

Accelerated Telomere Shortening in Leukocyte Subpopulations of Patients With Coronary Heart Disease

Role of Cytomegalovirus Seropositivity

Ioakim Spyridopoulos, MD; Jędrzej Hoffmann, MD; Alexandra Aicher, MD; Tim H. Brümmendorf, MD; Hans W. Doerr, MD; Andreas M. Zeiher, MD; Stefanie Dimmeler, PhD

Background—Shortening of mean telomere length (TL) in white blood cells is correlated with the development of coronary heart disease (CHD) and with increased mortality due to infectious disease. The goal of the present study was to investigate whether telomere shortening in CHD is restricted to specific peripheral blood lymphocyte and/or myeloid cell subpopulations. Results were correlated to TL in CD34⁺ hematopoietic peripheral blood stem cells and progenitor cells obtained from the same individual patients.

Methods and Results—TL was measured by multicolor flow cytometry–fluorescent in situ hybridization in 12 leukocyte subpopulations after immunomagnetic bead sorting. We investigated TL in 14 young (mean age 25 years) and 13 older (mean age 65 years) healthy male volunteers and in 25 age-matched patients with CHD (mean age 65 years). We show that TL in granulocytes and monocytes mirrors TL of CD34⁺ peripheral blood stem cells and progenitor cells extremely well ($r=0.95$, $P<0.0001$) in patients and in healthy adults. TL was ≈ 0.5 kilobases (kb) shorter in leukocytes from patients with CHD than in their age-matched control subjects. This difference was identical for CD34⁺ peripheral blood stem cells and progenitor cells, monocytes, granulocytes, B lymphocytes, and CD4⁺ T cells, including their memory and naïve subpopulations. Surprisingly, only in cytotoxic CD8⁺ T lymphocytes, we found a substantially increased TL deficit of 1.0 kb in CHD patients as opposed to control subjects. Further analysis revealed that TL shortening was particularly pronounced in CD8⁺CD28⁻ T cells obtained from cytomegalovirus-seropositive CHD patients, whereas such a difference was not observed in healthy cytomegalovirus-positive as opposed to cytomegalovirus-negative control subjects. Finally, TL shortening of CD8⁺CD45^{RA+} T cells was correlated with the decrease in left ventricular function in CHD patients ($r=0.629$, $P=0.001$).

Conclusions—Telomere shortening in patients with CHD could potentially be attributed to either inherited TL shortening or acquired accelerated telomere shortening restricted to the hematopoietic system, which affects the baseline TL of all peripheral blood cell populations, including peripheral blood stem cells and progenitor cells. In addition, cytomegalovirus-seropositive patients but not healthy control subjects exhibited further shortening of their cytotoxic T lymphocytes. Surprisingly, TL shortening of CD8⁺ T lymphocytes in CHD patients demonstrated a very strong correlation with cardiac dysfunction, which suggests a mechanistic link between CHD and immunosenescence. (*Circulation*. 2009;120:1364-1372.)

Key Words: telomere ■ coronary disease ■ cytomegalovirus ■ aging ■ immunology ■ stem cells ■ T cells

Individuals with shorter telomeres of their peripheral blood leukocytes carry a higher risk for dying of infectious and cardiovascular disease.¹ Naturally, both disease entities appear to represent different mechanisms in which telomere biology is involved. On closer examination, inflammation plays a crucial role in the development of atherosclerosis.²⁻⁴ Next to oxidized cholesterol, one of the proposed stimuli of inflammation is infectious pathogens such as cytomegalovi-

rus (CMV). Despite a high prevalence, the association between the presence of serum antibodies against CMV and atherosclerosis in humans is still debated.⁵⁻⁷ It is also highly debated whether telomere shortening in patients with coronary heart disease (CHD) is an acquired or an inherited condition. In vitro studies suggest that increased oxidative stress, such as that which is present in patients with CHD, leads to accelerated telomere shortening.⁸ Brouillette and

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From the Departments of Cardiology and Institute for Cardiovascular Regeneration, Centre of Molecular Medicine (I.S., J.H., A.A., A.M.Z., S.D.) and Institute of Medical Virology (H.W.D.), Johann Wolfgang Goethe University of Frankfurt, Frankfurt, Germany; Department of Oncology and Hematology (T.H.B.), University Cancer Center Hamburg-Eppendorf, Hamburg, Germany; Department of Hematology and Oncology (T.H.B.), University Hospital Aachen, Aachen, Germany; and Institute of Human Genetics (I.S.), Newcastle University, Newcastle Upon Tyne, United Kingdom.

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Correspondence to Ioakim Spyridopoulos, MD, Institute of Human Genetics, Newcastle University, International Centre for Life, Central Parkway, Newcastle Upon Tyne, NE1 3BZ, United Kingdom. E-mail ioakim.spyridopoulos@newcastle.ac.uk

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coworkers,⁹ however, found that leukocyte telomere shortening already existed in healthy offspring of patients with CHD compared with age-matched control subjects. This single study provides strong arguments in favor of a genetic link for CHD-associated telomere shortening, called the “telomere hypothesis.” Van der Harst and coworkers¹⁰ have recently shown that telomere length (TL) is shorter in patients with congestive heart failure than in age-matched control patients. Telomere shortening was also related to the severity of disease. We have shown previously that ischemic cardiomyopathy is associated with selective impairment of progenitor cell function in the bone marrow and in the peripheral blood, which may contribute to an unfavorable left ventricular remodeling process.¹¹

Clinical Perspective on p 1372

The goal of the present study was to gain a deeper insight into the mechanisms involved and the cell populations responsible for telomere shortening in CHD. We used a different approach than Brouillette et al by determining TL separately for all relevant leukocyte subpopulations, including CD34⁺ hematopoietic peripheral blood stem and progenitor cells (PBPCs), and compared them between patients with CHD and age-matched healthy control subjects.

Methods

Please see the online-only Data Supplement for details of the methods used in the present study.

Patient Population

We analyzed 25 men (mean age 64.8±3.4 years) with angiographic documentation of CHD and healed myocardial infarction (at least 3 months after infarction). All patients included in the study had impaired left ventricular systolic function (ejection fraction <55%) as confirmed by echocardiography. None of the patients were affected by neoplastic, autoimmune, or chronic infectious disease. All subjects with recent infections were also excluded.

Healthy Volunteers

Fourteen young (mean age 26.7±1.8 years) and 13 older (65.1±2.1 years) healthy volunteers were included in the present study after informed consent was obtained. The health status of young and elderly control subjects was determined on the basis of both medical history questionnaire and blood tests, which comprised complete blood cell counts, glucose, hemoglobin A_{1c}, urea, creatinine, electrolytes, high-sensitivity C-reactive protein, liver function tests, high-density lipoprotein and low-density lipoprotein cholesterol, triglycerides, and N-terminal pro-brain natriuretic peptide (NT-proBNP). Additionally, all elderly control subjects underwent extensive clinical testing that included 2-dimensional, M-mode, and Doppler echocardiography, as well as stress electrocardiography for the exclusion of subclinical heart disease.

Determination of CMV Status

The CMV serostatus of 13 elderly healthy control subjects and 20 CHD patients was determined from serum samples with the Enzygnost Anti-CMV/IgG enzyme immunoassay (Dade Behring, Marburg, Germany) according to the manufacturer's instructions.¹² For the determination of human CMV DNA load in peripheral blood, we used a TaqMan real-time quantitative polymerase chain reaction assay.¹³

Isolation of Leukocyte Subpopulations

Peripheral blood EDTA samples (100 mL) were collected from CHD patients and healthy volunteers (EDTA S-Monovette 9 mL, catalog No. 02-1066-001, Sarstedt, Nümbrecht, Germany). Peripheral blood mononuclear cells were obtained after density gradient centrifuga-

tion with Ficoll-Hypaque (catalog No. L6115, Biochrom, Berlin, Germany). After 2 washes with PBS, peripheral blood mononuclear cells were resuspended in ice-cold MACS buffer (Miltenyi Biotec, Bergisch Gladbach, Germany) that contained PBS with 0.5% FCS and 2 mmol/L EDTA (Titriplex III 1.1%). The cells were counted in trypan blue with a Neubauer hemocytometer. For isolation of different mononuclear cell subpopulations, we designed a cell-fractionation strategy based on immunomagnetic sorting (MACS; see online-only Data Supplement Figure I). All separation steps were performed with LS columns (catalog No. 130-042-401) placed in the QuadroMACS Separator (MACS MultiStand, catalog No. 130-042-303).

Flow–Fluorescent In Situ Hybridization and Multicolor Flow–Fluorescent In Situ Hybridization

For analysis of mean TL in subpopulations of peripheral leukocytes, we used either the standard flow cytometry–fluorescent in situ hybridization (flow-FISH) protocol (for purified CD14⁺ monocytes, CD8⁺CD28⁺CD56⁻ T cells, and CD8⁺CD28⁻CD56⁻ T cells) or a multicolor flow-FISH approach (CD15⁺ granulocytes, CD45^{RO+} T cells, CD45^{RA+} T cells, and CD19⁺ B cells; for CD34⁺ progenitor cells, see the online-only Data Supplement, Figure II) as previously described with minor modifications.^{14,15} Mean TL in subpopulations of peripheral blood leukocytes was measured with flow-FISH in accordance with previously published protocols.¹⁶ All monoclonal antibodies used for multicolor flow-FISH in the present study were conjugated with heat-stable Alexa Fluor 647 (AF647) fluorochrome. The interindividual variability of TL was 5.5%, and intraindividual variability was 1.0% (online-only Data Supplement Figure III).

Telomerase Enzyme Activity Measurement

Telomerase enzyme activity was measured with a commercially available polymerase chain reaction–based assay (TRAPEze ELISA telomerase detection kit, Chemicon International, Temecula, Calif; No. S7750) according to the manufacturer's protocol. Telomeric repeat-amplification protocol assays were performed with biotin-labeled TS primers as described previously.¹⁷

Statistical Power Analysis

We calculated the effect size (Δ) between CHD patients and age-matched control subjects that could be detected with 80% probability at a 5% significance level for each of the main cell populations: CD14 0.66 kilobases (kb), CD15 0.64 kb, CD34 0.69 kb, CD19 0.93 kb, CD3 0.88 kb, CD4 0.81 kb, and CD8 1.02 kb. From these calculations, we concluded that because the actual differences in TL were smaller (0.5 kb), the lack of a significant difference between TL of patients and age-matched control subjects for most cell populations could have been due to the limited sample size rather than the lack of a biological difference between the groups.

Statistical Analysis

Data depicted in box-and-whisker plots (Figures 1 and 2) are reported as median values, lower and upper quartiles, and 10%/90% percentiles to visualize dispersion. In the text, data are reported as mean±SE. Comparison of 3 means was performed by ANOVA, followed by a Tukey post hoc test for comparison of any 2 groups (patients versus age-matched control subjects and young versus elderly healthy volunteers). Comparison of 2 groups was calculated with an unpaired *t* test if normal probability plots (P-P plots) demonstrated approximate normality. For multiple testing (ANOVA, 2-factor design), the general linear model tool in SPSS (SPSS Inc, Chicago, Ill) was used. The linear relationship between 2 variables was calculated by the bivariate correlation procedure with the Pearson correlation coefficient. C-reactive protein and NT-proBNP levels were analyzed as continuous variables and were log transformed to take into account their skewed distribution. Comparison of slopes and intercepts in Figure 3 was performed by the ANCOVA procedure in GraphPad Prism version 5 for Macintosh (<http://www.graphpad.com>). All statistical tests except ANCOVA were performed with SPSS version 15.0 for Windows. Power analysis was

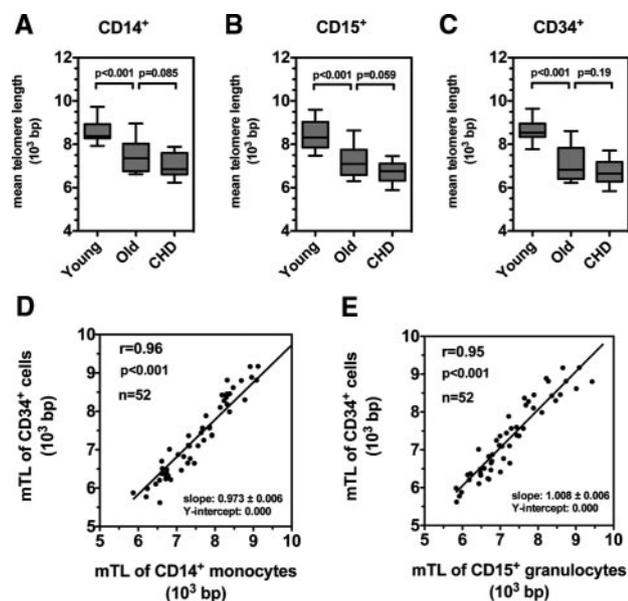


Figure 1. Telomere shortening in CHD is present in myeloid cells and CD34⁺ progenitor cells. Mean TL in CD14⁺ monocytes (A), CD15⁺ granulocytes (B), and CD34⁺ PBPCs (C) is shown for each cohort (n=14 young and healthy volunteers, n=13 elderly and healthy volunteers, and n=25 patients with CHD). Scatter-plots for TL of CD34⁺ PBPCs vs CD14⁺ monocytes (D) and CD15⁺ granulocytes (E), respectively, include the Pearson correlation coefficient (r) for the entire study population (n=52). mTL indicates mean telomere length.

performed with StatMate 2 for Macintosh by GraphPad Software Inc (San Diego, Calif).

Results

Study Population

We measured TL in 3 different study populations. To monitor the influence of chronological aging on TL in leukocyte

subpopulations, we first analyzed 14 young (27±2 years old) and 13 elderly (65±2 years old) completely healthy volunteers (Table). Healthy volunteers were all subjected to blood tests, echocardiography (elderly volunteers), and a full ECG stress test to exclude individuals with structural or subclinical heart disease. Twenty-five patients with CHD were age matched (65±3 years old) with these volunteers and underwent extensive testing for laboratory chemistry, echocardiography, and coronary angiography. All patients had had a myocardial infarction in the past.

TL in Myeloid Blood Cell Populations Is Shorter in Patients With CHD

We isolated CD14⁺ monocytes by immunomagnetic bead sorting and subjected these cells to the flow-FISH protocol. In addition, we used the multicolor flow-FISH protocol to determine TL in CD15⁺ granulocytes. Owing to the very limited number of CD34⁺ cells in immobilized peripheral blood, 100 mL of peripheral blood had to be taken to first enrich cells with CD34⁺ PBPCs and then process them with the multicolor flow-FISH, with a second antibody raised to a different epitope. As expected, TL of all 3 populations was 1.2 kb shorter in older than in younger healthy volunteers (P<0.001; Figure 1A through 1C). In addition, TL in CHD patients was ≈0.5 kb shorter in monocytes (P=0.085; Figure 1A) and granulocytes (P=0.059; Figure 1B) than in age-matched healthy control subjects. We also found an excellent correlation between TL in CD34⁺ PBPCs and their peripheral blood derivatives, monocytes (Pearson's correlation coefficient 0.96, P<0.001; Figure 1D) and granulocytes (Pearson correlation coefficient 0.95, P<0.001; Figure 1E), independent of age and CHD. This proves that the TL of peripheral blood granulocytes and monocytes reflects the TL of CD34⁺ PBPCs.

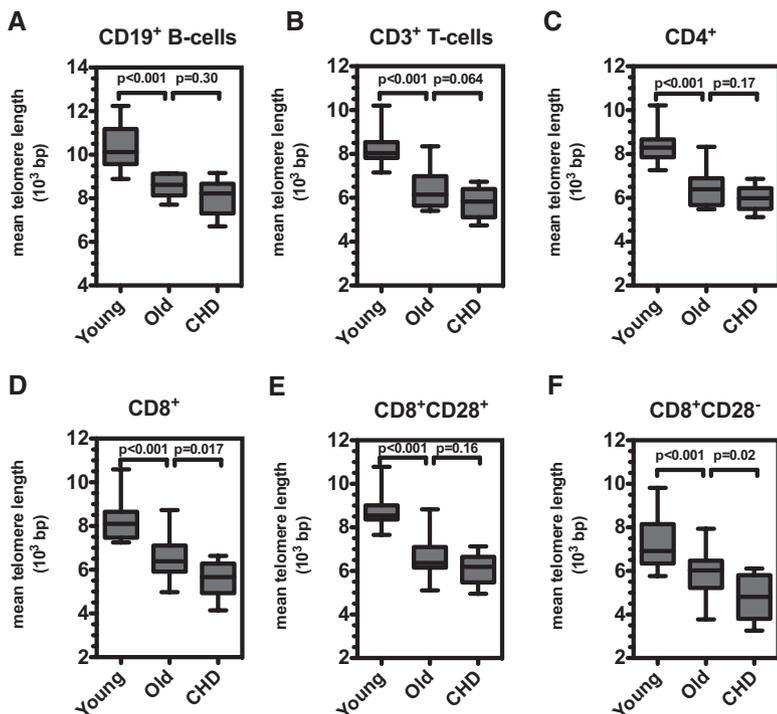


Figure 2. Telomere shortening in CHD is present in lymphoid cells. Mean TL in CD19⁺ B lymphocytes (A), CD3⁺ T cells (B), CD4⁺ T cells (C), CD8⁺ T cells (D), CD8⁺CD28⁺ T cells (E), and CD8⁺CD28⁻ T cells (F) is shown for each cohort (see legend to Figure 1).

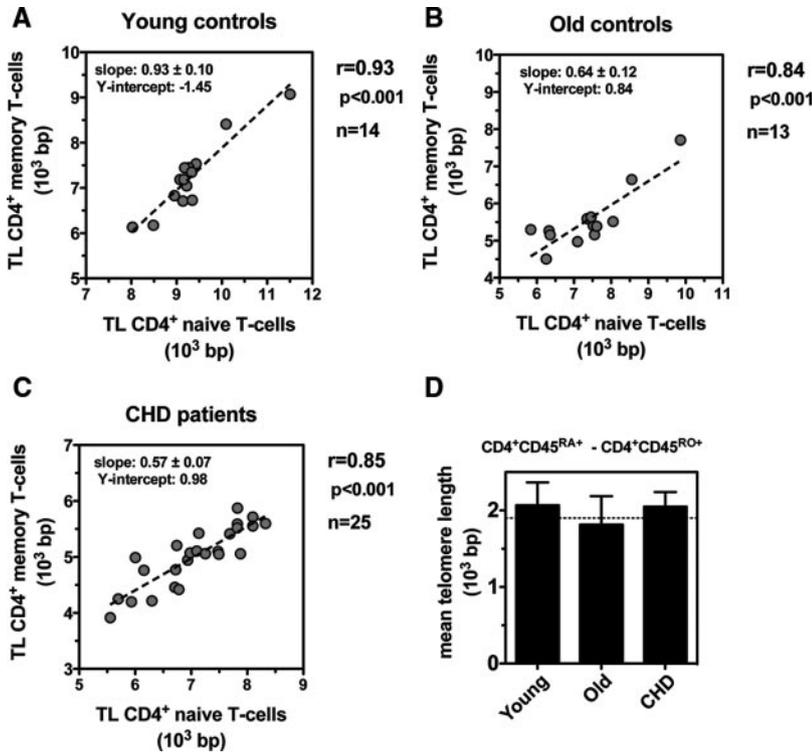


Figure 3. Telomere shortening in CD4⁺ memory T cells is independent of age or CHD. TL in CD4⁺ naive (CD45^{RA+}) vs CD4⁺ memory (CD45^{RO+}) T cells is shown for young and healthy volunteers (A; n=14), elderly control subjects (B; n=13), and patients with CHD (C; n=25). The Pearson correlation coefficient (*r*) is depicted. Slopes between young and elderly healthy control subjects (*P*=0.08, ANCOVA) and those between CHD patients and age-matched control subjects (*P*=0.58, ANCOVA) did not differ significantly, whereas intercepts between the latter 2 groups did show a significant difference (*P*=0.003). D, Mean TL difference between naive and memory CD4⁺ T cells is shown. All bars are mean±SEM.

Lymphocyte Telomere Shortening in CD8⁺ T Cells Is Accelerated in CHD

There was no difference in the absolute number of lymphocytes, CD4 helper cells, or CD8 cytotoxic lymphocytes among the 3 groups (online-only Data Supplement Table I). Although not statistically significant, we noted a trend toward an increased CD4/CD8 ratio with age and CHD (*P*=0.25). TL in all lymphoid subpopulations was 1.8 kb shorter in the older control group than in the younger volunteers (*P*<0.001; Figures 2A through 2D and 3A and 3B). Similar to our findings in myeloid cells, TL in CD4⁺ T cells (Figure 2C) and CD19⁺ B lymphocytes (Figure 2A) was ≈500 base pairs (bp) shorter in

CHD patients than in control subjects, although this difference did not reach statistical significance. Surprisingly, the TL difference in CD8⁺ cytotoxic T lymphocytes between CHD patients and healthy control subjects exceeded that in other populations (1.0 kb, *P*=0.017; Figure 2D). This was mainly attributed to TL shortening in “senescent” CD8 T cells, which lack the costimulatory receptor CD28 (CD8⁺CD28⁻; *P*=0.02; Figure 2F). Finally, we compared naive and memory CD4 T cells in patients with CHD and young and elderly control subjects (Figure 3A through 3D). Memory CD4 T cells (CD4⁺CD45^{RO+}) were all ≈2 kb shorter than their naive counterparts (CD4⁺CD45^{RA+}), independent of the presence of CHD or age.

Table. Clinical Baseline Characteristics

	Young Control Subjects	Elderly Control Subjects	CHD	<i>P</i> , Elderly Subjects vs CHD
n	14	13	25	
Age, y	26.7±1.8	65.1±2.1	64.8±3.4	0.93
HDL cholesterol, mg/dL	58.8±8.6	70.0±20.3	42.5±11.7	<0.001
LDL cholesterol, mg/dL	96.5±20.9	129.2±37.5	74.5±28.6	<0.001
Diabetes mellitus, n (%)	0	0	10 (40)	0.008
Hemoglobin A _{1c} , %	4.7±0.2	5.6±0.4	6.9±2.0	0.038
Smoking, n (%)	1 (7.1)	0 (0)	14 (56)	<0.001
hs-CRP, mg/dL	0.18±0.26	0.12±0.13	1.22±1.68	
log(hs-CRP), mg/dL	-0.98±0.41	-1.06±0.33	-0.32±0.71	0.001
Ejection fraction, %	...	65.2±1.6	29.6±11.1	<0.001
NT-proBNP, ng/mL	25±17	69±38	4680±8526	
Log(NT-proBNP), ng/mL	1.3±0.3	1.8±0.2	3.3±0.5	<0.001

hs-CRP indicates high-sensitivity C-reactive protein.

One-way ANOVA was performed for comparison of all 3 groups, followed by Tukey post hoc test to compare elderly control subjects and CHD patients. For categorical variables, a χ^2 test was performed.

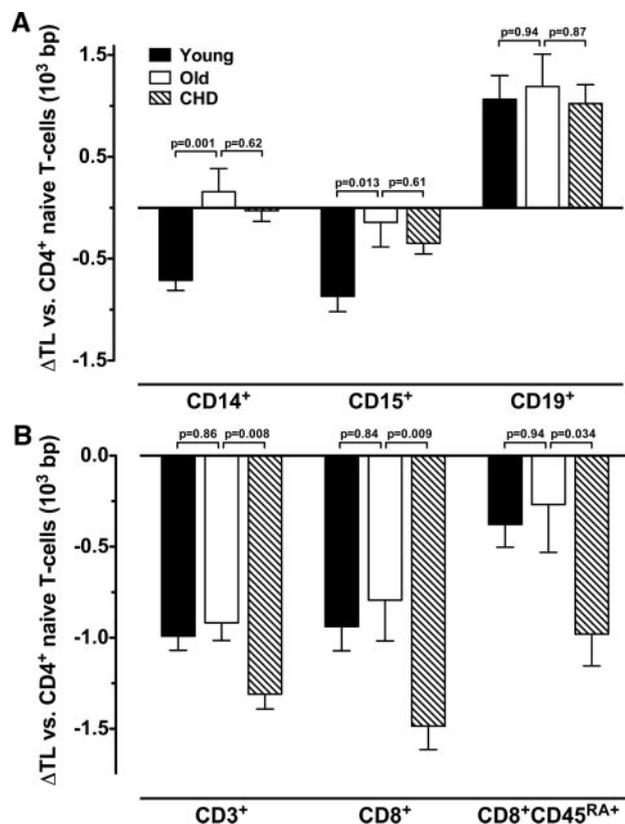


Figure 4. Accelerated telomere shortening in CD8⁺ T cells occurs in CHD patients. TL of naïve CD4 T lymphocytes was chosen as an internal standard and compared with TL of all other cell populations, rendering $\Delta TL = TL_x - TL_{CD4naive}$. All bars are mean \pm SEM. *P* values are derived from a Tukey post hoc test after ANOVA testing.

Telomere Shortening in CHD Affects All Cells Equally

Figure IVA in the online-only Data Supplement and Figure 4A and 4B herein summarize TL shortening in healthy volunteers across all main leukocyte subpopulations, which suggests that TL decreases at a similar rate in B and T lymphocytes (≈ 50 bp/year). Telomere attrition in both granulocytes and monocytes occurs more slowly with age (≈ 30 bp/year). Thus far, the results of the present study strongly suggest that in healthy males, age-dependent telomere shortening is primarily dependent on the TL of myeloid and lymphoid progenitors. Figure IVB in the online-only Data Supplement demonstrates that leukocyte telomere shortening in patients with CHD is essentially due to identical attrition rates in granulocytes, monocytes, B lymphocytes, and CD4 T cells. Interestingly, cytotoxic T lymphocytes represented the only exception to this pattern (Figure 4B). When naïve CD4 T cells were used as an internal standard, the difference in TL between these cells and cytotoxic CD8⁺ T lymphocytes (CTLs) increased from 0.79 ± 0.81 kb in age-matched healthy volunteers to 1.49 ± 0.65 kb in CHD patients ($P=0.009$), which suggests a different mechanism for telomere shortening in CD8 CTLs from patients with CHD.

CMV-Positive Patients and Control Subjects Show Increased Numbers of CD28-Negative T Cells

Recent publications have shown that chronic infection with CMV can lead to T-cell memory inflation and hence an

accumulation of CMV-specific CD8⁺CD28⁻ T cells with shorter telomeres.¹⁸ Because we had found accelerated telomere shortening specifically in CD8⁺CD28⁻ T cells from patients with CHD (Figure 2F), we retrospectively analyzed frozen plasma from patients and control subjects in the present study for CMV IgG antibody. Thirteen of 20 patient samples were positive for CMV, whereas 5 of 13 control samples appeared positive (χ^2 test $P=0.05$; online-only Data Supplement Table II). Acute or reactivated CMV infection was ruled out by analysis of CMV-specific DNA copy number, which was negative in all participants. Age distribution was similar among CMV-negative and -positive subjects. The CD4/CD8 ratio was lower in CMV-positive control subjects (1.3 ± 0.9 versus 2.1 ± 1.0) and patients (1.8 ± 1.2 versus 2.8 ± 1.2 , $P=0.044$; online-only Data Supplement Table II). CMV-positive patients also had a higher C-reactive protein level (1.45 ± 1.5 versus 0.37 ± 0.35 mg/dL, $P=0.03$; online-only Data Supplement Table II) and a lower ejection fraction ($25.5 \pm 9.5\%$ versus $37.1 \pm 11.5\%$, $P=0.04$; online-only Data Supplement Table II). In both groups, seropositive patients and control subjects revealed an increase in the absolute number of CD28-negative T-helper cells and in cytotoxic T lymphocytes (Figure 5A). CMV-seropositive patients with CHD showed a significant increase in CD4⁺CD28⁻ cells (75 ± 85 versus 4 ± 2 cells/ μ L, $P=0.01$), CD8⁺ T cells (566 ± 298 versus 331 ± 137 cells/ μ L, $P=0.03$), and CD8⁺CD28⁻ CTLs (320 ± 243 versus 67 ± 39 cells/ μ L, $P=0.003$; Figure 5A). Using 2-way ANOVA testing, we found a CMV-dependent increase in CD8⁺ T cells ($P=0.009$), CD8⁺CD28⁻ CTLs ($P=0.002$), and CD4⁺CD28⁻ cells ($P=0.006$) but not in total CD4⁺ T cells ($P=0.82$; online-only Data Supplement Table III), which reflects the general increase in CD28-negative T cells among CMV-seropositive individuals, both patients and control subjects.

CMV-Seropositive Patients but Not Control Subjects Show TL Attrition in CD8⁺ CTLs

We reanalyzed our existing data on TL in different leukocyte populations in reference to CMV status (Figure 5B). Surprisingly, healthy control subjects did not show any difference in TL from lymphocyte subsets between seropositive and seronegative subjects, as Figure 5B shows. In contrast, CMV-positive CHD patients had significantly shorter telomeres in their CD8⁺ T cells (5.18 ± 0.99 versus 6.00 ± 0.55 kb, $P=0.025$; Figure 5B) and CD8⁺CD28⁻ CTLs (4.37 ± 0.98 versus 5.52 ± 0.57 kb, $P=0.006$; Figure 5B) but not in other lymphocyte populations, including CD8⁺CD28⁺ CTLs. TL in CD34⁺ progenitor cells and in CD15⁺ granulocytes was unaffected in CMV-positive patients (Figure 5B).

Left Ventricular Function Strongly Correlates With TL in Lymphocytes

Finally, we attempted to evaluate whether shortening of TL from CMV-seropositive patients could have any effect on the underlying disease in patients with CHD. Because all patients had had a myocardial infarction in the past, we wanted to know whether TL in any cell population was associated with the extent of left ventricular dysfunction in this group. To our surprise, TL from all leukocyte populations, which presum-

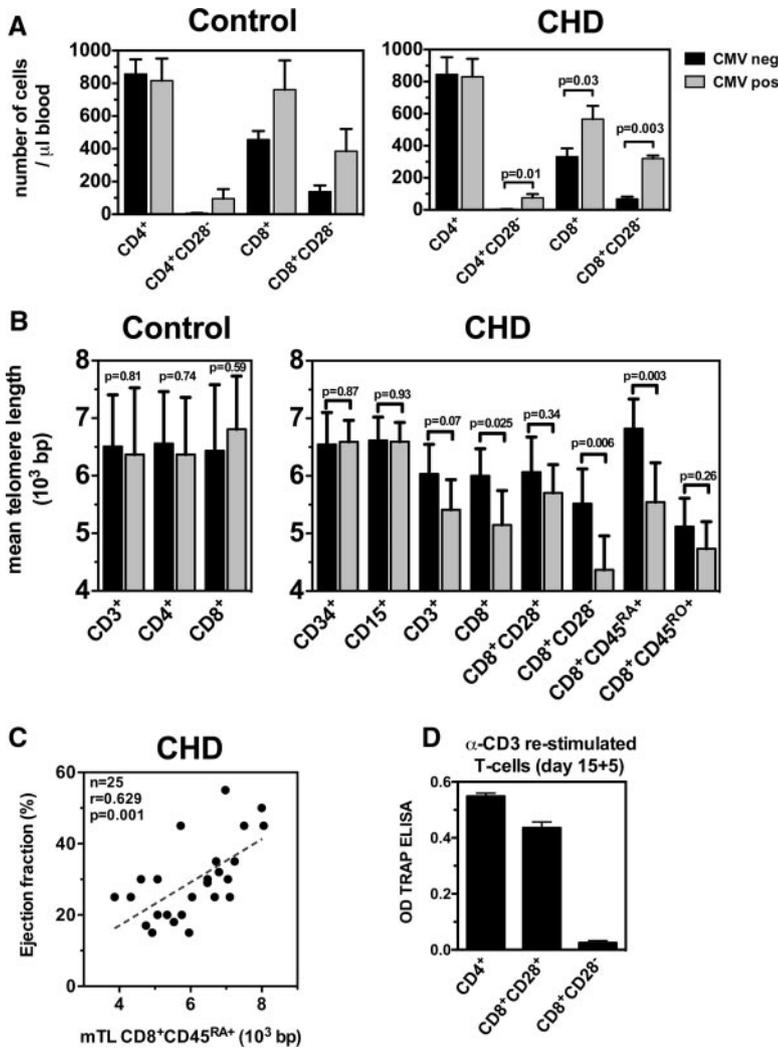


Figure 5. CMV seropositivity in CHD patients affects telomere shortening in cytotoxic T cells. A, The absolute numbers of CD4 and CD8 (sub) populations are indicated for CHD patients (n=20) and age-matched control subjects (n=13). CMV-seronegative (CMV neg) individuals are represented by solid black bars and seropositive individuals (CMV pos) by gray-shaded bars. P values were calculated from an unpaired t test. B, Mean TL for CHD patients (n=20) and age-matched control subjects (n=13) is shown separately for seronegative (solid black bars) and seropositive (gray shaded bar) individuals. P values were calculated from an unpaired t test. C, Scatterplot for left ventricular ejection fraction vs mean TL of CD8⁺CD45^{RA+} T cells in patients with CHD (n=25). r Indicates Pearson correlation coefficient. D, Telomerase activity in CD4⁺ T cells, CD8⁺CD28⁺ T cells, and CD8⁺CD28⁻ T cells (n=4). T cells were isolated on day 0 and incubated in α-CD3-coated wells. At day 15, cells were replated and restimulated with α-CD3. Telomerase activity was measured on day 20. OD TRAP ELISA indicates absorbance units as determined by the TRAPEZE® ELISA Telomerase Detection Kit.

ably contained CMV-specific lymphocytes, held a positive correlation with ejection fraction (online-only Data Supplement Table IV). In contrast, TL of myeloid cell populations or age both did not show any correlation with ejection fraction. The greatest correlation between TL and ejection fraction was seen for the CD8⁺CD45^{RA+} lymphocyte population (Pearson correlation coefficient 0.629, P=0.001; Figure 5C), which largely overlaps with the CD8⁺CD27⁻CD28⁻CD45^{RA+} cell population in CMV-positive patients, the phenotype of CMV-specific CTLs.¹⁹ These results suggest a link between CD8⁺ T cells and the deterioration of myocardial function in CMV-seropositive patients with CHD. Interestingly, CD8⁺CD28⁻ cells could not reactivate telomerase on repeated ligation of the T-cell receptor with anti-CD3 (Figure 5D).

Discussion

Our investigations on TL distribution in leukocyte subpopulations of patients with CHD as opposed to young and elderly healthy control subjects yielded 3 important novel results. First, leukocyte TL in patients with CHD was 500 bp shorter than in age-matched control subjects, a difference that appeared to be highly conserved throughout the hematopoietic system. In addition, TL in cytotoxic CD8⁺ T cells (CTLs) was 1000 bp shorter than in control subjects. We found the most

pronounced degree of telomere shortening in CD8⁺CD28⁻ T cells in CMV-seropositive CHD patients, but not in CMV-seropositive healthy volunteers. Finally, TL shortening of CD8⁺ T cells demonstrated a strong correlation with the decrease in left ventricular function in CHD patients. The results of the present study strongly suggest that telomere shortening in leukocyte subpopulations from patients with CHD is either inherited or reflects accelerated telomere shortening in common precursors of the leukocyte subpopulations, including PBPCs, the hematopoietic stem cell compartment. Whether in patients with CHD, the copresence of CMV accelerates immunosenescence and imposes on the course of CHD itself will need to be demonstrated in future studies.

We present for the first time a detailed analysis of TL in 12 leukocyte subsets, including CD34⁺ PBPCs, from young healthy volunteers and from elderly patients with CHD and their age-matched healthy control subjects. The results of this investigation could ultimately enable us to define the primary affected cell population accountable for accelerated telomere shortening in patients with established CHD. To achieve this goal, a large amount of peripheral mononuclear cells had to be gathered to harbor a sufficient number of CD34⁺ PBPCs that could be subjected to TL measurement. Furthermore, our previously used flow-FISH method^{20,21} had to be refined to

allow measurement of TL in conjunction with determination of surface antigens (multicolor flow-FISH). To limit the number of study participants required, we included only male subjects and performed extensive noninvasive testing for each volunteer before their inclusion in the study, which greatly facilitated characterization and homogeneity of the study populations. The results revealed a number of unexpected findings, all relevant to the interpretation of TL data in patients with CHD. The majority of telomere studies performed thus far on patients with cardiovascular disease have used terminal restriction fragment analysis methods on the total leukocyte population, which does not allow for a sophisticated analysis of leukocyte subpopulations.

Age-Dependent Influence on Leukocyte Subpopulations

It has been shown in multiple studies that leukocyte TL declines with age.^{22–24} We and others have also shown previously that TL shortening in lymphocytes occurs at a higher rate than in granulocytes.^{25–27} We confirm in the present study that lymphoid cell populations clearly demonstrate a faster rate of telomere shortening than myeloid cells during the same life span. Interestingly though, B lymphocytes, CD4 T cells, and CD8 T cells all showed the same rate of telomere shortening in healthy individuals. These results suggest that despite the complexity of the immune system, with clonal expansion and contraction of subpopulations, there is a common denominator for basic TL. The results of the present study strongly suggest that telomere shortening of a common progenitor of B lymphocytes and T lymphocytes in a healthy person determines basic TL. The generation of CD4 memory T cells leads to a substantial shortening of 2000 bp, which corresponds to ≈ 20 cell divisions. This TL difference was almost identical in young, elderly, and unhealthy study participants and was independent of the basic TL. These findings suggest that telomere shortening in CD4 memory cells reflects a general mechanism that is solely dependent on proliferation kinetics rather than on disease or age.

CD34⁺ PBPCs in CHD

Vaziri and coworkers²² showed that candidate human stem cells with a CD34⁺CD38^{lo} phenotype that were purified from adult bone marrow have shorter telomeres than cells from fetal liver or umbilical cord blood. Together with findings by Notaro and coworkers²⁸ that demonstrated that concomitant with their proliferation, hematopoietic progenitor cells lose TL, these findings indicate that the origin of leukocyte telomere shortening occurs at the level of their bone marrow-residing precursors. Therefore, it would appear very attractive to determine the TL of hematopoietic progenitor cells by obtaining TL of peripheral leukocyte populations. Although 1 study has found a strong correlation between PBPCs and peripheral blood leukocytes, these data were generated in mobilized patients with malignancies.²⁹ We have shown previously that TL in mononuclear bone marrow cells correlates extremely well with peripheral blood granulocytes in patients with CHD.²¹ In the present study, we were able to find a very strong correlation ($r > 0.95$) between TL in PBPCs and monocytes or granulocytes. Our findings in completely healthy volunteers were also independent of age, showing for

the first time that TL of peripheral myeloid cells can be used as an indicator for TL in hematopoietic progenitor cells in healthy persons.

Telomere Hypothesis

Patients with CHD have been shown to have shorter telomeres than age-matched healthy control subjects.³⁰ Therefore, the finding that patients in the present study had telomere shortening of ≈ 500 bp compared with their age-matched control group is well in line with previous reports. It has been debated intensely whether this difference in TL is the result of increased oxidative stress over time in patients with severe atherosclerosis, which would render TL a surrogate parameter for “cumulative oxidative stress.” Nevertheless, triggered by several recent publications, another pathophysiological concept has rapidly gained importance, the so-called telomere hypothesis that shorter telomeres will contribute in part to the pathogenesis of CHD.^{9,31} In the first randomized case-control study, Brouillette and colleagues³¹ have demonstrated the correlation between TL and the risk of developing CHD. In favor of the telomere hypothesis is another of their studies showing that mean TL in healthy offspring of subjects with CHD is shorter than in offspring of healthy control subjects.⁹ The present data (Figure 4; online-only Data Supplement Figure IV) point in the same direction, using a very different experimental approach. We found that TL was shortened by ≈ 500 bp in CHD patients compared with healthy age-matched individuals in the present study. Interestingly, this difference was maintained throughout most cell types (with cytotoxic T cells as the only exception), independent of their myeloid or lymphoid origin. If the difference of 500 bp were simply due to accelerated aging, we would expect a similar constellation in TL differences, such as between young and old healthy individuals, in whom lymphoid cell populations show faster telomere attrition than myeloid cells. This was not the case, however, because we found that except for CD8⁺ T cells, all cell populations appeared to have similar shortening, independent of their myeloid or lymphoid origin. We believe, therefore, that the present data argue strongly in favor of leukocyte TL shortening as a predisposing parameter in CHD rather than a coincidental surrogate.

Telomere Shortening of CD8⁺ CTLs and Role of CMV

Accelerated telomere shortening of CD8⁺ T cells on top of “inherited” shortening in patients with CHD in the present study occurred mainly within the CD8⁺CD28⁻ subpopulation. CD8⁺ T cells of this phenotype circulate at high frequencies in CMV-infected people.³² Most CMV-specific CD8⁺ memory T cells are reexpressing CD45^{RA+} and lack the costimulatory molecules CD27 and CD28.^{19,33} Therefore, we retrospectively analyzed the CMV status of our patients and control subjects and found that seropositive patients but not seropositive control subjects showed accelerated telomere shortening in CD8⁺CD28⁻ or CD8⁺CD45^{RA+} T cells, respectively. Different possibilities arise from this finding: (1) CMV seropositivity could represent a predisposition for CHD; (2) the shorter basic TL in patients with CHD could trigger a different response to chronic CMV antigen presentation; and

(3) more likely, in patients who survive the stress situation of an acute myocardial infarction, a different and stronger response of the immune system is triggered. In favor of the latter hypothesis is our finding that ejection fraction was lower in CMV-positive patients and correlated strongly with TL of CD8⁺CD45^{RA+} T cells. Interestingly, all cell populations that are thought to contain predominantly CMV-specific cells demonstrated a positive correlation with ejection fraction (online-only Data Supplement Table IV). This could suggest that patients with larger infarcts (and hence lower ejection fraction) had a stronger CMV-related reaction. In line with other studies, we also found an increased number of CD4⁺CD28⁻ and CD8⁺CD28⁻ T cells in CMV-seropositive control subjects and CHD patients.³⁴ Nevertheless, because of the very small proportion of CD28-negative CD4⁺ T cells compared with CD8⁺CD28⁻ T cells in CMV-seropositive CHD patients, the presumed telomere loss in these cells cannot affect the TL of CD4 cells to the extent it does in CD8 cells. A landmark study by Fletcher and coworkers³⁵ investigated the phenotype (CD27⁻CD28⁻) and TL of CMV-specific CD4⁺ T cells. Those authors found that the latter differentiate more rapidly than other populations of memory T cells, which suggests that the extent of CMV reactivation might correlate with the extent of telomere loss.

A significant number of clinical studies have been published in the past that investigated the controversial association between CHD and CMV infection.^{36–38} Interestingly, the coexistence of inflammation in addition to seropositivity for CMV, reflected by elevated serum levels of either C-reactive protein or interleukin-6, represented the most important predictor of survival. Patients who were CMV seropositive without coexisting inflammation basically failed to show an adverse prognosis. In the present study, patients were proven to already have rather advanced disease, reflected by previous myocardial infarction, severely reduced left ventricular function, and elevated NT-proBNP serum levels. In addition, high-sensitivity C-reactive protein levels were higher in the CMV-seropositive patients. Our understanding is, therefore, that CMV seropositivity in otherwise healthy individuals does not confer a higher risk for the development of CHD or associated complications unless there is ongoing and persistent reactivation of the virus. In this case, one would expect either increased levels of C-reactive protein or interleukin-6 or other evidence of systematic inflammation. The present study investigated the TL of CD8⁺ T cells and their subpopulations. Accelerated telomere shortening in lymphocytes could be the result of persistent (eventually lifelong) reactivation/proliferation of these cells and therefore represents an ideal parameter to understand cumulative CMV burden. Future prospective studies with sufficient statistical power will be required to prove our hypothesis.

Study Limitations

The lack of a significant difference between the TL of patients and age-matched control subjects for several cell populations could have been due to the limited sample size rather than the lack of biological difference between the groups. The study also was not powered for the discrimination of TL between CMV-seropositive and -seronegative subpopulations. Future studies

must include patients on a larger scale to gain more evidence, potentially including CMV-specific CTLs. Only approximately 70% of our measurements of CD34⁺ PBPCs gated for TL analysis were performed on more than 1000 events, a number assumed to be necessary to obtain a reliable TL measure (online-only Data Supplement Figure V). Nevertheless, the lack of correlation between the small amount of gated cells and a potential increase/decrease in the TL of PBPCs does not suggest largely incorrect assumptions of TL in the residual 30% of measurements on fewer than 1000 events.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Previous studies have provided evidence of an inverse association between telomere length in peripheral blood leukocytes and cardiovascular morbidity or mortality. Using a complex cell-fractionation protocol, we were able to measure telomere length in leukocyte subpopulations of patients with coronary heart disease and age-matched control subjects. Our results show that in patients with coronary heart disease, almost all leukocyte populations, including peripheral blood stem cells and progenitor cells, are affected equally by telomere shortening, which argues in favor of an inherited rather than an acquired cause. The mechanism by which shorter telomere length and the risk of developing coronary heart disease are linked remains to be investigated. It can be speculated whether impaired telomere biology in bone marrow-residing precursors could impose on vascular repair mechanisms. Finally, telomere length specifically in CD8⁺ cytotoxic T cells was shorter in cytomegalovirus-seropositive than cytomegalovirus-seronegative patients with coronary heart disease and was associated with decreased left ventricular function. Future research should investigate whether in patients with coronary heart disease, the coexistence of cytomegalovirus accelerates immunosenescence and imposes on the course of coronary heart disease.

Accelerated Telomere Shortening in Leukocyte Subpopulations of Patients With Coronary Heart Disease: Role of Cytomegalovirus Seropositivity

Ioakim Spyridopoulos, Jędrzej Hoffmann, Alexandra Aicher, Tim H. Brümendorf, Hans W. Doerr, Andreas M. Zeiher and Stefanie Dimmeler

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SUPPLEMENTAL MATERIAL.

METHODS

Determination of CMV status

The CMV serostatus of 13 old healthy controls and 20 CAD patients was determined from serum samples using the Enzygnost Anti CMV/IgG enzyme immunoassay (Dade Behring, Marburg, Germany) according to the manufacturer's instructions.(1) For the determination of human CMV (HCMV) DNA load in peripheral blood we used TaqMan real-time quantitative PCR assay.(2) Briefly, the double-stranded DNA was extracted from EDTA blood sample by using a QIAamp DNA Blood Mini Kit (Cat. No. 51 106, Qiagen Inc., Mississauga, Ontario, Canada). Prior to DNA extraction, the defined amount of mouse CMV DNA (MCMV) was added to the sample as internal standard. Quantitative PCR was performed in a real-time format on the 7900 ABI Prism Sequence Detector (Applied Biosystems). A primer set (HCMV-Forward: 5'-CGTTGGTGTGTAGCAACTGGC-3') and (HCMV-Reverse: 5'-TGTGCTCAAAGAGGTTCGAGTTCG-3') was designed to amplify a 140-bp fragment in the UL89 region of the HCMV genome. The primers (MCMC-Forward: 5'-CGTTAGTGTGTAGCAGCTGGC-3') and (5'-Reverse: CGTGCTCAAAGAGGTTCGAGTTC-3') were used to target the 140 bp fragment of the MCMV genome as internal standard. The TaqMan probes (PE Applied Biosystems) selected between both primers were fluorescence labeled with 6-carboxy fluorescein (FAM) (for HCMV DNA) or VIC[®] (for MCMV DNA) at the 5'-end as the reporter dye, and 6-carboxytetramethylrhodamine (TAMRA) at the 3'- end as the quencher. The sequences of fluorophore probes were as follows. For HCMV-probe: (5'-VIC-CGCGAAGGTGTGGCGGCAG TAMRA-3') and for MCMV-probe: (5'-FAM-CGGCCCGAGAACATCCGCTTG TAMRA-3'). A volume of 40 µl of the PCR mixture containing 10 µl of eluted DNA, 25 µl of PCR QuantiTect PCR Mix (Cat. No. 204343, Qiagen), 10.2 µl QIAgen water, 5 µM concentrations of each primer and 5 mM of each fluorophore probe, was added to PCR tubes. The TaqMan cycling conditions were: 15 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, annealing and extension for at 60°C for 60 s. The emission data measured during amplification cycles were analysed by using SDS software.

Quantification of CD4 and CD8 T cell counts

In order to obtain the percentages and the absolute numbers of T cell subsets, we first determined the total number of lymphocytes per μL of peripheral blood (ADVIA 120 hematology analyzer, Bayer Healthcare, IR 397). Subsequently 100 μl of EDTA peripheral blood were stained with appropriate amounts of monoclonal antibodies: FITC-conjugated anti-CD3 (Cat. No. 555332, BD), PE-conjugated anti-CD4 (Cat. No. 555347) or PE-conjugated anti-CD8 antibody (Cat. No. 555635, BD) and APC-conjugated anti-CD28 (Cat. No. 559770, BD). Following 30 minutes incubation at 4 °C in the dark, erythrocytes were lysed (FACS lysing solution, Cat. No. 349202, BD) and probes were washed twice with PBS/1% BSA. Finally, cells were fixed in PBS/1% paraformaldehyde (Cat. No. 29447, BDH Chemicals Ltd) and proceeded for flow cytometric analysis (FACS Calibur, BD). For each sample 20,000 total events were recorded and lymphocytes were identified based on the forward and side scatter profiles. The gates and quadrants were set based on the appropriate IgG1 isotype controls. The spectral compensation was set up at the beginning of each measurement by the additional staining of cells with FITC-conjugated anti-CD4 (Cat. No. 555346, BD) and PE-conjugated anti-CD8 antibodies (Cat. No. 555635, BD). The absolute numbers of CD4⁺ and CD8⁺ T cell subsets were calculated by multiplying their percentages derived from FACS by the absolute count per μL of each population.

Isolation of leukocyte subpopulations

Peripheral blood EDTA samples (100 ml) were collected from CAD patients and healthy volunteers (EDTA S-Monovette 9 ml, Cat. No. 02-1066-001, Sarstedt, Nümbrecht, Germany). Peripheral blood mononuclear cells (PBMCs) were obtained after density gradient centrifugation using Ficoll-Hypaque (Cat. No. L6115, Biochrom, Germany). After two washes with PBS, the PBMCs were resuspended in the ice chilled MACS buffer containing PBS with 0.5 % FCS and 2 mM EDTA (Tritriplex III 1.1 %). The cells were counted in trypan blue using a Neubauer hemocytometer. For isolation of different mononuclear cell subpopulations, we designed a cell fractionation strategy based on the immunomagnetic sorting (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) (see supplementary Figure 1). All separation steps were performed by using LS columns (Cat. No. 130-042-401) placed in the QuadroMACS Separator (MACS MultiStand Cat. No. 130-042-303). Briefly, starting with the less frequent leukocyte populations, we firstly enriched the CD34⁺ peripheral progenitor cells using Direct CD34 Progenitor Cell Isolation Kit (Cat. No. 130-046-702) according to manufacturer's instructions. The enriched CD34⁺ cells were

washed, resuspended in RPMI 1640 medium (Cat. No. 21875034, Gibco) containing 10% FCS (Cat. No. CC-4101C, Lorna, Walkersville, USA) supplemented with 10% horse serum (Cat. No. 800-3675296, Jackson Immunoresearch) and stored in refrigerator for few hours until subjected to multicolor flow fish staining. In the next step, CD8⁺ cells were isolated from the CD34⁻ cell fraction with the CD8 Multisort Kit (Cat. No. 552-01). After removal of CD8-Beads from the cell surface, the CD8⁺ cells were depleted from NK cells by using CD56 MicroBeads (Cat. No. 130-050-401). The negatively selected CD8⁺CD56⁻ were highly purified cytotoxic T cells as confirmed by flow cytometry (percentage of CD3⁺CD8⁺ over 98%). For further isolation of cytotoxic T cell subpopulations we stained the CD8⁺CD56⁻ cells with biotin-conjugated anti-CD28 monoclonal antibody (Cat. No. 555727, BD) and subsequently sorted them into CD28⁺ and CD28⁻ cells using Anti-Biotin Microbeads (Cat. No. 130-090-485). Purity of CD8⁺CD28⁺CD56⁻ and CD8⁺CD28⁻CD56⁻ cells was over 95% (CD8/CD28 Simultest, Cat. No. 340031, BD). In parallel, CD4⁺ T cells were enriched from CD8⁻ cell fraction using CD4 Microbeads (Cat. No. 130-045-101) following depletion of monocytes with CD14 MicroBeads (Cat. No. 130-050-201). Purity of enriched CD4⁺CD14⁻ T helper lymphocytes (percentage of CD3⁺CD4⁺) and CD14⁺ monocytes (percentage of CD45⁺CD14⁺) was over 98%. The remaining mononuclear cell flow through, depleted from T cells and monocytes (CD4⁻CD8⁻CD14⁻), was further used to detect the B cell population. The sorted cell populations were resuspended in PBS containing 10% FCS and stored at 4°C until subjected to multicolor flow fish procedure. Finally, peripheral blood leukocytes were obtained from 5 ml EDTA blood, directly before proceeding to multicolor flow fish protocol. Red blood cells were lysed by incubating EDTA blood with ammonium chloride solution (Cat. No. 07850, Stem Cell Technologies, Vancouver, Canada) in a 1:4 ratio 2 times for ten minutes on ice and the nucleated cells were then recovered by centrifugation. Cells were once washed with PBS containing 0.1% BSA, counted with hemocytometer, and directly processed on the basis of multicolor flow fish protocol.

Flow-fluorescent in situ hybridization (Flow-FISH)

For analysis of mean telomere length in subpopulations of peripheral leukocytes we used either the standard flow FISH protocol (for purified CD14⁺ monocytes, CD8⁺CD28⁺CD56⁻ T cells and CD8⁺CD28⁻CD56⁻ T cells) or multicolor flow-FISH approach (for CD34⁺ progenitor cells, CD15⁺ granulocytes, CD45^{RO+} T cells, CD45^{RA+} T cells and CD19⁺ B cells) as previously described with minor modifications.(3, 4) Mean telomere length in subpopulations of peripheral blood leukocytes were

measured using flow FISH in accordance to previously published protocols.(5) In brief, the cell aliquots containing 2×10^5 human cells and 1×10^5 bovine thymocytes as internal control, were placed into 1.5 ml polypropylene tubes (Cat. No. 7080.1, Roth, Germany). Since less than 2×10^5 enriched CD34⁺ cells per tube were available, a higher volume of calf thymocytes was added to each tube giving a total amount of 3×10^5 cells pro tube. After one wash with PBS (Cat. No. H15-002, PAA Laboratories, Pasching, Austria), the cells were resuspended in 300 μ l of hybridization mixture containing 20mM TRIS, pH 7.1 (Cat. No. T-1503, Sigma), 20 mM NaCl, 1% BSA, 75% deionized (using Resin, Cat. No. 142-6425, Bio-Rad) and ultrapurified formamide (Cat. No. 15515-026, Invitrogene, Carlsbad), and either 0.3 μ g/ml telomere specific N-terminal fluorescein isothiocyanate (FITC)-conjugated (C₃TA₂)₃ PNA probe (Panagene, Korea) or an equivalent amount of blank solution for background control (1:1 H₂O/N,N Dimethyl-Formamide (Cat. No. 321537643, Merck) solved in TE buffer (Qiagen). After an initial reaction time of 10 min at room temperature, the DNA in the samples was denatured in an 87°C water bath for 15 min and then hybridized for 2 hours at room temperature in the dark. In order to remove excess unbound PNA probe, the samples were washed three times with 1 ml wash buffer 1 containing 75% Formamid (Cat. No. A16682, Applichem), 10 mM TRIS pH 7.1, 1% BSA, 1% Tween20 (Cat. No. P-1379, Sigma) and one time with 1 ml wash buffer 2 containing PBS, 1% BSA, 1% Tween, 10 mM HEPES (Cat. No. H4034-100G, Sigma) and 5% glucose (Cat. No. G5400, Sigma). After each wash step, cells were spun down in Heraeus Biofuge primo R centrifuge at 2000 g for 5 min for the buffer 1 washes and for 10 min for the buffer 2 wash. Finally, cells were resuspended in 300 μ l of DNA counterstain solution containing PBS, 0.1% BSA, 10 μ g/ml RNase A (Qiagen), and 0.06 μ g/ml propidium iodide (PI, Cat. No. P-1304MP, Molecular Probes). The samples were stored at 4°C in the dark and subjected to flow cytometric analysis within the next 12 hours.

Multicolor flow-FISH

All monoclonal antibodies used for multicolor flow-FISH in this study were conjugated with heat stable Alexa Fluor 647 (AF647) fluorochrome. Anti-human CD3-AF647 (Cat. No. 557706) and AF647 Mouse IgG1 Isotype Control (Cat. No. 557714) were purchased from BD Biosciences, anti-human CD15 (Cat. No. 301911) and AF647 Mouse IgM κ Isotype Control (Cat. No. 401618) were purchased from BioLegend (San Diego, CA, USA). Anti-human CD19-AF647 (Cat. No. MCA1940A647), anti-human CD45RA-AF647 (Cat. No. MCA88A647), purified anti-human CD45RO (Cat. No. MCA461G) and purified anti-human CD34 (Cat. No. MCA1578) were obtained

from AbD Serotec (Oxford, UK). Anti-human CD45RO-AF647 and anti-human CD34-AF647 were prepared by labelling the purified anti-human CD45RO and anti-human CD34 monoclonal antibodies with a Zenon Alexa Fluor 647 Mouse IgG_{2a} labelling kit (Cat. No. Z25108, Invitrogen, Paisley, UK) and Zenon Alexa Fluor 647 Mouse IgG₁ Labelling Kit (Cat. No. Z25008, Invitrogen, Paisley, UK), respectively. Prior to starting the study experiments, we extensively tested each AF647-conjugate for heat and formamide fixation stability by comparing the immunofluorescence intensity of the surface stained cells before and after incubation at 87°C and hybridization steps. Aliquots of 2-4 x 10⁶ leukocytes, PBMCs and immunomagnetic sorted cell populations were transferred into 1.5 ml tubes and kept on ice (for enriched CD34⁺ cells, the aliquots were 0.5-1.0 x 10⁶). After washing once with PBS (10 min at 1800 rpm at 4°C), cells were resuspended in 100 µl PBS and stained with an appropriate amount of AF-647 monoclonal antibodies: 15 µl AF647 Mouse IgG1 Isotype Control (PBMCs), 15 µl anti-human CD3-AF647 (PBMCs), 15 µl anti-human CD15-AF647 (Leukocytes), 20 µl anti-human CD45RA-AF647 and 20 µl CD45RO-AF647 (CD4⁺ and CD8⁺ T cells), 15 µl anti-human CD19-AF647 (resting CD4⁻CD8⁻CD14⁻ mononuclear cells) and 20 µl CD34-AF647 (enriched CD34⁺ cells). After incubation time of 30 min at 4°C in the dark the cells were washed with PBS and resuspended in 180 µl PBS. The water soluble cross-linker Bis(sulfosuccinimidyl) suberate (BS³; Cat. No. 21580, Pierce Biotechnology, Rockford, IL, USA) was used to cross link the antibodies to the cell surface. 20 µl of 10 mM BS³-solution were added to the cells and incubated on ice for 30 min under light protection. Excess BS³ reagent was quenched by adding 1 ml of stop buffer containing 100 mM Tris-HCl, pH 7.0 and 150 mM NaCl, and the cells were further incubated in ice for 20 min. The cells were washed once with PBS and resuspended in 1 ml PBS. Since the washing steps required during cell isolation, surface staining and cross-linking, led to cell losses, all cell populations were recounted before proceeding to the in situ hybridization.

Determination of telomere length in CD34⁺ peripheral progenitor cells (PBPC)

The CD34 molecule is a transmembrane glycoprotein, which is expressed in hematopoietic stem and progenitor cells and on vascular endothelial cells. Peripheral CD34⁺ cells are commonly used in hematopoietic stem cell transplantation. This, however, requires a mobilization from bone marrow into peripheral blood by growth factor stimulatory treatments. Since peripheral blood, which is not mobilized contains a very low percentage of CD34⁺ cells (approximately 0.05 - 0.2%), their efficient isolation from unmobilized donors (e.g. using immunomagnetic bead sorting) may

provide difficulties. In this study we applied, for the first time, a combination of immunomagnetic cell sorting and multicolor flow-FISH to specifically analyse the mean telomere length in PBPC in unmobilized donors. For the additional staining of immunomagnetically enriched CD34⁺ cells we used a AF647-self-conjugated monoclonal antibody (Clone: 581) recognizing the CD34 Class III epitope which is different from the CD34 Class II epitope recognized by the magnetic beads-conjugated antibodies (Clone: Q-Bend) (see supplementary Figure 2). Class III CD34 epitopes have a broader distribution on normal hematopoietic progenitor cells than do class I and II epitopes. (6, 7) This step appeared to be necessary to overcome the common problem of insufficient purity of isolated low frequent peripheral CD34⁺ cells from unmobilized donors. The possible contamination of enriched CD34⁺ cells with other cell types, like monocytes or lymphocytes, would impair the reliable estimation of mean telomere length by using common methods including standard flow-FISH.

Analysis of flow-FISH and multicolor flow-FISH

Data acquisition of all samples was performed on FACSCalibur (Becton Dickinson). The analysis of data was performed using Cell Quest Pro software (Becton Dickinson). Briefly, human diploid cells were distinguished from calf thymocytes by gating on the basis of propidium iodide fluorescence (channel FL3, linear scale) and size (forward scatter). The human cells were further gated based on size (forward scatter) and granularity (side scatter) allowing the discrimination between myeloid and lymphatic cells. Finally, in the cell populations processed with multicolor flow fish protocol, AlexaFluor-647 fluorescence corresponding to cell surface marker was acquired (channel FL4 on a logarithmic scale), allowing an additional discrimination between positive and negative subpopulations. The telomere fluorescence was measured in the FL1 channel on a linear scale. To correct for the cell autofluorescence in FL1, we analysed samples with and without telomere specific probe, and subtracted then the mean fluorescence of unstained samples from the mean fluorescence hybridized cells. 20,000 events were measured in each specimen. Reproducibility was evaluated by performing duplicate samples, both with and without PNA probe, for each cell type, yielding a standard deviation of repeat measurements below 1% (< 100 base pairs). The calf thymocytes, which served as internal control in every single measurement were used to analyse the hybridization efficiency and to express the telomere length in kilobases. Mean telomere length of leukocyte subpopulations was calculated as molecular equivalents of sluble fluorochrom (MESF) in patient samples / MESF in thymocytes times mean telomere length of thymocytes determined directly by Southern Blotting. In order to test

linearity of the flow cytometer, we used five populations of FITC-labeled calibration beads (Quantum FITC MESF kit - Low Level, Cat. No. 824, Bangs Laboratories, Fishers, IN, USA) each having a fluorescence corresponding to different known amounts of molecular equivalents of soluble fluorochrome (MESF). Briefly, the calibration beads suspended in PBS with 0.1% BSA (two drops of beads in 300 ml fluid) were run in FACS and about 5,000 events were acquired in a FCS/SSC dot plot using identical FL1 settings as for telomere fluorescence measurements. The slope was calculated from the MESF values versus the FL1-fluorescence values of the five bead populations. Finally, we also calculated the variability of the Flow-FISH method by analysing the variability measurements of our internal control (bovine thymocytes) between patients (inter-individual variation) as well as between cell populations (intra-individual variation). We used 53 measurements of mean fluorescence activity (MESF), each performed on a different day. Inter-individual variability was 5.5%, and intra-individual variability 1.0% (see supplementary Figure 3).

RESULTS

Supplementary table S1

Lymphocyte characteristics of the complete study population

	Young Controls	Old Controls	CHD	p value* one-way ANOVA
N	14	13	25	
Total lymphocyte count [/ μ l]	1831 \pm 499	1815 \pm 443	1611 \pm 457	0.28
Total CD4 lymphocytes [/ μ l]	863 \pm 300	841 \pm 260	807 \pm 339	0.87
Total CD8 lymphocytes [/ μ l]	582 \pm 165	574 \pm 300	462 \pm 269	0.29
CD4 / CD8 ratio	1.52 \pm 0.41	1.80 \pm 0.97	2.26 \pm 1.36	0.14
Total CD4 ⁺ CD28 ⁻ lymphocytes [/ μ l]	5 \pm 4	41 \pm 88	49 \pm 72	0.17
Total CD8 ⁺ CD28 ⁻ lymphocytes [/ μ l]	112 \pm 87	233 \pm 231	222 \pm 221	0.22

*One-way ANOVA was performed for comparison of all 3 groups. Post-hoc tests were omitted due to the lack of statistical significance in the ANOVA p value.

Supplementary table S2

Baseline characteristics of CMV substudy

	Controls (n=13)		Cont vs CHD	CHD (n=20)		CMV pos vs CMV neg
	CMV neg	CMV pos		CMV neg	CMV pos	
N	8	5		7	13	
Age	64.8±2.6	65.6±1.1	0.79	65.5±3.0	64.3±3.8	0.82
Ejection fraction (%)	64.3±1.2	66.0±1.7	<0.001	37.1±11.5	25.5±9.5†	0.26
NT-proBNP (ng/ml)	54±23	90±48	0.015	2161±3018	3976±4197	0.43
log (NT-proBNP)	1.7±0.2	1.9±0.3	<0.001	3.1±0.5	3.4±0.5	0.13
LDL cholesterol (mg/dl)	131±47	126±16	<0.001	75±19	78±35	0.92
HbA1c (%)	5.6±0.5	5.5±0.2	0.66	7.1±1.3	7.0±2.5	0.90
Total lymphocytes (/μl)	1700±366	2000±535	0.18	1506±406	1734±487	0.12
CD4/CD8	2.1±1.0	1.3±0.9	0.18	2.8±1.2	1.8±1.2	0.044
hsCRP (mg/dl)	0.14±0.16	0.08±0.04	0.038	0.37±0.35	1.45±1.5‡	0.18
log (hsCRP)	-1.0±0.4	-1.1±0.2	0.003	-0.6±0.4	-0.2±0.8	0.50

All indicated p-values (Cont vs CHD as well as CMV positive vs CMV negative) were calculated by 2-way ANOVA (see Statistical methods section). † p=0.04 vs. CMV negative (only CHD group, t-test), ‡ p=0.03 vs. CMV negative (only CHD group, t-test)

Supplementary table S3**CD4 and CD8 populations in CMV substudy**

	Controls (n=13)		Cont vs CHD	CHD (n=20)		CMV pos vs CMV neg
	CMV neg	CMV pos		CMV neg	CMV pos	
N	8	5		7	13	
CD4 lymphocytes (/μl)	857±252	816±302	0.99	846±281	830±404	0.82
CD8 lymphocytes (/μl)	457±147	761±400	0.11	332±566	298±152	0.009
CD4 ⁺ CD28 ⁻ lymphocytes (/μl)	7±4	96±130	0.66	4±2	75±85	0.006
CD8 ⁺ CD28 ⁻ lymphocytes (/μl)	138±108	385±304	0.37	68±39	320±243	0.002

All indicated p-values (Cont vs CHD as well as CMV positive vs CMV negative) were calculated by 2-way ANOVA (see Statistical methods section).

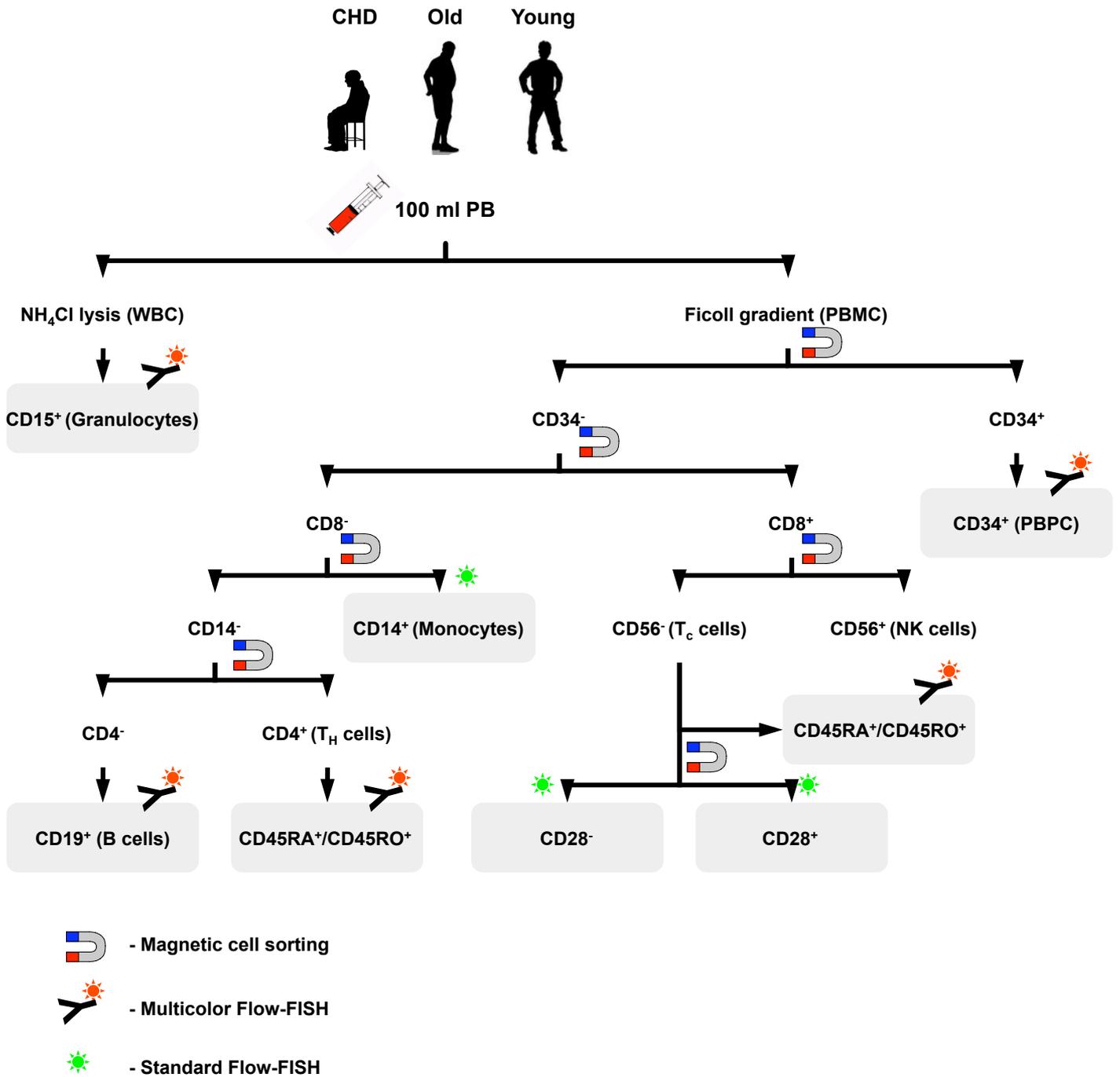
Supplementary table S4

This table lists Pearson's correlation coefficient to explore the linear relationship between ejection fraction (EF) and different parameters in cellular subpopulations from patients with CHD (n=25). Cell populations, which are thought to contain predominantly CMV-specific cytotoxic T-cells in CMV-seropositive individuals, are expressed in bold typeface.

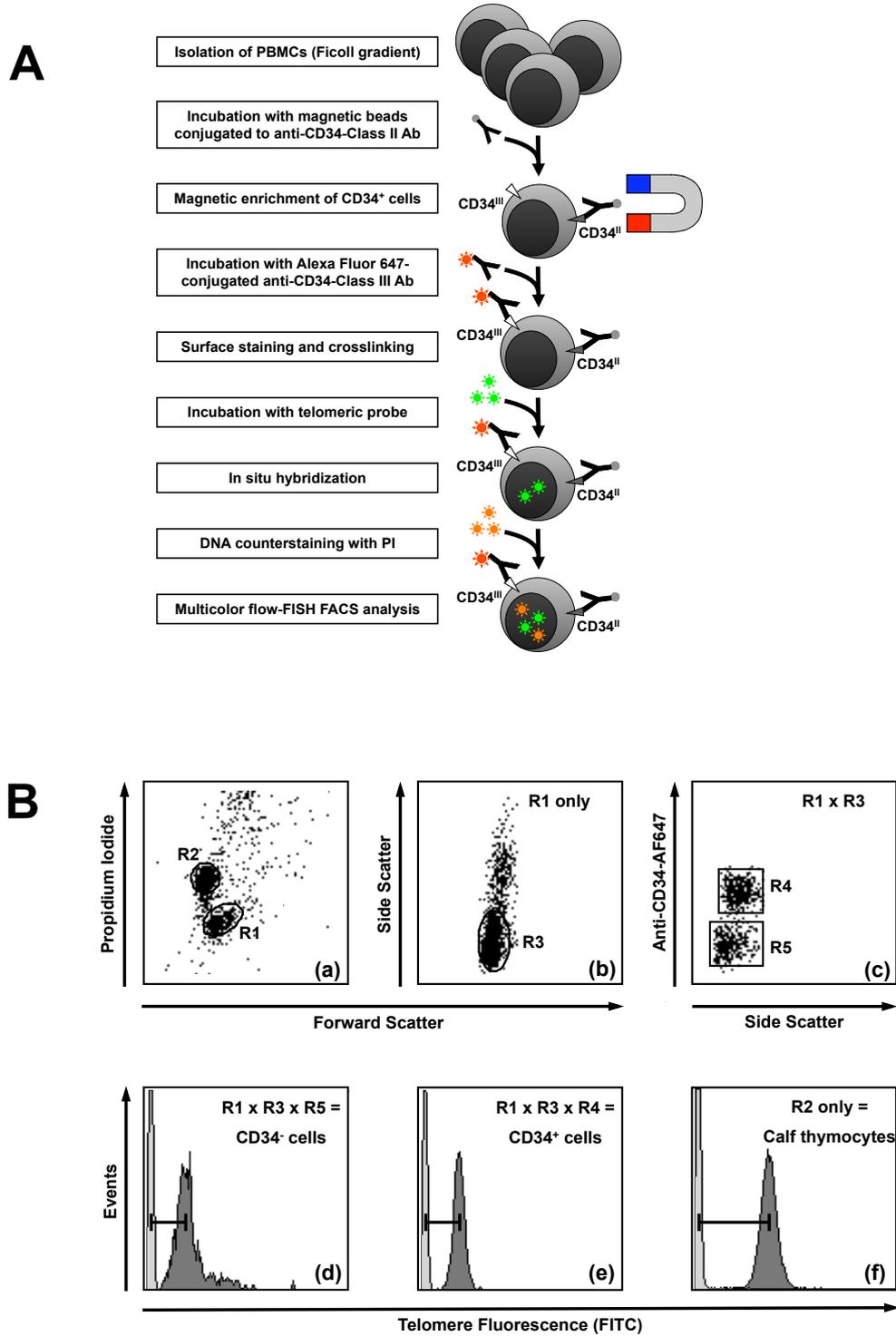
Correlation with ejection fraction	r	p
NT-proBNP	-0.467	0.019
mTL CD8 ⁺ CD45 ^{RA+}	0.629	0.001
mTL CD8 ⁺	0.556	0.004
mTL CD8 ⁺ CD28 ⁻	0.555	0.005
mTL CD3 ⁺	0.465	0.019
mTL CD8 ⁺ CD28 ⁺	0.384	0.064
mTL CD4 ⁺ CD45 ^{RA+}	0.369	0.070
mTL CD8 ⁺ CD45 ^{RO+}	0.346	0.17*
mTL CD14 ⁺	0.374	0.065
mTL CD4 ⁺	0.355	0.082
mTL CD34 ⁺	0.211	0.31
mTL CD4 ⁺ CD45 ^{RO+}	0.210	0.31
mTL CD15 ⁺	0.149	0.48
mTL CD19 ⁺	-0.128	0.54
number of CD8 ⁺ CD28 ⁻ cells/ μ l	-0.509	0.011
number of CD4 ⁺ CD28 ⁻ cells/ μ l	-0.255	0.23

* (n=17)

Supplementary Figure 1



Supplementary Figure 2



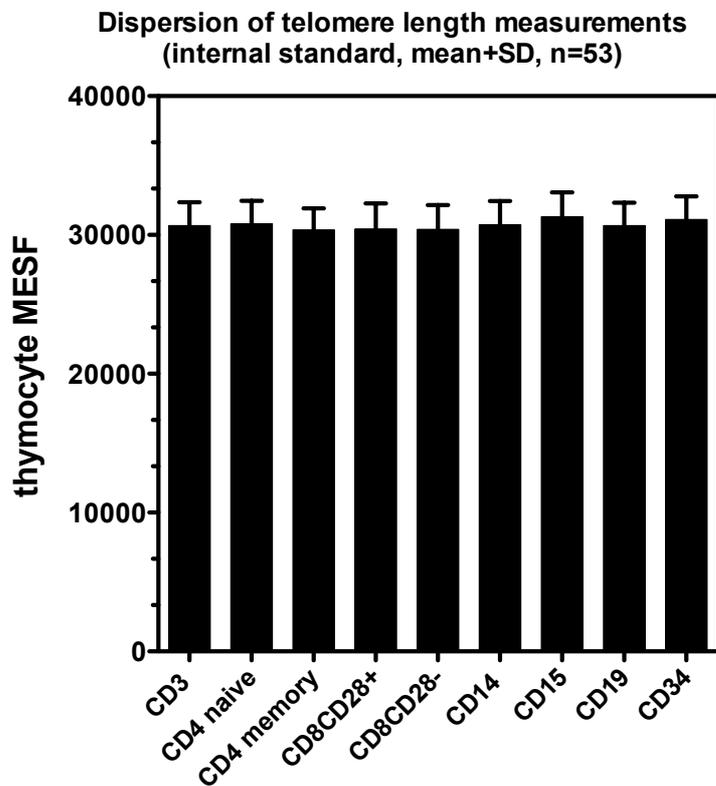
Supplementary Figure 3

Variability / Dispersion of telomere length measurements (Thymocytes)

	n	MESF mean	std-deviation	Variability
CD3	53	30655	1700	5,5%
CD4 naive	53	30788	1669	5,4%
CD4 memory	53	30354	1558	5,1%
CD8 CD28 pos	51	30425	1843	6,1%
CD8 CD28 neg	52	30387	1768	5,8%
CD14	53	30735	1710	5,6%
CD15	53	31301	1760	5,6%
CD19	53	30666	1647	5,4%
CD34	53	31078	1674	5,4%
		30710 average		5,5%
	all groups	318 stddev		
		1,0%		
	without CD15 (lysis)	244 stddev		
		0,8%		

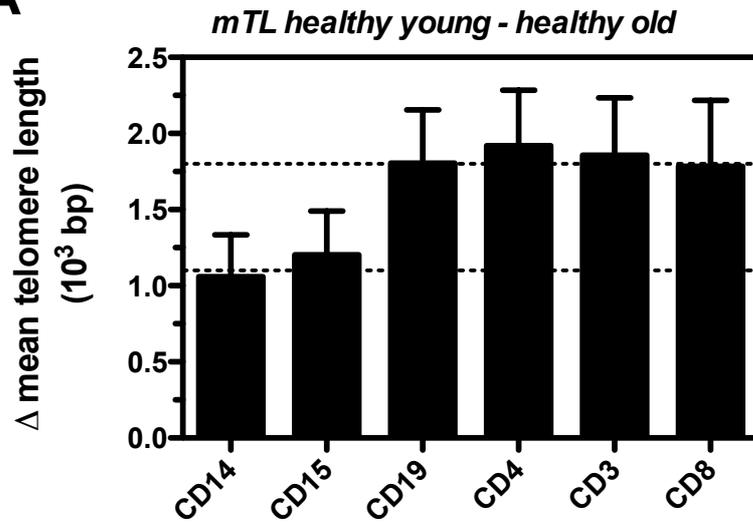
Thymocytes:

Inter-assay	5,5%
Intra-assay with CD15 (lysis)	1,0%
Intra-assay without CD15 (lysis)	0,8%

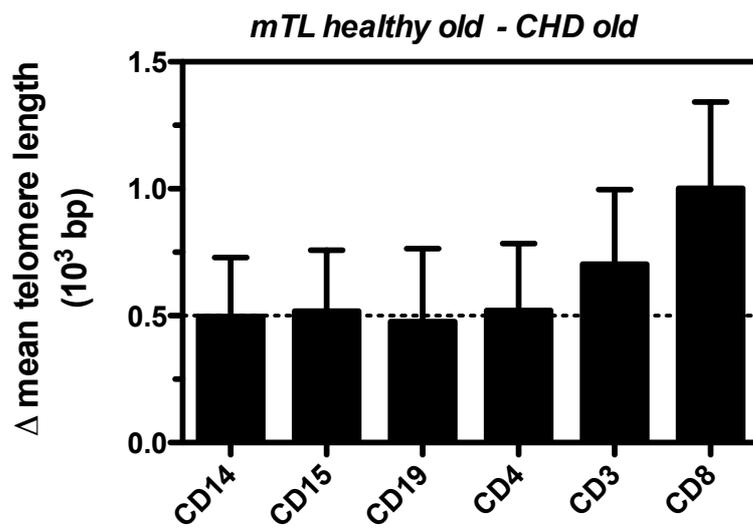


Supplementary Figure 4

A



B



Supplementary Figure 5

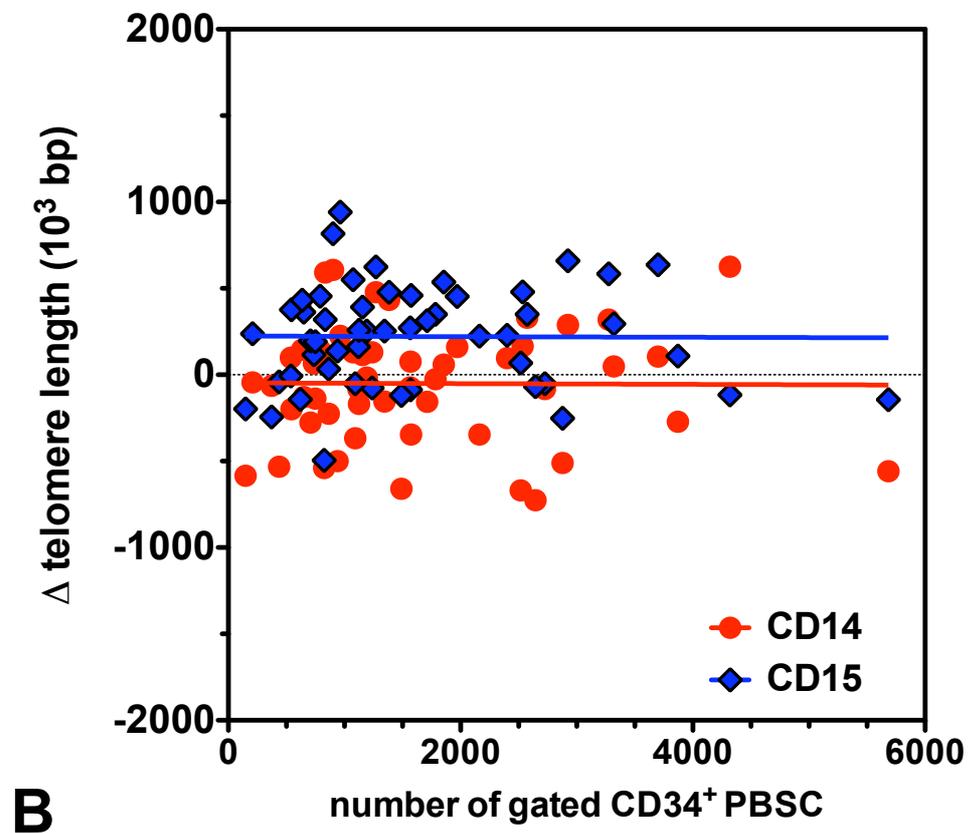
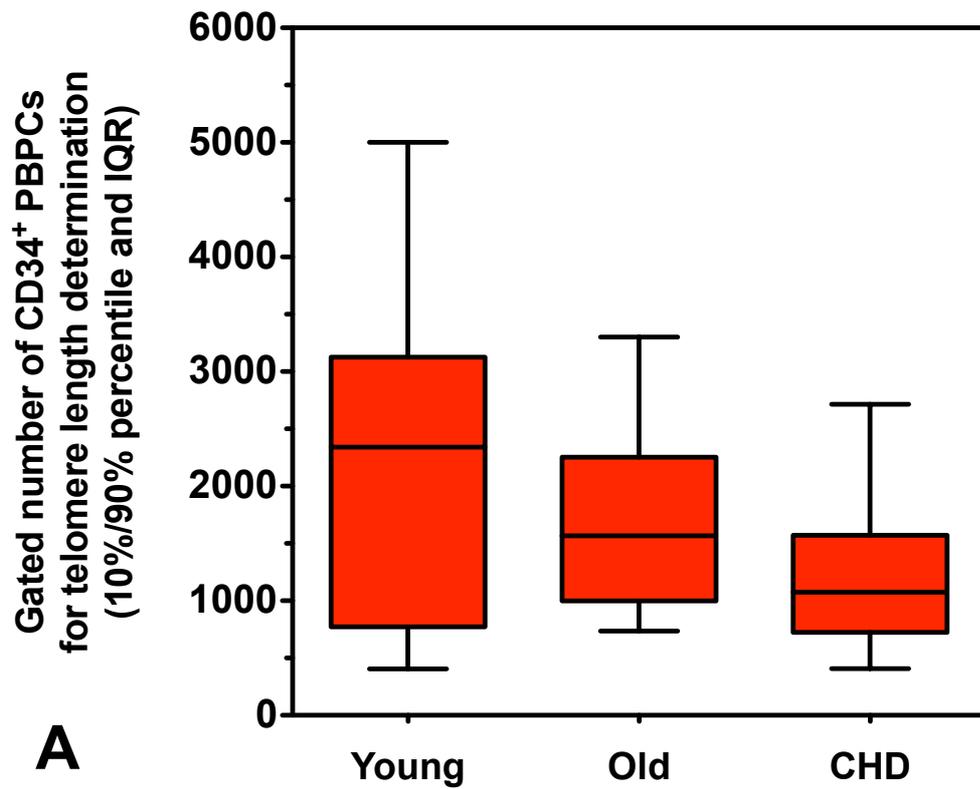


FIGURE LEGENDS

Supplementary Figure 1

100 ml of peripheral blood (PB) were obtained from CAD patients and healthy volunteers in EDTA-coated tubes. Peripheral white blood cells (WBCs) were isolated from 5 ml EDTA blood sample after red blood cell lysis with ammonium chloride (NH_4Cl) and further subjected to telomere length (TL) analysis of $\text{CD}15^+$ granulocytes using multicolor flow-FISH. Peripheral blood mononuclear cells (PBMCs) were obtained after Ficoll density gradient centrifugation and immunomagnetically sorted into $\text{CD}34$ -positive ($\text{CD}34^+$) and $\text{CD}34$ -negative ($\text{CD}34^-$) populations. The enriched $\text{CD}34^+$ cells were further processed with multicolor flow-FISH using second anti- $\text{CD}34$ antibody to specifically detect the TL of $\text{CD}34^+$ peripheral blood progenitor cells (PBPCs). The $\text{CD}34^-$ cells were separated into $\text{CD}8^+$ and $\text{CD}8^-$ cells using $\text{CD}8$ Multisort Kit. After removal of $\text{CD}8$ -Beads from the cell surface, the $\text{CD}8^+$ cells were depleted from $\text{CD}56^+$ NK cells. The selected $\text{CD}8^+\text{CD}56^-$ cytotoxic T cells (T_c) were finally sorted into $\text{CD}28^+$ and $\text{CD}28^-$ cell subpopulations with the subsequent TL analysis using standard flow-FISH. Additionally, an aliquot of $\text{CD}8^+\text{CD}56^-$ cytotoxic T cells was stained with anti- $\text{CD}45\text{RA}$ antibody to analyse TL by means of multicolor flow-FISH. In parallel, the $\text{CD}14^+$ monocytes were depleted from $\text{CD}8^-$ cells and subjected to standard flow-FISH analysis. $\text{CD}4^+$ T helper cells (T_H) were enriched from $\text{CD}8^-\text{CD}14^-$ cell fraction and TL of naive and memory cell subpopulations was detected with multicolor flow-FISH using anti- $\text{CD}45\text{RA}$ and anti- $\text{CD}45\text{RO}$ antibodies, respectively. The remaining mononuclear cell flow trough ($\text{CD}4^-\text{CD}8^-\text{CD}14^-$) was further processed to detect the TL of B cell population with the multicolor flow-FISH using anti- $\text{CD}19$ antibody.

Supplementary Figure 2

Example of multicolor flow-FISH data analysis of $\text{CD}34^+$ peripheral progenitor cells from an unmobilized young healthy donor (27 years old). The human diploid mononuclear blood cells (R1) (containing immunomagnetically enriched $\text{CD}34^+$ cells and contamination of other mononuclear cells, such as lymphocytes and monocytes) could be distinguished from calf thymocytes, our internal control (R2) by using forward scatter and PI staining (a). The gated cells in R1 were further separated on the basis of size and granularity (lymphocyte gate R3 containing $\text{CD}34^+$ progenitor cells was separated from monocytes) (b). Pure $\text{CD}34^+$ cells and contaminating lymphoid cells were further discriminated within gate R3 based on the staining with

anti-CD34-AF647-antibody (R4 and R5, respectively) (c). Telomere length analysis of CD34⁻ cells (d), CD34⁺ progenitor cells (e) and calf thymocytes (f) were performed with and without FITC-(C₃TA₂)₃ PNA probe (dark and light grey peaks, respectively) to allow subtraction of autofluorescence of cells from telomere fluorescence (difference indicated by horizontal bars).

Supplementary Figure 3

Calculation of the variability of the Flow-FISH method by analysing the variability measurements of the internal control (bovine thymocytes) between patients (inter-individual variation) as well as between cell populations (intra-individual variation). We used 53 measurements of mean fluorescence activity (MESF), each performed on a different day. Inter-individual variability was 5.5%, and intra-individual variability 1.0%.

Supplementary Figure 4

The telomere hypothesis in CHD. The mean telomere length difference between healthy young (n=14) and healthy old (n=13) volunteers (**A**) as well as between healthy old and CHD patients (n=25, **B**) is presented for 6 different leukocyte populations. All bars are mean±SEM.

Supplementary Figure 5

Gated number of CD34⁺ cells per subject with a measurement of telomere length (Figure 1C-E). **A.** Approximately 70% of our measurements were performed on more than 1000 events, which are necessary to obtain a reliably telomere length measure (based on M. Blasco and P. Lansdorp). Interestingly, the total number of CD34⁺ PBPCs gated for telomere length analysis was significantly lower in the CHD population as compared to the young controls (p<0.05). Therefore we definitely assume a trend towards less PBPCs in CHD patients, even after adjusting for age. **B.** Scatterplot of arithmetic difference between PBPC telomere length and CD14⁺ monocyte telomere length against the number of counts. The linear regression curves demonstrate the lack of correlation between the small amount of gated cells and a potential increase/decrease in the telomere length of PBPCs.

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