Variations in mitochondrial genome as potential prognostic markers in sickle cell disease

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Abstract

Alterations in the mitochondrial genome integrity, including changes in mitochondrial DNA copy number (mtDNA-CN) and accumulation of mtDNA mutations, are associated with aging and diverse disorders, often linked to underlying systemic inflammation and metabolic stress. In sickle cell disease (SCD), inflammation drives the pathology, resulting in organ damage and early mortality. The prognostic role of mitochondrial genomic variation in SCD is largely unexplored. This study investigated whole blood-derived mtDNA alterations, including mtDNA-CN and mtDNA mutations, in adults with SCD, sickle trait, and healthy controls, and examined their associations with age and mortality in SCD. We also assessed mtDNA heteroplasmy distribution across tissues in a humanized mouse model of SCD. Elevated mtDNA-CN and mtDNA heteroplasmy burden were observed with increasing genotype severity across all cohorts (HbAA, HbAS < HbSB+ < HbSC < SCA [HbSS & HbSβ0]). In sickle cell anemia (SCA) patients, mtDNA mutation burden - including mtDNA heteroplasmy and mtDNA deletions increased with age, whereas mtDNA-CN level declined, indicating progressive deterioration of mtDNA integrity with age. In SCD patients, specific mtDNA variants showed strong positive correlations with mortality risk, lower mtDNA-CN correlated with higher National Institutes of Health (NIH) risk scores, and nuclear variants *CYB5R3*^{T117S} and *PIEZO*^{1E756del} influenced mtDNA mutation burden without affecting the NIH risk score. Consistent patterns of mutational load were observed across specific regions in mitochondrial genome in both humans and mice, suggesting potential mtDNA mutational hotspots. We conclude that variations in the mitochondrial genome are potential prognostic markers for SCD.

Introduction

While the sickle pathology is initiated by polymerization of hemoglobin S (HbS), the multiple end-organ damage is inflicted by years of ongoing inflammation and vasculopathy,[1,2] evidenced by the presence of multiple proinflammatory markers.[3] Degenerative changes in sickle cell disease (SCD) lack pathognomonic features and resemble those in non-SCD individuals, but occur earlier, indicating "accelerated" aging,[4] likely driven by the underlying ongoing inflammation. This convergence of chronic systemic inflammation and aging has been referred to "inflamm-aging" that contributes to the pathogenesis of age-related diseases.[5] One common feature of human aging and degenerative disease is an accumulation of cells with mitochondrial dysfunction,[6] linked to alterations in mitochondrial DNA copy number (mtDNA-CN), and accumulation of mtDNA heteroplasmy.[7,8] mtDNA heteroplasmy refers to the co-existence of mutated and wild-type mitochondrial DNA within a cell or individual, where the accumulation of mutated mtDNA beyond a critical threshold can disrupt mitochondrial function and lead to significant physiological dysfunction.[9] Mitochondria have a crucial role in oxidative metabolism and the synthesis of adenosine triphosphate (ATP) via oxidative phosphorylation (OXPHOS), but they are also a potent source of inflammatory triggers, particularly circulating cell-free mtDNA (cf-mtDNA).[10] Of relevance, we previously showed that paired plasma samples of SCD patients contained significantly elevated and hypomethylated cf-mtDNA during acute pain compared with steady state, which were not only fragmented and proinflammatory but also were linked to abnormally retained mitochondria in enucleated

mature sickle red blood cells (RBC).[11] Mitochondria that are retained in the sickle RBC are depolarized, in a state that is associated with increased mitochondrial reactive oxygen species (mROS) generating more oxidative stress and mitochondria dysfunction.[12]

It is unclear whether changes in mtDNA integrity, such as heteroplasmy and mtDNA-CN, directly drive mitochondrial dysfunction in SCD or result from chronic oxidative and metabolic stress, potentially creating a vicious cycle that worsens the disease. Nonetheless, these mitochondrial genomic changes may serve as biomarkers of the 'inflamm-aging' process and disease severity in SCD, as demonstrated in many other diseases.[9] mtDNA-CN, an indicator of mitochondrial biogenesis and function, is linked to aging-related diseases and serves as a biomarker in conditions like cognitive impairment, cardiovascular disease prognosis, and cancer-related metabolic changes.[13-15] Conversely, mtDNA heteroplasmies and deleterious variants predict mortality risk and disease progression in cancers, myeloid neoplasms, neurodegenerative diseases, and renal dysfunctions.[16-19] Collectively, these mtDNA signatures provide valuable insights into disease progression and potential as prognostic biomarkers.

This study analyzed mtDNA from blood-derived whole-genome sequences (WGS) of 1,043 individuals, including SCD patients with various genotypes, carriers of the sickle allele (HbAS), and healthy ethnic-matched controls (HbAA) from three cohorts. We investigated associations between mtDNA variation and demographic factors, hemoglobin level, hydroxyurea (HU) treatment, α-thalassemia status, relevant nuclear variants, disease severity (National Institutes of Health [NIH] phenotypic risk score), and mortality. Tissue-specific mtDNA heteroplasmy was also assessed in humanized Townes SCD mice.

mtDNA variation correlated with SCD genotype severity, with higher mtDNA-CN and mutation burden seen in more severe genotypes across all three human cohorts. mtDNA variants were associated with increased mortality, while mtDNA-CN was inversely associated with NIH risk score. Certain nuclear variants influenced mtDNA heteroplasmy burden. In mice, mtDNA heteroplasmy varied iacross tissues and genotypes. Analysis of mtDNA mutation patterns across mitochondrial genome suggested the presence of mutational hotspots. Overall, these findings indicate that mtDNA-CN and heteroplasmy burden may serve as biomarkers and prognostic indicators in SCD.

Methods

Human subjects

Adult human participants enrolled under Institutional Review Board-approved protocols (*clinicaltrials gov. Identifier: NCT00011648, NCT00081523*, and *NCT03685721*) in three cohorts were included in this study. All participants were of African descent. Individuals with HbSS and HbS-β-thalassemia^0 (HbSB0) genotypes were combined as sickle cell anemia (SCA), the most severe form of SCD. Cohort 1 included a total of 673 SCD patients (554 SCA, 91 HbSC, 25 HbS-β-thalassemia$^+$ [SB+], and 3 other SCD genotypes); cohort 2 included 171 individuals (113 SCA, 30 HbAS, 16 HbAA, and 12 other SCD genotypes); cohort 3 included 199 individuals (96 SCA, 47 HbAS, 44 HbAA, and 12 other SCD genotypes). DNA from each human subject was extracted using peripheral blood buffy coat and was subjected to WGS. Survival analysis was based on cohort 1 where samples were collected over 23 years (February 2001-July 2024), with a mean follow-up time of 6.26 years and a maximum follow-up duration of 20.16 years. Details on human cohorts, sample distributions, and their demographics are described in *Online Supplementary Appendix*; *Online Supplementary Figure S1A; Online Supplementary Table S1*.

Mouse samples

Eighteen Townes humanized mouse model of SCD- littermates, equally distributed by sex and across three genotypes (AA, AS, SS), were included in the study. All experimental protocols were approved by the NIH Clinical Center Animal Care and Use Committee (DPM 23-01). Genomic DNA was extracted from nine tissue samples per mouse (total 162 samples) from which mtDNA was enriched and subjected to next-generation sequencing (NGS). Details on mouse sample distribution and generation of humanized mouse model of SCD are described in *Online Supplementary Appendix*; *Online Supplementary Figure S1B*. mtDNA was enriched from mouse genomic DNA samples by long-range polymerase chain reaction (LR-PCR) and confirmed by agarose gel electrophoresis, as detailed in *Online Supplementary Appendix; Online Supplementary Figure S2*.

Assessment of mitochondrial DNA copy number, mitochondrial DNA heteroplasmy and large mitochondrial DNA deletions

We applied the equation given below to quantify the mtDNA-CN for the human samples.[20, 21]

mtDNA copy number per cell = 2X (mitochondrial coverage/autosomal coverage)

Mouse samples were excluded from the mtDNA-CN analysis as mtDNA was enriched prior to NGS.

Variant calling was done using MitoHPC workflow (https://github.com/dpuiu/MitoHPC) with LoFreq variant caller tool (version 2.4) and VEP/106 annotation. Nuclear sequences of mitochondrial origin (NuMT), coverage depth threshold, and variant allele frequency percent (VAF %) filters were applied to eliminate false positives, homoplasmies, and low-confidence heteroplasmies. Details on NGS and analysis, variant calling, heteroplasmy detection pipeline, and identification of large mtDNA deletions (≥100 bp) from WGS BAM files using an R-based pipeline, are described in detail in *Online Supplementary Appendix*.

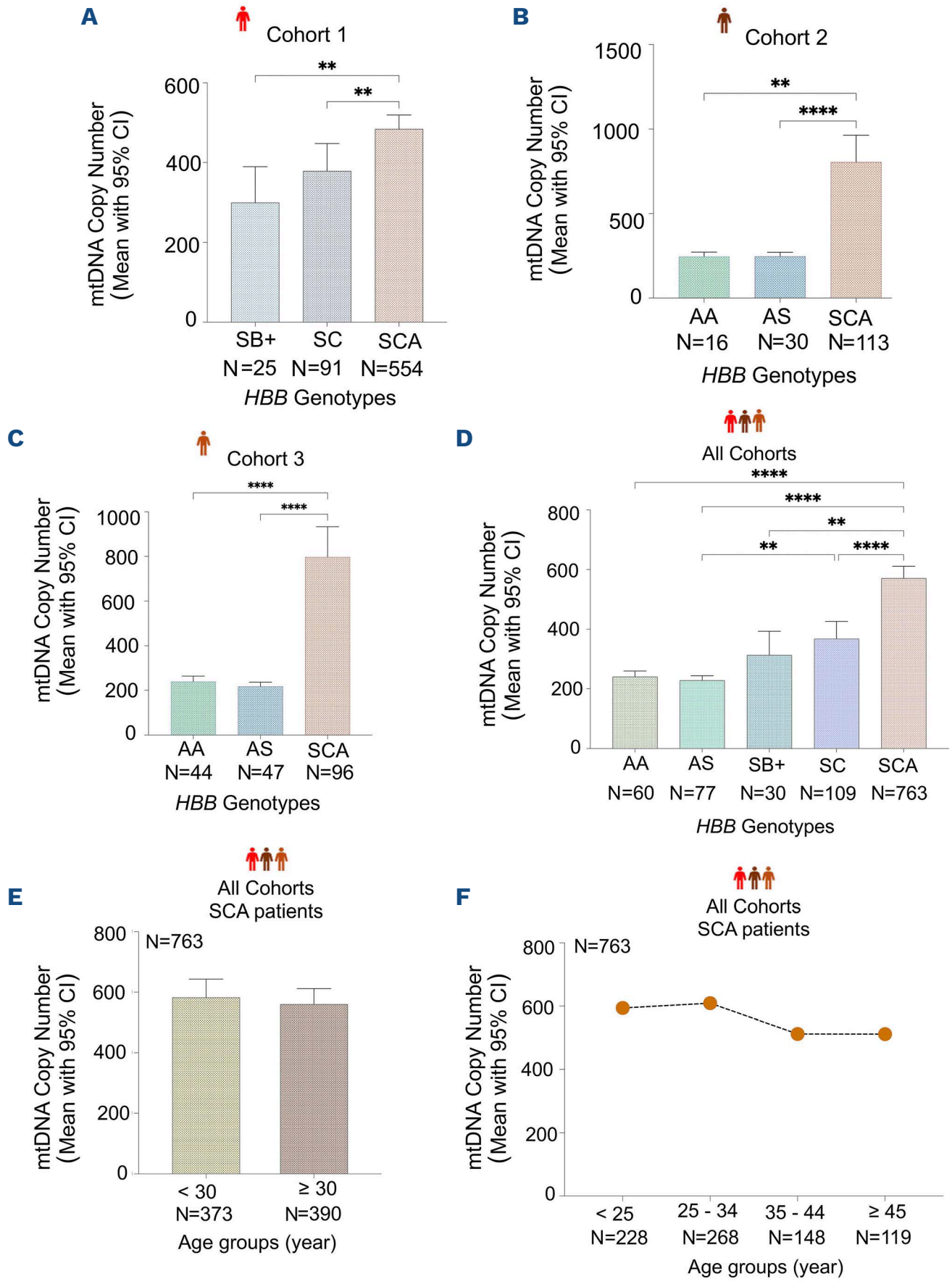


Figure 1. Mitochondrial DNA copy number increased with genotypic severity and declined with age. (A) Mitochondrial DNA copy number (mtDNA-CN) across genotypes in cohort 1. Mean mtDNA-CN in sickle cell anemia (SCA) (N=554) compared with hemoglobin (Hb)SC (N=91) was 483.9 *versus* 379.2 (**P=0.0017), and with HbSB+ (N=26), was 483.9 *versus* 299.4 (**P= 0.0010). (B) mtDNA-CN across genotypes in cohort 2. SCA (N=113) had significantly higher mean mtDNA-CN (804.7) compared to HbAS (N=30, mean mtDNA-CN 246.3) (****P<0.0001), and HbAA (N=16, mean mtDNA-CN 244.9) (**P=0.0019). (C) mtDNA-CN across genotypes in cohort 3. mtDNA-CN was significantly higher among SCA (N=96) (mean=796.7) compared to HbAS (N=47) (mean =217.1) (****P<0.0001) and HbAA (N=43) (mean=239) (****P<0.0001). (D) Comparison of mtDNA-CN levels across combined genotypes in cohorts 1, 2, and 3. Among the total samples (N=1043) from 3 human cohorts combined, SCA (N=763) had significantly higher mtDNA-CN compared to other genotypes: SC (mean=367.8, N=109) (****P<0.0001), SB+ (mean=313.30, N=30) (**P=0.0017), AS (mean=228.5, N=77) (****P<0.0001), and AA (mean=240.6, N=60) (****P<0.0001). (E) mtDNA-CN number stratified by age among the SCA patients (N=763) combined from cohorts 1, 2 and 3. Mean mtDNA-CN was higher in the younger age group (<30 years, N=373) (mean= 582.3) compared to the older group (≥30 years, N=390) (mean=559.8); (P=not significant). (F) mtDNA-CN levels among the SCA patients (N=763) across 4 age groups among the SCA patients (N=763) combined from cohorts 1, 2, and 3. mtDNA-CN level decreased gradually with age: <25 years (N=228, mean=594.0), 25-34 years (N=268, mean=609.3), 35-44 years (N=148, mean=512.3), and ≥45 years (N=119, mean=512.1). Statistical significance (P value) was assessed by Kruskal-Wallis test (between 3 or more groups) or Mann Whitney test (between 2 groups). If no other indication, results were not significant. CI: confidence interval.

Statistical analysis

We used the Cox proportional hazards model to analyze the relationship between mtDNA heteroplasmy and all-cause mortality. Overall phenotype risk was calculated using the NIH risk score for SCD. Comparison between two groups was conducted using Mann Whitney test, and three or more groups using Kruskal-Wallis test. Data were analyzed using GraphPad Prism (version 10.2.0), R (version 4.2.1), and Microsoft Excel.

All details are provided in the *Online Supplementary Appendix*.

Results

Mitochondrial DNA copy number level altered with genotypic severity and age

mtDNA-CN was estimated from WGS data as the ratio of mitochondrial to nuclear genomic reads. Across all three cohorts, mtDNA-CN increased with genotypic severity, with SCA showing significantly higher levels than other genotypes. In cohort 1, SCA group had significantly higher mtDNA-CN (mean=483.9) than HbSC (mean=379.2; *P*=0.0017), and HbSB+ (mean=299.4; *P*=0.001) (Figure 1A). In cohort 2, SCA showed significantly higher mtDNA-CN (mean=804.7) than HbAS (mean=246.3; *P*<0.0001) and HbAA (mean=244.9; *P*=0.0019), and a similar pattern was observed in cohort 3, where SCA had higher mtDNA-CN (mean=796.7) than HbAS (mean=217.1; *P*<0.0001) and HbAA (mean=239; *P*<0.0001) (Figure 1B, C). When all three cohorts were combined, mtDNA-CN increased with genotypic severity: HbAA, HbAS< HbSB+ < HbSC< SCA; and SCA (N=763) had significantly higher mtDNA-CN (*P*<0.001) compared to all other genotypes (Figure 1D).

We next examined whether mtDNA-CN level varies with age. Among all subjects combined from the three cohorts (N=1043), mtDNA-CN decreased with increasing age (*Online Supplementary Figure S3*). Further, when all SCA patients combined from three cohorts (N=763) were dichotomized into two age groups, the older group (≥30-year) had lower mean mtDNA-CN (559.8) compared to the younger (<30-year) age group (582.3), the difference was not significant (Figure 1E). Stratifying the SCA genotypic group into four age groups further shows an overall decrease in mtDNA-CN with age, with mean mtDNA-CN values of 594.0, 609.3, 512.3, and 512.1 in the <25, 25-34, 35-44, and ≥45-year age groups, respectively (Figure 1F).

Mitochondrial DNA heteroplasmy burden increased with genotypic severity and age

mtDNA heteroplasmies were detected using LoFreq variant caller. Potential homoplasmies (VAF >90%) were removed, and to ensure consistent detection of heteroplasmic variants across different cohorts with varying mtDNA sequencing depths, lower VAF (%) thresholds were defined based on the mean mitochondrial coverage for each cohort (*Online Supplementary Table S2; Online Supplementary Figure S4A-C*).

The total mtDNA heteroplasmy level per individual tended to increase with genotypic severity across all three cohorts, although the trend was not statistically significant (*Online Supplementary Figure S5A-C*). The increase in the burden of heteroplasmies was particularly noted in non-synonymous (NS) mutations across the three cohorts (*Online Supplementary Figure S5D-F; Online Supplementary Table S3*). When all three cohorts were combined (N=1,043), mean NS heteroplasmy burden increased with genotypic severity: AA (0.01) < AS (0.03) < SB+ (0.03) <SC (0.08) < SCA (0.12) (*Online Supplementary Figure S5G*). Although the differences across individual genotypes were not statistically significant (*Online Supplementary Figure S5D-F*), the mean NS heteroplasmy burden was found to be significantly higher in SCA when compared to other two major genotypes combined in each cohort (Figure 2A-D). In cohort 1, mean NS heteroplasmy was significantly higher in SCA at 0.11 compared to less severe genotypes (HbSB+ & HbSC) combined at 0.05 (*P*=0.02) (Figure 2A). In cohort 2, SCA showed significantly higher NS heteroplasmy burden (mean=0.14) than HbAS & HbAA combined (mean=0.02) (*P*=0.04) (Figure 2B). Similarly, in cohort 3, SCA had higher NS heteroplasmy burden (mean=0.10) than HbAS & HbAA combined (mean=0.03), with the difference approaching statistical significance (*P*=0.08) (Figure 2C). When subjects from all three cohorts were combined (N=1,043), total SCA patients (N=763) showed a significantly higher mean NS heteroplasmy burden at 0.12 compared to all other non-SCA individuals (N=280) at 0.05 (*P*=0.001) (Figure 2D).

We further analyzed whether NS heteroplasmy burden varied with age. Among the total SCA patients (N=763), the mean NS heteroplasmy burden was significantly higher in the older age group (≥30 years) compared to the <30 years age group (0.14 *vs*. 0.09; *P*=0.03) (Figure 2E). Further stratification into four age groups showed a gradual increase in NS heteroplasmy burden with age, with mean NS heteroplasmy burden of 0.10, 0.11, 0.11, and 0.17 in the <25, 25-34, 35-44, and ≥45-year age groups, respectively (Figure 2F).

Mitochondrial DNA copy number and mitochondrial DNA heteroplasmy variations were independent of hydroxyurea treatment and cell counts

We evaluated if HU treatment affects mtDNA heteroplasmy burden and mtDNA-CN level in cohort 1 which includes only SCD patients (N=673). HU treatment had no impact on heteroplasmy burden, but mtDNA-CN was found to be slightly increased upon HU treatment (*Online Supplementary Table S4; Online Supplementary Figure S6A, B*). When mtDNA-CN level was compared by genotypes within the HU-treated and non-HU treated groups separately, mtDNA-CN increased with genotypic severity in both the groups, mean mtDNA-CN was significantly higher in SCA than SC

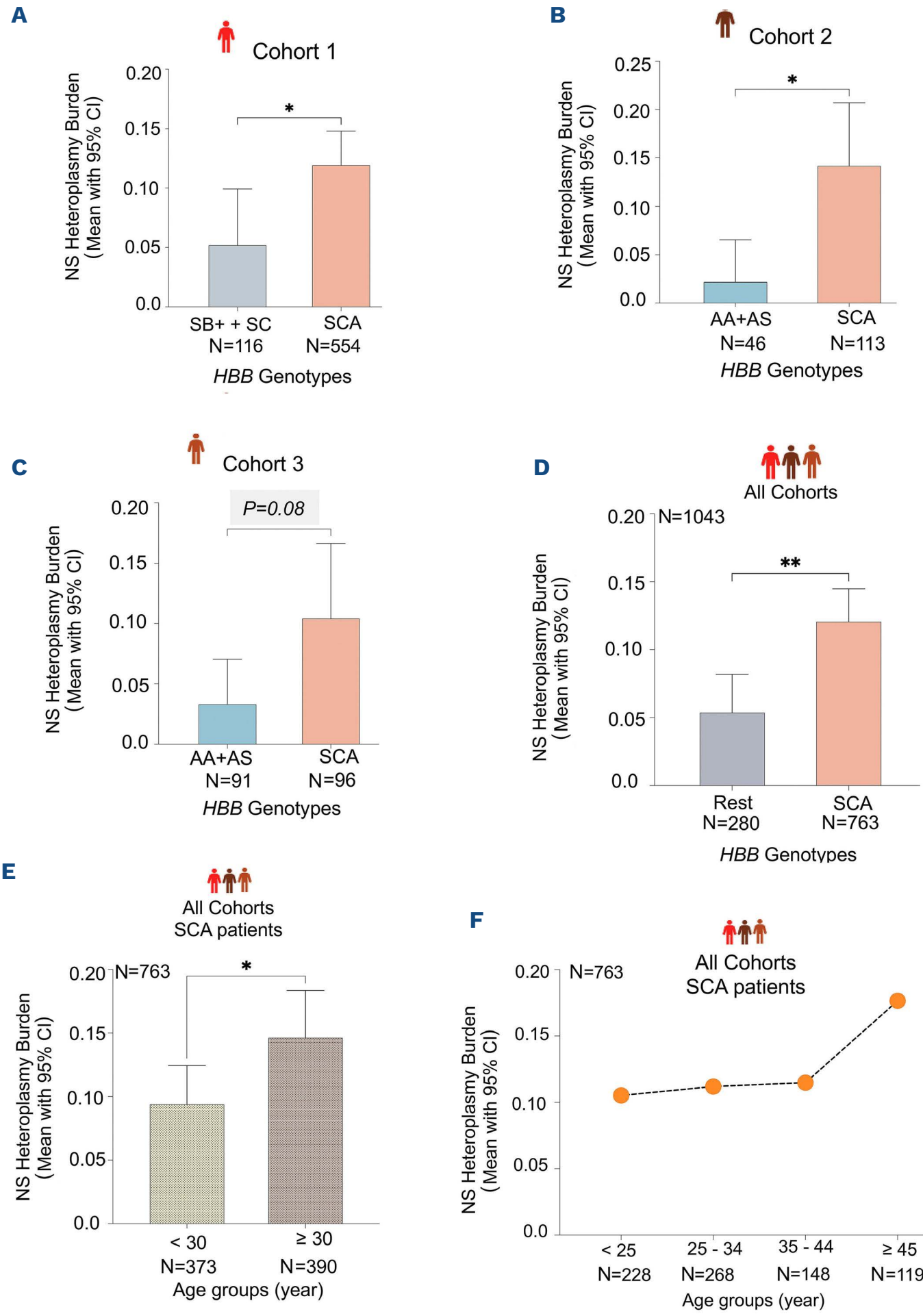


Figure 2. Burden of non-synonymous mitochondrial DNA heteroplasmy increased with severity of genotypes and age. (A) Burden of non-synonymous (NS) heteroplasmy across genotypes in cohort 1 sickle cell anemia (SCA) (N=554) group had higher burden of NS heteroplasmy (mean=0.11) compared to hemoglobin (Hb)SB+ and HbSC genotypes together (N=116, mean=0.05) (*P=0.0263). (B) Burden of NS heteroplasmy across genotypes in cohort 2. The burden of NS heteroplasmy was higher in SCA (N=113, mean=0.14) compared to HbAS & HbAA together (N=46, mean=0.02) (*P=0.0434). (C) Burden of NS heteroplasmy across genotypes in cohort 3. SCA (N=96) had higher burden of NS heteroplasmy (mean=0.10) compared to HbAS and HbAA together (N=91, mean=0.03) (P=0.05826) (mean=0.03). (D) Burden of NS heteroplasmy across genotypes in combined human cohorts (cohort 1, cohort 2, and cohort 3 combined). SCA subjects (N=763) combined from all cohorts had significantly higher burden of NS heteroplasmy (mean=0.12) compared to the rest of the genotypes together (N=280, mean=0.05) (**P=0.0017). (E) NS heteroplasmy burden with

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age among the SCA patients (N=763) combined from 3 human cohorts. NS heteroplasmy burden increased in the older age group (≥30 years, N=390, mean=0.14) compared to the younger age group (<30 years, N=373, mean=0.09) (*P=0.0397). (F) NS heteroplasmy burden across 4 age groups among the SCA patients (N=763) combined from 3 human cohorts. NS heteroplasmy increased with age: <25 years (N=228, mean=0.10), 25-34 years (N=268, mean=0.11), 35-44 years (N=148, mean=0.11), and ≥45 years (N=119, mean=0.17). Statistical significance (P value) was assessed by Kruskal-Wallis test (between 3 or more groups) or Mann-Whitney test (between 2 groups). If no other indication, results were not significant (P≥0.05). CI: confidence interval.

and SB+ in the non-HU treated group (*Online Supplementary Figure S6C*).

Whole blood-derived buffy coat primarily consists of leucocytes containing both nuclear and mitochondrial DNA, but it can also include traces of platelets and reticulocytes that contain mitochondrial DNA and lack nuclear DNA and thereby may influence the mtDNA-CN estimations. To assess whether genotype-wise variation in mtDNA-CN was confounded by platelet or reticulocyte count, multivariate regression analysis was performed in cohort 1. Platelet and reticulocyte counts exhibited negligible effects on mtDNA-CN variation with regression coefficients of 0.12 and 0.07, respectively, whereas genotype (regression coefficient =97.78; P=0.03) contributed to mtDNA-CN variation to a much greater extent (*Online Supplementary Table S5; Online Supplementary Figure S6D*).

Sex differences and hemoglobin levels did not affect mitochondrial DNA copy number and mitochondrial DNA heteroplasmy burden, whereas α-thalassemia specifically impacted mitochondrial DNA copy number

We evaluated whether sex differences influenced mtDNA-CN and mtDNA heteroplasmy. All participants (N=1,043) from three human cohorts were stratified by sex. No significant differences were observed between males and females in mtDNA-CN levels, total mtDNA heteroplasmy burden, or non-synonymous (NS) heteroplasmy burden. Similarly, focusing on the total SCA patients (N=763) from the three human cohorts, no significant sex-based differences were detected (*Online Supplementary Figure S7*).

Next, we assessed whether Hb levels were associated with mtDNA readouts. This analysis was restricted to the SCA genotypes in cohort 1 to ensure a clinically homogeneous phenotype. Spearman correlation analysis revealed no significant association between Hb levels and mtDNA-CN, total mtDNA heteroplasmy, NS mtDNA heteroplasmy (*Online Supplementary Figure S8*).

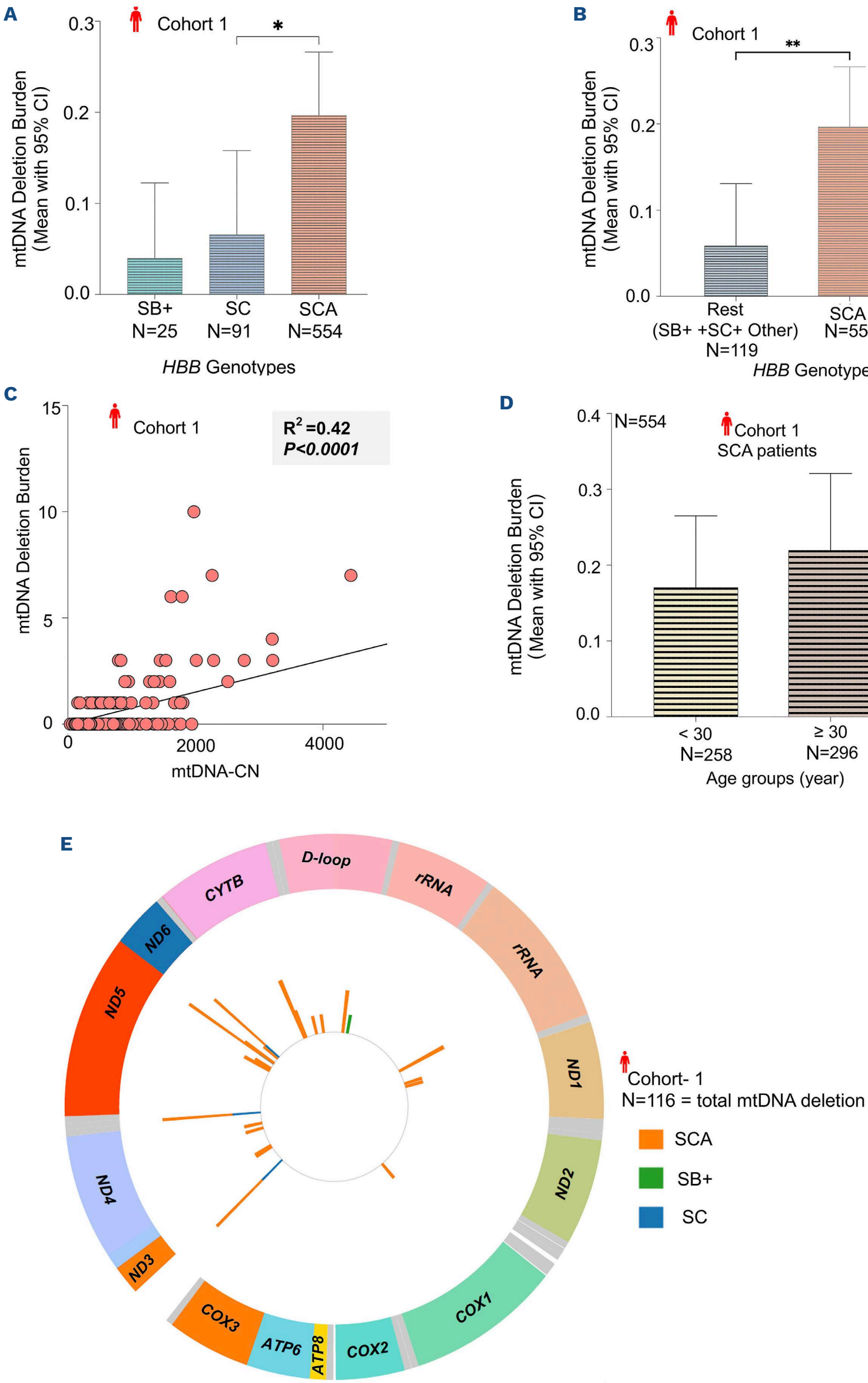
We further examined whether α-thalassemia status was associated with mtDNA-CN levels and mtDNA heteroplasmy burden in SCD (*Online Supplementary Table S6; Online Supplementary Figure S9*). Among the SCD patients in cohort 1 (N=673), α globin genotyping data were available for 632 patients. Spearman correlation analysis demonstrated a significant positive association between mtDNA-CN and α globin gene count (Spearman r=0.089; P=0.02; N=632). In contrast, neither total mtDNA heteroplasmy nor NS heteroplasmy showed a significant association with α globin gene count. The patients were further stratified into four groups based on α globin genotypes: -α/-α (2 normal α globin genes), -α/αα (3 normal α globin genes), αα/αα (4 normal α globin genes), and αα/ααα or more (normal α globin gene ≥5). mtDNA-CN increased with increasing α globin gene count in all SCD patients and SCA patients. Patients with -α/-α (2 globin genes) had significantly lower mtDNA-CN compared to αα/αα (wild-type [WT]) among both SCD patients (P=0.029) and SCA patients (P=0.038) (*Online Supplementary Figure S9B, C*). No differences were found in total mtDNA heteroplasmy burden or NS heteroplasmy burden according to number of α globin genes (*Online Supplementary Figure S9D, E*).

Mitochondrial DNA deletion burden increased with genotypic severity, mitochondrial DNA copy number, and age

A total of 116 mtDNA deletions (≥100 bp) were identified in cohort 1 which included 673 SCD patients. In cohort 2 and cohort 3, no deletions ≥100 bp were detected; all observed deletions were <100 bp and were excluded from analysis, as such small deletions are likely mapping artifacts. In cohort 1, the mean mtDNA deletion (≥100 bp) burden increased with genotypic severity, with SCA having the highest burden compared to SC (0.19 *vs*. 0.06; P=0.04) and SB+ (0.19 *vs*. 0.04; P=not significant) (Figure 3A). mtDNA deletion burden was significantly higher in SCA than all non-SCA genotypes combined (mean of 0.05; P=0.008) (Figure 3B). The mtDNA deletion burden increased with mtDNA-CN, showing a positive correlation (R^2=0.42; P<0.0001) (Figure 3C). Among the SCA patients in cohort 1, the deletion burden also rose with age, being higher in those aged ≥30 years (0.21) compared to those <30 years (0.17), although the difference was NS (Figure 3D). Protein coding mtDNA genes including *ND3, ND4, ND5, ND6*, and *CYTB*, showed a higher deletion burden, with SCA exhibiting the greatest burden across genotypes (Figure 3E).

Mitochondrial DNA heteroplasmy burden was predominantly composed of transition mutations enriched in the D-loop and complex I regions

In cohort 1 (SCD patients, N=673), we identified 268 heteroplasmic occurrences, with the displacement loop (D-loop) region contributing the most (36.94%) followed by complex I mutations (23.51%) (*Online Supplementary Table S7*). Among SCA patients (N=554), complex IV (10.1%), tRNA (13.2%), and complex V (3.5%) regions displayed higher burden compared to the other genotypes (*Online Sup-*



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Figure 3. Burden of mitochondrial DNA deletions among the sickle cell disease patients in cohort 1. (A) Burden of mitochondrial DNA (mtDNA) deletions (>100 bp) increased with genotypic severity in cohort 1. Sickle cell anemia (SCA) subjects (N=554) had higher burden of mtDNA deletions (mean=0.19) compared to other genotypes: SC (N=91, mean=0.06) (**P*=0.0467), SB+ (N=25, mean=0.04) individually (B) Comparison among SCA (N=554) and rest of the samples combined (rest, N=119) in cohort 1 (N=673) showed that SCA subjects (N=554) had a significantly higher burden of mtDNA deletions (mean=0.19) compared to the rest (mean=0.05) (***P*=0.008). (C) Pearson correlation between mtDNA deletions and mtDNA copy number (mtDNA-CN) in cohort 1 (N=673). mtDNA deletions displayed a positive correlation (R^2=0.42; ***P*<0.0001) with mtDNA-CN. (D) Burden of mtDNA deletions with age among the SCA patients (N=554) in cohort 1. mtDNA deletions burden was higher in the older age group (≥30 years; N=296, mean=0.21) compared to the <30 years age group (N=258, mean=0.17). (E) Distribution of mtDNA deletions burden across mito-genome. Circular representation (Circos plot) of the human mitochondrial genome where the outer most ring represents different mito-genes indicated in different color. Inner bars indicate genomic locations with mtDNA deletion burden, and the bar height reflects burden frequency. Bar colors represent genotypes (SCA, orange; SC, blue; SB+, green). A higher burden of mtDNA deletions was observed in protein-coding genes: *ND3, ND4, ND5, ND6, CYTB*. The overall pattern of mtDNA deletion burden was similar between genotypes; with SCA having higher burden. Statistical significance (*P* value) was assessed by Kruskal-Wallis test (between 3 or more groups) or Mann-Whitney test (between 2 groups). If no other indication, results were not significant (*P*≥0.05).

plementary Table S7; Online Supplementary Figure S10). Of the 268 occurrences, 165 were insertions deletions (Indel) and 103 were single nucleotide polymorphisms (SNP), predominantly transition-type substitutions (98/103, 95.2%) compared to transversions (5/103, 4.8%) (*Online Supplementary Table S8*).

Specific mitochondrial DNA variants were associated with increased all-cause mortality in sickle cell disease

In cohort 1 (SCD patients, N=673), we explored the impact of mtDNA heteroplasmy on survival using Cox proportional hazards model. Samples were collected over 23 years (February 2001-July 2024), with a mean follow-up time of 6.26 years and a maximum follow-up duration of 20.16 years (*Online Supplementary Table S9*). While not statistically significant, there was a trend indicating increased risk of all-cause mortality with higher mtDNA heteroplasmy (hazard ratio [HR]=1.01). Categorization by cumulative burden of heteroplasmy further increased the risk (HR=1.17 for 1 heteroplasmy *vs*. none; HR=1.3 for 2 heteroplasmies *vs*. none) (*Online Supplementary Table S10A*). This trend persisted after adjusting for age, suggesting a potential association despite limited sample size. Additionally, seven mito-variants were linked to higher mortality risk, with HR from 22.09 to 153.98 (*P*<0.05), mainly in the genes involved in oxidative phosphorylation (complex I, complex IV, and complex V) (Table 1). The highest-risk variant, MT: 8483 (HR=153.98; *P*=0.0008) was in the *ATP8* gene (complex V). Of note, six of these seven mito-variants were found in HbSS patients and one in HbSC patient (*Online Supplementary Table S10B*).

In keeping with the inverse association between mtDNA-CN and age, there was a negative trend between mtDNA-CN and mortality (HR=0.964), consistent after corrected for age (*Online Supplementary Table S10C*).

Correlation of mitchondrial DNA genomic variants with National Institute of Health risk score

We evaluated the relationship between mtDNA metrics and phenotypic severity in SCD using the NIH risk score, a previously published phenotypic risk prediction model integrating nine clinical and laboratory variables.[22] A total of 452 SCD patients in cohort 1 with complete data for all component variables required to calculate NIH risk score were included in this analysis.

Spearman correlation analysis revealed no significant association between NIH risk score and mtDNA-CN, total mtDNA heteroplasmy, or NS heteroplasmy (*Online Supplementary Table S11; Online Supplementary Figure S11A, C, E*). Stratification of the patients into two risk score groups: high risk (NIH risk score >3) and low risk (NIH risk score ≤3), showed that patients with high risk (N=71) had significantly lower mean mtDNA-CN than those with low risk (N=381) (mean mtDNA-CN 348.40 *vs*. 459.48; *P*<0.001) (*Online Supplementary Figure S11B*), consistent with the inverse trend between mtDNA-CN and mortality observed in survival analysis. In contrast, neither total mtDNA heteroplasmy nor NS heteroplasmy differed significantly between the two groups (*Online Supplementary Figure S11D, F*).

Specific nuclear variants impacted mitochondrial DNA heteroplasmy burden

We examined a panel of nuclear genetic variants with known relevance to SCD red cell biology, oxidative stress, mitochondrial function, and hemolytic process- including *SOD2*^{V16A}, *PIEZO1*^{E756del}, *CYB5R3*^{T117S}, *G6PD*^{A376G}, and *G6PD*^{G202A} (*Online Supplementary Table S12*), to assess their associations with mtDNA variants in SCD patients (cohort 1; N=673). Minor allele frequency (MAF) of these genetic variants ranged from 0.11 to 0.42 among the patients (*Online Supplementary Table S13*). For association analyses, variant status was encoded as 0 (WT), 1 (heterozygous), 2 (homozygous). Spearman correlation identified two variants that were significantly (*P*<0.05) associated with mtDNA heteroplasmy. *PIEZO1*^{E756del} showed a weak but statistically significant positive correlation with total mtDNA heteroplasmy (Spearman r=0.077; *P*=0.045). Similarly, *CYB5R3*^{T117S} was positively correlated with total

mtDNA heteroplasmy (Spearman r=0.11; *P*=0.002) and NS heteroplasmy (Spearman r=0.09; *P*=0.009) (*Online Supplementary Figure S12; Online Supplementary Table S14*). The remaining variants showed no significant associations, and mtDNA-CN was not significantly associated with any of these variants (*Online Supplementary Figure S12; Online Supplementary Table S14*).

To further investigate these associations, patients were stratified into WT or mutant (heterozygous and homozygous) groups. Both mutant groups carrying *PIEZO1*^{E756del} and *CYB5R3*^{T117S} exhibited higher total mtDNA and NS heteroplasmy burden than WT individuals (Figure 4). Specifically, in the *PIEZO1*^{E756del} group, mean mtDNA heteroplasmy was 0.49 in mutant versus 0.36 in WT (*P*=0.045); in *CYB5R3*^{T117S}, mean total mtDNA heteroplasmy was 0.48 in mutant versus 0.33 in WT (*P*=0.002). NS heteroplasmy was also higher in *PIEZO1*^{E756del} mutant group, 0.14 *versus* 0.09 in WT (*P*=not significant) and *CYB5R3*^{T117S} mutant (0.15 *vs*. 0.07 in WT; *P*=0.010). The remaining variants showed no significant differences in mtDNA heteroplasmy between WT and mutant groups. mtDNA-CN levels did not differ between the mutant and WT groups for any of the variants (*Online Supplementary Table S15*).

None of the nuclear variants showed any significant association with NIH phenotypic risk score among the SCD patients in cohort 1 (*Online Supplementary Table S16*).

Sickle cell disease mice exhibited tissue-specific and genotype-dependent mitochondrial DNA heteroplasmy burden

Of the 162 mouse tissue samples, one sample (AA, male, lung) was removed from analysis due to low coverage (*Online Supplementary Figure S4D*). mtDNA was enriched prior to NGS to achieve a uniform average mito-coverage across the tissues (*Online Supplementary Figure S4E*). A total of 11 heteroplasmic mito-variants with 106 total heteroplasmic occurrences were detected across 161 mouse tissue samples (*Online Supplementary Table S17*).

Similar to observations in humans, mtDNA heteroplasmy burden increased with increasing severity of the genotypes, with HbSS mice showing the highest mean total mtDNA heteroplasmy burden (0.76) followed by HbAS (0.70) and HbAA (0.51) (Figure 5A). mtDNA heteroplasmy burden differed between tissues within and across genotypes, with spleen having the highest overall mtDNA heteroplasmy burden (17/106 occurrences) (Figure 5B). Among the 11 mito-variants, the variant at mito-position 9,820 (MT: 9820, *mt-tr*) was the most prevalent (53/106 occurrences) shared between all three genotypes (*Online Supplementary Table S17;* Figure 5C). Four variants - MT:6753 (Phe476-Leu in *COX1*), MT:6974 (intergenic), MT:1220 (intergenic), and MT:425 (D-loop) were found only among the SS mice (Figure 5C), which occurred 22 times across the tissues with spleen having the highest burden (Figure 5D; *Online Supplementary Table S18*). In all three genotypes, female mice tended to have higher mtDNA heteroplasmy burden than males (*Online Supplementary Figure S13*); however, the differences were not statistically significant, the small sample size was insufficient for a reliable and robust comparison.

Mitochondrial genome displayed potential mutation hotspots

We compared mtDNA heteroplasmy distribution across the 37 mitochondrial genes in the three human cohorts and mouse samples using NCBI reference sequences (human: NC_012920.1 and mouse: NC_005089.1). The analysis showed a similar distribution of mtDNA heteroplasmy load across cohorts (Figure 6) and across genotypes (*Online Supplementary Figure S14A*). The D-loop, *RNR1, RNR2, ND1, CO1, ND5, ATP6*, and *CYTB* genes displayed higher heteroplasmy burdens compared to other mitochondrial genes, suggesting shared hotspots in the mitochondrial genome for both human and mouse samples (*Online Supplementary Figure S14B*).

Table 1. Specific mito-variants associated with higher hazard ratio for all-cause mortality.

Mito-variants	Hazard ratio			Significance *P* value		Mutation type	Base change	Impact	Gene affected	Functional category
	HR	HR.L	HR.R	*P*	*P* adj.					
MT: 8483	153.98	17.20	1,378.18	6.66E-06	0.000839	SNP	A/G	Coding	*ATP8*	Complex V
MT:16015	40.65	5.36	308.04	0.0003363	0.021190	INS	Indel	Non-Coding	*MT-TT*	MT- tRNA
MT: 8156	32.54	4.34	243.78	0.0007002	0.029408	SNP	A/G	Coding	*ND2*	Complex I
MT: 4916	26.37	3.55	195.62	0.0013740	0.042967	SNP	A/G	Coding	*ND2*	Complex I
MT: 4658	24.05	3.25	177.81	0.0018338	0.042967	SNP	T/C	Coding	*ND2*	Complex I
MT: 4695	23.04	3.12	170.01	0.0020980	0.042967	SNP	G/T	Coding	*COX2*	Complex IV
MT: 6158	22.09	3.00	162.82	0.0023870	0.042967	SNP	A/G	Coding	*COX1*	Complex IV

HR: hazard ratio; HR.L: lower limit of the hazard ratio confidence interval; HR.R: upper limit of the hazard ratio confidence interval; adj.: adjusted; MT: mito-variant; SNP: single nucleotide polymorphism; INS: insertion; Indel: insertions/deletions.

Discussion

Mitochondrial dysfunction and chronic inflammation are two determinants of the complex aging process and have been implicated in the pathogenesis of a variety of diseases.[4,5,23,24] Inflammatory processes, both acute and chronic, play a key role in SCD pathology leading to high background levels of ROS and oxidative stress that contribute to mitochondrial dysfunction and mtDNA damage, generating more oxidative stress driving the pathological inflammatory cycle in SCD. Here we provide a comprehensive analysis of alterations in mitochondrial genome in SCD, integrating human cohorts and mouse models. This study demonstrates the impact of two key defects in mitochondrial genomic alterations - changes in mtDNA content (mtDNA-CN) and accumulation of mtDNA mutations in SCD.

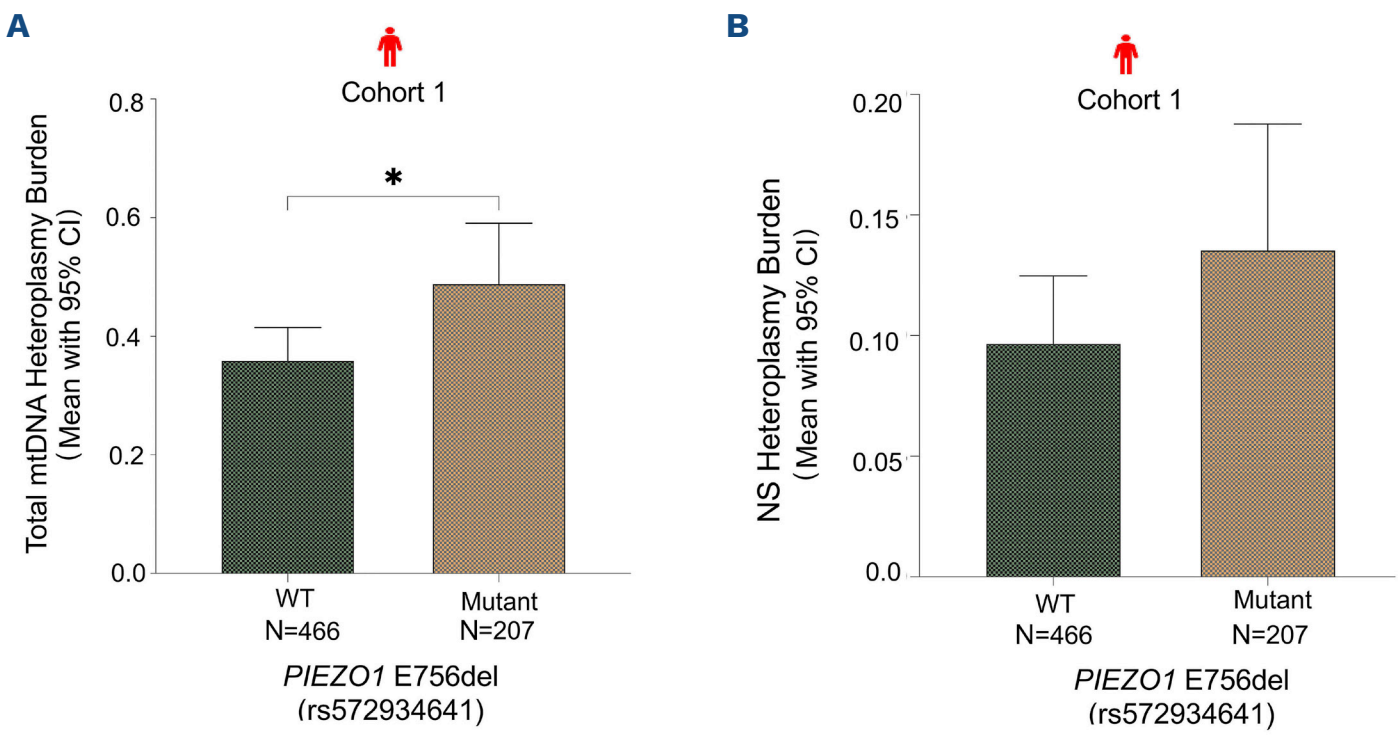


Figure 4. Association of nuclear variants *PIEZO1*^{E756del} (rs572934641) and *CYB5R3*^{T117S} (rs1800457) with mitochondrial DNA heteroplasmy burden. Sickle cell disease (SCD) patients in cohort 1 (N=673) were stratified into 2 groups: wild-type (WT) and mutant (heterozygous and homozygous). Mean of mitochondrial DNA copy number (mtDNA-CN), total mtDNA heteroplasmy, and non-synonymous (NS) heteroplasmy was compared between the 2 groups. (A) Mean total mtDNA heteroplasmy burden was significantly higher in *PIEZO1*^{E756del} mutant (N=207, mean=0.39) compared to WT (N=466, 0.36); *P*=0.045. (B) Mean NS heteroplasmy burden was higher in *PIEZO1*^{E756del} mutant (N=207, mean=0.14) compared to WT (N=466, 0.09); *P*=not significant. (C) Mean total mtDNA heteroplasmy burden was significantly higher in *CYB5R3*^{T117S} mutant (N=356, mean=0.48) compared to WT (N=317, 0.33); *P*=0.002. (D) Mean NS heteroplasmy burden was significantly higher in *CYB5R3*^{T117S} mutant (N=356, mean=0.15) compared to WT (N=317, mean=0.07); *P*=0.010. Statistical significance (*P* value) was assessed by Mann-Whitney test. If no other indication, results were not significant (*P*≥0.05). CI: confidence interval.

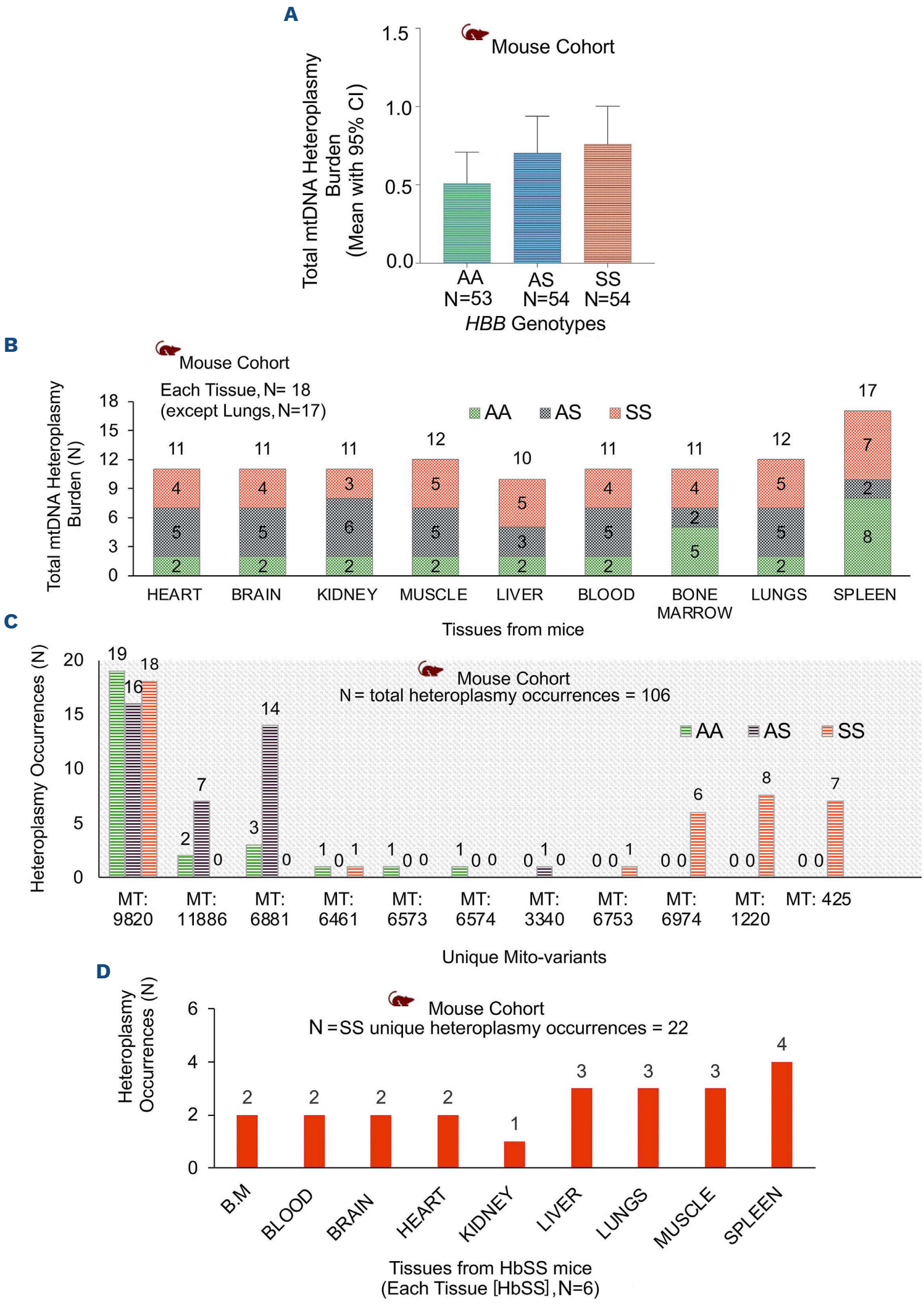


Figure 5. Mitochondrial DNA heteroplasmy burden among the mouse samples. In total 106 heteroplasmic occurrences originating from 11 unique mito-variants were obtained among all mice samples (N=161, 1 sample removed due to dropped coverage). (A) Mitochondrial DNA (mtDNA) heteroplasmy burden among 3 genotypes. Hemoglobin (Hb)SS mice (N=54) showed highest mtDNA heteroplasmy (mean=0.76), followed by HbAS (N=54; mean=0.70), and HbAA (N=53, 1 sample removed due to dropped coverage; mean=0.51) (*P*=not significant). (B) Tissue-wise comparison of mtDNA heteroplasmy burden across three genotypes. The number above each bar in the graph indicates the total heteroplasmic burden for the respective tissue, while the number within each bar represents the genotype-specific heteroplasmic burden within each tissue. The burden of mtDNA heteroplasmy varied across

Continued on following page.

different tissues both within and across genotypes. Spleen had the overall highest burden (N=17) of mtDNA heteroplasmy. (C) The distribution of 11 unique mito-variants across 3 genotypes. The number above each bar in the graph indicates the occurrences of the respective mito-variants in each genotype. (D) The distribution of the HbSS unique variants (MT: 6753, MT: 6794, MT: 1220, and MT: 425) across all 9 tissues. The number above each bar in the graph indicates the occurrences of HbSS unique variants across tissues. Statistical significance (*P* value) was assessed by Kruskal-Wallis test (between 3 or more groups) or Mann-Whitney test (between 2 groups). If no other indication, results were not significant ($P \geq 0.05$).

We showed for the first time that the mtDNA-CN level and mtDNA heteroplasmy burden per individual increased progressively from HbAA, HbAS < HbSβ+ thalassemia < HbSC < SCA (HbSS & HbSβ0), with genotypic groups that are associated with increasing phenotypic severity.

In general, cell types with high ATP demand exhibit higher mtDNA copy numbers.[25,26] Given that ATP depletion is a characteristic feature of SCD,[27, 28] progressive increase in mtDNA-CN with genotype severity could reflect an adaptive response to meet heightened energy requirements and mitigate oxidative stress, particularly in the more severe HbSS and HbSβ^0 (SCA) genotypes. Consistent with this, elevated mtDNA content is a recognized adaptive mechanism against oxidative stress,[29,30] as also evidenced by an increase in mtDNA-CN in whole blood from patients with transfusion-dependent β-thalassemia, a condition associated with iron overload and oxidative injury.[31] NS heteroplasmy burden was significantly higher among SCA patients across three cohorts in this study, indicating an accumulation of potentially deleterious mutations in coding regions of mtDNA, which are likely to impact mitochondrial function, including ATP production, and contribute to SCD pathophysiology. In keeping with other studies, we also found a higher heteroplasmic burden in the D-loop.[32,33]

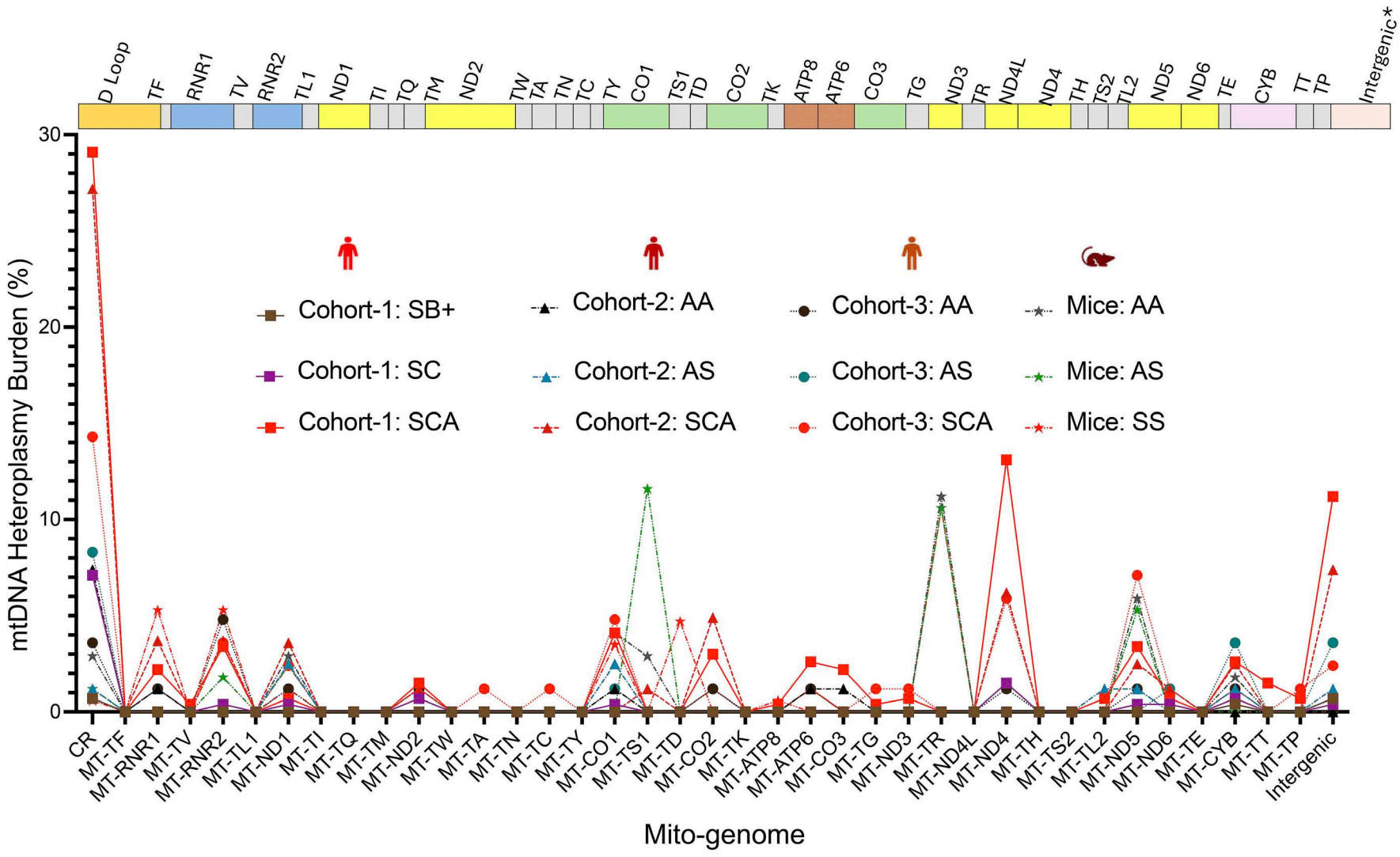


Figure 6. Pattern of mitochondrial DNA mutation load in mito-genome across cohorts and genotypes. The burden of mitochondrial DNA (mtDNA) heteroplasmy across the mito-genome was assessed across 4 cohorts (3 human cohorts and 1 mouse cohort) and each genotype within respective cohorts. All 4 cohorts were subjected to same variant calling pipeline (LoFreq variant caller, variant allele frequency [VAF]: 1-90%, coverage depth >1,000x) to obtain mtDNA heteroplasmic variants. mtDNA heteroplasmic load across the mitochondrial genes was quantified and visualized for each genotype within their respective cohorts. The top bar with different colors represents the mitochondrial genome, consisting of 39 regions - 37 mitochondrial genes, the D loop, and intergenic regions. All the heteroplasmies originating from the unspecified stretch of sequences between 2 genes are grouped as “Intergenic” and represented at the right side end of mitogenome bar in the figure. *D-loop, RNR1, RNR2, ND1, CO1, ND5, ATP6,* and *CYT-B* exhibited higher mtDNA heteroplasmy burden compared to the rest of the other genes in the mito-genome, consistent across the cohorts and genotypes.

D-loop, being the control region of mtDNA replication initiations is more likely to be exposed to oxidative stress and prone to accumulate mutations, contributing to its higher mutational load.[33,34] Among the coding mutations, we found a higher ratio of transition to transversion mutations consistent with other reports.[35] The low abundance of transversion mutations, which are mainly caused by oxidative damage and are deleterious, is known to result from repair mechanisms by mitochondrial 8-oxoguanine DNA glycosylase which corrects the oxidative lesions, and strong purifying selection in mitochondria that hinders their clonal expansion.[36,37] Large mtDNA deletions have long been associated with oxidative stress, aging, and many disease conditions including Kearns-Sayre syndrome, Pearson syndrome, metabolic diseases, and cancers.[38] In the current study, SCA patients exhibited an increased burden of mtDNA deletions, predominantly affecting genes involved in oxidative phosphorylation- *ND3, ND4, ND5, ND6*, and *CYTB*. Consistent with our findings, increased mtDNA deletion burden has also been found in transfusion-dependent β-thalassemia associated with iron overload and oxidative stress.[31]

HU affects mtDNA-CN and mitochondrial genome stability in model organism such as yeast.[39] HU, standard-care therapy for SCD, reduces acute pain and improves overall clinical outcomes but also acts as a myelosuppressive agent.[40,41] In this study of SCD patients, many of whom are on long-term HU therapy, we explored HU's impact on the mitochondrial genome. HU treatment did not influence the increase in mtDNA heteroplasmy burden associated with genotypic severity. Although mtDNA-CN slightly increased with HU treatment, a genotype-specific increase of mtDNA-CN was observed in both HU treated and untreated (P<0.001) groups. The genotype-dependent increase was independent of contributions from non-nucleated, mtDNA-containing cell types like platelets and reticulocytes, and was not influenced by sex or Hb levels. Together, these findings suggest that intrinsic genotypic severity primarily drives the increase in mtDNA-CN, with a positive correlation with mtDNA deletion burden indicating a compensatory response to mtDNA damage. The number of α globin genes did not have an impact on mtDNA heteroplasmy, but was associated with lower mtDNA-CN in both SCD and SCA patients, potentially reflecting the established beneficial effect of α-thalassemia co-inheritance in ameliorating outcomes of SCD.[42,43] The increased burden of non-synonymous mtDNA heteroplasmy in SCD patients with *PIEZO1*^{E756del} [44-46] and *CYB5R3*^{T117S} variants underscores their impact on mitochondrial dysfunction in SCD. *PIEZO1*^{E756del} promotes RBC dehydration and calcium overload, disrupting mitochondrial dynamics, while *CYB5R3*T^{117S}[47] impairs NADH-dependent redox balance and HU-mediated HbF induction; together exacerbating SCD severity.

In patients with sickle cell anemia, mtDNA heteroplasmy, particularly deleterious non-synonymous variants, and mtDNA deletion burden tended to increase with age, indicating cumulative mitochondrial damage. Concurrently, mtDNA-CN declined with age, and reduced mtDNA-CN was associated with higher NIH risk score, further strengthening the compensatory role of mtDNA-CN in SCD. This study also found that higher mtDNA heteroplasmy burden correlates with increased all-cause mortality, independent of age, and specific mito-variants have greater impact on mortality than cumulative heteroplasmies. These findings align with a study using data from the UK Biobank showing a dose-response relationship between mtDNA heteroplasmy burden and mortality risk.[16] Other studies have also linked specific mito-variants to increased mortality risks in breast cancer,[48] dementia and stroke among elderly individuals.[49] In SCD mice, despite a limited sample size, we observed varying mtDNA heteroplasmy across tissues, a phenomenon also reported in other studies.[36,50] Notably, splenic mtDNA heteroplasmy was highest in HbAA controls and lowest in HbAS mice, highlighting the complexity of mtDNA dynamics across tissues. Given the exploratory nature of the mouse study and limited sample size, tissue specific differences across genotypes would require validation in larger cohorts. The elevated splenic heteroplasmy observed in HbAA may be contributed by mouse-specific splenic environment, and splenic erythrophagocytosis process that may influence mtDNA dysfunction independent of disease genotype. Such fluctuations could be better attenuated in larger sample cohorts.

We also found that certain mitogenome regions consistently showed high mutation loads across genotypes in both humans and mice, indicating recurrent mutational hotspots. Several studies have demonstrated that these hotspots are more prone to accumulating mtDNA mutations, and are linked to disease prognosis, oxidative stress, and inflammation.[51-54]

This study, though limited by sample size, includes SCD patients, sickle carriers, and ethnically matched healthy controls from a single center. We are mindful that our results represent a global assessment of mtDNA defects, without specifying cell type contributions. In conclusion, our findings suggest that variations in mitochondrial genome integrity, such as alterations in mtDNA-CN and mtDNA heteroplasmy burden, could serve as potential biomarkers for SCD. A recent study in SCD mice[55] demonstrated increased clonal hematopoiesis and mutational burden with aging in SS mice, especially in the hematological malignancy related genes, underscoring their prognostic relevance in SCD. Likewise, mtDNA genome variations - including changes in mtDNA-CN, mtDNA heteroplasmy burden, and specific mtDNA variants could serve as biomarker of disease severity and accelerated aging in SCD, with a potential utility for assessing individual risk prognosis and inform clinical management. mtDNA alterations may serve as prognostic markers and reflect mitochondrial dysfunction in SCD progression. Elevated oxidative stress and ROS in SCD cause

cumulative mtDNA damage, impair mtDNA and mitochondrial function, and further increase ROS production, creating a vicious cycle that worsens disease severity. Future longitudinal studies are needed to clarify the mechanisms and rate of dysfunctional mtDNA accumulation, identify critical heteroplasmy thresholds, and determine how mtDNA hotspots are linked to SCD pathophysiology.

Disclosures

No conflicts of interest to disclose.

Contributions

SLT and LT conceived and designed the study. RR performed the mtDNA studies and data analyses in human and mouse samples. HL and SG provided statistical and bioinformatics support. NA provided updated survival data. KL provided support with mouse sample collection, and reviewing manuscript. MA and LT performed initial human mtDNA studies. XW provided support with mouse sample collection, laboratory experiments and nuclear variant analysis. YL provided support with NGS sequencing. CL, SK and ZMNQ provided SCD mouse support and mouse sample collection. CD performed whole-genome sequencing studies. JL provided support with bioinformatics. SLT supervised the study. RR wrote the first draft of the manuscript. SLT edited and wrote the manuscript. All authors reviewed the final manuscript.

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Artificial Intelligence (AI)- ChatGPT (OpenAI, San Francisco, CA, USA) assisted in constructing and debugging the R-based pipeline used for mitochondrial DNA deletion detection from whole genome sequence data. All AI-assisted code outputs were critically reviewed, verified, and validated by the authors for accuracy and scientific integrity.

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Data-sharing statement

Deidentified individual participant data in the reported results will be made available 3 months after publication for a period of 5 years after the publication date. Original data will be available from the corresponding author. Original data are available at dbgap.ncbi.nlm.nih.gov/home.

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