Leukocyte Telomere Length Is Associated With High-Risk Plaques on Virtual Histology Intravascular Ultrasound and Increased Proinflammatory Activity

Patrick A. Calvert, Tze-Vun Liew, Isabelle Gorenne, Murray Clarke, Charis Costopoulos, Daniel R. Obaid, Michael O’Sullivan, Leonard M. Shapiro, Duncan C. McNab, Cameron G. Densem, Peter M. Schofield, Denise Braganza, Sarah C. Clarke, Kausik K. Ray, Nick E.J. West, Martin R. Bennett

Objective—Leukocyte telomere length (LTL), a marker of cellular senescence, is inversely associated with cardiovascular events. However, whether LTL reflects plaque extent or unstable plaques, and the mechanisms underlying any association are unknown.

Methods and Results—One hundred seventy patients with stable angina or acute coronary syndrome referred for percutaneous coronary intervention underwent 3-vessel virtual histology intravascular ultrasound; 30,372 mm of intravascular ultrasound pullback and 1096 plaques were analyzed. LTL was not associated with plaque volume but was associated with calcified thin-capped fibroatheroma (OR, 1.24; CI, 1.01–1.53; P=0.039) and total fibroatheroma numbers (OR, 1.19; CI, 1.02–1.39; P=0.027). Monocytes from coronary artery disease patients showed increased secretion of proinflammatory cytokines. To mimic leukocyte senescence, we disrupted telomeres and binding and expression of the telomeric protein (protection of telomeres protein-1), inducing DNA damage. Telomere disruption increased monocyte secretion of MCP-1, IL-6, and IL-1β and oxidative burst, similar to that seen in coronary artery disease patients, and lymphocyte secretion of IL-2 and reduced lymphocyte IL-10.

Conclusion—Shorter LTL is associated with high-risk plaque morphology on virtual histology intravascular ultrasound but not total 3-vessel plaque burden. Monocytes with disrupted telomeres show increased proinflammatory activity, which is also seen in coronary artery disease patients, suggesting that telomere shortening promotes high-risk plaque subtypes by increasing proinflammatory activity. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: atherosclerosis ■ intravascular ultrasound ■ virtual histology

Telomeres are DNA–protein complexes protecting the ends of eukaryotic chromosomes. Conventional polymerases cannot replicate the telomere ends; therefore, incomplete replication shortens telomeres with each division. Additionally, DNA damaging factors such as oxidative stress cause further telomere attrition. A typical DNA damage response is activated when telomeres shorten to critical lengths, causing cellular senescence. Because telomere shortening in untransformed cells is usually progressive, a tissue’s mean telomere length (TL) indicates its replication history, and thus its biological age.

Studies have identified an inverse relationship between peripheral leukocyte telomere length (LTL) and coronary artery disease (CAD), premature myocardial infarction (MI),2–4 and cardiovascular disease mortality. Mean LTL in patients with CAD either before MI or with premature MI was 0.3 Kb shorter than controls,2,3 corresponding to a biological age of 11 years older. LTL is associated with cardiac events in both small6 and large patient cohorts.7 The precise contribution of inheritance to LTL is unknown, although there is a moderate correlation between generations (r=0.37; P=0.002).7 An association between LTL and atherosclerosis may reflect various mechanisms. TL is determined by both genetic9,10 and acquired mechanisms, with the latter particularly associated with disease. Thus, subjects born with a shorter TL may be predisposed to age-related diseases, including atherosclerosis. Alternatively, multiple postnatal factors associated with atherosclerosis, such as traditional risk factors, may accelerate leukocyte turnover, causing telomere loss with disease progression. In both these scenarios, LTL is associated with extent of disease and consequent cardiovascular events. However, it is unclear whether the association between shorter LTL and cardiac events just reflects disease burden, or whether shorter LTL...
is associated with a high-risk plaque phenotype and the mechanisms underlying any association.

Postmortem studies suggest that 60% to 70% of MI are caused by rupture of thin-capped fibroatheromata (TCFA), in which a thin fibrous cap containing macrophages separates a necrotic core from the lumen, frequently without significant vessel stenosis.\textsuperscript{11–13} Although histology remains the gold standard for plaque classification, other imaging modalities have been assessed against histology to noninvasively identify plaque subtypes. Of these, radiofrequency or virtual histology intravascular ultrasound (VH-IVUS), which is based on spectral analysis of ultrasound backscatter, is currently the best-validated standard for plaque classification, other imaging modalities to histologically defined TCFA, these VH-IVUS–identified TCFA (VHTCFA) are more frequent in acute coronary syndrome than stable angina,\textsuperscript{17,18} and they show the highest risk for subsequent cardiovascular events in prospective studies.\textsuperscript{19} We therefore examined whether LTL is associated with plaque extent or plaque composition on VH-IVUS, and how leukocyte telomere damage might promote high-risk plaques.

**Patients and Methods**

**VH-IVUS Patients**

The Virtual Histology in Vulnerable Atherosclerosis study research protocol was approved by the Cambridgeshire Research and Ethics Committee-3 (07/Q0106/47); all participants gave written informed consent before enrollment. Patients referred for PCI with either stable angina or troponin-positive acute coronary syndrome were recruited. Exclusion criteria were previous revascularization or unsuitable for 3-vessel VH-IVUS and acute coronary syndrome, active inflammatory condition, any form of surgery in the 3 months before enrollment.

**VH-IVUS Acquisition**

VH-IVUS acquisition and core laboratory analyses were described online.

**Coronary Artery Bypass and Normal Patients**

For cytokine validation studies, consecutive patients with CAD undergoing coronary artery bypass grafting (≥2 vessels) at Papworth Hospital, Cambridge, and age- and sex-matched controls with no CAD history were recruited.

**Plaque Classification**

Plaques were classified as described online and shown in Figure 1, consistent with subsequently published VH-IVUS classification.\textsuperscript{20} Culprit lesions were defined according to electrocardiographic criteria (ST segment shift or “T” wave inversion) and angiographic appearances (point of angiographic maximal stenosis, luminal irregularities consistent with ulceration, or filling defects consistent with thrombus) at PCI. Remodeling index and thrombus were determined according to established definitions.\textsuperscript{21} The minimal lumen area was defined as the IVUS frame with the smallest minimal lumen area. The worse necrotic core area frame was defined as the VH-IVUS frame with the highest necrotic core area.

**Leukocyte Isolation and Characterization**

Leukocytes were separated from erythrocytes by Dextran sedimentation into neutrophils and peripheral blood mononuclear cells by Percoll gradient centrifugation, and into CD3\textsuperscript{+} T lymphocytes and CD14\textsuperscript{+} monocytes by MACS isolation (Miltenyi Biotec). Isolation purity was confirmed by hematoxylin and eosin staining and CD3/CD14 immunocytochemistry of cytospins.

**Cytokine Assays**

Culture supernatants were assayed for IL-1β, IL-2, IL-6, IL-10, IL-12p70, IL-18, interferon-γ, tumor necrosis factor-α, and MCP-1 using eBioscience Flow Cytomix assays (eBioscience) on a Becton Dickinson FACScalibur flow cytometer (Becton Dickinson). Data were analyzed using Flow Cytomix Pro 2.2 software (eBioscience). IL-4 was assayed by enzyme-linked immunosorbent assay (Peprotech EC).

**Statistical Analysis: Clinical and VH-IVUS Parameters and LTL**

Clinical and VH-IVUS parameters of shorter LTL (lower) and the longer LTL (upper) tertiles were compared using 2-tailed unpaired \( t \) test, Mann Whitney \( U \) test, or \( \chi^2 \) test as appropriate. Each parameter

\[\text{Statistical Analysis: Clinical and VH-IVUS Parameters and LTL}\]

**Assessment of TL**

LTL was assayed using quantitative polymerase chain reaction amplified against 36B4, a single-copy gene encoding acidic ribosomal phosphoprotein PO.\textsuperscript{6} Telomere-to-36B4 ratios were compared to reference DNA of known telomere length (by Southern blotting) derived from human smooth muscle cells.

**Time-Lapse Videomicroscopy and Senescence-Associated \( \beta \)-Galactosidase Activity**

Telomestatin was obtained from Dr Kazuo Shin-Ya (Biological Information Research Center, Tokyo, Japan) and dissolved in DMSO. Cells were incubated with increasing concentrations of telomestatin or DMSO, and cell proliferation, apoptosis and Senescence-associated \( \beta \)-galactosidase activities were determined as described.\textsuperscript{22}

**Western Blotting**

Western blotting was as described previously.\textsuperscript{22}

**Reactive Oxygen Species Assay**

Monocytes were incubated with serum and phenol red-free RPMI 1640 containing 10 μmol/L dichlorofluorescein diacetate \( \pm 2 \mu g/mL \) lipopolysaccharide (LPS). Plates were read on a BioTek Synergy HT fluorescence plate reader with Gen5 software (BioTek Instruments Limited) at 485/530 nm at 0 to 90 minutes.

**Figure 1. Single-frame examples of plaque classification by virtual histology intravascular ultrasound (VH-IVUS). VH-IVUS classification of human atherosclerotic plaques.**

This figure shows single-frame examples of plaque classification by virtual histology intravascular ultrasound (VH-IVUS). VH-IVUS classification of human atherosclerotic plaques.
Table 1. Grayscale Intravascular Ultrasound Parameters and Leukocyte Telomere Length

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Upper Tertile of Leukocyte Telomere Length (n=56)</th>
<th>Lower Tertile of Leukocyte Telomere Length (n=56)</th>
<th>P</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-vessel total plaque volume (mm³)</td>
<td>1050 (762–1405)</td>
<td>1235 (933–1526)</td>
<td>0.058</td>
<td>1.00 (1.00–1.00)</td>
<td>0.10</td>
</tr>
<tr>
<td>Culprit vessel plaque volume (mm³)</td>
<td>387 (301–504)</td>
<td>523 (320–710)</td>
<td>0.015</td>
<td>1.002 (1.00–1.003)</td>
<td>0.024</td>
</tr>
<tr>
<td>3-vessel total N of plaques</td>
<td>6.0 (4.0–8.0)</td>
<td>6.0 (4.0–9.0)</td>
<td>0.64</td>
<td>1.05 (0.92–1.19)</td>
<td>0.52</td>
</tr>
<tr>
<td>Thrombus in any vessel</td>
<td>7 (13%)</td>
<td>6 (11%)</td>
<td>0.82</td>
<td>0.88 (0.27–2.80)</td>
<td>0.82</td>
</tr>
<tr>
<td>Plaque rupture in any vessel</td>
<td>8 (14%)</td>
<td>9 (16%)</td>
<td>0.73</td>
<td>1.20 (0.43–3.39)</td>
<td>0.73</td>
</tr>
<tr>
<td>MLA frame lumen area (mm²)</td>
<td>2.65 (0.65)</td>
<td>2.54 (0.58)</td>
<td>0.35</td>
<td>0.73 (0.39–1.39)</td>
<td>0.35</td>
</tr>
<tr>
<td>MLA frame remodeling index</td>
<td>0.98 (0.19)</td>
<td>1.05 (0.27)</td>
<td>0.094</td>
<td>4.77 (0.75–30.37)</td>
<td>0.10</td>
</tr>
<tr>
<td>MLA frame vessel area (mm²)</td>
<td>12.24 (5.54)</td>
<td>14.60 (6.07)</td>
<td>0.038</td>
<td>1.08 (1.00–1.18)</td>
<td>0.045</td>
</tr>
<tr>
<td>MLA frame plaque area (mm²)</td>
<td>9.60 (5.43)</td>
<td>12.07 (5.98)</td>
<td>0.027</td>
<td>1.09 (1.01–1.17)</td>
<td>0.034</td>
</tr>
<tr>
<td>MLA frame plaque burden (%)</td>
<td>75.8% (8.4)</td>
<td>79.9% (8.7)</td>
<td>0.016</td>
<td>1.06 (1.01–1.11)</td>
<td>0.020</td>
</tr>
</tbody>
</table>

CI, confidence interval; MLA, minimum luminal area; OR, odds ratio.
Parameters were compared using the unpaired t test, Mann Whitney U test, or χ² test as appropriate. Each parameter was then tested for an association with lower leukocyte telomere length tertiles using univariate logistic regression.

Results

We used grayscale IVUS to determine plaque burden in all 3 vessels, the culprit vessel, and the culprit plaque (minimal lumen area, plaque area, and remodeling index), together with IVUS features of plaque rupture and thrombosis. Importantly, 3-vessel plaque volumes and total number of plaques were not significantly different between lower and higher LTL tertiles. A number of grayscale IVUS parameters were increased in the lower LTL tertile, including culprit vessel plaque volume (P=0.015), minimum luminal area frame vessel area (P=0.038), minimal lumen area frame plaque area (P=0.027), and minimal lumen area frame plaque burden (P=0.016), and they were also associated with lower LTL tertile on univariate logistic regression (Table 1).

3-Vessel VH-IVUS Parameters and LTL

We analyzed the association between LTL and VH-IVUS–identified plaque composition and plaque subtype (Figure 1), in all 3 vessels and in the culprit vessel alone. Total fibroatheromata (VHThCFA + VHTCFA) number was greater in the lower LTL tertile vs upper tertiles (6.0 [4.0–8.0] vs 5.0 [3.0–6.0]; P=0.027; Table 2). After univariate analyses, the 3-vessel VH-IVUS factors associated with the lower LTL tertile included total calcified VHTCFA (OR, 1.24; CI, 1.01–1.53; P=0.039) and total VHThCFA + VHTCFA number (OR, 1.19; CI, 1.02–1.39; P=0.027).

Culprit Vessel VH-IVUS Parameters and LTL

The culprit vessel VH-IVUS parameters that were greater in the lower LTL tertile included the necrotic core volume (59 mm³ [CI, 35–111 mm³] vs 47 mm³ [CI, 29–74 mm³]; P=0.019), VHTCFA number (2.0 [CI, 1.0–3.0] vs 1.0 [CI, 1.0–2.0]; P=0.022), and worst necrotic core area frame plaque area (13.4% [5.1%] vs 11.3% [4.5%]; P=0.022; Table 2). After univariate logistic regression, culprit vessel necrotic core volume (OR, 1.01; CI, 1.00–1.01; P=0.013) and worse necrotic core area frame plaque burden (OR, 1.11; CI, 1.01–1.21; P=0.028) were associated with lower LTL tertile. After multivariable adjustment, culprit vessel VHTCFA number (OR, 1.75; CI,
Table 2. Virtual Histology Intravascular Ultrasound Parameters and Leukocyte Telomere Length

<table>
<thead>
<tr>
<th>VH-IVUS Parameters</th>
<th>Upper Tertile of Leukocyte Telomere Length (n=56)</th>
<th>Lower Tertile of Leukocyte Telomere Length (n=56)</th>
<th>P</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-vessel VH-IVUS parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-vessel necrotic core volume (mm³)</td>
<td>117 (64–182)</td>
<td>132 (83–214)</td>
<td>0.20</td>
<td>1.00 (1.00–1.01)</td>
<td>0.50</td>
</tr>
<tr>
<td>3-vessel necrotic core percentage</td>
<td>11.0% (7.3–16.1%)</td>
<td>11.3% (8.4–14.8%)</td>
<td>0.73</td>
<td>2.41 (0.01–5446)</td>
<td>0.82</td>
</tr>
<tr>
<td>3-vessel N of VHThCFA</td>
<td>4.0 (2.0–5.0)</td>
<td>4.0 (2.0–6.3)</td>
<td>0.43</td>
<td>1.08 (0.91–1.24)</td>
<td>0.28</td>
</tr>
<tr>
<td>3-vessel noncalcified VHThCFA</td>
<td>1.0 (0–2.0)</td>
<td>1.0 (0.8–3.0)</td>
<td>0.666</td>
<td>1.29 (0.96–1.74)</td>
<td>0.087</td>
</tr>
<tr>
<td>3-vessel calcified VHThCFA</td>
<td>3.0 (1.0–4.0)</td>
<td>3.0 (2.0–6.0)</td>
<td>0.061</td>
<td>1.24 (1.01–1.53)</td>
<td>0.039</td>
</tr>
<tr>
<td>3-vessel N of fibroatheromas (VHTCFA + VHThCFA)</td>
<td>5.0 (3.0–6.0)</td>
<td>6.0 (4.0–8.0)</td>
<td>0.027</td>
<td>1.19 (1.02–1.39)</td>
<td>0.027</td>
</tr>
<tr>
<td>3-vessel N of fibrocalcific plaques (VHFCa)</td>
<td>0 (0–1.0)</td>
<td>0 (0–1.3)</td>
<td>1.0</td>
<td>0.93 (0.72–1.20)</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Culprit-vessel VH-IVUS parameters

| Culprit vessel necrotic core volume (mm³) | 47 (29–74) | 59 (35–111) | 0.019 | 1.01 (1.00–1.01) | 0.013 |
| Culprit vessel NC percentage | 12.2% (8.0–15.9%) | 12.5% (9.0–16.5%) | 0.45 | 24.23 (0.02–30876) | 0.38 |
| Culprit vessel N VHThCFA | 1.0 (1.0–2.0) | 2.0 (1.0–3.0) | 0.022 | 1.25 (0.98–1.61) | 0.077 |
| MLA frame NC Area (mm²) | 1.55 (1.75) | 1.96 (1.56) | 0.21 | 1.17 (0.91–1.49) | 0.22 |
| MLA frame NC percentage | 20.4% (11.0%) | 19.7% (10.4%) | 0.76 | 0.99 (0.96–1.03) | 0.76 |
| WNCA frame plaque area (mm²) | 11.3% (4.5%) | 13.4% (5.1%) | 0.022 | 1.11 (1.01–1.21) | 0.028 |
| WNCA frame plaque burden | 70.2% (10.9%) | 73.2% (9.7%) | 0.13 | 1.03 (0.99–1.07) | 0.13 |
| WNCA frame NC Area (mm²) | 2.93 (1.88) | 3.55 (1.96) | 0.10 | 1.19 (0.96–1.47) | 0.11 |
| WNCA frame NC percentage | 34.3% (11.0%) | 34.1% (8.9%) | 0.95 | 1.00 (0.96–1.04) | 0.95 |

Parameters were compared using the unpaired t test, Mann Whitney U test or χ² test as appropriate. Each parameter was then tested for an association with lower leukocyte telomere length tertiles using univariate logistic regression.

1.22–2.51; P=0.002) and worse necrotic core area frame plaque area (OR, 1.11; CI, 1.00–1.23; P=0.047) were independently associated with lower LTL tertile.

Monocytes From CAD Patients Show Increased Secretion of Proinflammatory Cytokines

To examine how lower LTL might be associated with unstable plaques, we determined cytokine secretion from patients with CAD vs age- and sex-matched controls with no CAD history. LTL was significantly shorter in CAD patients vs controls (P<0.01) by ~0.45 kb. In subfractions, neutrophils, monocytes, and lymphocytes from the same patient had near-identical LTL (P=0.8, not shown); however, neutrophil TL and monocyte TL were significantly shorter in CAD patients vs controls, with a similar trend in lymphocytes (Figure 2A). To examine monocyte and lymphocyte function, we assayed ROS generation and cytokine release in response to LPS. Monocytes from CAD patients had higher baseline and LPS-induced ROS generation, increased MCP-1 and IL-6 secretion with or without LPS, increased LPS-induced IL-1β secretion, and increased LPS-induced tumor necrosis factor-α (Figure 2B–F). In contrast, there was no difference in secretion of interferon-α, IL-2, IL-10, IL-4, or tumor necrosis factor-α from lymphocytes of CAD patients vs controls (Supplemental Figure I available online at http://atvb.ahajournals.org).

Telomere Disruption Promotes Proinflammatory Cytokine Secretion

These results demonstrate that lower LTL is associated with high-risk plaques and increased monocyte proinflammatory cytokine secretion; however, whether telomere shortening or disruption produces a similar proinflammatory profile is unknown. Cultured human monocytes and lymphocytes show minimal proliferation without specific mitogens or activation. However, both telomere shortening and telomere disruption induce a DNA damage response (DDR) that mimics replicative senescence. We therefore used the G-Quadruplex ligand telomestatin to examine the functional effect of aging/telomere dysfunction in human leukocytes. Telomestatin promotes a particular 3-dimensional DNA structure at G-rich sequences, particularly telomeres, blocking the single-strand conformation, thereby inducing telomere shortening and premature growth arrest. Telomestatin also reduces binding of POT-1 to telomeres and inactivates POT-1 telomeric function, generating a dysfunctional and unprotected telomere. Telomestatin-induced features of senescence in replicating human aortic vascular smooth muscle cells, including a significant dose-dependant reduction in vascular smooth muscle cells proliferation, increased apoptosis (Supplemental Figure II A, B, available online at http://atvb.ahajournals.org), and expression of senescence-associated β-galactosidase (Supplemental Figure IIC available online at http://atvb.ahajournals.org).
Because monocyte/macrophages show limited proliferation and express endogenous β-galactosidase activity, we examined POT-1 localization by immunocytochemistry and expression of POT-1 and the DNA repair protein H2AX by Western blotting in telomestatin-treated monocytes and T-lymphocytes from healthy donors. Untreated cells showed discrete foci of nuclear-localized POT-1, consistent with location at telomeres; telomestatin significantly decreased nuclear POT-1 foci in human monocytes and lymphocytes (Figure 3A, Supplemental Figure III available online at http://atvb.ahajournals.org), with lymphocytes requiring a higher concentration. Telomestatin reduced POT-1 expression and increased H2AX phosphorylation, suggesting that POT-1 loss from telomeres activates a DDR in both cell types (Figure 3B).

Although in proliferating vascular smooth muscle cells and fibroblasts the DDR induces growth arrest and premature senescence, mediated in part by p53-induced p21 expression, and the cdk inhibitors p16 and p15, the senescence response of human monocytes and lymphocytes after DNA damage is unclear. Telomestatin reduced POT-1 expression and increased H2AX phosphorylation, suggesting that POT-1 loss from telomeres activates a DDR in both cell types (Figure 3B).

To examine the effect of telomestatin on monocyte and lymphocyte function, we assayed ROS generation and cytokine release in response to LPS. Telomestatin dose-dependently increased monocyte ROS release, augmented by LPS (Supplemental Figure IV available online at http://atvb.ahajournals.org), and increased MCP-1, IL-6, and IL-1β secretion from monocytes, a pattern similar to that seen in CAD patients vs controls, but reduced tumor necrosis factor-α secretion (Figure 4A–D). Telomestatin increased secretion of the proinflammatory cytokine IL-2 from stimulated lymphocytes and reduced the anti-inflammatory cytokine IL-10. Telomestatin did not affect interferon-γ release, but it again reduced tumor necrosis factor-α secretion (Figure 4E–H).

**Discussion**

Previous studies have shown that shorter LTL is associated with cardiovascular events in both primary and secondary cardiovascular disease prevention populations. However, it is unclear whether increased total plaque burden underlies this association, or whether shorter LTL predisposes to specific high-risk plaque phenotypes.

Using VH-IVUS, we show that LTL was not associated with plaque volume or 3-vessel number of plaques, all measures of total coronary plaque burden. In contrast, lower LTL tertile was associated with 3-vessel calcified VHUTCFA...
Figure 4. Effects of telomestatin on cytokine secretion from monocytes and lymphocytes. Secretion of cytokines from monocytes (A–D) and lymphocytes (E–H) after treatment with telomestatin with or without lipopolysaccharide. Data are means±SEM (n=3). *P<0.05, **P<0.01 vs control (no telomestatin).

This is the first report to our knowledge of an association between LTL and either grayscale or VH-IVUS findings. LTL was associated with specific high-risk plaque subtypes in 3-vessel and culprit vessel analyses, particularly calcified VHTCFA and 3-vessel VHTCFA + VHThCFA numbers, and was not associated with lower-risk subtypes such as pathologic intimal thickenings and fibrocalcific plaques. Culprit vessel VHTCFA number was 1 of only 2 factors independently associated with shorter LTL in a study population in which many conventional cardiovascular risk factors were not, suggesting that VH-IVUS plaque identification is more closely associated with this DNA-based cardiovascular risk predictor, perhaps because it represents a “downstream” marker of risk. In recent studies, VHTCFA have been shown to be prospectively associated with multiple adverse cardiovascular events, demonstrating that VH-IVUS can identify high-risk plaques. Similarly, culprit vessel necrotic core volume and worst necrotic core area frame plaque area were associated with shorter LTL, reinforcing previous studies linking necrotic core parameters to conventional cardiovascular risk factors, and high-risk features in acute coronary syndrome. To determine how short LTL could be associated with vulnerable plaques, we examined the cytokine secretion from monocytes and lymphocytes. Monocytes from CAD patients showed reduced TL length, increased ROS generation, and increased proinflammatory cytokine secretion (MCP-1, IL-6, IL-1, tumor necrosis factor-α) vs controls. Telomestatin, an agent that induces telomere dysfunction and DDR, induced multiple features of replicative senescence in proliferating cells. In both lymphocytes and monocytes, telomestatin reduced POT-1 expression and telomere-located POT-1 and also induced DDR, as determined by H2AX phosphorylation. The downstream proteins involved in the DDR in monocytes and lymphocytes differed; specifically, lymphocytes showed reduced rather than increased expression of p27, p21, and p15 (Figure 3B). It is possible that lymphocytes were not undergoing cell cycle transit at the time of telomestatin treatment, which may impair a G1/S growth arrest response. Alternatively, p27, p21, and p15 may not be part of the growth arrest response of lymphocytes after telomestatin-induced DNA damage. Telomestatin induced a predominantly proinflammatory phenotype in leukocytes, as demonstrated in monocytes by increased ROS generation and MCP-1, IL-6, and IL-1β secretion, similar to that seen in CAD patients, and in lymphocytes by increased IL-2 and reduced IL-10 secretion. In contrast, telomestatin reduced monocyte tumor necrosis factor-α secretion, suggesting that the increased tumor necrosis factor-α secretion seen in CAD monocytes may be multifactorial.

Interestingly, the difference in LTL in lymphocytes between CAD patients and controls did not reach statistical significance. There are a number of possibilities for this observation. First, the numbers of CAD patients and controls in this group are too small to determine differences in lymphocytes that were smaller than that seen in other cells. In other studies, TL was shorter in leukocytes from CAD patients than in their age-matched controls in all subfractions studied, including CD34+ peripheral blood stem cells and progenitor cells, monocytes, granulocytes, B lymphocytes, and CD4+ T cells, including their memory and naive subpopulations. Alternatively, circulating lymphocytes are released from sites outside of the bone marrow, and it may be that progenitor cells or lymphocytes at these sites are not affected to the same extent as the bone marrow. It should also be noted that lymphocytes from CAD patients did not show a similar proinflammatory phenotype to that seen after telomestatin.

Senescent cells in culture develop a complex senescence-associated secretory phenotype or senescence-messaging secretome, becoming capable of secreting a wide range of proinflammatory cytokines, including IL-6 and IL-8. The senescence-associated secretory phenotype can significantly affect local nonsenescent cells, for example, by promoting cell proliferation, migration, and differentiation, and also reinforce senescence arrest by local autocrine or paracrine mechanisms. Recent studies have shown that persistent DNA damage (rather than transient growth arrest) induces cytokine secretion (particularly IL-6 and IL-8), requiring the multiple DNA damage response proteins. Our studies sug-
gest that DNA damage in leukocytes directly promotes inflammation and further leukocyte migration.

Both aging and atherosclerosis are associated with DNA damage and changes in monocyte and lymphocyte subsets and function. Elderly subjects show a significant expansion of CD14+CD16− circulating monocytes, which have increased constitutive production of classically activated (M1) monocytic cytokines, including IL-1β and IL-6. A similar proinflammatory phenotype of lymphocytes has been described as CD3+CD28 in elderly patients. Both M1 and alternatively activated (M2) macrophages are present in atherosclerosis, but hypercholesterolemia and proinflammatory factors such as LPS, IL-1β, and interferon-γ all promote the M1 phenotype. The increased secretion of MCP-1, IL-6, and IL-1β seen after telomestatin and in CAD patients suggests that TL shortening may promote a M2–M1 transition. Thus, accelerated leukocyte aging may have numerous functional consequences, including a proinflammatory phenotype, which may accelerate atherosclerosis. Although numerous studies have identified that telomere shortening is associated with atherosclerosis, our study demonstrates that telomere shortening may be causal in atherosclerosis and identifies 1 potential mechanism by which this might occur. Our data support the general theory of TL shortening in aging and also indicates that TL shortening in leukocytes may directly promote accelerated aging of the leukocytes and age-related vascular disease.

**Limitations**

Previous epidemiological or therapeutic studies that lacked imaging showed an association between LTL and cardiovascular events using study populations of many thousands of patients compared with our smaller study numbers (n = 170). However, we find that VHTCFA number was independently associated with shorter LTL, and that LTL is associated with family history of MI, demonstrating that 170 patients are sufficient to demonstrate strong associations. Although VH-IVUS is the best-validated method of identifying TCFA in vivo, it has some technological limitations. Foremost is its 100- to 150-μm resolution, which is inadequate to resolve the 65-μm cap in the histology definition of TCFA, such that VH-IVUS will overestimate TCFA number with some histology-identified thick-cap fibroatheroma classified as VHTCFA. Despite these limitations, VHTCFA were associated with shorter LTL.

In conclusion, we show that specific high-risk plaque subtypes and composition rather than total coronary plaque burden are associated with shorter LTL, a marker of leukocyte senescence, providing a plausible mechanism to link leukocyte ageing and the vulnerable plaque phenotype.

**Sources of Funding**

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**References**


Leukocyte Telomere Length Is Associated With High-Risk Plaques on Virtual Histology Intravascular Ultrasound and Increased Proinflammatory Activity

Patrick A. Calvert, Tze-Vun Liew, Isabelle Gorenne, Murray Clarke, Charis Costopoulos, Daniel R. Obaid, Michael O'Sullivan, Leonard M. Shapiro, Duncan C. McNab, Cameron G. Densem, Peter M. Schofield, Denise Braganza, Sarah C. Clarke, Kausik K. Ray, Nick E.J. West and Martin R. Bennett

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Supplemental material

**VH-IVUS acquisition and core-lab analysis**

VH-IVUS was performed in all main coronary arteries (target vessels before and after PCI and also non-target vessels) following administration of glyceryl trinitrate. Data was acquired with the 20MHz Eagle-Eye Gold catheters (Volcano Corporation, Rancho Cordova, USA) using a motorized pullback at 0.5 mm/s from the most distal safe position up to the guide catheter. Greyscale data was captured at 30 frames/s. Backscatter data was captured at the ‘R’ wave peak using electrocardiographic triggering to adjust for catheter oscillation during myocardial contraction. Data was acquired on the S5 consul software version 3.1 (Volcano Corporation) and analysis performed offline by the Krakow Cardiovascular Research Institute (KCRI) core-lab, using the Volcano Image Analysis Software (VIAS) version 3.0.394. The kappa coefficient of agreement for plaque classification for intra-observer variability was 0.91 (interval 6 months) and inter-observer variability was 0.73 indicating excellent reproducibility. The investigators had direct access to the primary data in addition to the analysis from KCRI.

**Plaque classification**

Plaques were classified as below, consistent with subsequently published VH-IVUS classification\(^1\).

For three consecutive frames,

1. **Plaque**: plaque burden of >40% vessel cross-sectional area.

2. **Fibroatheroma (VHFA)**: plaque burden >40%, confluent necrotic core (NC) >10% plaque cross-sectional area.
3. Thin-capped fibroatheroma (VHTCFA): Fibroatheroma (above) with NC in contact with vessel lumen, subdivided into calcified (dense calcium >10% plaque cross-sectional area) and non-calcified VHTCFA.

4. Thick-capped fibroatheroma (VHThCFA): Fibroatheroma not fulfilling VHTCFA conditions, subdivided as above.

5. Fibrocalcific plaque (VHFCa): Plaque with dense calcium >10% plaque cross-sectional area, not meeting fibroatheroma definition.

6. Pathological intimal thickening (VHPIT): Plaque not meeting fibroatheroma or calcific plaque definitions and predominantly fibrous tissue.

References
### Supplemental Table I. Clinical Parameters and Leukocyte Telomere Length (LTL)
Parameters were compared using the unpaired *t*-test, Mann Whitney U test or chi-squared test as appropriate. Each parameter was then tested for an association with lower LTL tertiles using univariate logistic regression. m (months), LDL (low density lipoprotein), HDL (high density lipoprotein), MI (myocardial infarction), GP2b3a inhibitor (glycoprotein 2b3a inhibitor), OR (odds ratio), CI (confidence interval).

<table>
<thead>
<tr>
<th>Clinical Parameters</th>
<th>Upper tertile of LTL (n=56)</th>
<th>Lower tertile of LTL (n=56)</th>
<th>p value</th>
<th>Univariate Analysis</th>
<th>Odds ratio (OR)[95% CI]</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>59.9 (11.0)</td>
<td>63.1 (10.2)</td>
<td>0.12</td>
<td></td>
<td>1.03 [0.99-1.07]</td>
<td>0.13</td>
</tr>
<tr>
<td>ACS presentation</td>
<td>25 (45%)</td>
<td>23 (41%)</td>
<td>0.70</td>
<td></td>
<td>1.05 [0.48-2.31]</td>
<td>0.91</td>
</tr>
<tr>
<td>Percentage Male</td>
<td>42 (75%)</td>
<td>48 (86%)</td>
<td>0.15</td>
<td></td>
<td>2.00 [0.76-5.23]</td>
<td>0.16</td>
</tr>
<tr>
<td>Statin treatment for &gt;3 months</td>
<td>18 (32%)</td>
<td>20 (36%)</td>
<td>0.91</td>
<td></td>
<td>1.05 [0.48-2.31]</td>
<td>0.91</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.5 (1.5)</td>
<td>5.6 (1.1)</td>
<td>0.95</td>
<td></td>
<td>1.01 [0.71-1.44]</td>
<td>0.95</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>2.5 (1.1)</td>
<td>2.3 (0.9)</td>
<td>0.57</td>
<td></td>
<td>0.88 [0.56-1.37]</td>
<td>0.59</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.2 (0.4)</td>
<td>1.1 (0.3)</td>
<td>0.56</td>
<td></td>
<td>0.69 [0.20-2.38]</td>
<td>0.55</td>
</tr>
<tr>
<td>Total cholesterol : HDL ratio</td>
<td>4.3 (1.4)</td>
<td>4.2 (1.8)</td>
<td>0.70</td>
<td></td>
<td>0.95 [0.71-1.26]</td>
<td>0.70</td>
</tr>
<tr>
<td>Smoked in the past 3 m</td>
<td>14 (25%)</td>
<td>13 (23%)</td>
<td>0.74</td>
<td></td>
<td>0.86 [0.36-2.06]</td>
<td>0.74</td>
</tr>
<tr>
<td>Never Smoked</td>
<td>15 (27%)</td>
<td>11 (20%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex-smoker &gt; 3 m</td>
<td>27 (48%)</td>
<td>32 (57%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>28 (50%)</td>
<td>29 (52%)</td>
<td>0.93</td>
<td></td>
<td>1.04 [0.49-2.20]</td>
<td>0.93</td>
</tr>
<tr>
<td>Previous MI</td>
<td>1 (2%)</td>
<td>5 (9%)</td>
<td>0.093</td>
<td></td>
<td>5.41 [0.61-47.93]</td>
<td>0.13</td>
</tr>
<tr>
<td>Family history of MI</td>
<td>19 (34%)</td>
<td>26 (46%)</td>
<td>0.017</td>
<td></td>
<td>1.74 [0.79-3.80]</td>
<td>0.17</td>
</tr>
<tr>
<td>Diabetes</td>
<td>7 (13%)</td>
<td>8 (14%)</td>
<td>0.75</td>
<td></td>
<td>1.19 [0.40-3.55]</td>
<td>0.75</td>
</tr>
<tr>
<td>Thrombolysis on this admission</td>
<td>6 (11%)</td>
<td>7 (13%)</td>
<td>0.77</td>
<td></td>
<td>1.19 [0.37-3.80]</td>
<td>0.77</td>
</tr>
<tr>
<td>GP2b3a inhibitor on this admission</td>
<td>6 (11%)</td>
<td>5 (9%)</td>
<td>0.75</td>
<td></td>
<td>0.82 [0.23-2.85]</td>
<td>0.75</td>
</tr>
</tbody>
</table>
Supplemental Figure I. Lymphocytes from CAD patients show no change in cytokine secretion (A-E) Baseline and LPS-induced secretion of cytokines from lymphocytes from CAD patients (n=18) or control subjects (n=9). Data are means.
Supplementary Figure II. Telomestatin induces growth arrest and a dose-dependent apoptosis in VSMCs

Human VSMCs were incubated with increasing doses of telomestatin and % cumulative cell proliferation (A) or apoptosis (B) assayed by time lapse videomicroscopy. (C) Graph of SAβG expression in human VSMCs treated with 5 μM telomestatin or control. Data are means.
Supplemental Figure III. POT-1 nuclear foci in monocytes and lymphocytes with increasing concentrations of Telomestatin. Data are means, error bars indicate SEMs. n=3.

Supplemental Figure IV. Telomestatin increases reactive oxygen species generation in monocytes Monocyte reactive oxygen species generation ± LPS treatment with increasing concentrations of telomestatin. Data are means, (n=3).