Short Leukocyte Telomere Length Is Associated With Cardioembolic Stroke Risk in Patients With Atrial Fibrillation

Mikel Allende, PhD; Eva Molina, BSc; José Ramón González-Porras, MD, PhD; Estefanía Toledo, MD, PhD; Ramón Lecumberri, MD, PhD; José Hermida, MD, PhD

Background and Purpose—The risk of cardioembolic stroke in patients with atrial fibrillation (AF) cannot be accurately assessed and novel tools are needed to improve prediction. We hypothesize that telomere shortening constitutes a novel risk factor for cardioembolic stroke in patients with AF.

Methods—The peripheral blood leukocyte telomere length (LTL) was determined by real-time polymerase chain reaction in 187 patients with AF, 93 of them without stroke history and 94 of them having suffered 1 cardioembolic stroke. Percentiles were calculated according to LTL values in the nonstroke group to estimate the cardioembolic stroke risk associated with LTL using logistic regression models.

Results—Short LTL values were independently and dose-dependently associated with an increased risk of cardioembolic stroke, with an odds ratio (95% confidence interval) of 2.93 (1.24–6.94) and 6.26 (2.01–19.52), respectively, for sex, hypertension, diabetes mellitus, heart failure, and age-adjusted models using the LTL 10th and 5th percentile cut-offs, respectively.

Conclusions—Telomere shortening is associated with cardioembolic stroke risk in patients with AF. Prospective studies are encouraged to establish the value of LTL to improve prediction tools to categorize cardioembolic stroke risk in AF.

Key Words: atrial fibrillation • diabetes mellitus • heart failure • leukocytes • stroke

Atrial fibrillation (AF) is the major risk factor for cardioembolic stroke.1 CHADS2 (congestive heart failure, hypertension, age≥75 years, diabetes mellitus, stroke/transient ischemic attack [doubled]) or CHADS2-VASc (congestive heart failure, hypertension, age≥75 years [doubled], diabetes mellitus, stroke/transient ischemic attack [doubled], vascular disease, age 65–74 years, and sex category [female]), used to risk stratify patients with AF, are not accurate enough and >2% of patients per year are considered to be at low risk experience of stroke.2 Thus, novel tools to improve risk stratification are needed.

Telomeres are tandem TTAGGG repeats at the chromosome end to avoid unwanted events, such as chromosomal fusion. Their shortening is a consequence of cell division, and it has the consequence that, once a threshold is exceeded, cells enter a senescent/apoptotic state. Environmental factors such as oxidative stress or inflammation also contribute to shortening.3 Telomere length of peripheral blood leukocytes telomere length (LTL) adequately reflects telomere length of other cells, such as vascular cells.3,4 Importantly, short LTL has been associated with a variety of cardiovascular diseases.4–12 However, its association with the cardioembolic subtype of stroke has not been addressed. Because cardioembolic stroke risk increases with age in AF1 and, in turn, age influences telomere length,1 we hypothesized that short LTL is associated with cardioembolic stroke risk in patients with AF.

Methods

Study Population
A total of 204 patients with nonrheumatic AF, of whom 102 had never had a stroke and 102 had had 1 cardioembolic stroke, were consecutively recruited when attending the Anticoagulation Clinic of the University Hospital of Salamanca, Spain, between May 2009 and February 2010. The diagnosis, inclusion, and exclusion criteria are explained in the expanded Methods section of the online-only Data Supplement. The study was approved by the Institutional Review Boards of the Hospital of Salamanca and University of Navarra. All patients gave informed consent.

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LTL Measurement

The genomic DNA of peripheral blood leukocytes was isolated to measure LTL by a real-time polymerase chain reaction–based method\textsuperscript{13} as described in the expanded Methods section of this article.

Statistical Analysis

Continuous and categorical variables were compared between AF stroke and nonstroke patients using the Student’s \( t \) and the \( \chi^2 \) tests, respectively. Correlation was assessed using Spearman correlation coefficient.

A nonconditional logistic regression model was used to evaluate the LTL-associated stroke risk. We assessed the goodness of fit with the Hosmer–Lemeshow goodness-of-fit statistic. The main independent variable was LTL, of which the 10th and 5th percentiles according to the nonstroke group values were used as cut-offs. Univariate and sex, hypertension, diabetes mellitus, heart failure, and age-adjusted multivariate models were performed. Stata/SE 13.1 (StataCorp, College Station, TX) was used for the analyses.

Results

Table 1 shows the patients’ clinical characteristics. Seventeen patients were finally excluded because of lack of DNA. Patients with Stroke exhibited higher CHADS\textsubscript{2} values. There were no differences in sex, age, international normalized ratio, or creatinine.

Remarkably, LTL was significantly shorter in the patients with stroke (Table 1; Figure I in the online-only Data Supplement).

Because advanced age has been associated with telomere shortening,\textsuperscript{3} we studied their correlation in our population. We found no significant correlation between age and LTL (\( p=0.1; P=0.16) \). This unexpected result may be explained by the narrow range of age of our cohort.

To gain insights into the cardioembolic stroke risk associated with telomere shortening, nonconditional logistic regression models were applied, using the 10th and 5th percentiles of the nonstroke group LTL values as cut-offs. As shown in Table 2, LTL below the 10th percentile significantly increased the risk of cardioembolic stroke. Interestingly, the risk remained unchanged when sex and CHADS\textsubscript{2} components were used as confounders. It was noteworthy that the risk became higher when the 5th percentile was applied. Thus, short LTL independently and dose-dependently increases the cardioembolic stroke risk.

Discussion

New tools are needed to improve the assessment of cardioembolic stroke risk in patients with AF. We provide evidence that, among the patients with AF, cardioembolic stroke risk is dose-dependently associated with short LTL. Remarkably, the risk seems to be independent of traditional stroke risk factors.

The association between LTL and stroke has been a matter of controversy.\textsuperscript{8–12} However, those studies never specifically addressed the cardioembolic subtype: 2 reports excluded these patients,\textsuperscript{8,11} and the others included only a small proportion of them within the study population.\textsuperscript{9,10,12} Ours is the first study aimed to establish a relationship between LTL and this specific stroke subtype. Furthermore, our cohort presents an additional advantage: our control group consists of patients with AF, unlike the control groups of the cited studies that were made up of healthy subjects. Thus, we have found an independent association under rather severe analytic conditions.

Also controversial is the real role played by short telomeres in cardiovascular diseases: in spite of the fact that short telomeres seem to play a key role by driving cells into senescence and apoptosis,\textsuperscript{1,4} there are studies that do not support causality.\textsuperscript{14} Our finding that the cardioembolic stroke risk displayed a dose-dependency according to LTL values would provide support for the causative hypothesis.

Our study has limitations. Its retrospective nature precludes definitive conclusions about the prognostic power of LTL in predicting the cardioembolic stroke risk. Indeed, we cannot definitively establish that telomere shortening actively plays a role in the pathogenesis of cardioembolic stroke. However, our study was performed in a relatively small population. Nevertheless, we consider that the strength and dose-dependency of the observed results provide enough arguments to establish a sound relationship between LTL and cardioembolic stroke.

In summary, our findings indicate that short LTL was independently associated with higher cardioembolic stroke risk in patients with AF. Our findings encourage prospective studies to confirm that LTL constitutes an attractive biomarker to help in the decision to start anticoagulant treatment in patients with AF.

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### Table 1. Clinical Characteristics of the Patients With Atrial Fibrillation

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Nonstroke (n=93)</th>
<th>Stroke (n=94)</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>74.7±8.4</td>
<td>76.7±7.0</td>
<td>0.070</td>
</tr>
<tr>
<td>Sex (female), n (%)</td>
<td>43 (46.2)</td>
<td>51 (54.3)</td>
<td>0.201</td>
</tr>
<tr>
<td>CHADS\textsubscript{2}</td>
<td>1.47±0.84</td>
<td>1.72±0.87</td>
<td>0.047</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>62 (66.7)</td>
<td>73 (77.7)</td>
<td>0.093</td>
</tr>
<tr>
<td>Heart failure, n (%)</td>
<td>6 (6.5)</td>
<td>9 (9.6)</td>
<td>0.432</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>11 (11.8)</td>
<td>18 (19.2)</td>
<td>0.167</td>
</tr>
<tr>
<td>Previous stroke, n (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>...</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>1.00±0.26</td>
<td>0.98±0.35</td>
<td>0.642</td>
</tr>
<tr>
<td>INR</td>
<td>2.37±0.56</td>
<td>2.33±0.68</td>
<td>0.751</td>
</tr>
<tr>
<td>Time since atrial fibrillation, years</td>
<td>5.89±4.12</td>
<td>5.87±4.71</td>
<td>0.990</td>
</tr>
<tr>
<td>LTL (RU)</td>
<td>4.82±1.43</td>
<td>3.87±3.17</td>
<td>0.010</td>
</tr>
</tbody>
</table>

### Table 2. Risk of Cardioembolic Stroke in Patients With Atrial Fibrillation According to LTL

<table>
<thead>
<tr>
<th>LTL</th>
<th>OR (95% CI)</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10th percentile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude model</td>
<td>2.85 (1.23–6.59)</td>
<td>0.014</td>
</tr>
<tr>
<td>Adjusted model*</td>
<td>2.93 (1.24–6.94)</td>
<td>0.014</td>
</tr>
<tr>
<td>&lt;5th percentile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude model</td>
<td>5.64 (1.84–17.30)</td>
<td>0.003</td>
</tr>
<tr>
<td>Adjusted model*</td>
<td>6.26 (2.01–19.52)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

\( \text{CI} \) indicates confidence interval; LTL, leukocyte telomere length; and OR, odds ratio.

*Sex, hypertension, diabetes mellitus, heart failure, and age-adjusted models.
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Disclosures

None.

References

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EXPANDED MATERIALS AND METHODS

Cardioembolic stroke diagnosis criteria

Cardioembolic stroke was defined as the sudden onset of a focal neurologic deficit in a location consistent with the territory of a major cerebral artery and confirmed by imaging techniques (magnetic resonance imaging or X-ray computed tomography). The cardioembolic origin was allocated if atrial fibrillation (AF) was present at the time of stroke diagnostic and there was not present a carotid artery lesion occluding more than 50% of the lumen diameter in the side of the infarction according with the TOAST criteria (Trial of Org 10172 in Acute Stroke Treatment).1

Patients inclusion and exclusion criteria

The inclusion criteria were AF diagnosed by electrocardiography, which lasted more than three months and, in the case of the stroke patients, was diagnosed before or at the time of the stroke. Patients were excluded from the study if they met any of the following criteria: carotid artery lesion occluding more than 50% of the lumen vessel diameter in the side of the infarction, cancer in progress, leukocytosis (more than 7,000 cells/mL), leukopenia (less than 3,500 cells/mL), history of venous thromboembolism or acute coronary syndrome in the last three months, infection, autoimmune disease or surgery. Renal failure (creatinine value more than double the normal value), oral contraceptive use, hormonal therapy, and corticoid consumption were also exclusion criteria. Demographic variables [age, sex, AF diagnosis date, stroke date, CHADS2, international normalized ratio (INR) and creatinine] were collected at the time of the blood test.

Leukocyte telomere length (LTL) measurement

Genomic DNA of peripheral blood leukocytes was isolated using standard protocols to determine LTL using a real-time quantitative PCR (RT-PCR)-based method previously described by Cawthon.2 In brief, LTL was the ratio between the copy number of telomere repeats (T) and that of the single-copy gene ribosomal protein, large PO (RPLPO) (S), used as a quantitative control. The T/S ratio was calculated as follows: \[ \frac{2^{C_t(\text{telomeres})}}{2^{C_t(\text{single copy gene})}} = 2^{-\Delta C_t} \] (Ct, threshold cycle) because the amount of the PCR product approximately doubles in each cycle of the PCR. On the other hand, a calibration curve of the same DNA reference sample (from 50 to 3.125 ng/μL in 2-fold dilutions) was always included for each measurement as a standard to control the day-to-day variations.

RT-PCR was performed using an ABI-Applied Biosystems 7900 HT thermal cycler (Applied Biosystems, Foster City, CA, USA). QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA) was used as master mix. Standard curves with linearity \( R^2 > 0.98 \) were accepted. All samples were run in triplicate, and the ones exhibiting Ct differences among triplicates higher than 0.5 cycles were discarded. A no-template control was also included for quality control. The intra-assay coefficient of variation between triplicates for telomeres and for the single copy gene were 1.95 and 0.95% respectively. DNA reference sample was used to calculate the inter-assay coefficient of
variation, which was 1.34 and 1.08% for telomeres and for the single copy gene respectively. Thus, the small coefficients of variation support the reliability of this procedure.

SUPPLEMENTAL FIGURES

Figure I

Figure I. Distribution of leukocyte telomere length (LTL) in AF patients. LTL was calculated as the natural log of the ratio between the copy number of telomere repeats and that of the single-copy gen. RU, relative units.

ON LINE SUPPLEMENT REFERENCES
