Increased mitochondrial DNA deletions and copy number in transfusion-dependent thalassemia

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Introduction

β-Thalassemia is caused by mutations that reduce β-globin chain synthesis, leading to a failure of hemoglobin production (1). Many patients are dependent on regular blood transfusions to avoid severe morbidity and premature mortality. Iron overload develops secondary to transfusions, elevating the level of nontransferrin bound iron (NTBI) (2) and labile iron that can catalyze the Fenton reaction, leading to widespread oxidative damage to lipids, proteins, and nucleic acids (3–6). Serial assessment of iron burden combined with effective chelation therapy are necessary to minimize the risk of organ damage from excess iron (7).

At the cellular level, mitochondria are a major target for iron toxicity, observed in cell culture and animal models as depression of respiratory enzymes (8) and damage to mitochondrial DNA (mtDNA) (8–11). The mitochondrial genome is a small (16569-bp) DNA molecule present in multiple copies in each mitochondrion. It has increased vulnerability to oxidative damage owing to its proximity to ROS produced within mitochondria (9, 12, 13). The common 4977-bp mitochondrial deletion (ΔmtDNA4977) increases in frequency with oxidative stress (14) and accumulates in tissues with aging, serving as a biomarker of mtDNA damage (15). The abundance of mtDNA in the cell, which can be assessed by the ratio of mtDNA
to nuclear DNA (nDNA) copies, is altered in response to physiological demands and pathological states (16–19).

The understanding of mitochondrial dysfunction in individuals with thalassemia is hampered by the difficulty in obtaining tissue samples for enzymatic or respiratory assays (20). Unlike these conventional tests of mitochondrial function, mtDNA changes can be analyzed on a relatively smaller number of cells (18). This led us to examine the occurrence and significance of mtDNA copy number and mutation in blood cells from individuals with transfusion-dependent thalassemia.

**Results**

**Subjects.** Individuals with transfusion-dependent thalassemia (n = 38) and healthy controls (n = 24) were enrolled in the study. The median age was 29.5 years (ranging from 4–53 years) for thalassemia group and 25.5 years (ranging from 19–46 years) for controls. The clinical characteristics of the thalassemia group are described in Table 1.

The liver iron burden was mild (liver iron concentration [LIC] < 7 mg/g dry weight) in 37%, moderate (LIC 7–15 mg/g dry weight) in 6%, and severe (LIC >15 mg/g dry weight) in 47% of the group. There were no subjects in congestive heart failure at the time of study, but myocardial iron was detected (T2* < 20 ms) in 31%, and a history of cardiac failure or arrhythmia was present in 24%. A majority (61%) of the subjects were splenectomized, and this group was significantly older (mean ±SD, 35.0 ± 8.3 years) than the nonsplenectomized subjects (21.5 ± 8.2 years, P < 0.001). Comorbidities from iron toxicity were frequent, as shown in Table 1.

**mtDNA alterations in thalassemia and control groups.** The mean mtDNA/nuclear DNA (Mt/N) (±SE) was 187 ± 18 mtDNA copies per nDNA copy in the thalassemia group, which was 41% greater than the control group (133 ± 7 mtDNA per nDNA, P = 0.026, Figure 2A). Mt/N increased with age in thalassemia (r = 0.45, P = 0.005, Figure 2A) but not in the control group. The frequency of Mt/N also increased with age in thalassemia (r = 0.48, P = 0.004), without a significant change observed in the control group. Subjects with thalassemia who were < 20 years of age (n = 4) had noticeably low levels of Mt/N (< 3.5 mg/g dry weight) compared with those with intact spleen (150 ± 14 mtDNA per nDNA), but the difference between the means was not significant (P = 0.23). Since the splenectomized subjects were also older than those with intact spleen, it was not possible to control for splenectomy when evaluating the effect of age

**Table 1. Characteristics of Thalassemia Subjects, n = 38.**

<table>
<thead>
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<th>n</th>
<th>±SD</th>
<th>Range</th>
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<tr>
<td>Age (years)</td>
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<td>4–53</td>
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<tr>
<td>Years of transfusions</td>
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<td>20.4 ± 11.5</td>
<td>2–50</td>
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<td>&lt;7 mg/g</td>
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<td>37 (37)</td>
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<td>&gt;15 mg/g</td>
<td>18</td>
<td>47 (47)</td>
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<tr>
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<td>24 (24)</td>
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<tr>
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<td>12</td>
<td>32 (32)</td>
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<tr>
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<td>21 (21)</td>
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<tr>
<td>Hypogonadism</td>
<td>12</td>
<td>41 (41)</td>
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<td>Glucose tolerance (n = 32)</td>
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<tr>
<td>Numbers in parentheses represent percentages.</td>
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**Figure 1. Mitochondrial DNA (mtDNA) in leukocytes from subjects with thalassemia shows specific alterations.** (A) Increase in mtDNA copy number (P = 0.026) and (B) the frequency of 4977-bp mitochondrial DNA deletion (ΔmtDNA4977) (P < 0.001) in thalassemia compared with controls. The plots show median, with box extending from the 25th to 75th percentile and the whiskers extending from 10th to 90th percentile values, while mean values are shown as “+” and outliers as open circles. Unpaired 2-tailed Student’s t test on log-transformed data was used for statistical analysis comparing group means. The analysis included 24 control and 36 thalassemia subjects in the analysis of mtDNA copy number (A), along with 19 control and 33 thalassemia subjects in the analysis of frequency of ΔmtDNA4977 (B).
on Mt/N. However, the correlation of Mt/N with age was observed independently in subjects with intact spleen (r = 0.58) and those splenectomized (r = 0.38). The effect of splenectomy on ΔmtDNA4977 was marked, with a mean 3.0-fold increase in the 22 subjects who were splenectomized compared with 13 subjects with intact spleen (P = 0.001, unpaired t test with Welch’s correction). Iron overload and splenectomy had an additive effect on the effect on ΔmtDNA4977 (Figure 3). NRBC count was higher in splenectomized subjects (5.41 × 10³/μl ± 1.64 × 10³/μl versus 0.11 × 10³/μl ± 0.05 × 10³/μl, but the association between NRBC count and ΔmtDNA4977 was weak (r = 0.31, P = 0.09). The 6 subjects with active viral hepatitis did not differ in either Mt/N or ΔmtDNA4977 from the rest of the thalassemia group.

Iron overload. No association was found between Mt/N and serum ferritin, liver iron concentration, or cardiac iron measurements. There was also no association between ΔmtDNA4977 and LIC or serum ferritin data for the whole thalassemia group. However, as shown in Figure 3, the frequency of ΔmtDNA4977 varied according to splenectomy status and LIC. Subjects who were splenectomized and severely iron overloaded (LIC > 15 mg/g) demonstrated the highest values of ΔmtDNA4977. The mean (97.1 ± 19.3/1 × 10⁷ mtDNA) and median (106.9/1 × 10⁷ mtDNA) were 2.6-fold and 7.6-fold higher for this group compared with subjects who were not splenectomized and had LIC < 15 mg/g (mean 37.7 ± 28.3 and median 13.9/1 × 10⁷ mtDNA). Using one-way ANOVA, the difference among the groups was significant (P = 0.003), and

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**Figure 2.** Mitochondrial DNA (mtDNA) alterations increase with age in thalassemia. Individual values for thalassemia are plotted showing splenectomized (triangles) and nonsplenectomized (circles) subjects. The line (solid black) illustrates the relationship between age and (A) mtDNA copy number (n = 38, r² = 0.20, P = 0.005) and (B) the frequency of 4977-bp mitochondrial DNA deletion (ΔmtDNA4977) (n = 35, r² = 0.23, P = 0.004) obtained using a linear regression model. The broken line represents corresponding regression models derived from the control group; the slope in both analyses is not significantly different from 0. The individual values are not shown for control subjects.

**Figure 3.** High liver iron concentration and splenectomy worsen mitochondrial DNA (mtDNA) damage. Subjects with thalassemia (n = 35) were placed into 4 groups according to liver iron concentration (LIC) below 15 mg/g (low) or above 15 mg/g (high) and whether they were splenectomized or not. Symbols represent mean (+) and median (diamond) for each group. The differences among the group means were significant (P = 0.003) using one-way ANOVA. Using Tukey’s multiple comparisons test, the mean differences were statistically significant between groups “b” and “c” (P = 0.022) and groups “b” and “d” (P = 0.010). A borderline significant difference was found between groups “a” and “d” (P = 0.056).
there was a linear trend toward higher \( \Delta \text{mtDNA}_{4977} \) with increasing risk factors (Figure 3, \( P = 0.003 \)). When ferritin (< 2500 ng/ml or \( \geq 2500 \) ng/ml) was substituted for LIC in this analysis, a similar increasing trend in \( \Delta \text{mtDNA}_{4977} \) was observed (\( P = 0.011 \), one-way ANOVA).

Myocardial iron overload. A significant association was present between \( \Delta \text{mtDNA}_{4977} \) and cardiac iron overload (Figure 4, \( P = 0.019 \)). This analysis excluded 4 subjects who had received less than 6 years of transfusions, considered inadequate time for cardiac iron loading (21). Myocardial iron (T2* < 20 ms) was present in 0%, 22%, and 46% of subjects with \( \Delta \text{mtDNA}_{4977} \) frequency < 20, 20–40, and > 40/1 \( \times \) 10⁷ mtDNA, respectively (\( P = 0.025 \), \( \chi^2 \) test). All subjects with current cardiac dysfunction or a history of cardiac dysfunction (\( n = 9 \)) had \( \Delta \text{mtDNA}_{4977} > 20/1 \times 10^7 \) mtDNA.

mtDNA changes and glucose metabolism. Mt/N or \( \Delta \text{mtDNA}_{4977} \) values did not differ between the normal glucose tolerance, impaired glucose tolerance, and diabetic groups. Subjects with normal glucose tolerance or impaired glucose tolerance (\( n = 21 \), after excluding those with diabetes) were grouped according to Mt/N values above or below the median (189 mtDNA/nDNA). In this analysis, 5/10 subjects with low Mt/N had impaired glucose tolerance compared with 2/11 with high Mt/N (\( P = 0.18 \), Fisher’s exact test).

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**Figure 4.** Myocardial iron overload was linked with greater mitochondrial DNA (mtDNA) damage. (A) The association between cardiac MRI T2* in subjects on transfusions > 6 years and the 4977-bp mitochondrial DNA deletion (\( \Delta \text{mtDNA}_{4977} \)) was significant (\( n = 29 \), Pearson \( r = -0.43 \), \( P = 0.019 \)). All subjects with current or history of iron-induced cardiac dysfunction (solid symbols) had \( > 20 \Delta \text{mtDNA}_{4977} \) per 1 \( \times \) 10⁷ mtDNA copies. (B) The proportion of patients with MRI T2* < 20 milliseconds significantly increased with a higher frequency of \( \Delta \text{mtDNA}_{4977} \) when the 3 groups were compared using \( \chi^2 \) test for trend (\( n = 29 \), \( P = 0.025 \)).

**Figure 5.** Low mitochondrial DNA (mtDNA) copy number was associated with impaired glucose metabolism. (A) We used a 2-way repeated-measures ANOVA followed by Fisher’s least significant difference test to compare the group means over time. Data are presented as mean and ±SEM. No significant interaction between time and group was found. Subjects with mtDNA copy number below the median (solid symbols) had higher plasma glucose at baseline and at all time points following 75 g oral glucose challenge (\( P = 0.079 \)). (B) Matsuda insulin sensitivity index was significantly correlated with mtDNA copy number with Pearson \( r = 0.47 \) (\( P = 0.031 \)). There were 21 subjects included in both analyses.
The mean plasma glucose concentration was higher at all time points in the low Mt/N group during oral glucose tolerance test (OGTT) \((P = 0.079, \text{Figure 5A})\). The indices of insulin resistance were also influenced by Mt/N group assignment. In those with low Mt/N, the mean insulin sensitivity index (ISI) was 37% lower \((5.76 \pm 0.53 \text{ vs. } 9.11 \pm 0.95, P = 0.008)\), while mean homeostatic model assessment of insulin resistance (HOMA-IR) was 76% higher \((1.98 \pm 0.26 \text{ vs. } 1.12 \pm 0.16, P = 0.010)\). Correlation analysis showed a significant positive association between ISI and Mt/N \((P = 0.031, \text{Figure 5B})\). The indices of \(\beta\) cell function, insulin secretion at 30 minutes, or the glucose disposition index did not have a significant association with Mt/N.

**Discussion**

This study demonstrates the presence of marked alterations in mtDNA in blood cells of individuals with transfusion-dependent thalassemia. These changes in mtDNA were modified by iron overload and splenectomy status and were predictive of cardiac iron burden and abnormal glucose metabolism.

The increase in Mt/N observed in thalassemia is likely to be caused by iron-induced oxidative stress (3). Mt/N is an estimate of the number of mtDNA in a cell, which is tightly regulated through replication and mitophagy (22) under the influence of multiple signals from energy metabolism and the cell cycle (23). Mt/N is altered in pathological states such as inherited mtDNA mutations and is affected by the
Leukocytes from healthy individuals with elevated markers of oxidative stress show an increase in Mt/N (18). This increase in mitochondrial content is regarded as an adaptation to oxidant stress (23, 25) that is compromising cellular respiration. Thalassemia is associated with a marked pro-oxidant environment (3, 26), where the labile plasma iron (27) induces formation of ROS, to which the leukocytes are exposed. The increase in Mt/N with age in this study suggests that the duration of exposure to ROS may be a significant factor in inducing these changes. Mt/N also increases with age in healthy individuals (18) but by a much smaller magnitude (20%) compared with the nearly 2.5-fold rise observed in thalassemia. In contrast, individuals with inherited mitochondrial disorders demonstrate a decline in Mt/N in blood cells with increasing age (28).

The appearance of somatic mtDNA mutations secondary to oxidative damage, such as the ΔmtDNA<sup>4977</sup> deletion that removes several mitochondrial genes encoding for transfer RNAs (tRNAs) and respiratory complex subunits, is an expected consequence of aging (15, 29). Tissues with high energy demands and limited renewal capacity (muscle and brain cells) accumulate ΔmtDNA<sup>4977</sup> to a greater extent (15, 30), whereas leukocytes from healthy individuals do not show an age-dependent rise in ΔmtDNA<sup>4977</sup> (30). In our study population, a marked increase in ΔmtDNA<sup>4977</sup> was seen in adults with thalassemia. The paucity of ΔmtDNA<sup>4977</sup> in patients younger than 20 years was remarkable, and this threshold effect of age on the appearance of mtDNA damage may reflect poor control of systemic iron during adolescence. However, the frequency of ΔmtDNA<sup>4977</sup>, even among older subjects, remained well below the threshold of 90% abnormal mitochondrial genomes that starts to compromise oxidative phosphorylation (16). Other oxidative stress–induced mtDNA lesions that were not measured in this study may add to the loss of transcriptional efficiency (31) and may accumulate in tissues with low mitotic activity (endocrine cells and cardiomyocytes) to detrimental levels.

Splenectomy may play a role in increasing the extent of mtDNA damage in thalassemia. We observed splenectomy and iron overload to have an additive effect on increasing the frequency of ΔmtDNA<sup>4977</sup>. The distribution of iron in the body is altered by splenectomy, likely from a decrease in the iron storage capacity in the macrophage compartment (32). NTBI and extrahepatic iron were reported to be higher in splenectomized subjects with iron overload (33–35), which would increase the exposure of blood and BM cells to toxic iron species. Thus, mtDNA damage may be a surrogate marker for NTBI. While NTBI was not measured in this study, the significant association between ΔmtDNA<sup>4977</sup> and myocardial iron supports the hypothesis that mtDNA damage in leukocytes could be useful to identify and follow patients at risk of extrahepatic iron deposition. In contrast, while higher NTBI levels were reported in patients with clinical cardiac dysfunction (36), there are no data that directly link NTBI with degree of myocardial iron overload (37) or endocrine damage (36). This may be from the susceptibility of NTBI to vary with concurrent factors such as inflammation (13) or change rapidly with chelation therapy (38, 39). Thus, frequency of ΔmtDNA<sup>4977</sup> is the first biomarker discovered to be associated with myocardial iron overload in thalassemia.

We observed no relationship between ΔmtDNA<sup>4977</sup> and Mt/N, despite the fact that both of these abnormalities increased with age in thalassemia. In a previous study, healthy adults with detectable leukocyte ΔmtDNA<sup>4977</sup> had 5% higher Mt/N compared with those without the deletion (18). Our sample size was small to pick up this degree of difference, though a positive trend between Mt/N and ΔmtDNA<sup>4977</sup> was observed in the control group and thalassemia subjects with intact spleen. This relationship between mtDNA abundance and deletion frequency was reversed in the splenectomized group. We believe that other coexisting mtDNA lesions arising from oxidative stress could compromise replication of mtDNA (40). The exposure of cells to oxidant damage leads to rapid mutations in the displacement loop (D-loop) of mtDNA (41), a noncoding region critical for replication and transcription. A lack of compensatory increase in mitochondrial number among those with high ΔmtDNA<sup>4977</sup> in thalassemia could amplify the deleterious impact of heteroplasmy on cellular energy metabolism.

Iron overload in thalassemia is associated with early occurrence of insulin resistance with subsequent pancreatic damage and insulin deficiency leading to diabetes mellitus (42). HOMA-IR is a measure of hepatocyte sensitivity to insulin in the fasting state, while ISI measures whole-body insulin sensitivity following a glucose load (43). We found that subjects with thalassemia who had low Mt/N had insulin resistance by both of these indices. Since Mt/N increased with age in our study, as does the prevalence of impaired glucose tolerance (42), this inverse correlation was unexpected. However, adults with metabolic syndrome with insulin resistance were reported to have lower mitochondrial content in leukocytes (17).
While this study is limited by its cross-sectional design, we describe a new marker of mitochondrial damage in thalassemia that can be easily followed over time in clinical samples and can predict the risk of extrahepatic iron deposition. We propose the mtDNA damage to be secondary to oxidative stress from uncontrolled NTBI. With current technology, tracking of mtDNA damage over time may prove to be a more stable measure than NTBI. However, longitudinal studies are necessary to understand the influence of NTBI on mtDNA. It would be equally important to assess the reversibility of mtDNA damage in leukocytes with improvement in systemic iron overload. The possible link between mtDNA damage in leukocytes and mitochondria in the target organs for iron damage — liver, pancreas, pituitary, and heart — is unknown at this stage.

In conclusion, mtDNA alterations were prominent in individuals with thalassemia and had important associations with liver and cardiac iron, glucose metabolism, and splenectomy. mtDNA damage may reflect the chronic or intermittent exposure of cells to NTBI. Because of the ease of obtaining blood samples, the assessment of mtDNA damage in leukocytes may aid in the optimal management of iron overload in thalassemia.

Methods
Adults and children with transfusion-dependent thalassemia were recruited from the Thalassemia Clinic at UCSF Benioff Children’s Hospital Oakland between May 2011 and May 2013, along with healthy adults as controls. The participants who were excluded from analysis are shown in Figure 6. Clinical variables were collected from the clinical database. Liver iron concentration was measured with SQUID Ferritometer (44), and results were expressed per dry liver weight (45). Myocardial iron was measured with MRI T2* (46). Oral glucose tolerance test was performed after an overnight fast with 1.75 g/kg dextrose (up to 75 g) accompanied by blood sampling for plasma glucose and insulin at 0, 30, 60, and 120 minutes. Blood samples were drawn within 3 days of the next blood transfusions, and total DNA was isolated from 200 µl of whole peripheral blood using the QIAamp DNA Mini-Kit (Qiagen), with quality and quantity assessed by spectrophotometry and Qubit dsDNA HS Assay Kit (Invitrogen).

Mt/N assessment. Mt/N was quantified with an established duplex qPCR assay (47). Two adjustments were made to optimize the assay for analysis of whole blood samples. The final mitochondrial primer concentration was increased from 50–100 nM to improve the robustness of the assay. Also, in place of the commercial real-time PCR master mix, we used 10 µl of a custom mix with reagents at the following final concentrations: 250 mU/µl of AmpliTaq Gold (Roche Diagnostics), 200 µmol/l of each dNTP (Roche Diagnostics), 1× PCR Buffer II (Roche Diagnostics), 2.5 mmol/l MgCl₂ (Roche Diagnostics), 1× ROX dye (Invitrogen), and 4.2 µl molecular grade water. Samples were diluted to a DNA concentration of 1 ng/µl, with 4 ng analyzed per run. Each sample was run in triplicate. The DNA standard used was a prequantified, high molecular weight, human genomic DNA extract (G1471, Promega) diluted serially (1:1) from 26–0.2 ng. The 8 DNA standards were then run in triplicate to generate the standard curve used for absolute quantification. The ratio of one haploid nuclear copy per 3.3 pg of genomic DNA was used to calculate nDNA copy numbers (47). mtDNA copy numbers were calculated using a ratio of 200 mitochondrial copies per nuclear copy. This ratio was estimated from the average ΔCT between the mitochondrial and nuclear reactions from each dilution of the standard.

Relationship between blood cell count and mtDNA changes. The recent decline of splenectomy in the management of thalassemia led to many older subjects in this study who were splenectomized, while the younger were not. Leukocyte and platelet counts were higher with splenectomy and varied considerably, even within those with intact spleen. DNA from whole blood is a mixture of nDNA (derived from leukocytes and nucleated red blood cells) and mtDNA (derived in addition from reticulocytes and platelets). Variability in platelet count between subjects and the number of residual platelets following separation of peripheral blood mononuclear cells from whole blood are sources of error in real-time PCR quantification of mtDNA content (48). While we were able to achieve platelet-free PBMC separation using density centrifugation with sucrose gradient, characterization by flow cytometry revealed the continued presence of leukocyte-platelet conjugates (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/jci.insight.88150DS1) (49). In order to correct for the mtDNA contribution in measured Mt/N, the platelet contribution to Mt/N in whole blood was excluded using complete blood counts according to the following formula:
Corrected Mt/N = Measured Mt/N – [(platelet count/WBC count) × 4.1/2]

The factor of 4.1 is the average mtDNA copies per platelet based upon 40% contribution of platelet mtDNA to the measured Mt/N (48), which is approximately the same as the 4 mtDNA per platelet reported by Shuster, et al. (50); 2 is the correction for diploid nDNA in WBCs.

$\Delta mtDNA_{4977}$ frequency assessment. The primers and probe-targeting $\Delta mtDNA_{4977}$ described by Pogozelski, et al. (14), were used in a singleplex qPCR assay. qPCR conditions for amplification were the following: initial denaturation at 95°C for 10 minutes; 45 cycles of denaturation at 95°C for 15 seconds, and annealing and elongation at 60°C for 1 minute. The qPCR master mix contained 250 μmol/l of each primer, 200 μmol/l of the probe, 250 μU/μl of AmpliTaq Gold (Roche Diagnostics), 200 μmol/l of each dNTP (Roche Diagnostics), 1× PCR Buffer II (Roche Diagnostics), 2.5 mmol/l MgCl$_2$ (Roche Diagnostics), and 1× ROX dye (Invitrogen). TE-4 buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0; MO BIO Laboratories) was added as needed to achieve a total reaction volume of 20 μl per run. Ten million copies of the mitochondrial genome were analyzed per run. Each sample was run in triplicate. The deletion standard was generated from a sample confirmed with Sanger sequencing to contain the mtDNA$_{4977}$ deletion as follows. Amplicons were generated using primers flanking the deletion, and the deletion product was isolated using gel purification. Quantity was assessed with the Agilent DNA 1000 Kit (Agilent Technologies). Amplicon amounts of 5 million; 500,000; 50,000; 5,000; and 500 copies were run in duplicate, and amounts of 50, 10, 5, and 1 copy were run in sextuplets to generate the standard curve used for absolute quantification. The $\Delta mtDNA_{4977}$ frequency was calculated from the combined contribution from platelets and leukocytes. Results were not available for 3 subjects with thalassemia and 5 controls due to diluted DNA concentration.

Statistics. Descriptive statistics were computed for each subgroup including means and measures of variability. Values for Mt/N, $\Delta mtDNA_{4977}$, and myocardial MRI T2* were log transformed prior to analysis, since the measures had a skewed distribution. Means were compared using the 2-tailed Student’s t test or ANOVA models. Linear regression analyses and Pearson correlations were calculated to determine the association between mtDNA assay results and clinical variables. $P < 0.05$ was considered statistically significant. Analyses were performed using Prism 6.0a (GraphPad Software).

Study Approval. The study was approved by the IRB of UCSF Benioff Children’s Hospital Oakland. A written informed consent was obtained from all subjects prior to participation in the study.

Author contributions
AL and CC designed the research; EG and CC performed and interpreted the experiments; AL provided patients; and AL and EG collected and interpreted clinical data. All authors contributed to writing and reviewing the manuscript.

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