keywords
Ageing • Aneurysm • Arteries • Leucocytes • Vessels

Introduction
Aging is a significant risk factor for cardiovascular disease. Recent studies suggest that biological age rather than chronological age is a better predictor of vascular risk and benefit from therapeutic interventions that reduce vascular disease risk. However, these conclusions are predicated on the key assumption that telomere content in circulating blood leucocytes accurately reflects the biological age of the vascular wall. To date, this assumption has not been tested or validated.

Telomeres are found at the end of chromosomes and are characterized by tandem repeats of a specific DNA sequence (TTAGGG). Telomere attrition occurs naturally with each cell division because of incomplete replication of the telomere sequence. When the telomere is critically shortened, cell senescence and/or apoptosis can develop. Telomere length in human cells is in part genetically determined and the aforementioned process of telomere attrition and development of cellular senescence can be accelerated by direct oxidative damage to telomeric DNA. The link between telomere attrition and premature cellular senescence is the basis for using measurements of telomere DNA as an index of cellular age.

A small number of studies of human tissue from sites of vascular wall stress or pathology have demonstrated the presence of

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vascular cell senescence and/or telomere attrition.\textsuperscript{4–6} However, studying the relevance of vascular telomere attrition to clinical vascular disease has been hindered by the impracticality of obtaining human vascular tissue for routine clinical studies. Consequently, circulating blood leucocytes have been harvested as an alternative source of DNA for telomere quantification with the acknowledgement that the telomere content of the leucocyte may not be representative of telomere content in the vascular wall. Nevertheless, a reduction in blood leucocyte telomere content, relative to healthier controls, has been reported in many vascular disease states, including hypertension,\textsuperscript{7} coronary artery disease,\textsuperscript{8,9} cerebrovascular disease,\textsuperscript{10} obesity, and smokers.\textsuperscript{11} In addition, shorter telomere length in circulating leucocytes appears predictive of future coronary heart disease events\textsuperscript{12} particularly in middle-aged, high-risk men.\textsuperscript{2} However, until a direct link between telomere content of the leucocyte and the vascular wall in individual patients is confirmed, it is not possible to conclude that leucocyte telomere content is an index of vascular ageing. Alternative hypotheses for shorter telomere length in circulating leucocytes in the presence of vascular disease have been suggested which include that leucocyte telomere attrition is a consequence of a generalized inflammatory process accompanying vascular disease and/or that telomere content in the blood leucocyte has nothing to do with the vascular wall pathology or vascular ageing.\textsuperscript{8,11} The present study, thus, has the important objective of clarifying the relationship in humans between blood leucocyte telomere content and that of the vascular wall.

Methods

Study design and tissue collection

This study compared telomere DNA content in paired aortic tissue and blood samples from patients with aortic disease, i.e. abdominal aortic aneurysm (AAA) and a control group of people without aortic disease at the time of cadaveric organ donation. This study conforms with the principles outlined in the Declaration of Helsinki and was approved by the Leicestershire (UK) and Lincolnshire (UK) Research Ethics Committees.

Patients

Twenty consecutive patients under the age of 80 years, with asymptomatic AAA undergoing elective open repair were recruited into the study (mean age 66.6, SD 5.7, range 58–78 years). No patient refused consent. Paired AAA wall and blood samples were taken. The cross-sectional diameter of each AAA was measured from the most recent pre-operative computed tomogram (mean diameter 6.3 ± 0.31 cm). Blood samples were obtained pre-operatively and frozen in liquid nitrogen. AAA samples were obtained intra-operatively from the proximal portion of the infrarenal AAA sac. AAA samples were dissected free of luminal thrombus and adipose tissue and washed thoroughly to remove any attached blood cells before freezing in liquid nitrogen.

Controls

Samples from healthy aortic tissue and paired blood samples were obtained from cadaveric organ donors. We knew the mean age and age range of the AAA patients and therefore we asked the UK Human Tissue Bank to provide samples from people aged >55 years, to control for age. Consent for the use of the tissue for research was obtained by Transplant Coordinators. Renal allografts with attached aortic patches and paired whole blood were stored in the UK Human Tissue Bank, De Montfort University, Leicester and provided to the investigating group.

Samples from 13 organ donors who met the criteria were provided over a 3 year period but only 12 were analysed. One tissue sample was not included due to failure of DNA extraction from the tissue sample. The mean age of the 12 donor subjects was 63.2, SD 6.5, range 56–78 years. All donor aortic samples were macroscopically normal. The cause of death in the ‘normal aorta’ cohort was intracerebral haemorrhage in 11 and primary brain tumour in 1. Classification of a cause of death as intracerebral haemorrhage reflects spontaneous lethal haemorrhage, either an intracerebral bleed or subarachnoid haemorrhage.

The demographics of the AAA and normal aorta cohorts are shown in Table 1. As described earlier, we were able to balance groups for age and gender, and so there were no statistical differences in age and gender, or indeed other demographics, between the two groups (Table 1).

Quantitative PCR analysis of telomere DNA content

Telomeres were estimated using qPCR.\textsuperscript{14} The qPCR method is widely used for the assessment of telomeres\textsuperscript{14–17} and determines the telomere DNA content normalized against DNA for a single copy gene. This approach yields values in terms of telomere DNA repeat content and, in its present form, does not give a direct indication of the mean length of telomere DNA. Nevertheless, the qPCR telomere DNA content

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normal aorta (n = 12)</th>
<th>AAA (n = 20)</th>
<th>Difference (mean or proportion)</th>
<th>95% CI of difference</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (SD)</td>
<td>63.2 (6.5)</td>
<td>66.6 (5.7)</td>
<td>−3.4</td>
<td>−7.8 to 1.1%</td>
<td>0.13*</td>
</tr>
<tr>
<td>Gender distribution</td>
<td>7 males (58%)</td>
<td>14 males (70%)</td>
<td>−12%</td>
<td>−46 to 23%</td>
<td>0.70</td>
</tr>
<tr>
<td>Diabetes</td>
<td>2 (17%)</td>
<td>1 (5%)</td>
<td>12%</td>
<td>−12 to 35%</td>
<td>0.54</td>
</tr>
<tr>
<td>Hypertension</td>
<td>4 (33%)</td>
<td>12 (60%)</td>
<td>−27%</td>
<td>−61 to 8%</td>
<td>0.27</td>
</tr>
<tr>
<td>Current or ex-smoker less than 10 years</td>
<td>8 (67%)</td>
<td>12 (60%)</td>
<td>7%</td>
<td>−28 to 41%</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*Comparisons were made by independent t-test for age and Fisher’s exact test for categorical data.
DNA content correlates well with mean telomere DNA restriction fragment length by Southern blotting.\(^{1,16}\) Genomic DNA was isolated from whole blood (200 μL) and tissue (50mg tissue powdered in liquid nitrogen by mortar and pestle) using the QIAamp DNA Blood Mini and 'Mini' kits, respectively (Qiagen, Crawley, UK) and quantified using the Quant-it\(^{TM}\) PicoGreen\(^{\text{TM}}\) dsDNA assay kit (Invitrogen, Paisley, UK). PCR was performed in triplicate tubes using the MX4000 QPCR thermal cycler (Stratagene, La Jolla, CA, USA). Results for each PCR were expressed relative to a standard curve constructed using a reference DNA sample (isolated from whole blood). The standard curve for the genomic and telomere PCRs was comprised of five standards of 3.125–50 ng reference DNA. Telomere qPCR values were normalized against the value obtained for the genomic DNA PCR to yield a T/S ratio\(^{14}\) (Telomere repeat copy number vs. single copy gene copy number).

PCR of genomic DNA was performed using the Brilliant QPCR core reagent kit (Stratagene) with the following conditions: 15 ng template DNA; 2 mM MgCl\(_2\); 200 μM each dNTP; 100 nM 36B4d primer (36B4d: CCCATCTCATCATACACGGGTCAA); 100 nM 36B4u primer (36B4u: CAGCAAGTTGGAGGTGTACCC); 8% (v/v) glycerol; 3% (v/v) DMSO; 0.5X SYBR green; 30 nM 6-ROX dye; 1.25 units SureStart Taq DNA polymerase in a 25 μL reaction. Following activation of the DNA polymerase at 95 °C for 10 min, 40 cycles of 95 °C (15 s), 60 °C (60 s) and 72 °C (60 s) were performed. Results were normalized against the reference dye included in each reaction (30 nM 6-ROX dye).

PCR of telomere DNA was performed using the method of Cawthon\(^{14}\) and the AmpliTaq Gold QPCR system (Applied Biosystems, Warrington, UK) with the following conditions: 15 ng template DNA; 1.5 mM MgCl\(_2\); 200 nM each dNTP; 450 nM Tel1b primer (Tel1b: CGGTTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTG TT); 450 nM Tel 2b primer (Tel2b: GGGTTGCCTTACCTTAC TACCCCTTACCTTTACCTTT ACCCT); 2.5 mM DTT; 1% (v/v) DMSO; 0.4X SYBR green; 30 nM 6-ROX dye; 1.25 units AmpliTaq DNA polymerase in a 25 μL reaction. Following activation of the DNA polymerase at 93 °C for 15 min, 40 cycles of 95 °C (15 s) and 56 °C (60 s) were performed. Results were normalized against the reference dye included in each reaction (30 nM 6-ROX dye).

### Statistical analyses

Estimating that a meaningful correlation would be in the order of \(r = 0.65\), we calculated that the sample size to achieve significance (alpha 0.05) would be \(n = 20\) (power 0.9). At alpha 0.01 (power 0.9), sample size would be 28. Therefore, we aimed for collection of 30 paired samples of blood and artery wall. Our final collection was of 32 pairs. Despite the small sample size, formal tests of normality indicated no cause for concern and so two-sided parametric tests were applied for: (i) between group comparisons and (ii) correlations. Discrete variables (gender, diabetes, hypertension, and smoking) were presented as actual numbers (and percentages) and compared using Fisher’s exact test. Ages were presented as mean and standard deviation and compared using the independent t-test. Unadjusted telomere content ratios were compared using an independent t-test. Telomere content values were also adjusted for age and gender and adjusted means tested statistically and compared using a general linear model. Pearson’s correlation was used to correlate paired tissue and leucocyte data. No formal adjustments were made for multiple testing, but all \(P\)-values are interpreted with caution. Statistical significance was accepted when \(P < 0.05\).

### Results

#### Comparison of telomere DNA content of vascular tissue from patients with diseased vs. healthy aorta

The telomere DNA content in site-matched aortic wall biopsies was compared in cohorts with normal aorta and those with AAA. The telomere DNA content was significantly reduced in wall biopsies of AAA vs. normal aorta (normal aortic biopsy telomere DNA: 2.78 ± 0.21, vs. AAA biopsy telomere DNA: 2.15 ± 0.18, \(P = 0.03\), Figure 1). Because age and gender would probably have influenced the results, we adjusted for between group differences in these parameters. After adjusting for age and gender, there was some evidence that mean telomere DNA content was reduced in wall biopsies of AAA (2.17, 95% CI 1.77–2.56) vs. normal aorta (2.80, 95% CI 2.32–3.28, \(P < 0.05\)) a difference of 0.63 (95% CI 0.01–1.26). This indicates advanced biological ageing of the vascular wall of people with AAA when compared with the cohort of people with normal aorta, despite a similar mean chronological age for the two cohorts.

![Figure 1](image) Telomere DNA content in wall biopsies of abdominal aortic aneurysm (AAA) (n = 20) and normal aorta (n = 12) expressed as unadjusted T/S ratio. Bars represent the mean (normal aorta 2.78 ± 0.21, AAA 2.15 ± 0.18, \(P = 0.03\)).

#### Comparison of telomere DNA content of blood leucocytes from patients with diseased vs. healthy aorta

The telomere DNA content of blood leucocytes from patients with AAA was markedly reduced when compared with the blood leucocytes from patients with a normal aorta (normal aorta blood leucocyte telomere DNA: 1.27 ± 0.10 vs. AAA blood leucocyte telomere DNA: 0.82 ± 0.06, \(P < 0.001\), Figure 2). Adjusting for age and gender revealed no impact on this result. After adjusting for age and gender, there was strong evidence to suggest a reduction in the mean telomere DNA content in leucocytes of AAA (0.80 95% CI 0.65–0.96) vs. normal aorta (1.27 95% CI 1.08–1.46; \(P < 0.001\)) a difference of 0.47 (95% CI 0.22–0.71). This indicates that the presence of clinical vascular...
wall disease in humans was associated with reduced blood leucocyte telomere DNA content, despite similar mean age.

**Correlation of the telomere DNA content of the vascular wall with the telomere DNA content of circulating leucocytes**

The correlation of telomere DNA content between paired aortic wall and blood leucocytes samples was examined for patients with AAA and patients with normal aorta. There was a significant correlation between leucocyte DNA and vascular wall samples for each cohort (AAA; \( r = 0.44, P < 0.05 \) and normal aorta; \( r = 0.68, P < 0.02 \)). When this correlation was examined irrespective of disease status across all vessel wall and blood leucocyte pairs \((n = 32)\), and adjusting for age, there was strong evidence of a positive correlation between tissue and leucocyte telomere content \((partial correlation coefficient 0.62; P < 0.001, Figure 3)\).

**Correlation of the telomere DNA content with patient age**

There was no significant correlation between telomere content of the vessel biopsy and patient age \((AAA; r = -0.11, P = 0.64 \) and normal aorta; \( r = 0.25, P = 0.43 \)). Similarly, leucocyte telomere DNA content failed to correlate with patient age in both groups \((AAA; r = 0.39, P = 0.09 \) and normal aorta; \( r = -0.13, P = 0.68 \)). These results reflect the narrow age range of the groups studied \((AAA age range = 58–78 years and normal aorta age range = 56–76 years)\).

**Discussion**

Human donor arterial tissue has previously been shown to undergo age-dependent telomere attrition.\(^4,5\) Shorter telomeres in blood leucocytes from patients with various stages of coronary artery disease compared with disease-free controls have also been reported.\(^2,9\) Moreover, a recent report suggests shorter telomere DNA length is associated with CVD risk factors and also subsequent MI or stroke.\(^18\) Importantly, to date, no study has linked the magnitude of telomere attrition in blood leucocytes with that of the vascular wall in humans.

We demonstrate that telomere length is shortened in aneurysmal aortic tissue when compared to age- and site-matched tissue samples from morphologically normal aorta—consistent with advanced cellular ageing in the vascular wall of patients with AAAs \((Summarized in Table 2)\). We also demonstrate that the telomere lengths in blood leucocytes from patients with
Vascular telomere content based on leucocyte DNA

aneurysmal aortas were significantly shorter than those from people with morphologically normal aortas of similar age. The evidence for differences between telomere content of AAA and control leucocytes is stronger than for corresponding tissue samples presumably due to the heterogeneity of cell types within the vessels compared to white blood cells, this is reflected in the tighter 95% confidence intervals for the data for the latter. Importantly, using paired samples in the same patient, we demonstrate for the first time that there is a highly significant correlation between the telomere content of blood leucocytes and the vascular wall (Table 2). This relationship holds irrespective of whether or not there is overt vascular wall disease. Moreover, the presence of disease is associated with a shorter vascular and blood leucocyte telomere content. Importantly, our data suggest that shorter blood telomeres reflect shorter aortic telomeres and therefore that the measurement of telomeres in blood is an effective surrogate marker for vascular telomeres.

These findings are important because they provide the first demonstration that telomere attrition in blood leucocytes is indicative of similar changes in the vascular wall. These findings validate and support the conclusions of previous studies that have suggested that blood leucocyte telomere DNA content is likely to reflect vascular ageing in humans. The strength of the association, irrespective of the presence of overt vascular wall disease, suggests that telomere attrition in blood leucocytes and the vascular wall occurs in tandem and that the use of blood leucocyte telomeric DNA content is a convenient surrogate for vascular ageing in population studies.

The mechanisms linking telomere attrition to vascular disease have recently been reviewed.\(^1\) There are a number of potential explanations for our findings: (i) the strong association between human blood leucocyte and vessel wall telomere content may be genetically determined\(^{19-21}\) and shorter telomeres may predispose to the development of vascular disease. (ii) Conversely, the shorter telomeres in those with vascular disease may be acquired due to accelerated telomere attrition in the blood leucocyte and vessel wall occurring in tandem following exposure to vascular disease risk factors, e.g. hypertension, diabetes, dyslipidaemia, obesity, and smoking.\(^7,11,22-24\) Chronic inflammation and oxidative damage to DNA are a plausible common mechanism for the latter hypothesis.\(^1,3,25-27\) It is conceivable that the actual length of the telomere in the vessel wall has less to do with the process of ageing and is simply reflective of an inflammatory process that shortens telomeres in many tissues in parallel.\(^{28}\)

This study has some limitations. It was a small study for pragmatic reasons. Eleven of the 12 donor subjects who had normal aortas died of ‘intracerebral haemorrhage’. Information on the precise cause of haemorrhage is not available, but may be important because leucocyte telomere length has been associated with stroke. Moreover, subjects with normal aortas who suffered intracerebral haemorrhage must have had local intracerebral vascular lesions. However, these considerations do not invalidate our primary observation that the telomere length in the leucocyte is strongly correlated with that of the vascular wall, irrespective of whether the vascular wall from which the tissue is taken is overtly diseased or not. The strength of the correlations is striking, especially after consideration of the many factors that could independently influence the telomere length of the vascular wall and blood leucocytes.

We only sampled the proximal sac of the aneurysm, i.e. the site of localized vascular wall disease, which may not reflect the telomere content of the entire vessel in less diseased areas. Moreover, we were not able to fractionate the vessel wall samples to identify specific cell types. However, by also studying morphologically healthy aortas from cadaveric donors, we show a strong correlation between vascular wall and blood leucocyte telomere DNA content, irrespective of the presence of vascular disease. This strengthens the conclusions of our study. Many factors such as age, gender, smoking, hormone replacement therapy, body mass index, etc. have been reported to influence telomere length in large population studies.\(^7,11,18,22-24\) Our successful endeavours to age balance our study populations resulted in a narrow age range which prevented examination of the effects of age on telomere shortening in the two groups—much larger studies with a wider age range may be required to demonstrate such effects. With regards to the other variables known to influence telomere length, the overall sample size of our study, while sufficient to test our primary hypothesis, was insufficient to provide robust statistical power for sub-group analyses.

In conclusion, the present study is the first to demonstrate a strong association between blood leucocyte and vascular wall telomere DNA content and the impact of health and disease on this relationship. These findings are important because they strengthen the hypothesis linking accelerated vascular ageing and predisposition to vascular disease and also justify the use of blood leucocyte telomere DNA content as a surrogate for vascular ageing.

**Table 2** Summary of study data

<table>
<thead>
<tr>
<th>Age-adjusted parameter</th>
<th>Control Mean (95% CI)</th>
<th>AAA Mean (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic wall telomere content</td>
<td>2.80 (2.32–3.28)</td>
<td>2.17 (1.77–2.56)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Leucocyte telomere content</td>
<td>1.27 (1.08–1.46)</td>
<td>0.80 (0.65–0.96)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Partial correlation: leucocyte telomere vs. wall telomere</td>
<td>r = 0.62</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

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**Conflict of interest:** none declared.
References