Hemophilia is associated with accelerated biological aging

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Abstract

Hemophilia is a rare X-linked bleeding disorder caused by mutations in the *F8* or *F9* gene (hemophilia A or B), leading to deficient factor VIII or IX proteins, respectively. Hemophilia-related complications caused by bleeding into the joints (the hallmark of hemophilia) and age-related comorbidities occur frequently and impact the functionality and quality of life of persons with hemophilia (PwH). Given the chronic nature of hemophilia, we hypothesized that hemophilia has an association with accelerated biological aging. Therefore, we investigated biological aging biomarkers, i.e., telomere length and mitochondrial DNA (mtDNA) copy number with a quantitative polymerase chain reaction-based assay in PwH (N=99) and age- and sex-matched healthy controls (N=61). The association of telomere length and mtDNA copy number with hemophilia severity was investigated using ordinary least-squares linear regression models allowing for interactions with chronological age. Telomere length (6.09 [interquartile range (IQR), 4.79-7.68] kb *vs*. 10.07 [IQR, 7.93-12.66] kb; *P*<0.001) and mtDNA copy number (243.17 [IQR, 206.54-293.79] *vs*. 495.52 [IQR, 399.60-615.06]; *P*<0.001) were significantly lower in PwH compared to healthy controls. Persons with severe hemophilia had longer telomere length than those with non-severe hemophilia (6.29 [IQR, 5.36-8.05] kb *vs*. 5.12 [IQR, 3.97-6.58] kb), while no difference in mtDNA copy number was observed. We observed accelerated biological aging, indicated by shorter telomere length and lower mtDNA copy number, in PwH compared to healthy controls. Interestingly, telomere length shortening was more pronounced in non-severe than in severe hemophilia, which needs further investigations. Further research is needed to understand the underlying mechanisms of biological aging in hemophilia.

Introduction

Inherited hemophilia is a rare X-linked disorder of hemostasis characterized by bleeding. It is caused by mutations in the coagulation *F8* (hemophilia A) or *F9* (hemophilia B) genes.[1-3] The severity of hemophilia is determined by residual factor VIII or IX activity levels (severe <1%, moderate 1-5%, and mild 5-40%).[4] The hallmark of hemophilia is recurrent bleeding into joints and muscles. Bleeding into joints leads to swelling, synovitis, damage of the articular cartilage, and eventually to destruction of the whole joint and bone, also referred to as hemophilic arthropathy. With increasing age, the arthropathy progresses, leading to chronic pain and morbidity, impacting functionality and quality of life.[5,6] With the availability of safe and effective therapies for the treatment and prophylaxis of bleeds, the overall life expectancy and quality of life of persons with hemophilia (PwH) have increased.[7] Advanced chronological age is a risk factor for the development of comorbidities,[7,8] and age-associated diseases (e.g., cardiovascular and neurodegenerative diseases) may occur in addition to hemophilia-related conditions such as chronic arthropathy or chronic liver disease.[7,9]

Chronic diseases, e.g., atherosclerosis, cardiovascular diseases, chronic kidney disease and diabetes, have been found to be associated with accelerated biological aging.[10-15] Mechanisms involved in biological aging include chronic inflammation and oxidative stress.[16] In particular, increased reactive oxygen species (ROS) have been suggested to accelerate the aging process.[17]

Biological aging can be assessed with different biomarkers, such as DNA methylation (epigenetic age), histone modification, telomere length, mitochondrial function, and mitochondrial DNA (mtDNA) copy number.[10,18-20] ROS, which are mainly produced in the mitochondrial matrix, can lead to mitochondrial dysfunction, altered mtDNA copy numbers and energy production capacities.[21] While a higher mtDNA copy number in advanced age is linked to better health, low mtDNA copy numbers were found in patients with chronic kidney disease, cardiovascular diseases, di-

abetes, and venous thromboembolism (VTE), indicating accelerated biological aging.[14,22-24] Telomere length, another biomarker of biological aging, is also influenced by ROS. Telomeres are repeated sequences (TTAGGG) that protect the chromosome ends from the end-replication problem, i.e., during each cell division telomeres shorten instead of the chromosome endings.[25-27] A reduced telomere length was reported in diabetes, atherosclerosis, and other cardiovascular diseases.[10,12,15]

As hemophilia is considered a chronic disease, we hypothesized that PwH may exhibit accelerated biological aging. Therefore, we investigated two biomarkers of biological aging (human mtDNA copy number and absolute human telomere length) in PwH of different severity in comparison to age-matched healthy controls. Furthermore, we analyzed the association of the two biomarkers of biological aging with the severity of hemophilia.

Methods

Study population and blood sampling

We performed a case-control study at our hemophilia treatment center for adults (Division of Hematology and Hemostaseology, Department of Medicine I, Medical University of Vienna, Vienna, Austria). All PwH presented in this analysis participated in the Austrian Hemophilia Registry. During routine visits at our hemophilia treatment center, blood was collected and stored at the biobank of our institution. All study procedures were approved by the local ethics committee (EC No. 981/2011, 553/2010, AN2016-0271 369/4.5, F-10-16), and the study was conducted in accordance with the Declaration of Helsinki. Overall, we included 129 persons with hemophilia A or B of all grades of severity (severe, moderate, and mild). Additionally, 67 age-matched men (±5 years) without hemophilia, cardiovascular diseases or any other acute and chronic disease served as healthy controls (approval number by the local ethics committee: EC No. 039/2006).

All patients and healthy controls gave written informed consent before study inclusion. Blood sampling was performed with a 21-gauge butterfly needle in an EDTA VACUETTE tube. On the day of blood sampling, routine laboratory measurements (blood counts, coagulation, chemistry) were measured. In addition, aliquots of the whole blood samples were stored at -20°C for further analyses, which were performed in series.

DNA isolation

DNA was isolated from whole blood samples with the Promega Maxwell RSC Blood DNA Kit AS1400 for the Maxwell automated system.

Measurement of average human telomere length

A quatitative polymerase chain reaction (qPCR)-based method from ScienCell (AHTLQ #8918) was used to determine the human average telomere length. Besides the telomere primers, this kit included a single copy reference (SCR) primer set, which amplifies a 100 bp-long region of the human chromosome 17 and acts as a normalization reference. A standard serial dilution for the reference human genomic DNA sample was done. The qPCR was performed on a Bio-Rad CFX384 Real-Time System. The cycling conditions for both targets were an initial denaturation step at 95°C for 10 minutes (min) followed by 39 cycles of 95°C for 20 seconds (sec), 52°C for 20 sec, 65°C for 5 sec, and 95°C for 5 sec. The samples were run in duplicates within the same run. The δ CT method was used to calculate the average human telomere length, whereby the CT values of the samples were compared to the reference genomic DNA sample. Samples that received non or just one CT value were excluded.

Measurement of average human mitochondrial DNA copy number

The qPCR-based method from ScienCell (AHMQ #8948) was used to determine the absolute human mtDNA copy number. The mtDNA primer set contained complementary primers for the most conserved human mtDNA regions. Additionally, the SCR primer set served as a normalization reference, amplifying a 100 bp-long region of the human chromosome 17. A standard serial dilution for the reference human genomic DNA sample was done. The qPCR was performed on a Bio-Rad CFX384 Real-Time System with the following cycling conditions: an initial denaturation step at 95°C for 10 min followed by 36 cycles of 95°C for 20 sec, 52°C for 20 sec and 65°C for 5 sec, and 95°C for 5 sec. Within the same run, the samples were run in duplicates. Similar to the determination of average human telomere length, the δ CT method was used to calculate the average human mtDNA copy number. Samples were excluded if non or just one CT value was obtained.

Dot blot analysis of 8-oxoguanine levels

One μg DNA, extracted from whole blood, was blotted onto a 45 μm nitrocellulose membrane (Thermo Fisher), previously washed with 2x saline-sodium citrate buffer (SSC; Thermo Fisher). After washing with 2x SSC, the membrane was blocked in 5% dry milk TBS-T (50 mM Tris, 0.5 M NaCl, 0.1% Tween-20, pH 7.4) and incubated with mouse anti-8-oxoguanine (8-oxoG) monoclonal antibody (1:1,000, clone 483.15, MerkMillipore) overnight followed with anti-mouse IgG (H+L), horseradish peroxidase (HRP) conjugate (1:2,500, Promega) for 1.5 hours. Membranes were developed with Signal Fire ECL Reagent (Cell Signalling Technology), images acquired with Vilbert Fusion FX (Vilbert), and dots quantified with ImageJ (PMID: 22743772) gel analysis tool.

Statistical analysis

Categorical and continuous data were summarized by fre-

quencies (percentages) and median (interquartile range [IQR]), respectively. Univariable comparisons between groups were formally tested using the non-parametric two-sample Wilcoxon rank sum test with continuity correction. Correlations between groups were quantified by Spearman's rank correlation coefficient (ρ). To investigate the association of biological markers of aging with hemophilia severity, we regressed average telomere length and mtDNA copy number on study group (severe hemophilia, non-severe hemophilia, or healthy control), using ordinary least squares and allowing for an interaction with chronological age. All analyses were done using R version 4.4.1.[28] Statistical significance was defined as a $P \leq 0.05$.

Results

Study population

The study cohort consisted of 129 male PwH and 67 healthy age-matched male controls. For the final analysis, we included only those PwH and controls where more than one single read for the mtDNA copy number and telomere length was obtained. In 30 PwH (23%) and six (9%) controls, no data for both biomarkers could be obtained, and those were excluded from further analysis. Therefore, the final cohort consisted of 99 (77%) PwH and 61 (91%) healthy controls with sufficient reads for mtDNA copy number and telomere length. Of the 99 PwH, 88 (89%) had hemophilia A and 11 (11%) had hemophilia B. Overall, 66 (67%) had severe, and 33 (33%) non-severe hemophilia A or B 11 [11%] moderate, and 22 [22%] mild hemophilia). The median age of PwH and healthy controls was comparable (Table 1). Platelet counts were significantly higher in healthy controls than in PwH (232 [IQR, 201-264] x10^9/L *vs*. 207 [IQR, 175-240] x10^9/L; *P*=0.005). Laboratory parameters are shown in Table 1. Baseline characteristics divided into severity levels of people with hemophilia and healthy controls can be seen in the *Online Supplementary Table S1*. PwH can also be grouped according to their treatment regimens, with 45 PwH receiving prophylactic therapy and 54 receiving on-demand therapy (see Table 1). According to the severity levels, one of 33 non-severe PwH (3%) and 44 of 66 (67%) severe PwH received prophylaxis, and 32 of 33 (97%) non-severe PwH and 22 of 66 (33%) severe PwH received on-demand therapy (*Online Supplementary Table S1*). In general, the PwH receiving prophylaxis were chronologically younger than those treated with on-demand therapy (29 [IQR, 20-42] *vs*. 41 [IQR, 28-50] years; *P*=0.014).

Human telomere length in persons with hemophilia and healthy controls

Overall, average telomere length was significantly shorter in PwH compared to healthy controls (6.09 [IQR, 4.79-7.68] kb *vs*. 10.07 [IQR, 7.93-12.66] kb; *P*<0.001; Figure 1). The median telomere length in non-severe PwH was 5.12 [IQR, 3.97-6.58] kb compared to 6.29 [IQR, 5.36-8.05] kb in severe PwH (*P*=0.004; *Online Supplementary Figure S1*). No significant difference in the median telomere length was found between hemophilia A and hemophilia B (6.04 [IQR, 4.75–7.60] kb *vs*. 6.26 [IQR, 5.49–8.78] kb; *P*>0.05).

Chronological age and telomere length showed an inverse correlation (ρ=-0.402; *P*<0.001; Table 2) in PwH, (non-severe PwH ρ=-0.556; *P*<0.001; severe PwH ρ=-0.286; *P*=0.020; *Online Supplementary Table S2*), while evidence for a negative correlation in healthy controls was weak (ρ=-0.144; *P*=0.382; Figure 2).

Using linear regression, while mean telomere length displayed an inverse relationship with chronological age (*P*=0.012) and differed between groups (*P*=<0.001), we found no significant evidence for a difference in the change of telomere length over time between groups (*P*=0.537). Mean telomere length was lower in persons with non-severe

Table 1. Baseline characteristics of the study population.

Demographics and blood counts, median (IQR)	Persons with hemophilia N=99	Healthy controls N=61	*P*
Age in years	36.0 (24.0–45.5)	36.3 (27.0–53.0)	0.228
BMI kg/m²	24.3 (21.9-26.7)	24.7 (22.4-26.2)	0.866
Hemoglobin g/dL	15.1 (14.4-15.9)	14.9 (14.5-15.6)	0.352
Leukocytes x10^9/L	5.8 (4.8-6.6)	5.4 (4.8-6.7)	0.783
Neutrophils x10^9/L	3.2 (3.1-4.0)	3.0 (2.6-3.7)	0.165
Platelets x10^9/L	207 (178-240)	232 (206-269)	0.005
hsCRP mg/dL	0.11 (0.06-0.21)	0.09 (0.05-0.17)	0.554
Fibrinogen mg/dL	275 (253-322)	261 (235-302)	0.085
Treatment regimens, N (%) Prophylaxis On-demand	 45 (45.5) 54 (54.5)	 NA NA	 - -

BMI: body mass index; hsCRP: high-sensitivity C-reactive protein; IQR: interquartile range; NA: not applicable; *P* values were calculated using the Wilcoxon rank sum test.

(-4.33 kb; 95% confidence interval [CI]: -5.46 to -3.20 at the sample median age of 36 years) and severe hemophilia (-3.31 kb; 95% CI: -4.22 to -2.41 at the sample median age of 36 years) compared to healthy controls (Figure 2), whereas evidence for a lower mean telomere length in non-severe and severe hemophilia was weak (-1.01 kb; 95% CI: -2.13 to 0.10 at the sample median age of 36 years).

Further analysis between telomere length and laboratory data revealed a weak negative correlation with fibrinogen in PwH (ρ=-0.266; *P*=0.016) and healthy controls (ρ=-0.295; *P*=0.021; Table 2). Additionally, PwH being treated with prophylaxis had a significantly longer telomere length compared to PwH receiving on-demand therapy (6.29 [IQR, 5.43-8.01] kb *vs*. 5.56 [IQR, 4.11-7.30] kb; *P*=0.004).

Mitochondrial DNA copy number in patients with hemophilia and healthy controls

Median mtDNA copy number per diploid cell was significantly lower in PwH (243.17 [IQR, 206.54-293.79]) than in healthy controls (495.52 [IQR, 399.60-615.06]; *P*<0.001; Figure 3). Evidence for a significant difference in median mtDNA copy number between non-severe and severe PwH was weak (240.92 [IQR, 205.09-286.65] *vs*. 246.86 [IQR, 213.05-318.51]; *P*=0.383; *Online Supplementary Figure S2)*. The distribution of mtDNA copy number in persons with hemophilia A and hemophilia B was similar (median 242.44 [IQR, 211.43-293.66] *vs*. 243.17 [IQR, 175.15-292.53]; *P*=0.529). Similar to mean telomere length, mean mtDNA copy number differed between groups (*P*<0.001). However, evidence for an association with chronological age (*P*=0.714) or a difference in the change over time between groups (*P*=0.648) was weak. Mean mtDNA copy number were lower in persons with non-severe (-272.32; 95% CI: -221.95 to -322.70 at the sample median age of 36 years) and severe hemophilia (-256.31; 95% CI: -216.11 to -296.52 at the sample median age of 36 years) compared to healthy controls (Figure 4), with inconclusive evidence for a difference between non-severe and severe hemophilia (-16.01; 95% CI: -65.62 to 33.60 at the sample median age of 36 years).

Further analysis revealed a weak positive correlation between mtDNA copy number and platelet count in PwH (ρ=0.244; *P*=0.025; Table 3). No significant correlations between mtDNA copy number and other laboratory data were found in the healthy control cohort. Spearman's rank correlation coefficients between mtDNA copy number and

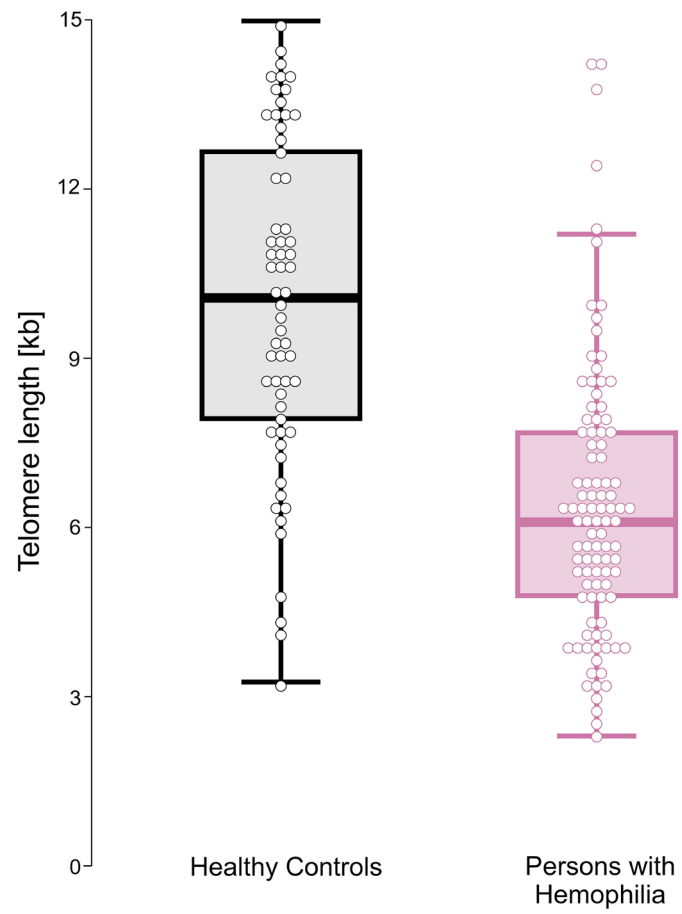

Figure 1. Distribution of telomere length in healthy controls and persons with hemophilia. Persons with hemophilia (median 6.09 [interquartile range (IQR), 4.79-7.68] kb) had significantly (*P*<0.001) shorter telomeres than healthy controls (median 10.07 [IQR, 7.93-12.66] kb).

Table 2. Spearman's rank correlation coefficients of telomere length with baseline characteristics of the study population.

	Persons with hemophilia N=99			Healthy controls N=61		
	Telomere length, per diploid cell			Telomere length, per diploid cell		
	N	ρ	*P*	N	ρ	*P*
Clinical data						
Age in years	99	-0.402	<0.001	61	-0.114	0.382
BMI kg/m²	52	-0.248	0.07	61	0.057	0.661
Laboratory data						
Hemoglobin g/dL	82	-0.006	0.957	61	0.052	0.69
Leukocytes x10⁹/L	85	-0.017	0.88	61	-0.207	0.109
Neutrophils x10⁹/L	85	-0.28	0.185	61	-0.024	0.855
Platelets x10⁹/L	85	-0.033	0.761	61	-0.077	0.553
hsCRP mg/dL	71	-0.199	0.075	61	-0.12	0.356
Fibrinogen mg/dL	82	-0.266	0.016	61	-0.295	0.021
8-oxoG	99	-0.050	0.621	60	-0.089	0.500

BMI: body mass index; hsCRP: high-sensitivity C-reactive protein; ρ: Spearman's rank correlation coefficient; 8-oxoG: 7,8-dihydro-8-oxoguanine.

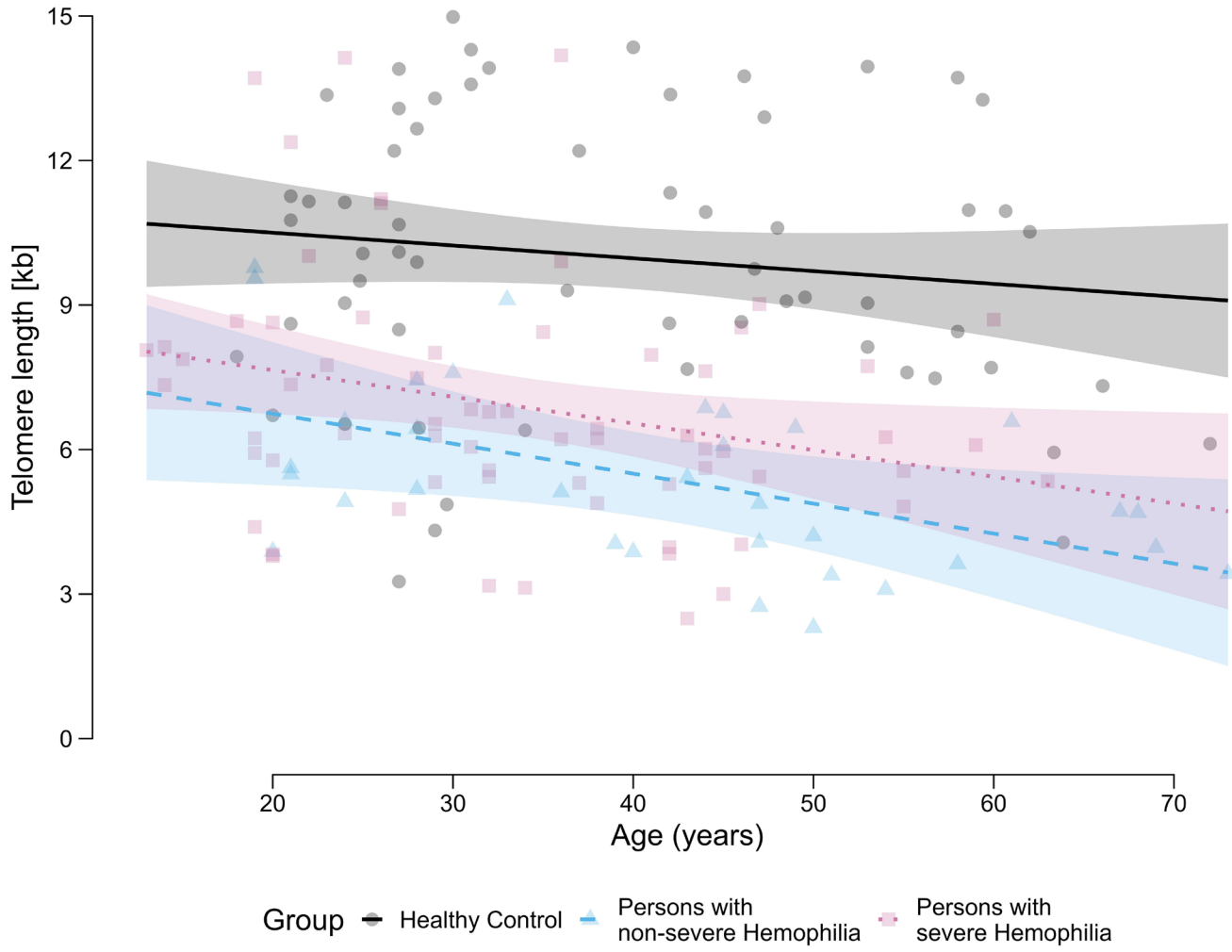

Figure 2. Linear regression of telomere length on study group (healthy controls, persons with non-severe hemophilia, and persons with severe hemophilia), allowing for an interaction with chronological age. The lines and shaded region depict regression lines and accompanying 95% confidence intervals for healthy controls (black), non-severe (blue), and severe hemophilia (purple), respectively.

laboratory data in hemophilia severity can be seen in *Online Supplementary Table S3*. Moreover, there was no significant difference in the mtDNA copy number between the two treatment groups (on-demand: 244.15 [IQR, 205.81-288.10] *vs*. prophylaxis: 236.79 [IQR, 212.90–309.29]; *P*=0.383).

8-oxoguanine levels

Furthermore, we also analyzed the extent of oxidative DNA damage in DNA isolated from whole blood samples via the oxidative stress marker 8-oxoG using dot blot analysis. It was found that PwH showing a borderline significant higher DNA oxidation signal than healthy controls (3.74 [IQR, 2.92-4.80] *vs*. 3.31 [IQR, 2.25-4.70]; *P*=0.058). According to the severity levels, severe PwH had higher DNA oxidation levels than non-severe PwH (3.94 [IQR, 3.13-4.97] *vs*. 3.26 [2.77-4.47], *P*=0.05). Additionally, severe PwH showed higher DNA oxidation levels than healthy controls (3.94 [IQR, 3.13-4.97] *vs*. 3.31 [IQR, 2.25-4.70]; *P*=0.021). However, the oxidation levels of non-severe PwH did not significantly differ from those of healthy controls.

We also performed further correlation analysis of the oxidative stress marker 8-oxoG, and found a positive correlation with CRP (all PwH: ρ=0.249; *P*=0.025; subgroup of severe PwH: ρ=0.384; *P*=0.005). However, no significant correlation between 8-oxoG and CRP was found for the non-severe subgroup and healthy controls (subgroup of non-severe PwH: ρ=-0.034; *P*=0.859; healthy controls: ρ=-0.139; *P*=0.289).

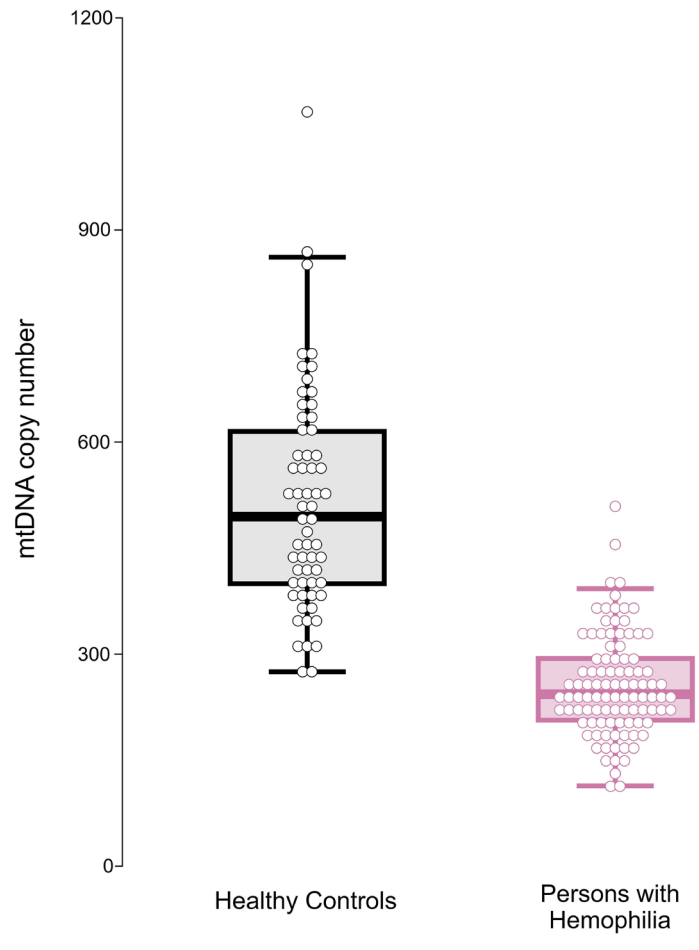

Figure 3. Distribution of mitochondrial DNA copy number in healthy controls and persons with hemophilia. Median mitochondrial DNA (mtDNA) copy number per diploid cell was significantly lower in persons with hemophilia (median 243.17 [interquartile range (IQR), 206.54-293.79]) than in healthy controls (median 495.52 [IQR, 399.60-615.06]; *P*<0.001).

Discussion

In our study, we investigated biomarkers of biological aging, specifically human average telomere length and mtDNA copy number, in PwH and age-matched healthy controls. Interestingly, telomere length and mtDNA copy number in PwH were reduced compared to healthy controls, indicating accelerated biological aging in PwH. Furthermore, telomere length among PwH differed between those with non-severe and severe disease, with persons with non-severe hemo-

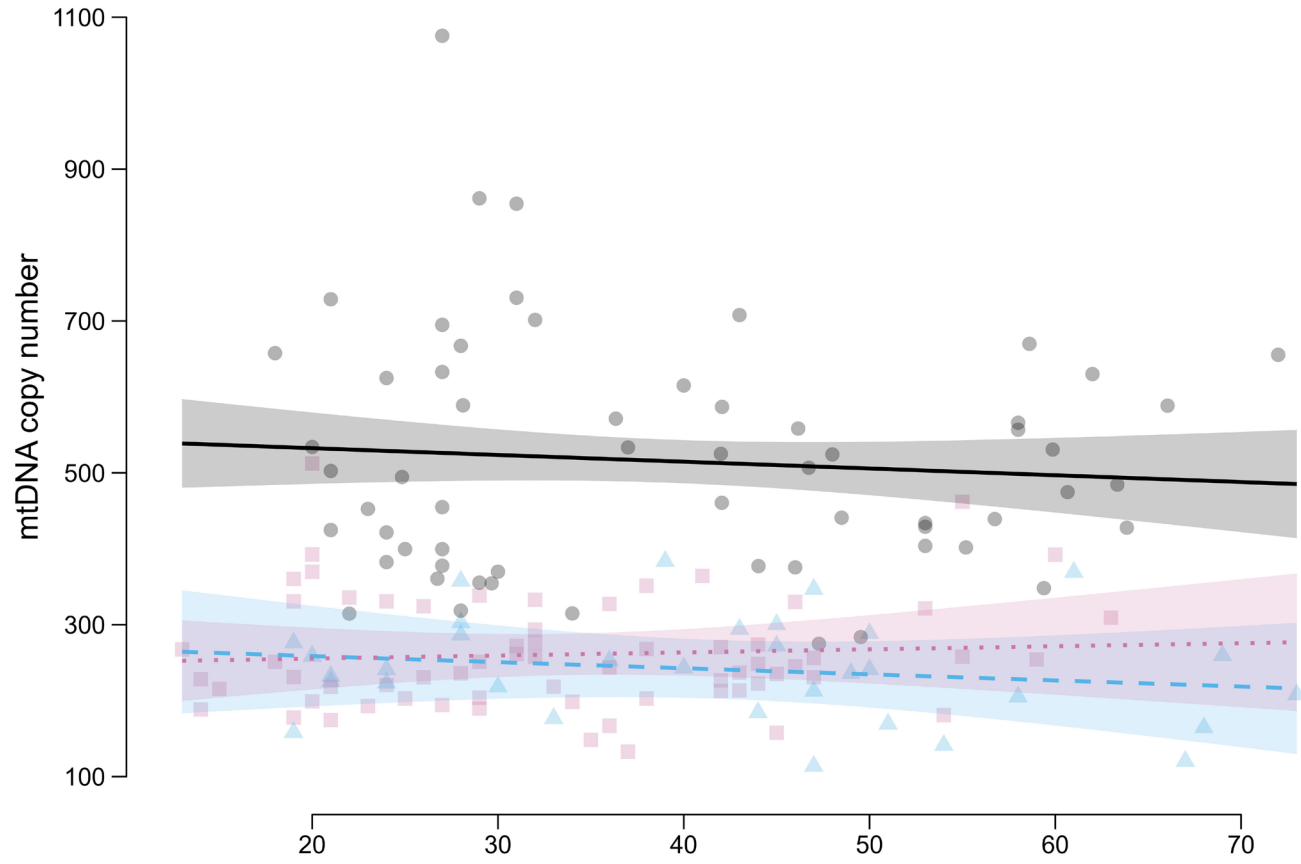

Figure 4. Linear regression of mitochondrial DNA copy number on study group (healthy controls, persons with non-severe hemophilia, and persons with severe hemophilia), allowing for an interaction with age. The lines and shaded region depict age-adjusted fitted values and accompanying 95% confidence intervals for healthy controls (black), non-severe (blue) and severe hemophilia (purple), respectively; mtDNA: mitochondrrial DNA.

Table 3. Spearman's rank correlation coefficients of mitochondrial DNA copy number with baseline characteristics of the study population.

	Persons with hemophilia N=99			**Healthy controls N=61**		
	mtDNA copy number, per diploid cell			**mtDNA copy number, per diploid cell**		
	N	**ρ**	***P***	**N**	**ρ**	***P***
Clinical parameters						
Age in years	99	-0.028	0.785	61	-0.009	0.946
BMI kg/m²	52	-0.000	0.999	61	0.137	0.293
Laboratory parameters						
Hemoglobin g/dL	82	-0.056	0.614	61	0.113	0.385
Leukocytes x10⁹/L	85	0.186	0.089	61	-0.188	0.147
Neutrophils x10⁹/L	85	0.082	0.703	61	-0.172	0.186
Platelets x10⁹/L	85	0.244	0.025	61	0.058	0.658
hsCRP mg/dL	71	0.110	0.328	61	-0.133	0.308
Fibrinogen mg/dL	82	0.160	0.150	61	0.049	0.705
8-oxoG	99	-0.168	0.096	60	-0.088	0.506

BMI: body mass index; hsCRP: high-sensitivity C-reactive protein; ρ: Spearman's rank correlation coefficient; 8-oxoG: 7,8-dihydro-8-oxoguanine; mtDNA: mitochondrrial DNA..

philia displaying slightly shorter telomeres.

A shorter telomere length, reflecting an advanced biological aging, has been reported in other diseases, such as chronic autoimmune diseases like multiple sclerosis. Hecker *et al*. found that shorter telomere lengths were also common compared to healthy controls and also correlated with disease progression and severity.[29] Another study of 237 patients with immune thrombocytopenia (ITP) found shortened telomere length in comparison to 200 healthy controls.[30] In patients with ITP, telomeres shorten with age. Unlike our study, which measured the absolute telomere length, both studies assessed the relative telomere length. In our study, we observed an inverse correlation of telomere length with chronological age within PwH, like in multiple sclerosis and ITP. As hemophilia is a chronic disease, our findings seem to confirm previous reports on accelerated biological aging in chronic diseases. To our knowledge, research on telomere length in bleeding disorders is lacking, and our observations are novel.

Interestingly, a telomere length below the threshold of ≤5 kb is called the "telomere brink", which has been associated with a high risk of mortality. This threshold is usually not met in healthy controls except for those of very advanced age.[31] While the average telomere length for PwH in our study was above 5 kb, it is worth mentioning that the average telomere length for persons with non-severe hemophilia fell below the telomere brink (non-severe: 5.12 [IQR, 3.97-6.58] kb *vs*. severe: 6.29 [IQR, 5.36-8.05] kb). This might also indicate accelerated biological aging in non-severe form of hemophilia, and not only in severe forms with comorbidities as hepatitis C and arthropathy.

The reasons for more pronounced biological aging in persons with non-severe hemophilia compared to those with severe hemophilia, as indicated by a lower telomere length, is difficult to explain on a mechanistical level. Possible reasons for this observed difference may be that persons with severe hemophilia benefit from regular medical contact and supervision, in general, to detect and treat comorbidities early. Also, prophylactic therapy to prevent bleeds and subsequent inflammatory processes, which is established in persons with severe hemophilia and which is not uniformly applied in those with non-severe hemophilia may contribute to overall improvements.[1,8,32] Given that telomere length naturally declines with age, it is plausible that the greater age in the non-severe group may account, at least in part, for their more pronounced telomere shortening compared to the severe group. This natural decline could overshadow other disease-specific mechanisms influencing telomere dynamics in hemophilia. Further studies with age-matched cohorts are necessary to disentangle the effects of age on disease severity.

Furthermore, in our study, PwH also had decreased mtDNA levels compared to healthy controls. In a case-control study by our group, mtDNA copy number was reduced in patients with recurrent VTE compared to healthy control subjects. While the mtDNA levels of the healthy controls decreased with chronological age, mtDNA levels in the youngest VTE patients (according to chronological age) were even lower than in older control subjects.[14] In general, higher mtDNA copy numbers have been associated with better health and less age-related diseases among the elderly.[33] A possible explanation for reduced mtDNA levels could be chronic inflammation. Low-grade inflammation plays a critical role in developing arthropathy in hemophilia.[34] Inflammation is also linked to impaired clotting in PwH and disrupted macrophage differentiation (more pro-inflammatory M2 macrophages).[35]

Another interesting study in the chronic disease setting investigated the epigenetic age as well as inflammatory/immunological proteins in patients with chronic kidney disease. Yusipov *et al*. found accelerated biological aging in patients compared to healthy controls. Additionally, inflammatory biomarkers were linked to accelerated aging processes, indicating that inflammation might play a role.[13] However, in our study this association was independent of inflammation, as hsCRP levels did not differ in PwH and health controls. Chronic kidney disease was associated with lower mtDNA copy number levels in another study.[23] Interestingly, patients with advanced chronic kidney disease also exhibit a high risk of bleeding.[36] The enhanced inflammatory status in chronic diseases can lead to mitochondrial dysfunction, which may reflect a causal relationship.

FVIII is known to play roles in processes such as inflammation, cellular signalling, and possibly vascular function. It has been suggested that FVIII deficiency could impact endothelial health, immune modulation, and other pathways potentially relevant to aging.[37,38] In our study, we observed no significant differences between HA and HB patients in terms of telomere length or mtDNA copy number. However, this subgroup analysis has limited statistical power due to the lower prevalence of HB in our cohort. Future studies with larger, more balanced cohorts could explore the differences more comprehensively and determine whether FVIII impacts age-related health outcomes.

Moreover, we could demonstrate that the oxidative stress marker 8-oxoG is elevated in PwH compared to healthy controls, suggesting increased oxidative DNA damage in PwH. This observation aligns with studies showing that 8-oxoG not only serves as a biomarker of oxidative stress but also plays an active role in modulating immune responses, like the increased production of pro-inflammatory mediators as IL-6, TNF, and IL-8.[39] Therefore, increased 8-oxoG levels in PwH may contribute to increased immune activation and inflammation, potentially worsening the chronic low-grade inflammation often observed in hemophilia. Additionally, while 8-oxoG is typically associated with oxidative damage, its role in signaling and inflammation may link oxidative stress to the accelerated biological aging seen in PwH.

There are some limitations of our study and investigations, which need to be addressed. Although DNA isolation was

successful in all cases, not all samples in our study had detectable mtDNA copy numbers and telomere length. Study participants with invalid or undetectable results for the two biomarkers were excluded from further analyses. However, the advantage of the methods used to determine both biomarkers is that they are relatively easy to perform, and only small amounts of DNA are needed for measurements. Furthermore, the number of persons with non-severe hemophilia was lower than the number of those with severe hemophilia. Therefore, comparisons between the subgroups of hemophilia need to be interpreted with caution. In addition, hemophilia A and B were not equally distributed due to the overall lower prevalence of persons with hemophilia B. Additionally, a possible limitation of our study is the age discrepancy between the non-severe and severe PwH subgroups (44 [IQR, 28–50] *vs*. 32 [23–43] years; *P*=0.014; *Online Supplementary Table S1*), which could contribute to the observed difference in telomere length shortening. However, we circumvented this discrepancy by adjusting for the chronological age in our linear regression model. Another limitation of our study is the lack of the formal joint assessment of all hemophilia patients during the time point of blood collection with the hemophilia joint health score (HJHS) or with ultrasound (e.g., HEAD-US). Therefore, we could not provide a detailed analysis to investigate the association of the joint status with biological aging. The strength of our study is that we have compared the results to a control cohort of healthy subjects, which was matched by chronological age. For the control group of our analyses, we included only people without comorbidities like cardiovascular diseases, atherosclerosis, diabetes, or osteoarthritis. Since these conditions can influence the senescence process. Excluding any comorbidities other than hemophilia itself provided in our view a more focused and meaningful analysis of biological aging. However, further research with a larger cohort is necessary to confirm our findings.

Conclusion

In summary, our study reports that telomere length and mtDNA copy number were significantly lower in PwH than in healthy controls, suggesting an increased biological aging in PwH. Further investigations are necessary to explore the role of mtDNA and telomere length and elucidate mechanisms of biological aging in hemophilia, especially to understand the differences between severe and non-severe hemophilia.

Disclosures

CA received personal fees for lectures and/or participation in advisory boards from Biotest, Bayer, CSL Behring, Novo Nordisk, Roche, Sobi, and Takeda. IP discloses personal fees for lectures from and/or participation in advisory boards at BMS/Pfizer, Daiichi Sankyo, Rovi and Sanofi. DK received personal fees for participation in advisory boards from CSL Behring. JG received personal fees for lectures from and/or participation in advisory boards at CSL Behring, Sobi, Novartis and Amgen.

Contributions

CA designed the study. MT, RV, MS, DK, JG, PH, IP contributed to data acquisition, data analysis, data interpretation, and manuscript writing. MT and RV drafted the first version of the manuscript. All authors revised the work critically for important intellectual content. All authors agreed to be accountable for all aspects of the work and approved publication.

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Data-sharing statement

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

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