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Accelerated cellular senescence as underlying mechanism for functionally impaired bone marrow-derived progenitor cells in ischemic heart disease

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ABSTRACT

*Background and aims***.** Bone marrow (BM)-derived progenitor cells are functionally impaired in patients with ischemic heart disease (IHD), thereby hampering the outcome of autologous stem cell therapy. In search for underlying mechanisms for this BM dysfunction, accelerated cellular senescence was explored.

Methods. We analysed telomere length of BM-derived mononuclear cells (MNC) by MMqPCR in patients with coronary artery disease (n=12), ischemic heart failure (HF; n=9), non-ischemic HF (n=7) and controls (n=10), and related it to their myeloid differentiation capacity. Expressions of senescence-associated genes *p53*, $p21^{Cip1}$ and $p16^{Ink4A}$; and telomere maintenance genes *TERT*, *TRF1/2, Sirt1* in BM-MNC were evaluated using qPCR. Pro-inflammatory cytokine levels (TNFα, IFNγ, IL-6) in BM were measured by MSD.

Results. BM-MNC telomere length was shortened in patients with IHD, irrespective of associated cardiomyopathy, and shortened further with increasing angiographic lesions. This telomere shortening was associated with reduced myeloid differentiation capacity of BM-MNC, suggesting accelerated senescence as underlying cause for progenitor cell dysfunction in IHD. Both $p16^{Ink4A}$ and $p21^{Cip1}$ were activated in IHD and inversely related to myeloid differentiation capacity of BM-MNC; hence, the BM-MNC functional impairment worsens with increasing senescence. While BM-MNC telomere attrition was not related with alterations in *TERT, TRF1/2* and *Sirt1* expression, IFNγ levels were associated with $p21^{Cip1}/p16^{Ink4A}$ upregulation, suggesting a link between inflammation and cellular senescence. Still, the trigger for telomere shortening in IHD needs to be elucidated.

Conclusions. Accelerated replicative senescence is associated with a functional impairment of BM-derived progenitor cells in IHD and could be targeted to improve efficacy of stem cell therapy.

Keywords:

Bone Marrow; Progenitor Cells; Heart Failure; Telomere length; Ischemic Heart Disease; Senescence.

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1 **INTRODUCTION**

2

3 In search for an explanation for the poor outcome of autologous stem cell therapy in ischemic heart
4 disease (IHD) and heart failure (HF), we have recently documented a functional impairment of bone
5 marrow (BM)-derived progenitor cells in patients with coronary artery disease (CAD) irrespective of
6 left ventricular dysfunction.[1] The underlying mechanisms, however, are still unclear. In the present
7 study, we hypothesize that a process of accelerated replicative senescence might contribute to BM
8 cell deterioration in IHD, and thereby hampers the clinical efficiency of autologous stem cell therapy
9 in this patient group. This hypothesis is based on previous research by Van der Harst et al.
10 demonstrating increased telomere shortening and senescence in circulating leukocytes from patients
11 with HF compared to healthy age and gender-matched controls. In this study, leukocyte telomere
12 shortening was also more pronounced in patients with ischemic HF than with HF of non-ischemic
13 etiology.[2] In addition, patients with CAD and acute myocardial infarction show shorter leukocyte
14 telomere lengths than healthy controls.[3-5]

15

16 Telomeres are TTAGGG repeats located at the distal ends of chromosomes that are important in the
17 maintenance of genomic stability during cell division. With each cell division, telomere length (TL)
18 shortens. The enzyme telomerase is able to add new telomere repeats onto the chromosome ends
19 and prevents loss of TL. The expression of telomerase, however, is rather low in the majority of
20 mature human somatic cells, whereas it is upregulated in committed progenitor cells, activated
21 immune cells and cancer cells [6, 7]. In addition to specific intrinsic genetic factors associated with the
22 process of biological aging, endogenous stimuli such as systemic chronic inflammation and mild
23 oxidative stress may aggravate telomere shortening and promote cellular senescence.[8] When a
24 critical TL is reached, cell cycle inhibitors *p53/p21*^{Cip1} and *p16*^{Ink4A} become activated, which halts cell
25 division and induces a state of replicative cell senescence. This telomere shortening mechanism
26 could, thereby, compromise the proliferative and differentiation potential of cells. [2, 8, 9]

1

2 The present study compares TL of BM-derived mononuclear cells (MNC) of patients with different
3 types of heart disease, including coronary artery disease (CAD), ischemic HF, non-ischemic HF, and
4 control subjects. Furthermore, the relationship between telomere shortening and BM-MNC
5 dysfunction is investigated. We evaluated the activation of senescence-associated genes $p16^{Ink4A}$,
6 $p21^{Cip1}$ and p51, as well as the expression of telomerase reverse transcriptase *(hTERT)*, telomeric
7 repeat binding-factor 1 *(TRF1)* and *2 (TRF2)*,[10] and Sirtuin1 *(SIRT1);*[11] all involved in telomere
8 maintenance.[8] Furthermore, chronic inflammation was explored as potential trigger for telomere
9 shortening in this study cohort.[12]

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1 **MATERIALS AND METHODS**

2

3 Detailed methods ARE available as Supplementary material.

4

5 **Study population**

6 Thirty-eight subjects were included in the study and divided into 4 groups according to the presence
7 of significant CAD and/or HF. Group 1 consisted of patients with CAD without HF (i.e. CAD group;
8 n=12), group 2 consisted of patients with HF due to ischemic cardiomyopathy (i.e. ischemic HF group;
9 n=9), group 3 were patients with HF due to other reasons (i.e. non-ischemic HF group; n=7) and
10 group 4 were healthy subjects without significant past history of cardiovascular disease (i.e. healthy
11 controls; n=10). All cardiovascular patients underwent coronary angiography and cardiac ultrasound
12 to determine the CAD complexity (Syntax score[13]) and left ventricular ejection fraction (LVEF),
13 respectively. The study complied with the Helsinki Declaration and was approved by the local Ethics
14 Committee. Written informed consent was obtained from all subjects.

15

16 **Sampling and isolation of BM-MNC**

17 In patients, BM (2x10ml) was aspirated by sternal puncture under general anesthesia prior to cardiac
18 surgery. In control subjects, BM was aspirated at the time of bone prelevation of the iliac crest prior
19 to jaw reconstruction.
20 The purity of BM aspirates by means of peripheral blood contamination was quantified as described
21 previously.[14] BM aspirates with a bone matrix vesicle-bound alkaline phosphatase <15% were
22 excluded from the study for quality reasons. From the 50 samples, 38 pure BM samples were
23 retained for further analyses. Next, BM-MNC were isolated by density gradient centrifugation
24 immediately after sampling.

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1 **Monochrome multiplex qPCR for TL measurement**

2 The average TL was measured in a gDNA sample extracted from BM-MNC using the monochrome
3 multiplex qPCR method, as originally developed by Cawthon et al.[15] and optimized by Van der Harst
4 et al.[16] Telomeric DNA was amplified using specific telomere primers (TelC and TelG, see
5 Supplementary material); the reference gene albumin using albumin primers (AlbUgc and AlbDgc).
6 The relative T/S ratio of the telomere (T) signal to the albumin (S) signal was calculated for each
7 sample, with all samples being compared to the same reference DNA sample. The mean T/S ratio is
8 considered to be proportional to the average BM-MNC TL.

9

10

11 **Real time qPCR for telomerase, regulators of telomerase activity and senescence-associated genes**

12 Expression of *hTERT*, the catalytic subunit of the enzyme telomerase and considered as a good
13 correlate of telomerase expression;[6, 17] *TRF1 and 2*, which are known regulators of the telomerase
14 activity; senescence-associated genes encoding for *p53, p21*^{Cip1} and *p16*^{Ink4A}; as well as *hSIRT1* were
15 all normalized to *GAPDH* and calculated by real time qPCR using the ΔΔCT method. A fold change of
16 ≥1.5 or ≤-1.5 was considered as relevant up- or downregulation.

17

18 **Colony-forming unit (CFU) assays**

19 Isolated BM-MNC were aliquoted in duplicate in Methocult H4535 Enriched without EPO medium
20 (Stem Cell Technologies, France) for induction of differentiation into the myeloid lineage
21 (granulocyte/macrophage- colony forming units, GM-CFU); as well as in Methocult H4434 Classic
22 medium (Stem Cell Technologies) for induction of differentiation into the erythroid lineage
23 (erythroid-burst forming units, BFU-E). After 14-day incubation, the GM-CFU and BFU-E numbers
24 were counted.

25

26 **Inflammatory cytokine levels in BM**

Levels of the inflammatory cytokines TNFα, IFNγ and IL-6 were measured in BM plasma using Meso Scale Discovery multiplex platforms (Meso Scale Diagnostics, USA).

Statistical analysis

Results are expressed as median (Interquartile range-IQR). IBM SPSS Statistics version 22.0 was used for statistical analysis. Normality of the variables was tested with Shapiro-Wilk and by visual Q-Q-plot inspection. Logarithmic transformation of skewed data was done to obtain a normal distribution. Comparison between different groups was performed using ANOVA (normally distributed data), Kruskal-Wallis (skewed data) or chi-square test (nominal data), as appropriate. To evaluate the specific influence of either IHD or HF, a two-way ANOVA was performed for each parameter with IHD, HF and the interaction between IHD and HF as independent factors. All models were adjusted for age. When the interaction between IHD and HF was not significant, it was removed from the model. Post-hoc pairwise comparison was obtained by Mann-Whitney U and adjusted with the Bonferroni-Holm correction for multiple testing. Correlations were evaluated by the Pearson or Spearman test, as appropriate. A p-value of <0.05 was considered statistically significant.

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1 **RESULTS**

2

3 **Patient characteristics**

4 Demographic and clinical characteristics of the subjects are depicted in Table 1. Groups were similar
5 with respect to age and gender. Syntax scores were similar between the ischemic HF and CAD
6 groups, indicating a comparable CAD complexity. Patients with ischemic HF showed significantly
7 lower LVEF compared to patients with non-ischemic HF (*p*=0.023), but NYHA functional class was not
8 different. Furthermore, four control subjects received ACE-inhibitors, beta-blockers and/or statins as
9 treatment for arterial hypertension or hypercholesterolemia respectively.

10

11 **Telomere length and CFU-capacity of BM-MNC**

12 Table 2A shows the results the TL of BM-MNC in the 4 groups. The statistical analysis by ANOVA is
13 shown in Table 2B, in which IHD, HF and the interaction between IHD and HF were entered in a two-
14 way ANOVA regression model as independent factors. This analysis revealed a significant shortening
15 in TL of BM-MNC in the presence of IHD (*p*=0.003; Fig. 1A); while the presence of HF did not show
16 any effect on the TL, nor did we find a correlation with LVEF. In addition, the BM-MNC TL shortens
17 further with increasing IHD complexity, as assessed by the syntax score (r=-0.530; *p*=0.004; Fig. 1B),
18 and is also inversely related with the 10-year risk for cardiovascular disease, i.e. the Framingham
19 score (r=-0.525; *p*=0.006; Fig. 1C). TL is strongly determined by the process of biological aging as also
20 represented in our study population: for the total cohort, shorter TLs were associated with increasing
21 age (r=-0.507; *p*=0.001; Fig. 1D).
22 In an additional ANOVA model, testing the effects of age, gender and the interaction between age
23 and gender on the aforementioned parameters, no significant effect of the interaction, nor of gender
24 was observed (data not shown). Age showed a significant effect on the TL and was therefore
25 included as covariate in the two-way ANOVA model testing the effects of IHD and HF.

1 Table 2 shows the CFU-capacity of BM-MNC in the 4 groups. Both myeloid (GM-CFU) and erythroid
2 (BFU-E) differentiation capacities were significantly reduced in the presence of IHD (p=0.009 and
3 p=0.041 respectively, Table 3), as illustrated in Fig. 1E and F. Shorter TL in BM-MNC was strongly
4 related to a reduced myeloid differentiation capacity (r=0.581; p=0.001; Fig. 1G).

5

6 **Activation of senescence-associated pathways p16 and p21 in IHD**

7 Since telomere shortening results in so-called replicative senescence, we analyzed the expression of
8 the downstream senescence-associated pathways $p53/p21^{Cip1}$ and $p16^{Ink4A}$, responsible for cell cycle
9 inhibition. On mRNA level, $p21^{Cip1}$ and to a lesser extent $p16^{Ink4A}$ were significantly up-regulated in
10 BM-MNC in the presence of IHD (Fig. 2A), but we did not detect any differences in p53 expression.
11 There was a significant negative relation between the myeloid differentiation capacity of BM-MNC
12 and the $p21^{Cip1}$ (Fig. 2B) and $p16^{Ink4A}$ (Fig. 2C) expressions, which was even more explicit in IHD
13 patients alone ($p21^{Cip1}$: r=-0.626; p=0.013; $p16^{Ink4A}$: r=-0.547; p=0.035).

14

15 **The role of telomerase activity, hSIRT1 and low-grade inflammation in BM-MNC telomere**
16 **shortening in IHD**

17 To explore the possibility whether a reduced telomerase activity contributes to the observed TL
18 shortening in BM-MNC of patients with IHD, the expression of human telomerase transcriptase
19 (*hTERT*), as well as of the regulators *TRF1 and 2* were analysed. No differences in expression of
20 *hTERT, TRF1 and 2* were observed in the presence of IHD (Fig. 2D).

21 Secondly, the expression of *hSIRT1*, a positive regulator of the TL [18], was evaluated in BM-MNC of
22 subjects with IHD and compared to subjects without IHD. Yet, we did not detect a significant
23 downregulation in the presence of IHD, nor find any correlation with TL or differentiation capacity of
24 BM-MNC (Fig. 2E).

25 Third, the levels of inflammatory cytokines IFNγ, TNFα and IL-6 were studied in BM plasma of a
26 subpopulation of 21 subjects (Table 3). Whereas levels of these inflammatory cytokine levels were

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1 not directly correlated with TL of BM-MNC, IFNy levels were positively related with *p53* (r=0.721; p<0.001), $p21^{Cip1}$ (r=0.458; p=0.037) and $p16^{Ink4A}$ (r=0.507; p=0.019) expressions in BM-MNC,

2

3 suggesting a link between inflammation and senescence.

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1 **DISCUSSION**

2

3 Thepresent study was designed to systematically evaluate TL in BM-MNC of patients with heart
4 disease and to relate it to their stem cell function. The following findings emerged from this study:
5 1. the TL is significantly shortened in BM-MNC of patients with IHD, with or without associated
6 cardiomyopathy and shortens further with increasing complexity of angiographic lesions.
7 2. This telomere shortening in IHD is strongly associated with a decreased myeloid differentiation
8 capacity of BM-MNC, which suggests replicative senescence as an underlying cause for progenitor
9 cell dysfunction in IHD.
10 3. The senescence-associated pathways $p21^{Cip1}$ and $p16^{Ink4A}$ are activated in BM-MNC of IHD patients
11 and are both inversely related to myeloid differentiation capacity of BM-MNC.
12 4. Higher IFNy levels in BM were related to increased $p53/\,p21^{Cip1}$ and $p16^{Ink4A}$ expression, suggesting
13 a link between inflammation and cell cycle inhibition.

14

15 **Telomere length of BM-MNC is incrementally shorter according to the severity of coronary**
16 **atherosclerotic lesions**

17 In this study, we provide the first evidence that TL is shorter in BM-MNC from patients with IHD
18 compared to non-IHD, and this both in patients with and without associated cardiomyopathy
19 (LVEF>45%). On the other hand, HF as such did not influence TL in BM-MNC. Interestingly, further
20 telomere shortening was associated with increasing complexity of coronary artery lesions, as well as
21 with higher Framingham risk factor scores, which portends clinically relevant prognostic information.
22 Our findings are in line with the work of Spyridopolous et al. who reported telomere shortening in
23 BM-MNC of patients with ischemic cardiomyopathy (previous MI, LVEF 35(25-46)%, NYHA I-III)
24 compared to a group of young controls. In contrast, the BM-MNC TL of patients with dilated
25 cardiomyopathy (LVEF 33(22-43)%, NYHA II-III) was not significantly reduced.[19] In addition, the

1 present study shows that patients with ischemic heart disease and a *normal* LVEF also have telomere
2 shortening of BM-MNC.
3 The observed telomere shortening in BM-MNC in the presence of IHD might underlie the earlier
4 observed reduced TL of circulating leukocytes in IHD patients. Van der Harst et al. already reported
5 reduced leukocyte TL in patients with ischemic cardiomyopathy (LVEF 27±8%, NYHA II/III/IV)
6 compared to patients with non-ischemic HF (LVEF 27±7%).[2] In line, Spyridopolous et al. observed
7 reduced TL of 12 different leukocyte subpopulations in CHD patients (LVEF 29.6±11%), all affected
8 equally, compared to controls.[20] Additionally, the TL of circulating granulocytes, and to a lesser
9 extent of circulating lymphocytes, even reflects the TL of myeloid BM-MNC in patients with CAD.[19]
10 The observation that TL is incrementally shorter according to the severity and complexity of CAD is
11 intriguing. In line, we observed a significant inverse relation between BM-MNC TL and Framingham
12 score, the calculated 10-year cardiovascular risk for cardiovascular diseases. Spyridopolous et al.
13 described shorter TL in 3-vessel disease compared to 1-vessel disease.[19] Van der Harst et al. observed
14 shorter TL in leukocytes with increasing extent of atherosclerotic manifestations (coronary, cerebral,
15 peripheral arteries) in patients with chronic heart failure (27±7%, NYHA II-IV).[2]
16 The association of telomere attrition and risk factors for atherosclerotic cardiovascular disease has
17 been a widely discussed topic. Some studies suggest that the process of telomere shortening starts
18 before the onset of overt atherosclerotic disease, implying a causal role for telomere shortening in
19 atherogenesis.[17] Indeed, many of the traditional cardiovascular risk factors such as hypertension,[21]
20 diabetes,[22] smoking, obesity, and physical inactivity [23] have been associated with shorter leukocyte
21 TLs in cross-sectional analyses. Even after correction for these risk factors, the relation between
22 atherosclerotic disease and leukocyte TL shortening was maintained.[5, 17] There is also emerging
23 evidence for the inheritance of shorter leukocyte TLs in the offspring of patients with coronary artery
24 disease compared to the offspring of healthy controls, but not for circulating CD34+ MNC. These data
25 lend some support to this telomere hypothesis, when considering familiar history of cardiovascular
26 disease as important risk.[8, 24, 25] The fact that we observe telomere shortening in BM-MNC of IHD

1 patients, containing cells which are involved in the processes of re-endothelialisation and
2 neovascularization, supports the telomere hypothesis, postulating that telomere shortening might be
3 causally linked to cardiovascular disease, [26] and therefore merits further investigation.[17]
4
5 **Telomere shortening is related to functional impairment of BM-MNC**
6 We previously reported a significant functional impairment of isolated BM-MNC in patients with
7 coronary atherosclerotic disease undergoing coronary artery bypass surgery, which was independent
8 of the degree of left ventricular dysfunction.[1] In particular, erythroid and myeloid differentiation
9 capacities of BM-MNC were reduced. The present data show that this reduced myeloid
10 differentiation capacity is strongly associated with TL in BM-MNC, suggesting accelerated telomere
11 shortening as an underlying trigger for BM-MNC dysfunction in IHD. Yet, the hallmark of senescence
12 is the inability to progress through the cell cycle.[27] Hence, senescent cells arrest growth with likely a
13 direct impact on cellular proliferation rate and differentiation capacity.
14
15 **Activation of both senescence-associated pathways p21 and p16 in BM-MNC of IHD**
16 Telomere shortening has been identified as a marker for cellular senescence.[28] Telomere shortening
17 induces DNA damage, which will in turn elicit DNA damage response signals. These signals trigger the
18 tumor suppressing pathways $p53/p21^{Cip1}$ and $p16^{Ink4A}/Rb$, resulting in mitotic arrest and consequently
19 cellular senescence.[8, 28] In this study, we observed a significant up-regulation of both cyclin-
20 dependent kinase inhibitors $\mathrm{p21}^{\mathrm{Cip1}}$ and $p16^{Ink4A}$ in isolated BM-MNC in the presence of IHD,
21 suggesting that BM-MNC senescence is induced by both pathways.
22 Moreover, we also found that the up-regulation of $p21^{Cip1}$ and $p16^{Ink4A}$ were both associated with
23 reduced myeloid differentiation capacity of BM-MNC. These results implicate that the functional
24 impairment of BM-derived progenitor cells increases with the degree of accelerated cellular
25 senescence induced by telomere shortening.
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1 **Underlying mechanisms for telomere shortening in ischemic heart disease**

2 Our results showed accelerated telomere shortening in BM-progenitor cells in the presence of IHD.
3 Theoretically, telomere attrition can be caused by multiple endogenous factors:

4 The most common inducer of telomere shortening is biological **aging**.[8] At present, TL has even
5 emerged as a possible biomarker for biological age, at least at the cellular level; with shorter
6 telomeres indicating increased biological age.[17] The results of the present study cannot be explained
7 by age because (1) patient groups were matched for age and (2) age was added as co-factor in the
8 regression model.

9 Secondly, the effect of **gender** has to be taken into account: age-adjusted leukocyte TL is longer in
10 women compared to men.[29] This gender-effect might be attributed to estrogen, which activates
11 telomerase by direct and indirect stimulation of *hTERT*.[30] Patient groups in this study were gender-
12 matched and no significant gender-effect was found on the BM-MNC TL. Also, no significant effect of
13 the interaction between age and gender was observed, implicating that there was no confounding
14 bias of the menopausal age of the included women in this study.

15 As mentioned above, several cardiovascular risk factors such as **diabetes** have been associated with
16 shorter leukocyte TLs.[22] Therefore, diabetes was assigned as exclusion criterion in our study.

17 As mentioned earlier, the enzyme **telomerase** is able to restore the telomeric repeats in high
18 proliferating cells.[6] Telomerase knockout mice models have shown accelerated telomere shortening
19 and premature aging.[31] In this regard, we tested whether a reduced telomerase activity could be an
20 underlying mechanism for the observed BM-MNC TL shortening in IHD. However, the expression of
21 the telomerase catalytic subunit hTERT was similar between subjects with and without IHD. In
22 contrast, Satoh et al. observed a reduced telomerase activity in circulating EPC with shortened TL of
23 CAD patients compared to healthy subjects. This discrepancy might be attributed to differences in
24 telomerase activity assessment technique, as well as in the studied cell type: BM-MNC is a
25 heterogenous pool of cell types, containing also more mature and differentiated cells that have
26 lower or no expression of the enzyme telomerase.

Palacios et al. identified SIRT1 as a positive regulator of the TL in embryonic fibroblasts *in vivo*, dependent on the telomerase activity. Overexpression of SIRT1 in a mouse model resulted in longer telomeres and vice versa.[18] Yet, in our patient groups, hSIRT1 was not differentially expressed.

Lastly, chronic inflammation is identified as important trigger of telomere shortening by inducing more telomere loss per cell division, and has also been implicated in the aging process.[32]

Whereas we did not find any correlation between the BM-MNC TL and inflammatory markers, higher levels of IFNγ in the BM were related to increased expression of *p53/ p21*^{Cip1} and p*16*^{Ink4A}, suggesting a link between inflammation and cell cycle inhibition.

Hence, in the search for causal factors of telomere shortening in IHD, several triggers deserve future exploration. First, the relation between oxidative stress and telomere shortening in progenitor cells and in cardiovascular disease has been suggested by Satoh et al., who observed a strong association between increased oxidative DNA damage and reduced TL, as well as reduced telomerase activity in circulating EPC of patients with CAD compared to controls.[33] Secondly, emerging evidence suggests an anti-senescence effect of nitric oxide (NO) on endothelial cells, partially by the activation of telomerase, as extensively reviewed by Hayashi et al.[34] A reduced NO bioavailability is a key characteristic for patients with atherosclerosis and/or IHD and contributes to both endothelial and progenitor cell dysfunction, since NO is critical for cell survival, proliferation and migration.[35-37] Both increased oxidative stress and reduced NO availability, as triggers of accelerated senescence, also fit in the theory of a BM microangiopathy in IHD patients, as deduced from patients with diabetes.[38] In diabetic patients, leukocyte telomere shortening has been described.[39] Additionally, the diabetes-induced microvascular disease of the BM niche, characterized by BM endothelial dysfunction, was shown to be associated with elevated levels of reactive oxygen species and reduced NO bioavailability.[40] Whether these mechanisms are responsible for the observed telomere shortening in BM-MNC of patients with IHD merits further investigation.

Future perspectives: rejuvenating the stem cell product

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1 Functional impairment of BM-derived progenitor cells by accelerated senescence will hamper the
2 clinical efficacy of autologous stem cell therapy.[1] Therefore, a rejuvenation treatment of the stem
3 cell product prior to administration may sound as an attractive option. In this regard, modulation of
4 cellular microRNAs implicated in the senescence pathways, could result in improved cell survival with
5 a direct beneficial impact on cardiac and/or vascular regeneration after administration in patients
6 with cardiovascular disease.[41] In a mouse model of AMI, inhibition of miR-34a (targeting *SIRT1*) by
7 locked nuclear acid-34a in BM-MNC resulted in better cell survival and improved cardiac function 2
8 weeks after intramyocardial injection.[42] In theory, the overexpression of miR-24 and/or miR-17-5p,
9 regulators of *p16* and *p21* expression respectively, may resolve in a 'younger' stem cell product.[43]
10 However, experimental evidence for this hypothesis is currently lacking. In addition, also
11 overexpression of *TERT* results in rejuvenated stem cells: transduction of mesenchymal stem cells
12 (MSC) with a lentiviral vector encoding for *TERT* enhanced cell survival, growth and myogenic
13 differentiation capacity. Transplantation of these rejuvenated MSC In a murine model of hindlimb
14 ischemia resulted in better therapeutic efficacy compared to non-treated MSC.[44] Yet, not only the
15 intrinsic stem cell function, but also other aspects of autologous stem cell therapy should be
16 addressed to improve the therapeutic efficacy. In particular, the poor engraftment of the
17 transplanted cells is a second major limitation of stem cell therapy. The majority of cells transplanted
18 in the hostile environment are lost due to either cell death or squeezing out of the injection site and
19 leakage to the nearby vasculature.[45] Less than 10% of the transplanted cells are actually retained at
20 the injection site 24 hours after intramyocardial delivery.[46] Hence, several innovative approaches to
21 improve homing to the ischemic region and better cell retention should be considered and applied.
22
23 **Limitations**
24 Differences in BM-MNC TL and gene expression between various underlying etiologies for
25 cardiovascular diseases were evident in our study; yet, our results need to be further confirmed in
26 larger studies.

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2 **Conclusions**

3 Accelerated telomere shortening in BM-progenitor cells is related to the earlier observed functional
4 impairment of progenitor cells in patients with IHD. Both *p16* and *p21* senescence-associated
5 pathways are activated in BM-MNC and related to the cellular dysfunction. The observation that TL is
6 incrementally shorter according to the severity of CAD is intriguing. Whether telomere shortening of
7 BM-MNC, with an important role in re-endothelialisation, also contributes to progression of
8 atherosclerotic disease remains to be elucidated. Future work should focus on additional potential
9 triggers for this observed BM-MNC telomere attrition in IHD, as well as on ex-vivo modulation to
10 rejuvenate senescent BM-MNC for their further application as effective stem cell product in
11 autologous stem cell therapy.

12

13

14 **CONFLICT OF INTEREST**

15 The authors declared they do not have anything to disclose regarding conflict of interest with respect
16 to this manuscript.

17

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22

23 **AUTHOR CONTRIBUTIONS**

24 EVC, EN, VH and CV contributed to the research hypothesis and study design. EN, as principal
25 investigator, performed the conceptional and technical elaboration. EVC provided conceptual
26 guidance for all study aspects. VH provided technical guidance in PCR and data analysis. EN and EVC

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1 conributed to the statistical data analysis. VVH performed the quality assessment of BM aspirates. IR
2 and DD collected BM samples from patients undergoing cardiac surgery. MD collected BM samples
3 from control subjects undergoing maxillofacial surgery. The manuscript was written by EN, EVC and
4 VH and commented on by all authors.
5

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9 measurement.
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FIG.S

Fig. 1. Telomere shortening and functional impairment of BM-MNC in IHD.

(A) The average telomere length (TL) of BM-MNC was significantly shortened in subjects with IHD (i.e. CAD and ischemic HF group, n = 12+9) compared to subjects without IHD (i.e. non-ischemic HF group and control subjects, n = 7+10). (B) TL shortens further with increasing IHD complexity, i.e. syntax score; (C) and with 10-year risk for cardiovascular disease, i.e. Framingham risk score. (D) For the total cohort, shorter TLs were strongly associated with increasing age. (E) *In vitro* myeloid (GM-CFU numbers) and (F) erythroid differentiation capacities of BM-MNC were decreased in the presence of IHD. (G) The reduced myeloid differentiation capacity of BM-MNC was related with a reduced TL. A *p*-value of <0.05 (ANOVA) was considered statistically significant.

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Fig. 2. Activation of senescence-associated pathways p16 and p21 and the role of telomerase activity and *hSIRT1* in BM-MNC telomere shortening in IHD.

(A) The expression of *p21*^{Cip1} (fold change 2.19±2.44 *vs.* 1.32±1.02) and *p16*^{Ink4A} (fold change 1.62±1.17 *vs.* 1.11±0.47) was upregulated in subjects with IHD (i.e. CAD and ischemic HF group, n=12+9) compared to subjects without IHD (i.e. non-ischemic HF group and control subjects, n=7+10), whereas the expression of *p53* (fold change -1.02±0.19 *vs.* 1.04±0.32) in BM-MNC did not differ. (B) For the total cohort, both *p21*^{Cip1} and (C) *p16*^{Ink4A} expression was inversely related to myeloid differentiation capacity of BM-MNC. (D) Relative expression of the catalytic subunit *hTERT* (fold change 1.08±0.64 *vs.* 1.19±0.73) and the telomerase regulators *TRF1* (fold change 1.25±0.42 *vs.* 1.03±1.03) and *2* (fold change 1.21±0.43 *vs.* 1.05±0.37), as well as of (E) *hSIRT1* (fold change -1.06±0.36 *vs.* 1.04±0.34) in BM-MNC was similar in subjects with IHD compared to subjects without IHD. A fold change of ≥ 1.5 or ≤ -1.5 was considered as relevant up- or down-regulation.

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TABLES

Table 1. Characteristics of the study population.

	CAD (n=12)	**Ischemic HF (n=9)**	**Non-ischemic HF (n=7)**	**Control subjects (n=10)**	***p*-value**
GENERAL					
Age (yrs)	65 (60-70)	62 (43-72)	59 (52-64)	55 (40-66)	0.284
Sex (M/F)	10/2	7/2	5/2	6/4	0.651
BMI (kg/m^2)	28 (25-32)	26 (22-30)	33 (21-35)	24 (23-26)	0.077
CARDIAC EVALUATION					
IHD	+	+	-	-	N.A.
HF	-	+	+	-	N.A.
LVEF (%)	59 (55-67)	24 (17-38)	40 (35-50)	N.A.	**<0.001**
NYHA I/II/III/IV	N.A.	0/3/4/2	0/3/4/0	N.A.	0.414
Syntax Score	17 (11-27)	20 (9-26)	N.A.	N.A.	0.983
Framingham Score	17 (12-26)	13 (3-24)	20 (3-14)	N.A.	0.182
MEDICATION (N)					
ACE-inhibition/ARB	6	5	5	2	0.179
Beta blockers	8	5	5	1	**0.028**
Diuretics	4	5	6	0	**0.003**
Statins	12	6	2	2	**0.001**
SURGERY (N)					
CABG	12	7	0	0	**<0.001**
MV surgery	0	1	7	0	**<0.001**
VAD	0	2	0	0	0.078
Maxillofacial	0	0	0	10	**<0.001**

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Data are presented as median (IQR). *p*-values for comparison between groups by ANOVA, Kruskal-Wallis or Chi-square test, as appropriate.

ACE, angiotensin-converting-enzyme; ARB, angiotensin II receptor blockers; BMI, body mass index; CAD, coronary artery disease; HF, heart failure; CABG, coronary artery bypass grafting; IHD, ischemic heart disease; LVEF, left ventricular ejection fraction; MV, mitral valve; NYHA, New York Heart Association class; VAD, ventricular assist device.

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Table 2. Telomere length and CFU-capacity of BM-MNC.

A. Data presented as median (IQR) for the different patient cohorts.

	CAD (n=12)	Ischemic HF (n=9)	Non-ischemic HF (n=7)	Control subjects (n=10)
Telomere length (T/S) BM-MNC	0.87 (0.74-0.99)	0.95 (0.83-1.15)	1.12 (1.04-1.19)	1.13 (1.00-1.21)
GM-CFU (N)	50 (21-63)	43 (34-76)	75 (58-102)	76 (54-86)
BFU-E (N)	27 (17-42)	33 (27-48)	44 (23-59)	41 (37-55)

B. Parameter estimates resulting from two-way ANOVA analysis. The regression model tested IHD and HF as independent factors, adjusted for age, for each parameter.

	IHD			HF			Model
Parameter	**Beta Coefficient**	**SE**	***p*-value**	**Beta Coefficient**	**SE**	***p*-value**	***p*-value**
Telomere length (T/S) BM-MNC	-0.170	0.052	**0.003**	0.077	0.051	0.138	**<0.001**
GM-CFU (N)	-26.432	9.360	**0.009**	6.627	9.073	0.472	**0.032**
BFU-E (N)	-11.019	5.166	**0.041**	4.536	5.035	0.375	0.159

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Abbreviations as in Table 1; BFU-E, eryhtroid-burst forming units; GM-CFU, granulocyte-macrophage colony forming units; SE, standard error.

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Table 3. Inflammatory cytokine levels in BM.

	CAD (n=5)	**Ischemic HF** (n=6)	**Non-ischemic HF** (n=4)	**Control subjects** (n=6)	***p-value***
BM IFNγ (pg/ml)	4.40 (3.11-4.92)	3.75 (0.48-5.64)	4.25 (2.42-9.72)	2.01 (0.20-3.41)	*0.152*
BM TNFα (pg/ml)	2.07 (1.50-2.82)	1.61 (1.10-2.89)	1.80 (1.53-2.07)	1.12 (1.05-1.61)	*0.065*
BM IL-6 (pg/ml)	0.47 (0.13-0.76)	0.88 (0.52-1.52)	0.84 (0.16-1.40)	0.30 (0.05-0.48)	*0.088*

Data are expressed as median (IQR). *p*-values for comparison between groups by Kruskal-Wallis.

Abbreviations as in Tables 1 and 2; IFNγ, interferon gamma; IL-6, interleukin 6; TNFα, tumor necrosis factor alpha.

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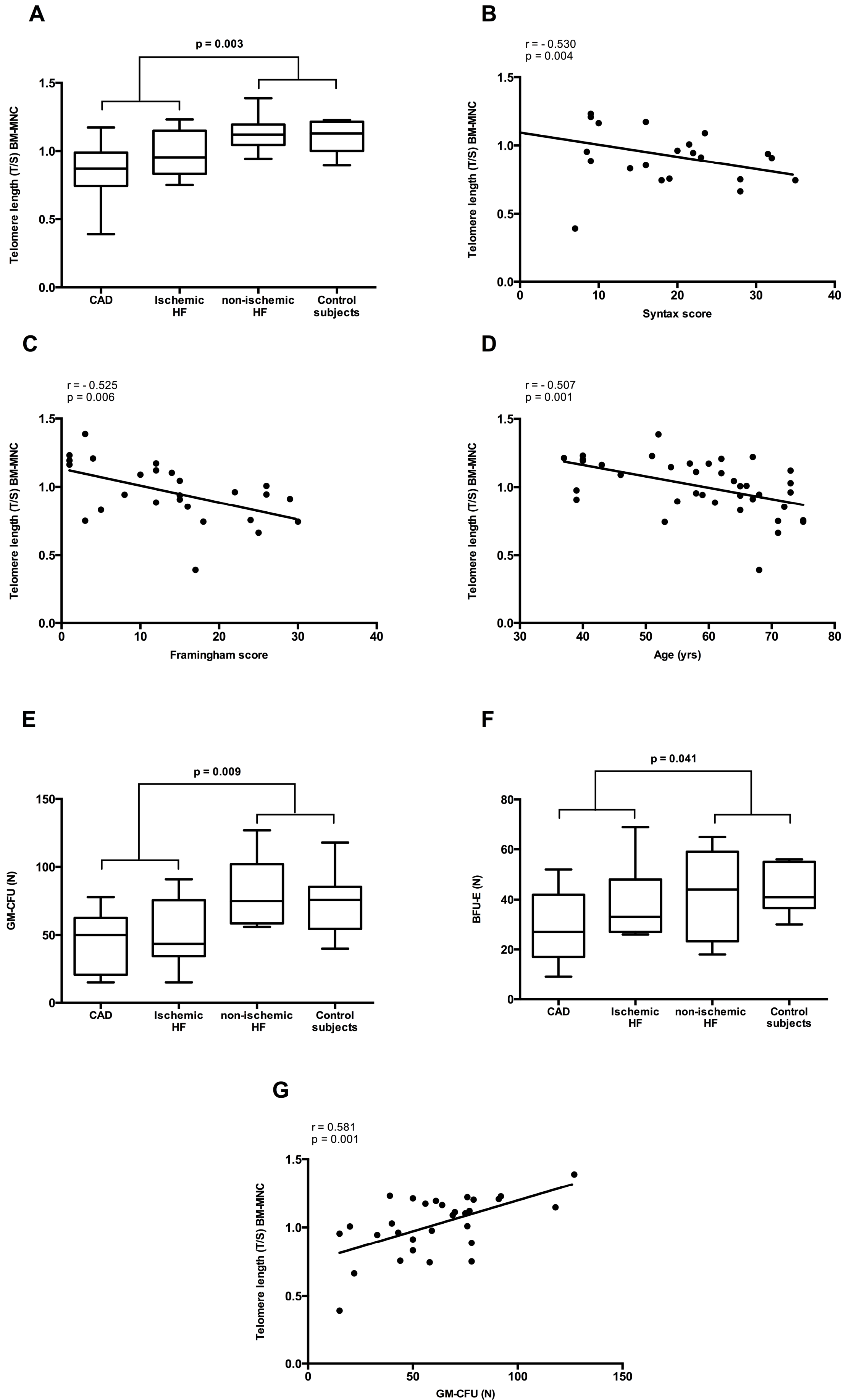

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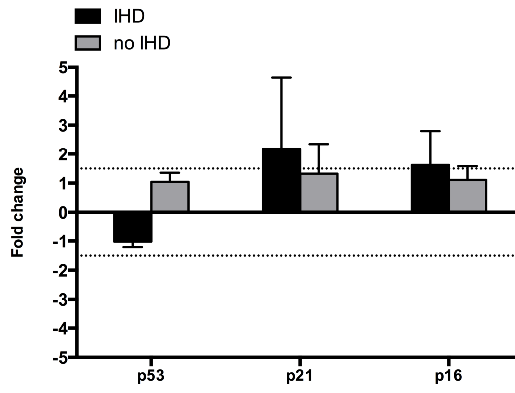

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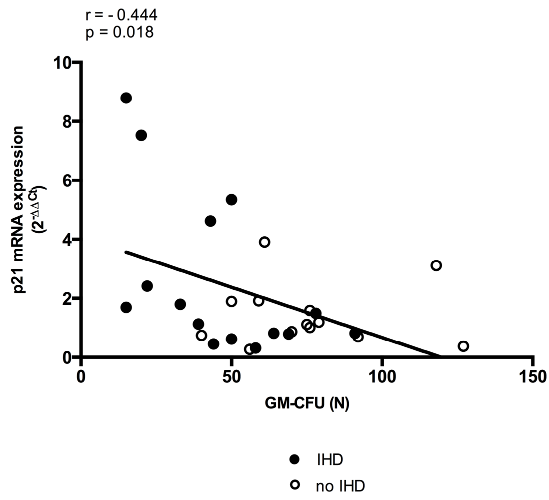

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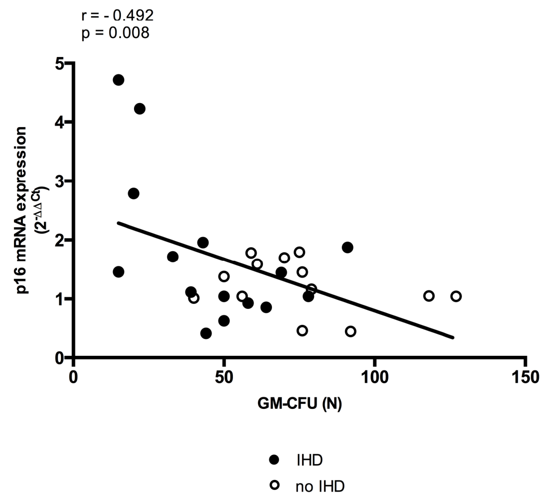

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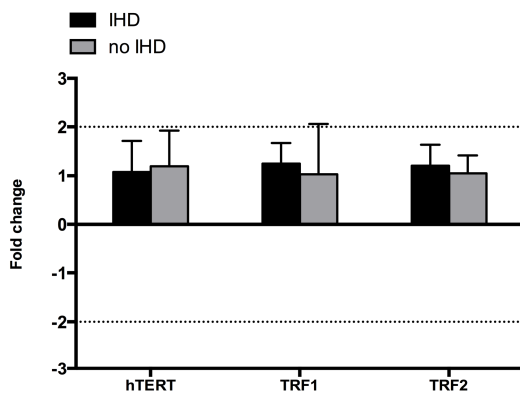

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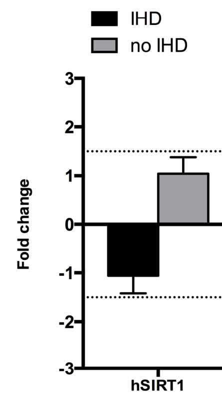

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HIGHLIGHTS

- Accelerated senescence is explored as cause for functionally impaired bone marrow-derived mononuclear cells (BM-MNC) in ischemic heart disease (IHD).
- BM-MNC telomere length is shortened in IHD, irrespective of associated heart failure (HF).
- BM-MNC telomere length shortens with increasing IHD complexity.
- Telomere shortening is related to functional impairment of BM-MNC.
- Senescence-associated genes p16 and p21 are upregulated in BM-MNC of IHD patients.

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