No Shorter Telomeres in Subjects With a Family History of Cardiovascular Disease in the Asklepios Study

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Objective—Shorter telomere length is associated with the occurrence of cardiovascular events, but the question of causality is complicated by the intertwined effects of inheritance, aging, and lifestyle factors on both telomere length and cardiovascular disease (CVD). Some studies indicated that healthy offspring of coronary artery disease patients exhibited shorter telomeres than subjects without a family history. Importantly, this result would imply that inheritance of shorter telomeres is a primary abnormality associated with an increased risk of CVD, the so-called Telomere Hypothesis of CVD. Therefore, we aimed at further validating the latter results in the large, population-representative Asklepios Study.

Methods and results—Peripheral blood leukocyte telomere length was measured using telomere restriction fragment analysis in the young to middle-aged (≥35–55 years old) Asklepios study population, free from overt CVD, and could be successfully combined with data from the Asklepios Family History Database for 2136 subjects. No shorter telomere length could be found in healthy subjects with a family history of CVD compared with those without.

Conclusion—These findings cast serious doubt on the hypothesis that telomere length is shorter in families with an increased risk of CVD and do not support the Telomere Hypothesis of CVD. (Arterioscler Thromb Vasc Biol. 2012;32:3076-3081.)

Key Words: Asklepios Study ■ cardiovascular disease ■ family history ■ telomere ■ telomere hypothesis of cardiovascular diseases

Telomeres are the protein-embedded DNA repeats composing and protecting the chromosomal termini. Telomere length (TL), that is, the number of repeats, decreases with cellular replication and functions as a mitotic clock, inducing replicative senescence on critical attrition.1 It has been proposed that this cellular mechanism might also be causally involved in aging associated diseases, such as cardiovascular diseases (CVD), the so-called Telomere Hypothesis of CVD.2 Indeed, shorter telomeres (typically measured in peripheral blood leukocytes) have been demonstrated in subjects with advanced (but not preclinical) atherosclerosis,3,4 coronary heart disease,5 coronary artery calcification,6 aortic valve stenosis,7 and chronic heart failure.8 However, a subject’s TL decreases with age and also several cardiovascular risk factors have been associated with shorter TL (most likely attributable to an accelerated telomere attrition rate), for example, male sex, inflammation, oxidative stress, obesity, smoking, and hypertension,9–12 suggesting that shorter telomeres in CVD might also be an epiphenomenon instead of a primary abnormality. On the contrary, several studies could identify an increased risk for myocardial infarction and heart disease in subjects with shorter telomeres,2,13,14 although also here it is unclear to what extent TL might have already been affected by the (hidden) disease status or by confounding variables at baseline.

In their study on the interplay between TL and CVD, Brouilette et al15 eliminated potential baseline effects using an elegant study design: because TL and CVD risk are to an important extent inherited,16–18 they evaluated TL in healthy offspring of subjects with and without a family history of coronary artery disease (CAD). Results from this pilot study (=100 subjects) indicated shorter telomeres in the offspring of CAD patients, suggesting that shorter inherited telomeres predispose to CVD. This finding supports the Telomere Hypothesis of CVD implying that shorter telomeres are a primary, heritable, abnormality causally associated with an increased risk of CAD/CVD.15

To some extent, these findings could be confirmed in several small studies, that is, by Wong et al19 (60 offspring included) and Dei Cas et al20 (82 offspring), but also in the larger Bruneck3 (800 offspring) and European Atherosclerosis Research Study II (EARSII)31 (765 offspring) studies. However, results were often only borderline significant, and could be prone to confounding effects. In addition, results were sometimes only
significant in a specific tissue\textsuperscript{19} or subpopulation.\textsuperscript{21} Because the validity of the causality hypothesis remains a central issue in contemporary TL research, these results definitely warrant additional validation in large, representative populations, such as the Asklepios Study cohort.

The Asklepios Study is a longitudinal study on successful aging focusing at the interplay between aging, hemodynamics, and inflammation in the development of CVD,\textsuperscript{22} of which the first round was completed between 2002 and 2004, and the second round was started in 2011. The presence of both TL data and the Asklepios Family History Database (Van daele et al, submitted manuscript) in a large population free of overt CVD allows for an accurate evaluation of the aforementioned results.

Materials and Methods

Study Subjects
For a full description of the Asklepios study and included subjects, we refer to the study protocol, rationale, and baseline characteristics.\textsuperscript{22} In summary, the Asklepios Study population consists of an extensively phenotyped random sample of 2524 white volunteers aged $\geq$55 to 55 years from the twinned Belgian communities Erpe-Mere and Nieuwerkerken, who were free from overt CVD and other major concomitant illness at the time of inclusion. The study complies with the declaration of Helsinki, the protocol was approved by the ethical committee of the Ghent University Hospital and all subjects gave written informed consent.

Family History of Cardiovascular Disease
Family history of CVD was based on the Asklepios Family History Questionnaire, providing information on the occurrence of CVD for 4 generations of the participant’s family. Because the participants had several days to complete the questionnaire, they could obtain additional information from family members. On completion, all questionnaires were reviewed by the study nurse together with the subject on the day their samples and other measurements were collected. For 373 volunteers (14.8%), questionnaires were not completed or considered to be unreliable because of insufficiently accurate knowledge of their family history and were excluded from further analysis. For more details we refer to Van daele et al (submitted manuscript) and Rietzschel et al.\textsuperscript{22}

The basic definition for family history of CVD is occurrence of premature CVD (<55 years for men and <65 years for women) in a first-degree relative (conventional family history, cFH), as commonly used in current guidelines.\textsuperscript{22} This definition classifies subjects into 2 classes: a large group with a negative family history (cFH-negative) and a smaller group with a positive family history (cFH-positive). In addition, to obtain a potentially higher sensitivity, we explored the effect of using an extended family history definition (eFH), which also incorporates information regarding late CVD (55 or 65 years old in men and women respectively) and occurrence of disease in second-degree relatives. In particular, we further divided the large group of cFH-negative subjects into an eFH low-risk ($\leq$1 second-degree relative with late-onset CVD; or no known CVD in any first- or second-degree relatives) and an eFH moderate risk class ($\geq$2 first-degree relative with late-onset CVD; or $\geq$2 second-degree relatives with late-onset CVD; or 1 second-degree relative with premature CVD). The cFH-positive group was further complemented with subjects with $\geq$2 second-degree relatives with premature CVD to obtain the eFH high-risk group. In addition, because the analyses by Brouilette et al\textsuperscript{15} only focused on CAD, abovementioned definitions were also restricted to the latter to obtain a cFH and an eFH of CAD.

Telomere Length and CVD Family History

Telomere Length Measurements
Whole blood was sampled in EDTA tubes and cooled at 4°C for at most 3 days before DNA isolation using the Puregene Genomic Purification Kit (Genta Systems, MN). DNA was long term stored at $-80$°C and 5 μg was used for Hinf1/Rsa1 restriction digest, followed by gel electrophoresis, Southern blotting, radioactive hybridization of the telomeric fragments and weight markers, phospho-imaging, and quantification.\textsuperscript{10}

Statistical Analysis
Statistical analyses were performed in IBM SPSS Statistics version 20 (Chicago, IL). The level of significance ($\alpha$) was set at 0.05 and all tests were 2-sided. The independent t test, 1-way ANOVA, and Pearson $r^2$ test were used to test for unadjusted differences between family history classes, whereas general linear (continuous dependent variables) and multinomial logistic regression (discrete dependent variable smoking status) models were used to test for adjusted differences. With $>2100$ subjects in the final dataset, the study was sufficiently powered to validate the previously reported family history effect, if present (more information in the discussion section).

Results

Baseline Characteristics
TL and reliable cFH family history data were both available for 2136 subjects. Baseline characteristics of this subset, stratified by cFH, are available in Table 1. When comparing the groups with a positive and a negative cFH of CVD, there was a significant difference in systolic blood pressure ($P=0.007$), a borderline significant difference in body mass index ($P=0.070$), and nonsignificant differences in total cholesterol ($P=0.490$) and smoking status (smokers, nonsmokers, and ex-smokers; $P=0.306$) between positive and negative family history groups after adjustment for age and sex (general linear models, except for smoking status: multinomial logistic regression). Paternal age at the offspring’s birth is a known determinant of TL\textsuperscript{24,25} and might also be affected by social class and therefore family history of CVD. Here, however, paternal age did not differ between cFH classes ($P=0.506$).

Family History and Telomere Length
TL was not significantly associated with the cFH of CVD ($P=0.608$, independent t test, Figure 1). Using a general linear model to adjust for known determinants of TL, cFH ($P=0.699$) remained nonsignificant, whereas age, sex, and paternal age at birth clearly were (Table 2) complying with results for the full population and literature.\textsuperscript{10,24,25} Results for the univariate analyses of the different variables are available in Table II in the online-only Data Supplement. Additional adjustment for other potential confounders in Table 1 did not substantially alter these results, neither did stratifying the analyses according to sex (data not shown).

Brouilette et al\textsuperscript{15} only considered families with CAD and controls. Therefore, the analyses were repeated using the cFH of CAD. Because the exact type of CVD could not always be pinpointed by the participants, results from 212 questionnaires were removed from the analysis, yielding a data set of 1924 subjects. Also here, general linear models, adjusting for age, sex, and paternal age at birth, did not yield significant differences in TL depending on cFH ($P=0.894$).
As outlined in the discussion section, several studies (including Brouilette et al) did not use a straightforward definition of family history. Therefore, it was evaluated whether a more eFH of CVD and CAD could improve sensitivity and provide support for the Telomere Hypothesis of CVD after all.

Extended Family History and Telomere Length

An eFH was defined as outlined in the Materials and Methods section. The most prominent characteristic of this extended history is that it attempts to increase sensitivity by splitting up the conventional negative family history group in a low and a moderate risk class, whereas the positive family history group largely corresponds with the high-risk class. The baseline characteristics of the population stratified by eFH have been summarized in Table I in the online-only Data Supplement.

When considering this eFH of CVD, there was a significant difference in systolic blood pressure \( (P=1.2E-4) \), and there were borderline significant differences in body mass index \( (P=0.071) \), total cholesterol \( (P=0.061) \), and a nonsignificant difference in smoking status (smokers, nonsmokers, and ex-smokers; \( P=0.157 \)), between risk groups (low, moderate, high) after adjustment for age and sex (general linear models, except for smoking status: multinomial logistic regression). In addition, after (subject’s) age and sex adjustment, there was a significant difference in paternal age at birth between eFH risk classes \( (P=4.4E-9, \text{ general linear model}) \), with a lower paternal age in the low-risk group. Note that differences were overall more prominent because of the creation of low and moderate risk classes in the eFH.

However, this increased sensitivity did not result in significant eFH-dependent TL differences \( (P=0.373, \text{ 1-way ANOVA, Figure 2}) \), also when a general linear model was used to further adjust for age, sex, and paternal age (eFH as a whole, \( P=0.558 \), Table 2), or when an eFH definition restricted to CAD was considered \( (P=0.892, \text{ general linear model}) \). Stratification by sex did not alter these conclusions (data not shown).

Discussion

Over the last decade, epidemiologic telomere biology research accumulated massive amounts of evidence demonstrating that TL is at least a biomarker for CVD, but left 1 central question unanswered: does shorter TL play a causal role in CVD, and aging diseases in general, the Telomere Hypothesis of CVD. Because the inheritance component is large for both CVD and TL, Brouilette et al evaluated this hypothesis in healthy offspring, thereby largely eliminating the aging and lifestyle components, and found significantly shorter telomeres in

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**Table 1. Baseline Characteristics of the Population Under Study**

<table>
<thead>
<tr>
<th></th>
<th>cFH Neg. (n=1695)</th>
<th>cFH Pos. (n=441)</th>
<th>P Value*</th>
<th>Total (n=2136)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>45.83 (6.00)</td>
<td>46.30 (6.01)</td>
<td>0.146</td>
<td>45.93 (6.00)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td>0.765</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>813 (48.0%)</td>
<td>208 (47.2%)</td>
<td></td>
<td>1021 (47.8%)</td>
</tr>
<tr>
<td>Female</td>
<td>882 (52.0%)</td>
<td>233 (52.8%)</td>
<td></td>
<td>1115 (52.2%)</td>
</tr>
<tr>
<td>Telomere length, kbp</td>
<td>7.87 (0.72)</td>
<td>7.85 (0.74)</td>
<td>0.608</td>
<td>7.87 (0.72)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.57 (4.07)</td>
<td>26.0 (4.36)</td>
<td>0.050</td>
<td>25.66 (4.14)</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>216.3 (36.4)</td>
<td>218.2 (37.5)</td>
<td>0.334</td>
<td>216.7 (36.6)</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>126.5 (14.1)</td>
<td>126.6 (14.4)</td>
<td>0.005</td>
<td>126.9 (14.2)</td>
</tr>
<tr>
<td>Paternal age at birth, y†</td>
<td>31.52 (6.56)</td>
<td>31.82 (6.64)</td>
<td>0.393</td>
<td>31.58 (6.58)</td>
</tr>
<tr>
<td>Smoking status‡</td>
<td></td>
<td></td>
<td>0.308</td>
<td></td>
</tr>
<tr>
<td>Non</td>
<td>887 (52.3%)</td>
<td>218 (49.4%)</td>
<td></td>
<td>1105 (51.7%)</td>
</tr>
<tr>
<td>Ex</td>
<td>485 (28.6%)</td>
<td>125 (28.3%)</td>
<td></td>
<td>610 (28.6%)</td>
</tr>
<tr>
<td>Active</td>
<td>323 (19.1%)</td>
<td>98 (22.2%)</td>
<td></td>
<td>421 (19.7%)</td>
</tr>
</tbody>
</table>

Data indicate mean (SD) or frequency (%). cFH indicates conventional family history; Neg., negative; Pos., positive; kbp, kilobase pairs; BMI, body mass index.

*Independent t test (continuous data) or Pearson χ² test (frequencies).
†Data available for 1674 (98.8%) cFH-negative and 437 (99.1%) cFH-positive subjects.
‡All sources of nicotin.
offspring of CAD patients, indicating causality. In sharp
contrast with Brouilette et al and subsequent studies, our
results do not support that subjects with a family history of
CVD/CAD are featured by shorter TL. This implies that, in the
Asklepios Study population, shorter inherited telomeres are
not a primary abnormality leading to CVD, and therefore our
conclusion does not support the Telomere Hypothesis of CVD.

Importantly, it should be noted that our results do not
imply that telomere biology cannot be causally involved in
CVD. A subject’s (average peripheral blood leukocyte) TL
at a given moment in time is the product of both the inher-
ited TL and telomere attrition during life. Whereas the family
history design only considers the former, it remains possible
that the impact of telomere attrition is far more important
leading to critically short telomeres in specific tissues with
cardiovascular events as immediate consequence (for an
overview of possible mechanisms, see eg). Also, the TL
hypothesis might still prove to be valid for other (aging asso-
ciated) diseases.

There are several discrepancies between the Asklepios
Study and the other aforementioned studies that could pro-
vide an explanation for the different conclusions. First, off-
spring in the Asklepios Study underwent vascular and cardiac
echography and exhibited subclinical atherosclerosis at most
(in the scanned regions), whereas the offspring included in
the other studies were only apparently healthy or, as in the
Bruneck Study, consisted of a random subset of the popu-
lation with over 10% of cardiovascular events during the 10
years of follow-up. It is therefore not unlikely that the family
history–TL association might be confounded by clinically rel-
vant CVD present at the time of inclusion in at least some of
these study populations.

Vice versa, although the presence of CVD in the affected family
members was validated in some studies (Brouilette et al), fam-
ily history of CVD/CAD was only self-reported in the Asklepios
study. However, previous studies showed that questionnaires
regarding family history of CVD can be considered to be gener-
ally accurate. In addition, participants had the time to contact
family members during questionnaire completion, the question-
naires were additionally reviewed by the study nurse (together
with the participant), and questionnaires deemed unreliable were
eliminated from the analyses.

Another major difference between the different studies was
the definition of family history of CVD/CAD. For example,
their recruitment history forced Brouilette et al to combine
2 different family history definitions; the EARSII study only
considered paternal history of myocardial infarction; Dei
Cas et al used a very high age cutoff (75 years old) for his-
tory of events or revascularization procedures. Whereas the
other studies used an ad hoc definition of family history,
the Asklepios Study family history was defined using well-
accepted guidelines (cFH). In addition, an eFH was also
considered to further increase sensitivity, but did not yield dif-
ferent conclusions.

The specific differences in study conception and recruitment
also affect the included populations. The Asklepios Study
population was, for example, older than the other populations

![Figure 2](image-url)
(with the exception of the Bruneck population), which implies a relatively smaller weight of inheritance compared with aging and lifestyle components on a subject’s (average leukocyte) TL, in line with the findings by Nordfjäll et al., although the impact of this difference is, in all likelihood, minimal at most. Indeed, telomere dynamics are similar and gradual in both age groups of adults, contrasting the rather dramatic effects in young children. This is also reflected by the very comparable telomere attrition rates in the Brouilette et al. and Asklepios populations (27 versus 25 bp/y), suggesting that a possible shift in the weight of inheritance is by far outweighed by the overall effect of inheritance on average TL. The same reasoning is valid for other known differences between populations, because their effect on TL is typically substantially smaller than that of age.

With the exception of the Brouilette et al. study, the reported associations between family history and TL were typically weak to even borderline (P values ranging from 0.01–0.05), suggesting the potential impact of confounding factors. For example, besides the Asklepios Study, none of the studies adjusted for paternal age at the offspring’s birth. The latter is known to have a large impact on TL, tends to depend on socioeconomic class, an important cardiovascular risk factor modulator and affects the family history itself (younger parents are still at lower risk for CVD). In addition, Wong et al. did not adjust for age and sex, 2 other important TL determinants.

On the contrary, one could argue that the Asklepios study lacks sensitivity to detect the TL—family history association. Here, it should be noted that all relevant P values were clearly nonsignificant (>0.5) and that the number of included subjects was higher than in the other mentioned studies together. Indeed, post hoc analysis revealed that, given the distribution of CVD family history in our study, the size of our sample was sufficient to reach 99% statistical power for detecting differences in TL as small as 0.20 kbp (at the 5% significance level), which is less than half the 472 bp difference observed by Brouilette et al. Note that our study even reached 74% power for detecting small differences in TL of 0.10 kbp (which are actually beyond typical measurement error). In addition, P values of the other factors/covariates in Table 2, as well as other published telomere biology findings in this population, indicate that the TL measurement methodology used in the Asklepios Study is sufficiently sensitive to identify significant minor differences in TL. For example, the reported family history difference in TL reported by Brouilette et al. was about 2.5 times the clearly significant difference between sexes (Table 2) in the Asklepios Study.

Finally, it should be noted that results in the aforementioned studies are not always consistent. For example, Wong et al. reported results with respect to the offspring TL only to be significant in the leukocyte fraction, but clearly not in the mononuclear cell and CD34+ cell fractions nor in buccal cells. In addition, in the EARSII study, results were highly significant in the Baltic subpopulation (which did not exhibit shorter TL), but clearly not in the other European subpopulations, yielding a borderline significant overall result. If the Telomere Hypothesis of CVD were true, one would expect to find significant results in all tissues and populations: all tissues have roughly the same TL through inheritance (cf.), and any causal association between shorter TL and CVD should be present throughout different populations.

In summary, we could not validate the finding that healthy subjects with a family history of CVD have shorter telomeres. These results do not confirm the hypothesis that shorter inherited TL is a primary abnormality leading to an increased risk of CVD, and therefore do not support the Telomere Hypothesis of CVD. Additional, carefully designed studies are required to evaluate the association between TL and family history of CVD, and to provide a definitive answer to the Telomere Hypothesis of CVD.

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Disclosures
None.

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