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ORIGINAL RESEARCH

Telomere Length Is Associated With Adverse Atrial Remodeling in Patients With Atrial Fibrillation

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BACKGROUND: Atrial fibrillation (AF) is the most common cardiac arrhythmia with a massive burden on global health. The prevalence of AF increases dramatically with age and can be up to 18% in patients older than 80 years. Telomeres, which are short, repeated DNA sequences at the end of chromosomes, are known to act as a biological aging marker. In this study, we investigated the relation of telomere shortening and AF in the context of atrial remodeling. Furthermore, we assessed changes in the gene expression profiles of patients with AF according to telomere length (TL) and left atrial fibrosis.

METHODS: We included 72 patients undergoing catheter ablation for AF. Bipolar voltage maps were obtained to determine left atrial low voltage areas as a surrogate for atrial fibrosis. TL was quantified and correlated to low voltage areas. 3′ mRNA sequencing was performed for gene expression profiling. Clonal hematopoiesis of indeterminate potential was assessed by next generation sequencing. Telomerase reverse transcriptase knockout (*Tert*$^{-/-}$) and telomerase RNA component knockout (*Terc*$^{-/-}$) mice were used to investigate the mechanistic impact of telomere shortening on atrial remodeling.

RESULTS: Patients with advanced left atrial fibrosis had shorter telomeres compared with patients with healthy left atria. Furthermore, there was a strong correlation between the extent of left atrial low voltage areas, TL, and outcome after catheter ablation of AF. 24 months after ablation, only 26.5% of patients with advanced fibrosis and short TL were in sinus rhythm compared with 62.5% of patients with no/low fibrosis and long TL. Gene expression profiles and clonal hematopoiesis of indeterminate potential frequency differed in patients with AF with short and long telomeres. Finally, atrial tissue of mouse models with shortened telomeres showed marked left atrial fibrosis and over-expression of fibrosis-related genes.

CONCLUSIONS: Telomere shortening is correlated with left atrial remodeling. Shorter telomeres are associated with a series of molecular events which could eventually lead to cardiac fibrosis and perpetuate AF.

Key Words: aging ■ atrial fibrillation ■ fibrosis ■ telomere

Atrial fibrillation (AF) is the most common arrhythmia in adults with a massive impact on global health. In Europe, around 8 million adults suffered from AF in 2016, which will presumably reach around 14 million by 2060. AF is one of the major causes for stroke, heart failure, cognitive dysfunction, cardiovascular morbidity and mortality.[1]

The prevalence of AF is clearly associated with age, affecting 0.12% to 0.16% of patients younger than 49 years and 13.5% to 17.8% of patients beyond

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CLINICAL PERSPECTIVE

What Is New?

- Patients with atrial fibrillation and advanced structural remodeling have shorter age-adjusted telomeres.
- Telomere shortening is associated with systemic inflammation and expansion of clonal hematopoiesis of indeterminate potential and may therefore be mechanistically linked to left atrial remodeling.

What Are the Clinical Implications?

- Telomere length can be used as a predictor for left atrial fibrosis.
- Presence of left atrial low-voltage areas and short telomeres are both associated with an unfavorable treatment outcome after catheter ablation for atrial fibrillation.

Nonstandard Abbreviations and Acronyms

CHIP	clonal hematopoiesis of indeterminate potential
LVA	low voltage area
SR	sinus rhythm
TERC	telomerase RNA component
TERT	telomerase reverse transcriptase
TL	telomere length

80 years of age.[2] The median age of diagnosis is approximately 75 years. Aging is a multifactorial biological process, leading to a progressive decline of physiological functions. A major hallmark of aging is systemic inflammation, also known as inflammaging, which can lead to uncontrolled activation of fibrotic pathways, with excessive accumulation of extracellular matrix (ECM) and organ dysfunction. Therefore, organ fibrosis, including cardiac fibrosis, can commonly be detected in the elderly.[3]

Atrial fibrosis, specifically left atrial fibrosis, is a hallmark of arrhythmogenic structural remodeling in patients with AF and is a major driver of the disease.[4] Fibrosis plays an important role in the onset and perpetuation of AF through structural and electrical remodeling processes. The mechanisms that lead to atrial fibrosis are complex and not yet completely understood. However, clinical observations suggest that the aging process plays a central role in its pathogenesis.

Telomeres are short repeated DNA sequences at the end of chromosomes that have a protective function by preventing DNA damage responses and thereby reducing the risk of apoptosis.[5] With increasing biological age, telomere shortening can be observed leading to chromosomal instability.[6,7] Therefore, telomere length (TL) is considered to be a surrogate marker representing cellular age and is also known to be associated with age-related diseases including those affecting the human heart.[5,8,9]

The aim of this study was to investigate the relationship between left atrial remodeling, biological aging, and chronic inflammation. In addition, we aimed to demonstrate a pathophysiological role of shortened telomeres in the development and perpetuation of AF.

METHODS

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

Patients

A total of 72 patients ≥18 years who presented for catheter ablation of symptomatic persistent AF at the university hospital RWTH Aachen were included in this study. All patients gave their informed consent. The study was approved by the local ethics committee (EK054/21).

Animals

All animal husbandry and experimental procedures were conducted in accordance with the German Animal Protection Law and approved by the local animal authorities and performed according to the guidelines of the institution. Two models of telomere shortened mice were used, telomerase reverse transcriptase knockout (*Tert*$^{-/-}$) and telomerase RNA component knockout (*Terc*$^{-/-}$). The *Tert*$^{-/-}$ mice were generated as described previously.[10] C57BL/6J mice with a null mutation in the Tert gene were crossed until the fourth generation (G4). The knockout *Terc*$^{-/-}$[11] G1 mice were bred with C57BL/6J mice to generate heterozygote *Terc*$^{-/+}$ mice. Subsequently, the Terc$^{-/+}$ mice were bred over multiple generations to generate wild-type *Terc*$^{+/+}$ mice and *Terc*$^{-/-}$ G1, G2, and G3. TL in leukocytes, spleen and thymus for both lines was assessed by fluorescence-in-situ hybridization.[12]

Mapping and AF Ablation

Catheter ablation for AF including high density bipolar voltage mapping has been described previously.[13] Shortly after transseptal puncture, sinus rhythm (SR) was restored by cardioversion if necessary. A high-density 3D anatomical voltage map was generated using the Carto3 system (Biosense Webster, Johnson and Johnson) and a Pentaray/Octaray mapping

catheter. A minimum of 10 000 voltage points were acquired per patient. An electrogram <0.1 mV during SR was identified as a low-voltage area (LVA), a known surrogate for atrial fibrosis.[14,15] The Carto-3 built-in software was used to exclude the pulmonary veins and the mitral annulus, and the percentage of LVAs from the atrial corpus surface was calculated. Maps were color-coded to distinguish substantial LVAs (<0.1 mV, shown in red) from normal voltage (>0.5 mV, shown in purple). Patients were categorized into 2 groups according to their extent of LVAs: 36 patients with no to less LVAs (<10%) and 36 patients with advanced to massive LVAs (≥40%). Patients with intermediate LVAs (11%–39%, n=41) were excluded from the study to avoid analyzing individuals undergoing a transitional phase of disease and to enhance the precision of the results.

Pulmonary vein isolation was performed with a QDOT micro ablation catheter (Johnson & Johnson), following the "very high-power short duration" protocol with 50 W energy delivery at the anterior wall and 90 W at the posterior wall. Prior to ablation, a 20 mL blood sample was collected from a peripheral vein. Patients were followed-up by 72-hour Holter-ECG recordings at 3, 6, 12, and 24 months post ablation. In addition, patients were instructed to seek ECG documentation when symptoms suggestive of AF occurred. Recurrences were defined as documented AF (12-lead-ECG or Holter-ECG with a duration >30 seconds after a blanking period of 3 months).

TL Quantification

TL measurement was carried single-blinded as described previously.[16] Briefly, DNA isolation from peripheral blood was performed using DNeasy blood and tissue kit (Qiagen, Hilden, Germany). 1.4 ng of genomic DNA per reaction was used in the absolute Human TL Quantification qPCR Assay Kit (ScienCell, Carlsbad, CA, USA) and FastStart Essential DNA Green Master (Roche, Basel, Switzerland). TL measurements are given in T/S ratios, which are calculated based on the number of copies of the telomere template (T) divided by the single copy reference template (S), an amplified 100 bp region on human chromosome 17. All steps were done according to the manufacturer's protocol. Peripheral blood samples of 104 healthy blood donors of various ages were used as control group for age-adaption.[17] Age-adapted TL is given as ΔTel T/S ratio. Southern blot was performed to validate the quantitative polymerase chain reaction (qPCR) results. Power analysis indicated a power of 1 showing that despite the unequal distribution of telomere lengths, the sample size was adequate to reliably detect the observed effect. The reliability and consistency of the qPCR measurements were insured through a validation process. The amplification efficiencies of primers ranged from 96% to 99.8%. The Inter-assay variability ranged from 0.54% to 3.4% and intra-assay variability ranged from 0.45% to 1.42% which indicate that the assay is both accurate and reliable. Additional methods can be found in Data S1.

Next-Generation Sequencing Analysis

250 ng genomic DNA from peripheral blood was used per test batch for next generation sequencing analysis. Targeted amplicon sequencing was done on a tabletop sequencer (MiSeqDx, Illumina, California, USA) using the MiSeq Reagent Kit V3 in the AmpliSeq workflow (Illumina), as previously described.[18] A self-designed gene panel was used to detect somatic mutations related to clonal hematopoiesis, including ABL, ASXL1, BARD, CALR, CBL, CEBPA, CHEK2, CSF3R, DNMT3A, ETNK1, ETV6, EZH2, IDH1, IDH2, JAK2, KIT, KRAS, MPL, NFE2, NRAS, PDGFRA, PTPN11, RUNX1, SETBP1, SF3A1, SF3B1, SH3B2 (LNK), SRSF2, TCF12, TET2, TP53, and U2AF1.[18] Raw data were analyzed with the Illumina RTA software (version 1.18.54) after sequencing 250 bp paired end and the SeqNEXT software (version 4.4.0, JSI medical systems GmbH, Ettenheim, Germany) was used for alignment and variant calling. A variant allele frequency (VAF) threshold of 2% and at least 10 reads (absolute) was chosen to detect clonal variants in our cohort.

Epigenetic Age Prediction With Pyrosequencing

Epigenetic age prediction was performed by targeted DNA methylation analysis at 3 cytosine guanine dinucleotides (CpGs), as described previously.[16,19] 300 ng of genomic DNA from peripheral blood was bisulfite converted with the EZ DNA Methylation Kit (Zymo Research, Irvine, USA). For pyrosequencing analysis, DNA methylation at the 3 age-associated cytosine guanine dinucleotides (CpG sites) that are associated with the *CCDC102B* (coiled-coil domain-containing protein 102B), *FHL2* (four and a half LIM domains protein 2), and *PDE4C* (phosphodiesterase 4C) were amplified using the PyroMark PCR kit (Qiagen), as described in detail before.[16,19] Pyrosequencing was then performed on the PyroMark Q48 Autoprep system (Qiagen) using the PyroMark Q48 Advanced Reagent Kit. The results were analyzed using PyroMark Q48 Advanced software. All steps were done according to the manufacturer's protocol. Epigenetic age was then calculated as follows:

$$\text{Predicted age (in years)} = 8.21 + 0.91\ \text{DNAm}^{FHL2} - 0.68\ \text{DNAm}^{CCDC102B} + 0.78\ \text{DNAm}^{PDE4C}$$

Histological Analysis

Atrial tissue from 3 months old *Terc*$^{-/-}$ and *Tert*$^{-/-}$ male mice (WT, second generation, and third generation), except for the *Tert*$^{-/-}$ mice (fourth generation) where the tissues were cryoprotected using optimal cutting temperature compound, was fixed in 4% paraformaldehyde and embedded in paraffin. The sections were cut into 4 μm sections and mounted on glass slides. Deparaffinization and rehydration were done in a series of xylol and ethanol. Heat induced antigen retrieval (citrate buffer) was followed.

For cryosections, the slides were fixed in 4% paraformaldehyde for 5 minutes and washed with 0.5% tween-PBS. After blocking the tissues with 5% BSA diluted in PBS, overnight incubation was done at 4 °C with primary antibodies against collagen I (1:100) (CL50151-AP, Cedarlane) and titin (1:100) (TTN-3y, Myomedix). The secondary antibodies used were FITC Affinipure donkey anti-rabbit IgG (1:33) (711–095-152, Jackson Immunoresearch) and Cy3 Affinipure goat anti-chicken IgY (1:150) (103–165–155, Jackson Immunoresearch). Mounting was done using Vectashield antifade mounting medium with DAPI (Vectashield, vector Laboratories). Imaging was performed using a Leica DM5500B microscope and quantified with Image J software.

RNA Isolation and Quantitative Real Time PCR

RNA was isolated from frozen left atrium tissue of mouse using RNeasy mini kit (Qiagen). cDNA was transcribed using the iScript cDNA synthesis kit (Biorad). Real time PCR quantification of fibrosis markers smooth muscle actin (*Acta2*), fibronectin (*Fn*), connective tissue growth factor (*Ctgf*) and collagen 1a (*Col1A2*) was done using the Takyon sybr green (Eurogentec) on the StepOnePlus system (Applied Biosystems). Results were presented as the fold change of target gene transcripts.

RNA Sequencing Profiling

RNA was isolated from the peripheral blood. 3′ mRNA sequencing libraries were prepared using the Collibri RNA Library Prep Kit for Illumina (Thermo Fisher Scientific). Libraries were assessed for quality, denatured, diluted, and loaded onto a NextSeq High Output v2.5 (75 cycles) flowcell. Single-end sequencing was performed with 75 cycles on the Illumina NextSeq platform according to the manufacturer's instructions.

Gene ontology analysis was performed to elucidate the functional roles of differentially expressed genes using the clusterProfiler package in R.[20] This analysis categorized genes into biological processes, molecular functions, and cellular components. Enriched gene ontology terms were identified based on adjusted *P* values.

Gene set enrichment analysis was performed using clusterProfiler version 4.6.2, employing hallmark gene sets from MSigDB.[21] Pre-ranked gene lists, generated from differential gene expression analysis, were put into clusterProfiler to identify enriched biological pathways. Enriched hallmark gene sets with a false discovery rate *q* value <0.05 were considered significant, offering insights into the biological processes associated with the observed transcriptional changes.

Statistical Analysis

For patient samples, Student *t* test, Fisher exact test or ANOVA was used. Correlation of clinical parameters of patients with AF according to TL and LVA was done by regression analysis. Power analysis was performed with the G*Power software.[22] Receiver operating characteristic (ROC) analysis was done to show the prediction value of LVAs and TL in atrial remodeling. The unadjusted risks were estimated using the Kaplan–Meier estimator with 95% CIs. For mice samples one-way ANOVA and Student *t* test was used. Patient sample values are represented as mean±SD and mice sample values are represented as mean±SEM. Data were analyzed using GraphPad Prism 10.

RESULTS

Characteristics and LVAs in Patients Undergoing AF Ablation

The study population comprised 72 patients with AF with symptomatic persistent AF who were scheduled for catheter ablation at the University Hospital RWTH Aachen. The mean age was 68.60±0.88 years, 66.67% were male and 33.33% were female, mean body mass index (BMI) was 28.30±0.55 kg/m^2 and the mean CHA_2DS_2-VASc score was 2.90±0.15. All clinical characteristics are listed in Table 1.

High density 3D-anatomical voltage maps were obtained, and LVAs were quantified as a surrogate for left atrial fibrosis. The mean percentage of LVAs (extent of fibrotic areas from the whole left atrial surface area) was 45%. We assigned the patients into 2 groups: 36 patients had no to low LVAs (<10% of the left atrial surface), while 36 patients had advanced to massive LVAs (≥40% of the left atrial surface). A representative image of a voltage map of a patient with minor LVAs and one with advanced LVAs is shown in Figure 1.

Left Atrial Fibrosis Is Associated With Shorter Telomeres

Telomere shortening was found in 62 of 72 patients (Figure 2A). Patients with advanced LVAs presented a higher prevalence of shorter telomeres, also when telomere length was age-adjusted (Figure 2B). Absolute

Table 1. Clinical Parameters of Patients With AF

Age, y	68.64±7.47
Male/Female	48 (66.67%)/24 (33.33%)
BMI, kg/m^2	28.34±4.73
EHRA-Score	2.48±0.65
CHA_2DS_2-VASc score	2.86±1.28
Left atrial diameter, mm	42.30±4.03
Systolic blood pressure, mm Hg	128.24±14.43
Diastolic blood pressure, mm Hg	78.96±9.72
Previous PCI	14 (19.40%)
Previous myocardial infarction	8 (11.10%)
Mitral valve disease (>first degree)	5 (6.90%)
LV ejection fraction, %	48.90±8.59
Previous stroke	4 (5.60%)

AF indicates atrial fibrillation; BMI, body mass index; EHRA, European heart rhythm association; LV, left ventricle; and PCI, percutaneous coronary intervention.

TL is shown in Figure S1. To validate the qPCR results, Southern blot analysis was performed to access telomere length in a subset of samples (Figure S2). Additionally, to rule out a potential influence of cytomegalovirus (CMV)—seropositivity on telomere length, CMV serostatus was obtained from all samples (Figure S3). In our cohort, CMV status had no influence on TL.

When TL was plotted against LVAs, 3 distinct groups of patients could be identified (Figure 2A). We clustered the patients in 3 groups: stage I: no/low fibrosis and long telomeres, stage II: no/low fibrosis and short telomeres, stage III: advanced fibrosis and short telomeres.

Separate binary logistic regression analyses were performed to correlate telomere length to clinical parameters. We found that shorter telomeres were more present in younger patients (62.61±6.93 years versus 69.61±7.13 years). Left atrial diameter was significantly enlarged in patients with shorter telomeres (42.70±3.12 mm versus 40.10±3.28 mm). Comorbidities specifically myocardial infarction (MI) and mitral valve disease were significantly more common in patients with AF with shorter telomeres (Table 2). Furthermore,

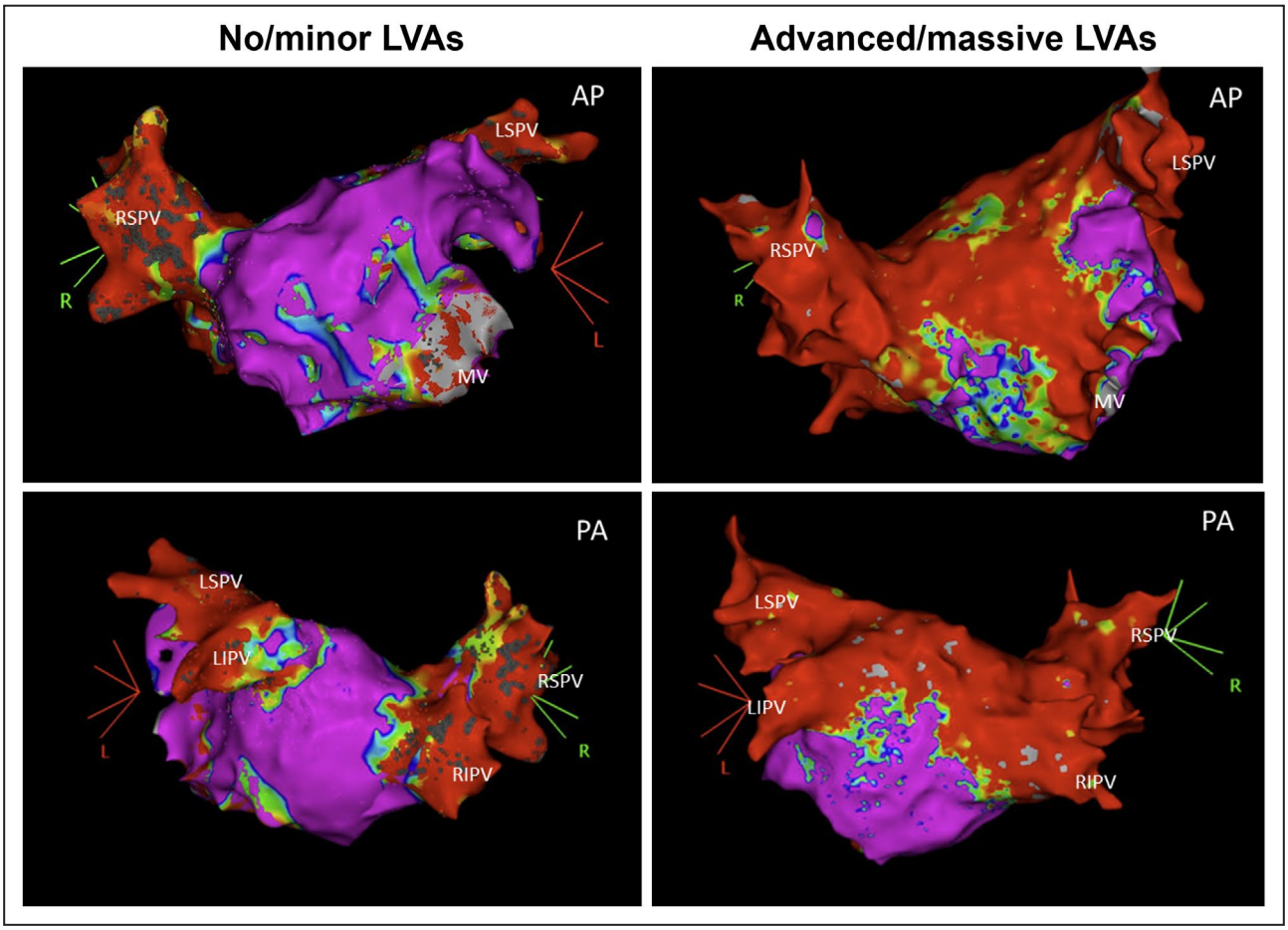

Figure 1. Representative bipolar voltage maps from patients undergoing catheter ablation.
Patients were assigned to 2 groups: no/minor low voltage areas <10% and extensive low voltage areas ≥40%. Red color indicates a low voltage area (<0.1 mV) as a surrogate for fibrosis, purple represents healthy atrial tissue. AP indicates anterior–posterior view; LIPV, left inferior pulmonary vein; LSPV, left superior pulmonary vein; MV, mitral valve; PA, posterior–anterior view; RIPV, right inferior pulmonary vein; and RSPV, right superior pulmonary vein.

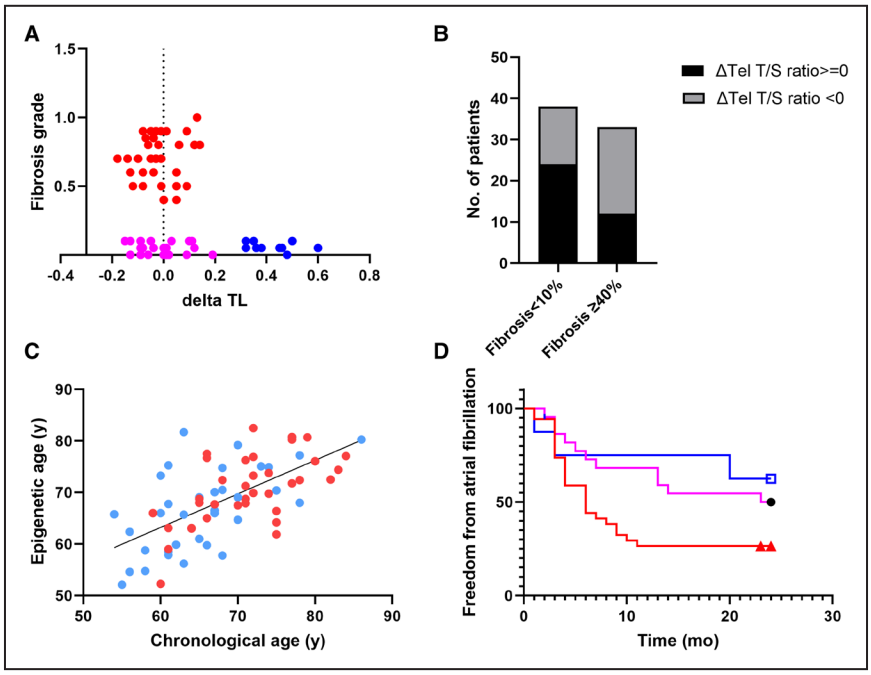

Figure 2. Patients with short telomeres show advanced atrial remodeling.
A, Age-adjusted telomere length (TL) correlates with low voltage area (LVAs). 49.29% of all patients with persistent atrial fibrillation had ΔTel <0 compared with age-adjusted individuals without atrial fibrillation. 60% of these patients with delta TL<0 had advanced LVAs. Patients could be assigned to 3 groups: stage I (long telomeres–no/low LVAs), stage II (short telomeres–no/low LVAs), stage III (short telomeres–advanced LVAs). **B**, Patients with shorter telomeres had more prominent LVAs, (P=0.033, Fisher exact test). **C**, Correlation of epigenetic age to chronological age. n=71–72. **D**, TL and fibrosis correlates with treatment outcome after catheter ablation. 62.5% of patient in stage I (long telomeres–no/low LVAs) were in stable sinus rhythm 24 months after ablation, whereas 50% of patient in stage II (short telomeres–no/low LVAs) and only 26.5% of patients in stage III (short telomeres–advanced LVAs) were in stable sinus rhythm 24 months after ablation. ΔTel age-adapted TL.

positive correlation was observed between epigenetic age prediction (as assessed by DNA methylation at 3 age associated cytosine guanine dinucleotide sites) and chronological age (Figure 2C). However, no correlation was found between epigenetic age and LVAs.

Ablation Success Is Positively Correlated to TL and LVAs

Left atrial remodeling is known to be a negative predictor for successful ablation in patients with AF. We therefore correlated AF-free survival with the presence of LVAs and ΔTL. Figure 2D shows the age-adjusted Kaplan–Meier curve on event-free survival after AF catheter ablation. 62.50% patients in stage I were in stable SR during the observation period of 24 months, whereas 50% of patients in stage II and 26.50% of patients in stage III were in SR 24 months after ablation.

We also correlated clinical parameters with the presence of LVAs and ΔTL (Table 3). The average age of patients in stages I, II and III was 62.60±13.32, 67.27±11.80 and 71.31±6.65 years, respectively. Left atrial diameter was greater in stage III (43.37±4.51) compared with stage II (41.68±3.12) or stage I (40.10±3.28). Patients in stage III had a higher prevalence of comorbidities (MI: 19.44%, mitral valve disease 11.11%) compared with the other 2 groups. Similarly, left ventricular ejection fraction (LVEF) was less in stage III (46.26±9.32) compared with stage II (51.60±7.30) and stage I (51.40±6.38).

ROC curve analysis was used to evaluate the predictive value of age, left atrial diameter, LVAs and LVAs with telomere length in determining recurrence of AF after ablation. TL, in particular in combination with LVAs, increased the predictive value of the test (Figure S4).

TL and LVAs Result in Distinct Transcriptome Patterns

Peripheral blood from all patients underwent 3′ mRNA sequencing. The expression profiles of the patients were clustered according to their clinical stage and the differentially expressed genes were subjected to gene ontology and gene set enrichment analysis to illustrate their

Table 2. Clinical Parameters of Patients With AF According to Telomere Shortening

	Patients with AF		
	Short telomere (n=62)	Long telomere (n=10)	*P* value
Age, y	69.61±7.13	62.60±6.93	0.01*
Male/Female	41 (66.13%)/21 (33.87%)	6 (60%)/4 (40%)	…
BMI, kg/m^2	28.46±4.51	27.59±4.49	0.58
CHA_2DS_2-VASc score	2.97±1.41	2.20±1.48	0.15
Left atrial diameter, mm	42.67±3.12	40.10±3.28	0.04*
Systolic blood pressure, mm Hg	128.97±2.92	123.90±3.78	0.25
Diastolic blood pressure, mm Hg	79.58±1.55	75.20±2.17	0.10
Previous PCI	14 (22.58%)	0	…
Previous myocardial infarction	8 (12.90%)	0	…
Mitral valve disease (>first degree)	5 (8.06%)	0	…
LV ejection fraction, %	48.48±1.46	51.40±2.02	0.23
Previous stroke	4 (6.45%)	2 (20%)	…
Blood parameters			
Erythrocytes	4.72±0.09	4.78±0.11	0.62
Hemoglobin	14.14±0.28	14.46±0.34	0.42
Hematocrit	42.05±0.72	42.59±0.85	0.59
Thrombocytes	229.34±21.19	253.50±19.63	0.29
Leukocytes	6.98±0.31	7.76±0.82	0.37
CRP	5.10±1.25	1.89±0.53	0.09
Glucose	109.73±3.93	111.70±13.62	0.89

P values were calculated using unpaired 2-tailed t test. AF indicates atrial fibrillation; BMI, body mass index; CRP, C-reactive protein; LV, left ventricle; and PCI, percutaneous coronary intervention.

*$P<0.05$.

functional associations. Comparing stage III with stage I, we found enrichment of biological processes related to myeloid cells (myeloid cell development, myeloid cell differentiation and myeloid cell homeostasis), erythrocytes (erythrocyte development, erythrocyte homeostasis, and erythrocyte differentiation), heme metabolism and the interferon gamma response pathway (Figure 3A). Comparison of stage III with stage II (Figure 3B) showed upregulation of 8 gene ontology terms related to ECM (collagen-containing ECM, ECM structural constituent, complex of collagen trimers, collagen-activated tyrosine kinase receptor signaling pathway, collagen trimer, ECM organization, ECM structural constituent conferring tensile strength, and collagen-activated signaling pathway). Comparing stage III with stage I, gene set enrichment analysis also showed more enrichment of the heme metabolism and interferon gamma response pathway (Figure 3C). Gene set enrichment analysis of stage III when compared with stage I also increasing enrichment of gene sets associated with epithelial mesenchymal transition, angiogenesis, and myogenesis (Figure 3D). The downregulated pathways are shown in Figure S5.

Clonal Hematopoiesis of Indeterminate Potential Is Associated With Telomere Length

Recent findings suggest an association between clonal hematopoiesis of indeterminate potential (CHIP), which refers to an age dependent expansion of leukocytes with preleukemic mutations, and the development of AF.[23] Therefore, we subjected blood samples of all 72 patients with AF to a next generation sequencing gene panel that detects somatic mutations related to clonal hematopoiesis.

Overall, the frequency of CHIP was 27.8% in our cohort, which is higher than that reported in an age-adjusted population.[24] The most prevalent mutations were found in DNMT3A (16.7%), TET2 (5.6%) and ASXL1 (4.2%, Figure 4A). In addition, we observed a higher frequency of CHIP in patients with shorter telomeres compared with patients with longer telomeres (Figure 4B).

Mouse Models With Short Telomeres Show Marked Left Atrial Fibrosis

TL is maintained by telomerase, a ribonucleoprotein complex that synthesizes telomere repeats at the end of chromosomes, in which the protein component TERT possesses catalytic activity, while the non-coding RNA TERC provides the template function for telomeric repeats. *Tert*$^{-/-}$ and *Terc*$^{-/-}$ mice are known to exhibit shortened telomeres and organ fibrosis,[5,25] however, atrial tissue of these lines has not been investigated so far.

We analyzed left atrial remodeling in the 2nd to 4th generation of *Tert*$^{-/-}$ and *Terc*$^{-/-}$ mice and found an enhanced degree of left atrial fibrosis compared with their wildtype littermates (Figures 5A and 5B, Figure S6). In addition, real time qPCR of atrial tissue with fibrosis markers *Acta2*, *Col1A2*, *Fn*, and *Ctgf* (Figure 5C) showed activation of molecular processes resulting in atrial remodeling.

DISCUSSION

AF is a complex disease with multiple interacting mechanisms. The development of left atrial fibrosis is a central common pathway and is associated with an unfavorable treatment outcome. There is a clear association between aging and the prevalence of AF, however, age-related mechanisms that drive the progression of AF remain largely unclear.

Our study shows that

1. Left atrial remodeling, in particular the presence of left atrial LVAs as a surrogate marker for fibrosis, is more frequent in patients with shorter telomeres.

Table 3. Clinical Parameters of Patients With AF According to Telomere Shortening and Fibrosis

	Patients with AF			
	Stage I (n=10)	Stage II (n=26)	Stage III (n=36)	*P* value
Age, y	62.60±13.32	67.27±11.80	71.31±6.65	0.002*
BMI, kg/m^2	27.59±4.49	28.97±4.51	28.10±5.03	0.07
CHA_2DS_2-VASc score	2.20±1.48	2.69±1.41	3.17±1.06	0.07
Left atrial diameter, mm	40.10±3.28	41.68±3.12	43.37±4.51	0.05*
Systolic blood pressure, mm Hg	123.90±11.97	129.12±14.58	128.86±15.11	0.59
Diastolic blood pressure, mm Hg	75.20±6.88	78.84±7.74	80.11±11.46	0.38
Previous PCI	0	2 (7.69%)	12 (33.33%)	…
Previous myocardial infarction	0	1 (3.85%)	7 (19.44%)	…
Mitral valve disease (>first degree)	0	1 (3.85%)	4 (11.11%)	…
LV ejection fraction, %	51.40±6.38	51.60±7.30	46.26±9.32	0.03*
Previous stroke	2 (20%)	2 (7.69%)	1 (2.78%)	…
Blood parameters				
Erythrocytes	4.78±0.33	4.77±0.46	4.68±0.62	0.77
Hemoglobin	14.46±1.07	14.42±1.40	13.94±1.54	0.36
Hematocrit	42.59±2.67	43.03±3.67	41.34±4.80	0.28
Thrombocytes	253.50±62.06	253.85±108.05	211.64±55.51	0.07
Leukocytes	7.76±2.58	7.02±1.60	6.95±1.45	0.39
CRP	1.89±1.68	3.70±6.36	3.34±5.07	0.59
Glucose	111.70±43.07	105.31±20.03	112.92±17.50	0.07

P values were calculated using one-way ANOVA. AF indicates atrial fibrillation; BMI, body mass index; CRP, C-reactive protein; LV, left ventricle; PCI, percutaneous coronary intervention.

*$P<0.05$.

2. Shorter telomeres together with advanced LVAs can act as a predictor for treatment outcome after catheter ablation for AF.
3. Systemic 3′mRNA-Seq profiles and CHIP frequency differ in patients with AF with short and long telomeres.
4. Mouse models with shorter telomeres show enhanced left atrial remodeling, suggesting that telomere shortening is actively involved in the pathogenesis of AF.

Aging is a biological process with a gradual decline of body functions which results in an increased risk for many human diseases.[26] Prevalence of AF is clearly associated with age, and its prevalence increases from 0.1% in individuals with <55 years up to 18% in individuals with >80 years.[2,27]

Telomere shortening is one of the hallmarks of aging.[28] As telomeres shorten, the DNA ends become exposed, initiating a DNA damage response. This response causes a cascade of molecular processes leading to cellular senescence.[5] TL is believed to be a marker of biological age, but it is not always concordant with the chronological age of an individual.[29]

It is known that patients with shorter telomeres have an increased risk of developing various cardiovascular disease such as coronary artery disease, myocardial infarction, atherosclerosis and congestive heart failure.[30] Telomere shortening has also been linked to (and can be used to screen for) hereditary and various acquired diseases that are associated with excess organ fibrosis, such as myeloproliferative neoplasms or chronic myeloid leukemia.[16,31–34] For example, shorter telomeres have been found in patients with liver fibrosis and type 2 diabetes mellitus[35] as well as in patients with idiopathic pulmonary fibrosis.[36] Our study shows that telomere length is associated not only with an increased risk of developing AF, but also with the presence of left atrial fibrosis, suggesting a pathomechanistic link between aging and atrial remodeling.

After a follow-up of patients for 24 months, we found that the group of patients with short telomeres and advanced fibrosis had a lower probability of event free survival compared with the other 2 groups. This is consistent with the study by Wang et al, which showed that patients with short telomeres had a higher probability of AF progression from paroxysmal to persistent AF compared with patients with long telomeres.[37]

In addition to TL, we performed epigenetic age prediction and determined DNA methylation at 3 age-associated cytosine guanine dinucleotide (CpG) sites. Interestingly, we found a good correlation between chronological and biological age. However, we did not find a correlation between epigenetic age and the

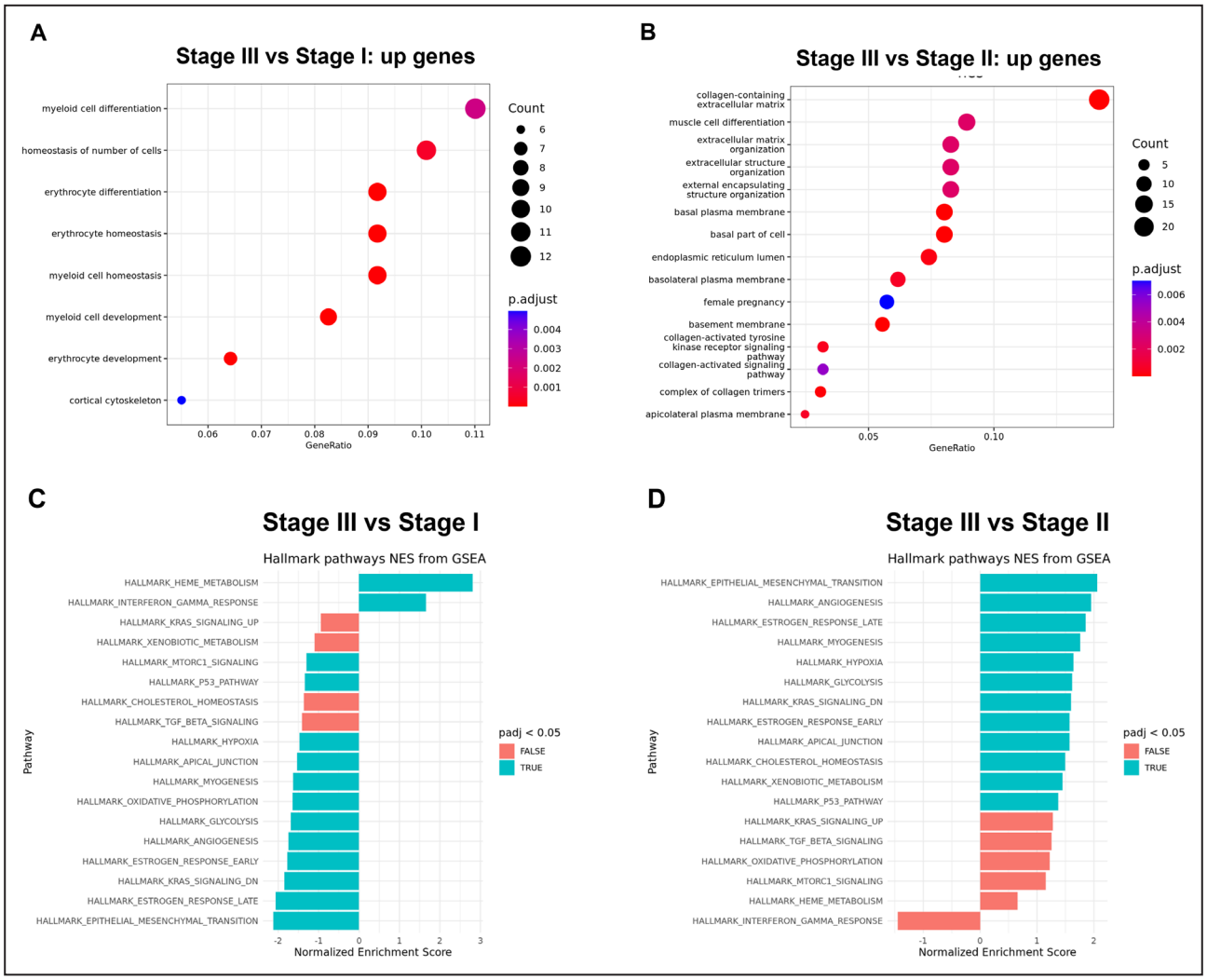

Figure 3. Gene ontology (GO) and gene set enrichment analysis of differentially expressed genes (DEGs) in different subgroups of patients according to telomere length (TL) and fibrosis.
A, GO comparison of DEGs of patients in stage III with stage I. **B**, GO analysis of DEGs of patients in stage III with stage II. The abscissa represents the gene ratio factor, and the ordinate represents the name of the GO terms. The size of each circle represents the number of DEGs enriched in the corresponding process and colors represent the *P* value. **C**, GSEA analysis of patients in stage III compared with stage I. **D**, GSEA analysis comparing patients in stage III vs stage II. n=72. *P*<0.05 was considered significant. GSEA indicates gene set enrichment analysis; and NES, normalized enrichment score.

presence of low-voltage areas. This suggests that, while epigenetic aging is a valuable marker for overall biological aging, it may not be influenced by a specific localized cardiac pathology. We therefore hypothesize that only telomere shortening, but not epigenetic age or DNA methylation contributes mechanistically to left atrial remodeling.

To further evaluate the molecular signaling mechanisms associated with telomere shortening, we analyzed the transcriptome in the blood of our cohort. Interestingly, we observed potential patterns of expression that appeared to vary according to the clinical stage. For instance, patients in stage III (advanced fibrosis–short telomeres) compared with patients in stage I (long telomeres–no LVAs) showed an apparent upregulation of transcripts that are often associated with myeloid cell populations. Myeloid cells originate from hematopoietic stem cells and play a crucial role in the immune system. They are activated during inflammation and tissue damage and induce interferon-gamma response during inflammation.[38,39]

When comparing patients with stage III (advanced fibrosis–short telomeres) to those with stage II (no fibrosis–short telomeres), the most significantly enriched gene families were associated with ECM and fibrosis. ECM is a network composed of collagen, elastic fibers, proteoglycans, hyaluronan and glycoproteins. ECM provides structural stability to cells and tissues. However abnormal accumulation of ECM components is involved in the pathological progression of fibrosis.[40] Our data

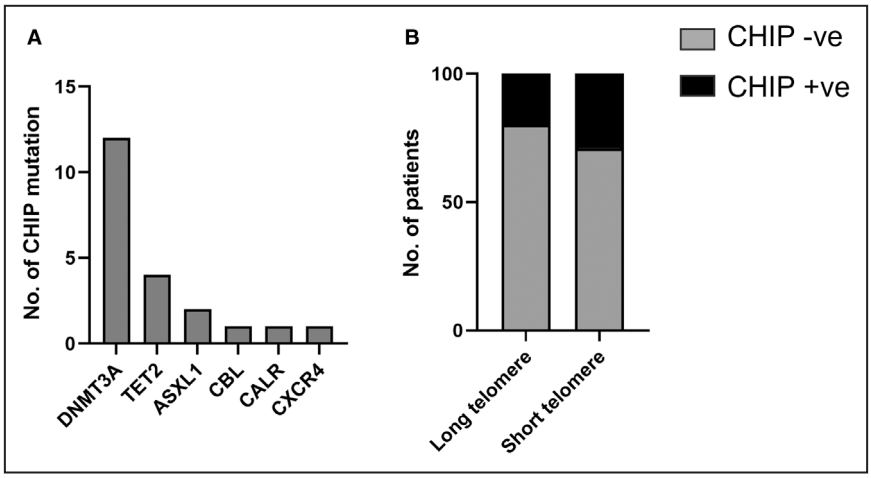

Figure 4. Clonal hematopoiesis of indeterminate potential in patients according to telomere length.
A, The most frequent somatic mutations were found in DNMT3A, TET2, and ASXL1. **B**, Clonal hematopoiesis of indeterminate potential frequency is higher in patients with atrial fibrillation with short telomeres compared with patients with long telomeres (shown in percentage), Fisher exact test, *P*=0.577. n=71. CHIP indicates clonal hematopoiesis of indeterminate potential.

suggest that patients in stage II (no fibrosis–short telomeres) have an intermediate phenotype on the molecular level, with activation of myeloid cells and inflammatory pathways without the presence/activation of ECM, whereas stage III patients (short telomeres–significant LVAs) demonstrate all features of full atrial myopathy. On the other hand, patients in stage I (long telomeres–no LVAs) do not exhibit any signs of inflammation or ECM development. In these patients, AF can be considered to be a pure electrical disease that can be treated by catheter ablation with a good outcome.

CHIP, another age-associated phenomenon which leads to a clonal expansion of blood cells with preleukemic mutations, has been recently linked to various cardiovascular diseases, including atrial fibrillation.[23] For example, somatic mutations in *DNMT3A* and *TET2* can be frequently found in patients with AF.[41]

Our study confirmed the higher-than-expected prevalence of CHIP in the AF cohort. In particular, we found a higher frequency of CHIP in patients with short telomeres compared with patients with long telomeres. Since CHIP is known to be associated with a pro-inflammatory state,[42] our study may provide a connection between aging, telomere shortening, chronic inflammation, development of left atrial fibrosis and atrial fibrillation.

To confirm that TL is mechanistically linked to left atrial remodeling, we investigated atrial tissue from *Terc*$^{-/-}$ and *Tert*$^{-/-}$ mice, which are established mouse models of short telomeres. With each successive generation, these lines undergo progressive telomere shortening, resulting in a reduction in telomere length.[10] Remarkably, we found that the third and fourth generation of *Tert*$^{-/-}$ mice exhibited increased atrial fibrosis in contrast to both wild-type and homozygous first- or second-generation *Terc*$^{-/-}$ and *Tert*$^{-/-}$ mice. Similar findings have been observed in lung[43,44] and kidney tissue,[44,45] as well as in cardiac (ventricular) tissue,[46] implying a substantial association between telomere shortening and increased fibrotic changes within the heart.

Based on studies associating telomere shortening with numerous age-related diseases, telomeres are being focused on as a target for novel therapeutic options. Adeno-associated virus serotype 9-mediated overexpression of TERT in a mouse model of myocardial infarction reduced the percentage of short telomeres, improved ventricular function, and reduced mortality of mice due to heart failure.[47] Several studies have been conducted with telomerase activators. One of these, TA-65, extracted from *Astragalus membranaceus*, demonstrated a reduction in short telomeres and DNA damage in *Tert*$^{-/-}$ mice.[47] Similarly, in cigarette smoke induced small airway remodeling, TA-65 treatment increased the expression of TERT, decreased Transforming growth factor beta (TGF-β1) expression, inhibited TGF-β1 induced myofibroblast differentiation and reactive oxygen species production.[48] These findings as depicted in Figure 6, strongly indicate the importance of telomerase as a promising target for future therapeutic intervention and might also be a powerful tool to combat AF.

We acknowledge several limitations in our study: TL in our patients was assessed at one distinct time point only; TL measurement at 2 different time points from

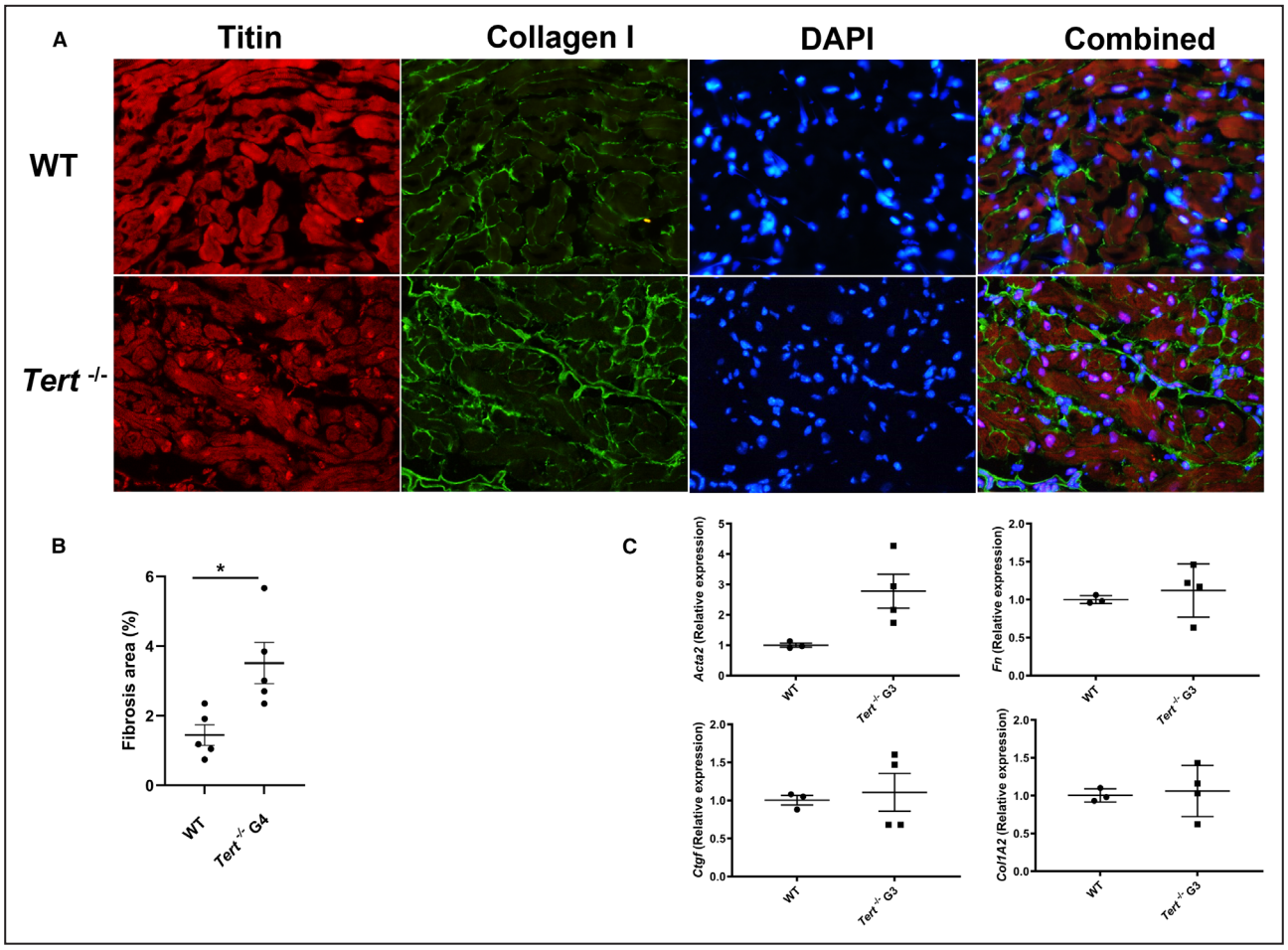

Figure 5. Left atrial fibrosis in mouse models with shorter telomeres.
A, Representative pictures of immunofluorescence staining with antibody against collagen I and Titin and counterstaining with DAPI. **B**, Quantification of collagen I (fibrosis) in wild-type (WT) and *Tert*$^{-/-}$ fourth generation mice, n=5 per group. **C**, Gene expression of profibrotic and fibrotic markers in left atrium of WT and *Tert*$^{-/-}$ third generation mice. n=3–4 per group, data are expressed as mean±SEM. **P*<0.05. Scale bars=50 μmol. *Acta2* indicates Alpha-smooth muscle Actin; *Fn*, fibronectin; *Ctgf*, connective tissue growth factor; and *Col1A2*, collagen type I alpha 2 chain.

the same patient would have been interesting but, unfortunately, these blood samples were not available.

We used PCR-based techniques to quantify telomere length, which has a lower accuracy than fluorescence-in-situ hybridization. However, we have previously compared our PCR approach to flow-fluorescence-in-situ hybridization,[49] the gold standard for telomere length measurement and could demonstrate that PCR and flow-fluorescence-in-situ hybridization correlate quite well with each other. We also evaluated several of our samples by southern blot and found a good correlation with the qPCR results.

We acknowledge TERT and TERC knock-out mice have different phenotypes and TERT also has non-nuclear functions[50,51] which might have influenced our findings. However, adult mice do not express telomerase in the heart. This renders them phenotypically TERT-null.[52] The extra-nuclear or mitochondrial function of TERT may become relevant and exert cardioprotective effects only if telomerase is ectopically reactivated or genetically overexpressed as done by the Bär group[53] or the Haendeler group[50] (but not in our study). We therefore believe that TL is mechanistically linked to atrial fibrosis.

Finally, there could be variations in telomere length between different cellular subtypes, such as CD4+ and CD8+ cells.[54] However, given that granulocytes generally constitute about 60% of blood cells, variations in smaller subsets like memory immune cells should have a minimal impact on the overall telomere length. In addition, cellular subsets correlate quite well with each other, reducing the potential bias that any single subset might have on the overall telomere length. In our cohort, this effect is balanced, and no patients had recognized hematological diseases that could bias the data.

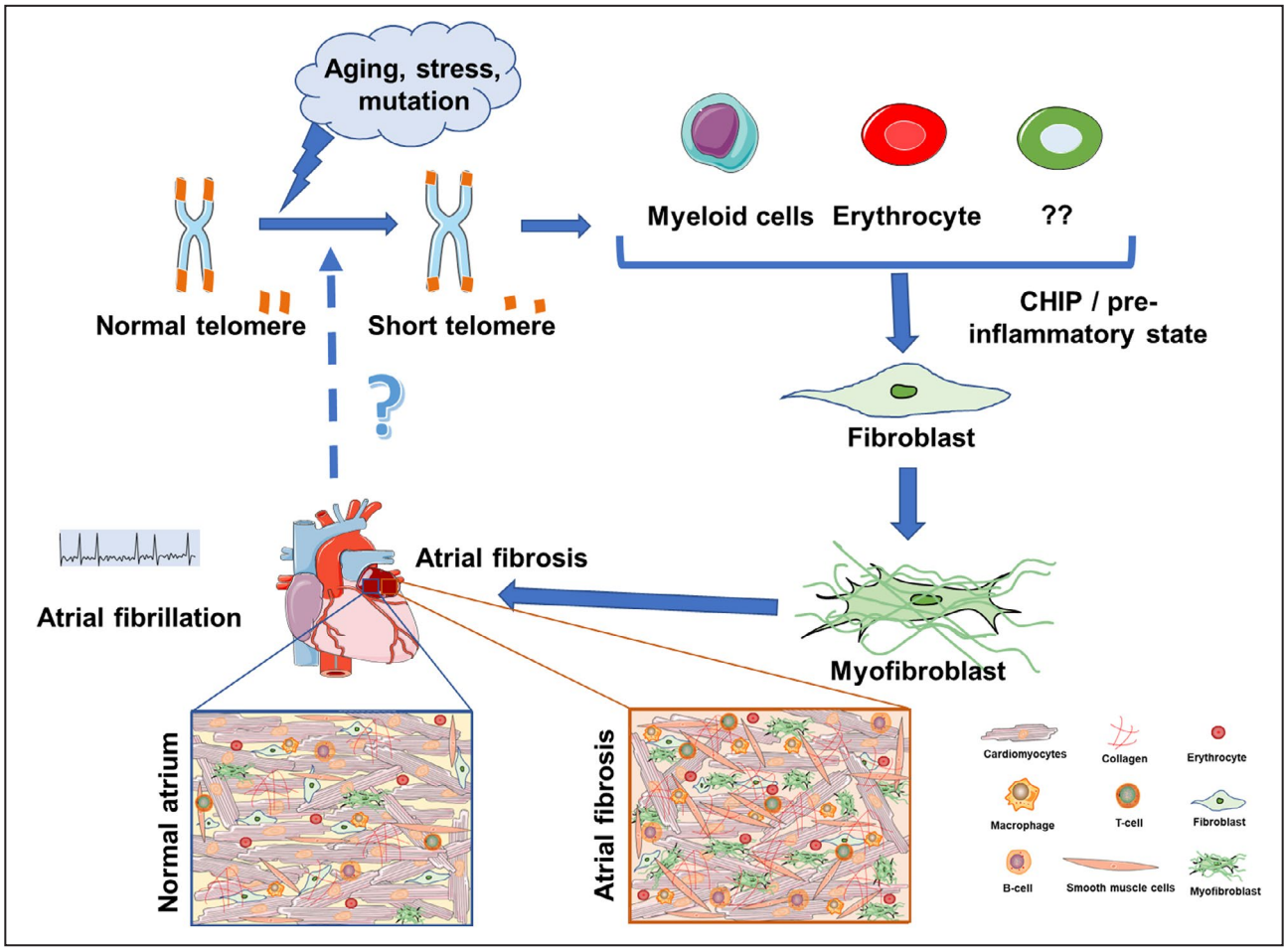

Figure 6. Schematic diagram. Telomere shortening and atrial fibrosis—proposed mechanism.
Telomeres shorten due to aging, stress or certain mutations. These shortened telomeres lead to an increased activity of myeloid cells or erythrocytes, which in turn induce inflammation with fibroblast to myofibroblast transformation. Myofibroblasts deposit extra cellular matrix resulting in atrial fibrosis. CHIP indicates clonal hematopoiesis of indeterminate potential.

ARTICLE INFORMATION

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Disclosures

Dr Wagner is cofounder of Cygenia GmbH (www.cygenia.com), which can provide service for epigenetic analysis to other scientists. V.T. contributes to this company, too. The remaining authors have no disclosures to report.

Supplemental Material

Data S1
Figures S1–S6

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