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ORIGINAL RESEARCH

Number and Replating Capacity of Endothelial Colony-Forming Cells are Telomere Length Dependent: Implication for Human Atherogenesis

Simon Toupance, PharmD, PhD*; Stéphanie Simonici, PhD*; Carlos Labat, BSc; Chloé Dumoulin, BSc; Tsung-Po Lai, PhD; Cécile Lakomy, BSc; Véronique Regnault, PhD; Patrick Lacolley, MD, PhD; Françoise Dignat George, PharmD, PhD; Florence Sabatier, PharmD, PhD; Abraham Aviv, MD; Athanase Benetos, MD, PhD; on behalf of the TELARTA consortium†

BACKGROUND: Short leukocyte telomere length (TL) is associated with atherosclerotic cardiovascular disease. Endothelial repair plays a key role in the development of atherosclerosis. The objective was to examine associations between TL and proliferative dynamics of endothelial colony-forming cells (ECFCs), which behave as progenitor cells displaying endothelial repair activity.

METHODS AND RESULTS: To isolate ECFCs, we performed a clonogenic assay on blood samples from 116 participants (aged 24–94 years) in the TELARTA (Telomere in Arterial Aging) cohort study. We detected no ECFC clones in 29 (group 1), clones with no replating capacity in other 29 (group 2), and clones with replating capacity in the additional 58 (group 3). Leukocyte TL was measured by Southern blotting and ECFCs (ECFC-TL). Age- and sex-adjusted leukocyte TL (mean±SEM) was the shortest in group 1 (6.51±0.13 kb), longer in group 2 (6.69±0.13 kb), and the longest in group 3 (6.78±0.09 kb) (P<0.05). In group 3, ECFC-TL was associated with the number of detected clones (P<0.01). ECFC-TL (7.98±0.13 kb) was longer than leukocyte TL (6.74±0.012 kb) (P<0.0001) and both parameters were strongly correlated (r=0.82; P<0.0001).

CONCLUSIONS: Individuals with longer telomeres display a higher number of self-renewing ECFCs. Our results also indicate that leukocyte TL, as a proxy of TL dynamics in ECFCs, could be used as a surrogate marker of endothelial repair capacity in clinical and laboratory practice because of easy accessibility of leukocytes.

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Key Words: aging ■ endothelial progenitor cell ■ telomere

Atherosclerosis develops at the blood-vascular wall interface, where the vascular endothelium perpetually interacts with circulating blood cells. As the vascular endothelium and blood cells share a common embryonic precursor, the hemogenic endothelium,[1] their telomeres might be of similar lengths. This premise could explain the association of short leukocyte telomere length (LTL) with atherosclerotic cardiovascular disease (ASCVD).[2,3]

Atherosclerosis largely reflects repeated injury-repair cycles of the endothelium.[4] Endothelial repair starts with the migration of surrounding mature

Correspondence to: Athanase Benetos, MD, PhD, Department of Geriatrics, University Hospital of Nancy, 54511 Vandoeuvre les Nancy, France. E-mail: a.benetos@chru-nancy.fr

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*S. Toupance and S. Simonici contributed equally.

†A complete list of the TELARTA consortium members can be found in the Appendix at the end of the article.

For Sources of Funding and Disclosures, see page 7.

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CLINICAL PERSPECTIVE

What Is New?

- Our results indicate that the obtaining, replating capacity, and number of endothelial colony-forming cells (ECFCs) are positively associated with telomere length measured in both leukocytes and ECFCs.
- We also found a strong correlation between telomere length in leukocytes and in ECFCs.

What Are the Clinical Implications

- Individuals with longer telomeres display a higher number of self-renewing ECFCs suggesting that they might have a better endothelial repair capacity.
- ECFCs telomere length-mediated compromised replicative potential may contribute to atherogenesis; the implication of telomere dynamics in ECFCs replicative capacity may be of potential interest in prevention and/or treatment of the atherosclerotic process.
- Telomere length in leukocytes, as a proxy of telomere length in ECFCs, could be used as a surrogate marker of endothelial repair capacity in clinical and laboratory practice because of easy accessibility of leukocytes.

Nonstandard Abbreviations and Acronyms

ECFCs	endothelial colony-forming cells
ECFC-TL	endothelial colony-forming cells-telomere length
LTL	leukocyte telomere length
MTL	muscle telomere length
PBMC	peripheral blood mononuclear cells
TELARTA	Telomere in Arterial Aging
TL	telomere length
VWF	von Willebrand factor

endothelial cells, but these terminally differentiated cells have low proliferative potential and thus limited ability to replace the damaged endothelium.[5] So-called circulating endothelial progenitor cells might facilitate the re-endothelialization of injured arteries by replacing dysfunctional endothelial cells or secreting paracrine factors that attenuate vascular injury.[6] Accordingly, some studies reported association between low endothelial progenitor cell count or function and ASCVD.[7–12] However, the majority of these cells might be myeloid cells with paracrine angiogenic activity.[13] By contrast, circulating endothelial colony-forming cells (ECFCs), which originate from the vascular wall stem cell niche, display a strong vasculogenic and repair activity.[14–17] ECFCs senescence or low replicative capacity might, therefore, compromise endothelial repair.[18,19] If such a phenomenon stems from short telomeres, it could partially explain the LTL-ASCVD connection. In this work we tested hypotheses that link telomere length (TL) with parameters of ECFCs. To this end, we detected and counted ECFCs by an ex-vivo clonogenic assay, and subsequently quantified their replating capacity, proliferative potential, senescence, and vasculogenic capacity. In addition, we quantified circulating levels of von Willebrand factor (VWF), an endothelial dysfunction marker.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Subjects, Tissue Biopsies, and Blood Samples

This research draws on the TELARTA (Telomere in Arterial Aging) study.[20] Briefly, the overall goal of the TELARTA study was to examine the role of TL dynamics in arterial aging and the development of atherosclerosis. Men and women were enrolled in Nancy and Marseille, France, where they were hospitalized for various surgical procedures from June 2014 to May 2016. Skeletal muscle in the surgical field was biopsied during surgery and blood collected.

All participants provided written informed consent approved by the Ethics Committee (Comité de Protection des Personnes) and the investigation conformed to the principles outlined in the Declaration of Helsinki. This clinical study has been registered in ClinicalTrials.gov under Unique Identifier: NCT02176941.

Findings are based on data derived from a subset of the TELARTA cohort, comprising 116 TELARTA participants who agreed to an additional blood sampling of 80 mL for ECFCs isolation.

ECFCs Characterization

ECFCs Isolation

Clonogenic assays were performed using heparinated blood according to Ingram et al[21] and international standards.[22] Briefly, peripheral blood mononuclear cells (PBMCs) were seeded on fibronectin-coated plates in EGM-2MV culture medium (Lonza, Basel, Switzerland) and ECFCs clones appeared as adherent cells with typical cobblestone morphology after 10 to 15 days in culture. Primary colonies were then detached from culture plates by trypsin/EDTA treatment and serially

expanded in EGM2-MV culture medium until passage 3 to enable quantifying ECFC-TL, proliferative potential, senescence, and vasculogenic capacity.

Detection, Counting, and Replating Capacity of ECFCs

ECFCs were characterized by the following 3 parameters: First, we detected the presence of clones (yes/no) using the ex-vivo culture method. Second, we counted the number of clones per million of cultured PBMCs (henceforth, number of clones). Third, we quantified the ability of the clones to proliferate in culture (replating capacity).

Proliferative Capacity, Senescence, and Vasculogenic Potential of ECFCs

Among the primary clones, only those showing replating capacity were expanded in sufficient quantity to permit measurements of (1) proliferative capacity by 5-bromo-2'-deoxyUridine (BrdU) incorporation (Roche Molecular Biochemicals, Mannheim, Germany), (2) senesence based on senescence associated ß-galactosidase (SA-ß-gal) activity (BioVision Research Products, CA, USA), where percentage of SA-ß-gal positive cells was counted in 10 randomly selected microscopic fields (magnification ×20), and (3) vasculogenic potential, using a three-dimensional spheroid assay.[23]

For quantifying vasculogenic potential, ECFCs were suspended in culture medium containing 0.2% (wt/vol) carboxymethylcellulose, which was then seeded in non-adherent wells at a density of 400 cells/well. Spheroids were generated overnight and then collected and embedded into collagen I gels. The spheroid containing gels were rapidly transferred into pre-warmed Labtek II slides and allowed to polymerize (30 minutes); thereafter, 100 µL of EGM2-MV were added on the top of the gels. Following 24 hours of culture, the spheroids were fixed for 30 minutes in 4% paraformaldehyde at room temperature. After washing, photographs were taken with a phase-contrast Leica DMI8 microscope. To measure the cumulative sprout length per spheroid in vitro, sprouts from 20 spheroids were evaluated and the mean cumulative sprout length per spheroid was calculated. In addition, the number of branch points and the number of sprouts were counted in 20 spheroids and calculated as mean number of branch points and mean number of sprouts per spheroid.

TL Measurements

We measured LTL, ECFC-TL, and muscle TL (MTL), as an internal reference of a minimaly proliferative tissue. DNA was extracted by the phenol/chloroform/isoamyl alcohol method from EDTA blood, culture-expanded ECFCs and skeletal muscle biopsies, respectively. After a DNA integrity control in 1% agarose gel, TL measurements were performed in duplicate by Southern blot of the terminal restriction fragments, as described previously.[24] Briefly, DNA samples were digested (37°C) overnight with the restriction enzymes Hinf I and Rsa I (Roche Diagnostics GmbH, Mannheim, Germany). Digested DNA samples and DNA ladders were resolved on 0.5% (wt/vol) agarose gels. After 23 hours, the DNA was depurinated, denatured, neutralized, and transferred onto a positively charged nylon membrane (Roche Diagnostics GmbH) using a vacuum blotter (Biorad, Hercules, CA, USA). Membranes were hybridized at 42°C with a digoxigenin (DIG)-labeled telomeric probe after which the probe was detected by the DIG luminescent detection procedure (Roche Diagnostics GmbH) and exposed on charge coupled device CCD camera (Las 4000, Fujifilm Life Sciences, Cambridge, MA, USA). Optical density values versus DNA migration distances obtained from raw data were converted to optical density/molecular weight (MW) ratios versus MW using a power function transformation of DNA migration distance (mm) in MW (kb) owing to DNA ladders. Mean terminal restriction fragment was calculated in the 3 to 20 kb range and inter-assay coefficient of variation for duplicate measurements on different membranes was 1.4%.

von Willebrand Factor

VWF level in citrated plasma was measured by ELISA kit (Asserachrom, Stago, Asnières, France) according to the manufacturer's instructions. Results are expressed as percentage using the human calibrator supplied within the kit.

Statistical Analysis

Continuous variables are presented as means±SD or mean±SE, and discrete variables as frequencies or percentages. Pairwise comparisons were performed using T tests. TL values (in leukocytes, ECFCs and muscle) are presented and compared with or without adjustment to age and sex. VWF was log-transformed to normalize its distribution. Bivariate relationships between continuous variables were determined using Pearson correlation coefficients. Comparison between groups 1, 2, and 3 were performed using trend ANOVA. Paired *t*-tests were used for TL comparison between tissues (leukocytes, ECFCs, and muscles). A $P<0.05$ was considered significant. Statistical analyses were performed using the NCSS 9 statistical software package (NCSS, Kaysville, UT, USA).

Table. Characteristics of the Subjects

	All Subjects
No. of subjects	116
Women	40%
Age, y	63±15
BMI, kg/m^2	27.0±6.4
LTL, kb	6.69±0.88
MTL, kb	8.58±0.78
VWF, %	160±165

Values are expressed as mean±SD or percentage. BMI indicates body mass index; LTL, leukocyte telomere length; MTL, muscle telomere length; and VWF, von Willebrand factor.

RESULTS

Subjects

Characteristics of the participants are summarized in Table. Participants' age range was 24 to 94 years. LTL was associated with age (r=–0.57; P<0.0001) and women had longer age-adjusted LTL than men (6.90±0.10 kb versus 6.56±0.08 kb; P<0.02). LTL was correlated with MTL (r=0.74, P<0.0001) and VWF (r=–0.19, P<0.04 after adjustment for age and sex).

We classified subjects into 3 groups by their ECFCs detectability and replating capacity (Figure 1).

1. Group 1 (n=29): no clone detected after culture of PBMCs.
2. Group 2 (n=29): clones detected with no replating capacity.
3. Group 3 (n=58): clones with replating capacity.

Characteristics of the participants in these groups are summarized in Table S1. The 3 groups showed no difference in age, sex, and body mass index. The number of clones was higher in group 3 than in group 2 (0.62±0.81 and 0.28±0.36 clones/million PBMCs, respectively; P=0.02 after adjustment for age and sex).

LTL Associations With ECFCs Detectability and Replating Capacity

Age- and sex-adjusted LTL was associated with clone detectability and replating capacity (Figure 2; ANOVA trend, P<0.05), with the shortest LTL in group 1 (undetected clones; 6.51±0.13 kb), a longer LTL in group 2 (detected clones; 6.69±0.13 kb), and the longest LTL in group 3 (detected clones showing replating capacity 6.78±0.09 kb). For group 3, we observed a trend between the number of clones and LTL (P=0.12).

Relationship of ECFC-TL With Other ECFC Parameters (Group 3)

The number of clones was positively correlated with ECFC-TL (P=0.03, Figure 3). A 1 kb increase in ECFC-TL was associated with an increase of 0.283±0.125 clones. No association was observed between ECFC-TL and BrdU incorporation, SA-ß-gal activity, and vasculogenic potential (Table S2).

Relationship of ECFC-TL With VWF (Group 3)

ECFC-TL was inversely correlated with VWF plasmatic values (R=–0.37, P=0.008, Figure 4). This association remained after adjustment to age and sex (P<0.02).

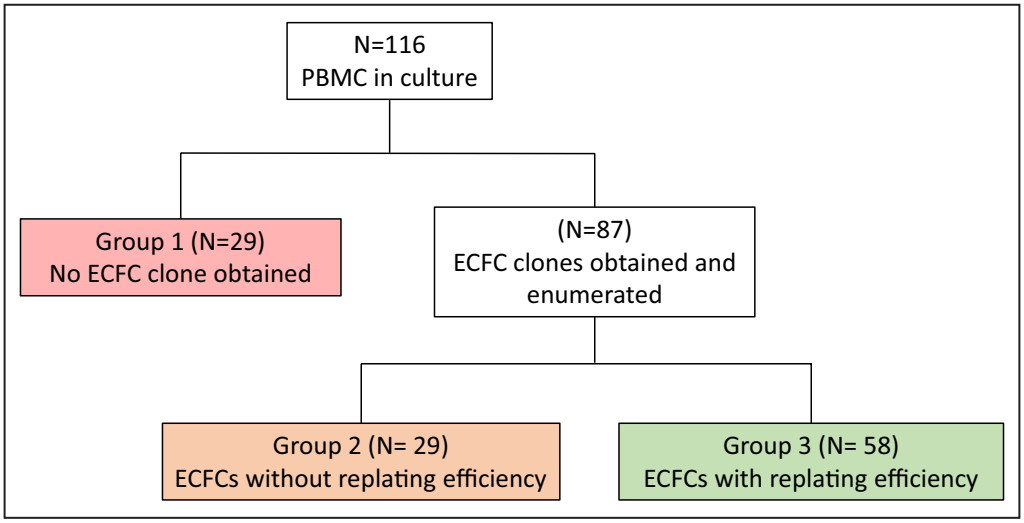

Figure 1. Classification of the subjects according to endothelial colony-forming cells detectability and replating capacity.
ECFCs indicates endothelial colony-forming cells; and PBMC, peripheral blood mononuclear cells.

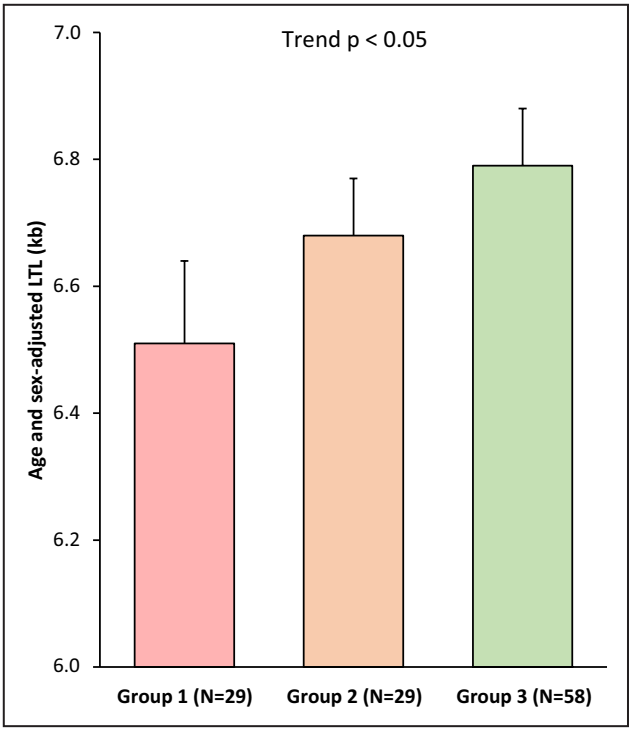

Figure 2. Leukocyte telomere length and endothelial colony-forming cells detectabity and replating capacity.
Age- and sex-adjusted leukocyte telomere length values in the 3 groups. Black lines and crossbars represent mean±SD, circles represent individual values, and *P* is from trend ANOVA. ECFCs indicates endothelial colony-forming cells; and LTL, leukocyte telomere length.

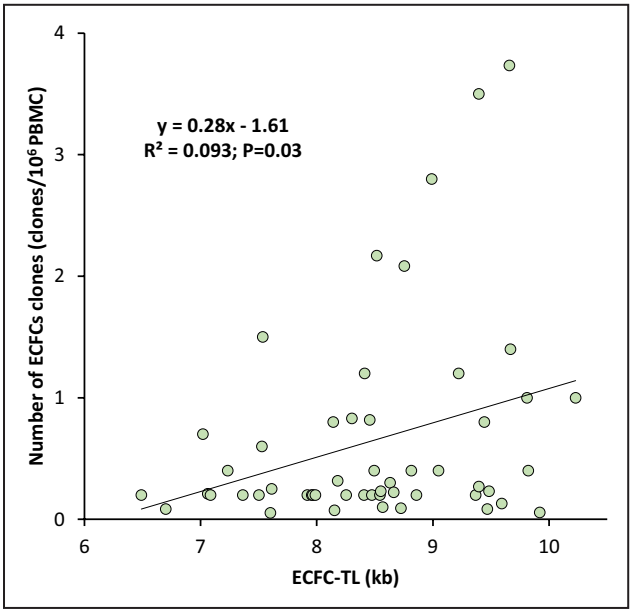

Figure 3. Endothelial colony-forming cells-telomere length and number of clones.
Number of endothelial colony-forming cell clones obtained from culture of peripheral blood mononuclear cells according to endothelial colony-forming cells-telomere length. R^2 and *P* are from Pearson correlation. ECFCs indicates endothelial colony-forming cells; ECFC-TL, endothelial colony-forming cells telomere length; and PBMC, peripheral blood mononuclear cells.

Relationship Between ECFC-TL, MTL, and LTL (Group 3)

ECFC-TL was shorter than MTL (Figure 5A, *P*<0.0001) and longer than LTL (*P*<0.0001). The individuals' values showed that ECFC-TL was always longer than LTL. ECFC-TL correlated with both LTL and MTL (*R*=0.79 and *R*=0.65, respectively, Figure 5B and 5C). Cross-sectional analysis showed that TL shortened with age as follows: ECFC=21.5 base pairs (bp)/year, muscle=17.9 bp/year, and leukocyte=35.8 bp/year (Figure S1).

DISCUSSION

The key findings of this research are as follows: (1) formation and number of ECFCs and their replating capacity are associated with TL (LTL and ECFC-TL), and (2) ECFC-TL largely reflects LTL and it shortens with age. It follows that individuals with shorter LTL, including older persons, have not only fewer circulating ECFCs, but also diminished TL-dependant ECFC replicative potential. This conclusion is consistent with the shorter LTL[2,3] and lower number of ECFCs[25–27] in individuals with ASCVD.

We found an association of TL with replating capacity of ECFCs but not with proliferation rate measured in self-replicating ECFCs (group 3). This is concordant with observations made in umbilical cord blood ECFCs where high proliferative potential ECFCs differ from low proliferative potential ECFCs by their number of population doublings and not their proliferation rate.[21,28] Interestingly, high proliferative potential ECFCs are associated with higher clonogenicity defined as increased initial number of isolated ECFCs,[29] a parameter we also found associated with ECFC-TL. These characteristics of high proliferative potential ECFCs are consistent with presence of long telomeres, known to postpone senescence, increase population doubling capacity, but not to increase proliferative rate of cells.[30,31]

The inverse association of VWF level with ECFC-TL is supported by previous finding of a higher level of VWF in individuals with shorter LTL.[32,33] The inverse association between LTL and VWF was attributed to increased leukocyte turnover in subjects with high VWF levels. However, our results suggest an alternative pathway. Given that 80% to 85% of circulating VWF is secreted by the endothelium[34,35] and as ECFC-TL strongly correlates with LTL, it is likely that endothelial cells with short telomeres secrete more VWF. This conclusion points, therefore,

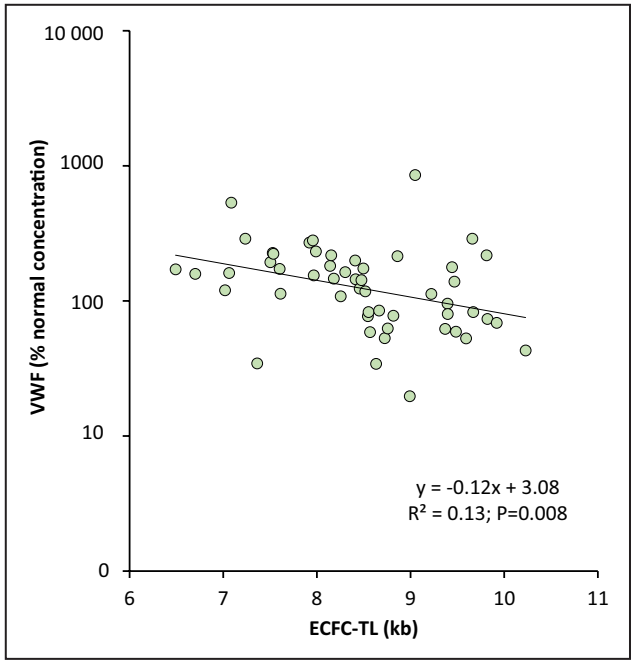

Figure 4. Endothelial colony-forming cells-telomere length and circulating von Willebrand factor levels.
Relationship between plasmatic levels of von Willebrand factor and ECFC-TL (n=52). R^2 and P are from Pearson correlation. ECFC-TL indicates endothelial colony-forming cells-telomere length; and VWF, von Willebrand factor.

to a potential role of ECFC-TL dynamics in the injury/repair of the vascular endothelium, the hallmark of atherogenesis.

Notably, findings of similar rates of age-dependant ECFC-TL and MTL shortening are consistent with the low replicative rate of ECFCs,[36] but more relevant for clinical settings is the correlation between ECFC-TL and LTL. Such a correlation suggests that LTL is a proxy for ECFC-TL, and hence endothelial repair capacity.

To our knowledge, our study is the first to show the association of LTL with the ECFCs and their TL dynamics, based on standardized methods of isolation and characterization of ECFCs. Moreover, we used Southern blotting, a precise method of TL measurement that generates data in absolute units of TL. We acknowledge also the limitations of the study. First, we could measure ECFC-TL only in subjects whose PBMCs generated clones with replating capacity (group 3). Second, the inference of rates of TL shortening is based on cross-sectional findings that may not accurately reflect interindividual variation based on longitudinal data. Third, the study provides mainly a descriptive association between replicative potential of cells and their TL, therefore molecular approaches such as transcriptomic profiles determination and telomere position effect assessment would be of interest to decipher molecular pathways and offer a mechanistic and integrated insight in future studies.

Finally, the link we observed between LTL and ECFC-TL could explain, in part, the association between short LTL and ASCVD[20,37] and the present study provides a new insight into the potential role of short TL in ASCVD. ECFCs are engaged in endothelial repair[15,26,38] and their TL-mediated compromised replicative potential may contribute to atherogenesis.

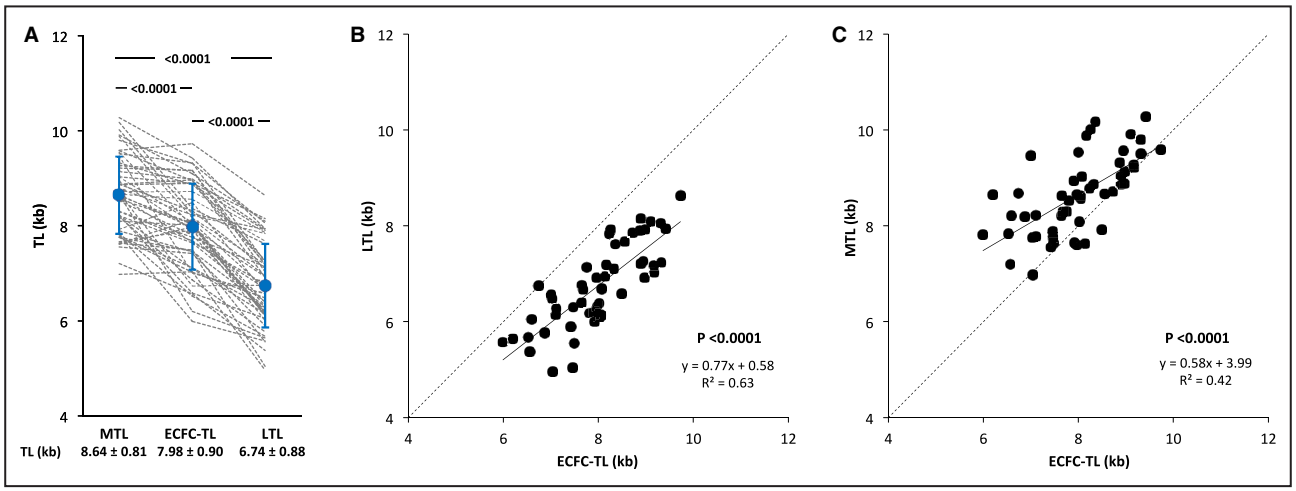

Figure 5. Relationship between endothelial colony-forming cells-telomere length (ECFC-TL), muscle telomere length, and leukocyte telomere length.
A, Mean and individual values of muscle telomere length, ECFC-TL, and leukocyte telomere length (n=52). Value are expressed as mean±SD (kb) and P are from paired t tests. **B**, Relationship between ECFC-TL and leukocyte telomere length. R^2 and P are from Pearson correlation. **C**, Relationship between ECFC-TL and muscle telomere length. R^2 and P are from Pearson correlation. ECFC-TL indicates endothelial colony-forming cells-telomere length; LTL, leukocyte telomere length; and MTL, muscle telomere length.

APPENDIX

Members of the TELARTA Consortium

Sylvie Gautier, Ghassan Watfa, Huguette Louis, Mélanie Folio, Margaux Schmitt, Oualid Ayad, Agnes Didier, Sandrine Vauthier, Nelly François, Sophie Visvikis-Siest, Maria G. Stathopolou, Masayuki Kimura, Pascal M. Rossi, Patricia Béranger, Serguei Malikov, Nicla Settembre, Jacques Hubert, Luc Frimat, Baptiste Bertrand, Mourad Boufi, Xavier Flecher, Nicolas Sadoul, Pascal Eschwege, Michèle Kessler, Irene P. Tzanetakou, Ilias P. Doulamis, Panagiotis Konstantopoulos, Aspasia Tzani, Marilina Korou, Anastasios Gkogkos, Konstantinos Perreas, Evangelos Menenakos, Georgios Samanidis, Michail Vasiloglou-Gkanis, Jeremy D. Kark, Simon Verhulst.

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Affiliations

From the Inserm, DCAC (S.T., C.L., C.L., V.R., P.L., A.B.); and CHRU-Nancy, Pôle Maladies du vieillissement, Gérontologie et Soins Palliatifs and Fédération Hospitalo-Universitaire CARTAGE-PROFILES (S.T., A.B.), Université de Lorraine, Nancy, France; Inserm, INRA, C2VN, Aix Marseille University, Marseille, France (S.S., C.D., F.D.G., F.S.); and Center of Human Development and Aging, Rutgers, The State University of New Jersey, New Jersey Medical School, Newark, NJ (T.L., A.A.).

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Disclosures

None.

Supplementary Material

Tables S1–S2
Figure S1

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SUPPLEMENTAL MATERIAL

Table S1. Patients characteristics in the 3 groups.

	Group 1	Group 2	Group 3	p
Number of subjects	29	29	58	
Women	34%	45%	40%	0.73
Age (years)	64 ± 16	59 ± 18	63 ± 13	0.44
BMI (kg/m^2)	28.2 ± 8.3	25.4 ± 6.7	27.1 ± 4.9	0.26
ASCVD (%)	41%	24%	40%	0.29
Hypertension (%)	52%	31%	49%	0.20
Diabetes (%)	21%	11%	19%	0.27
Dyslipidemia (%)	28%	10%	19%	0.25
Tobacco smoking (%)	38%	28%	47%	0.20

- Group 1 “No ECFC”: No clone detected after culture of PBMCs;
- Group 2 “ECFCs with no replating capacity”: Clones detected with no ability to expand in culture;
- Group 3 “ECFCs with replating capacity”: Clones detected showing the ability to expand in culture.

Values are expressed as mean +/- SD or %, p is from ANOVA or Chi-Square.

BMI= Body mass index; ASCVD= Atherosclerotic cardiovascular disease; ECFCs= Endothelial Colony Forming Cells; PBMC= peripheral blood mononuclear cells.

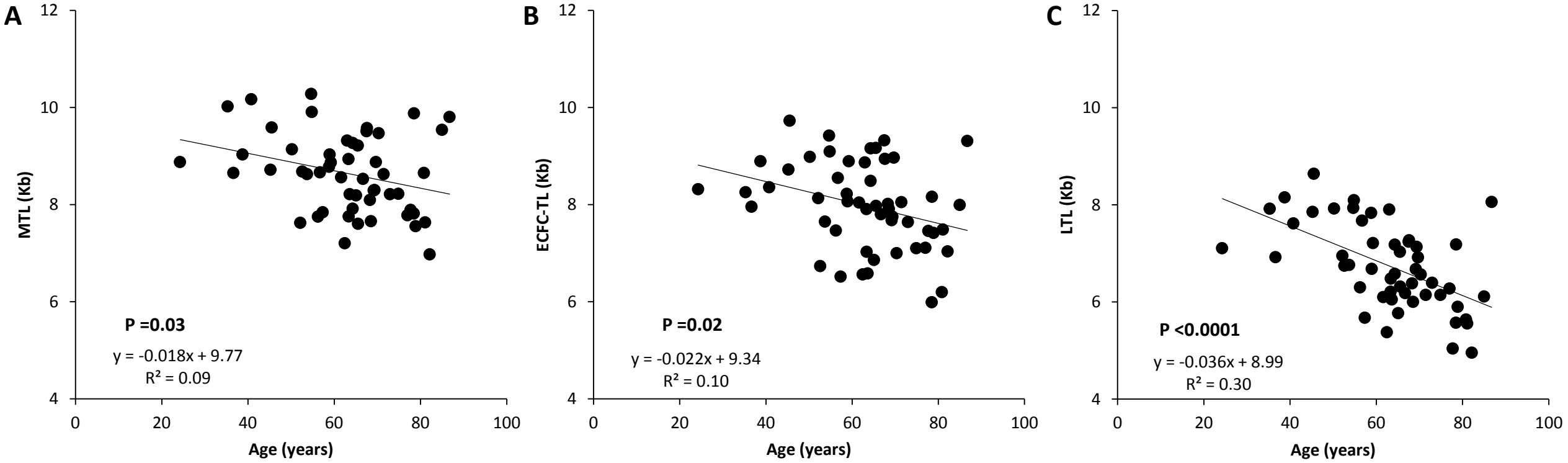
Table S2. ECFCs parameters and ECFC-TL.

	ECFC-TL (kb)	
	R	**P**
ECFCs Proliferation (BrdU incorporation, 10^6rlu/100ms)	0.16	0.26
ECFCs Senescence (SA-β-Gal positive cell %)	0.13	0.37
Sprout/spheroid	-0.10	0.49
branching points/spheroïd	0.01	0.93
Cumulative length of sprout and branching (mm)	-0.17	0.24

ECFCs parameters did not follow normal distribution. R and p are from Spearman rank correlation.

ECFCs= Endothelial Colony Forming Cells; BrdU= 5-bromo-2'-deoxyUridine; SA-β-Gal= senescence associated β-galactosidase; ECFC-TL= Endothelial Colony Forming Cells Telomere Length; kb= kilobase.

Figure S1. MTL, ECFC-TL, and LTL relation with age.



(A) MTL versus Age, (B) ECFC-TL versus Age and (C) LTL versus Age.

The slopes of the decreases in MTL (A) and ECFC-TL (B) are almost similar. The slope of the decrease in LTL (C) is double the slope of MTL (A). R^2 and p are from Pearson correlation.

MTL = muscle telomere length; ECFC-TL= Endothelial Colony Forming Cells telomere length; LTL= leukocyte telomere length; kb=kilobase.