Depletion of mitochondrial DNA in leukocytes harboring the 3243A>G mtDNA mutation

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ABSTRACT

**Background:** The 3243A>G MTTL1 mutation is the most common heteroplasmic mitochondrial DNA (mtDNA) mutation associated with disease. Previous studies have shown that the percentage level of mutated mtDNA decreases in blood as patients get older, but the mechanisms behind this remain unclear.

**Objectives:** To understand the dynamics of the process and the underlying mechanisms, we established an accurate fluorescent assay for 3243A>G heteroplasmy and determined the amount of mtDNA in blood with real-time PCR. We measured the amount of mutated and wild-type mtDNA at two time points in eleven subjects.

**Results:** Our observations indicate that the percentage level of mutated mtDNA decreases exponentially during life, and that peripheral blood leukocytes in patients harboring 3243A>G are profoundly depleted of mtDNA.

**Conclusions:** A similar decrease in mtDNA levels has been seen in other mitochondrial disorders, and in 3243A>G cell lines in culture, indicating that mtDNA depletion may be a common secondary phenomenon in a number of mitochondrial diseases. MtDNA depletion is not always due to mutation of a nuclear gene involved in mtDNA maintenance.

**KEY POINTS**

- The percentage level of the 3243A>G MTTL1 mutation in blood decreases exponentially during life.
- Peripheral blood leukocytes in subjects harboring 3243A>G are profoundly depleted of mitochondrial DNA (mtDNA).
- MtDNA depletion is not always due to mutation of a nuclear gene involved in mtDNA maintenance.
INTRODUCTION
The 3243A>G MTTL1 gene mutation of mitochondrial DNA (mtDNA) is the most common heteroplasmic pathogenic mtDNA mutation and is found in ~1 in 6000 of the general population.\(^1\) Although first described in mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS), the phenotypic spectrum is extremely diverse, including isolated diabetes and deafness, hypertrophic cardiomyopathy and retinitis pigmentosa.\(^2\) The clinical variability can be explained, in part, by tissue-specific differences in the percentage level of mutated mtDNA.\(^3,4\)

Intriguingly the percentage of mutated mtDNA is consistently lower in peripheral blood than in post-mitotic tissues such as skeletal muscle and brain.\(^3,5\) Serial measurements in the same subject have shown that the percentage level of the 3243A>G mutation in blood decreases over time,\(^6,7\) but the reasons for this are not clear. One possibility is that vegetative segregation in rapidly proliferating leukocyte precursors leads to high percentage levels of mutated mtDNA in some cells. This causes a biochemical defect of the respiratory chain, which either impairs the further proliferation of that cell lineage or leads to cell death.\(^7\) This would ultimately lead to a decrease in the percentage level of mutated mtDNA in the daughter cells present in the peripheral blood. However it is currently not known whether the biochemical defect is primarily due to high amounts of mutated mtDNA,\(^8\) low amounts of wild-type mtDNA \(^9\) or a combination of both.

To advance our understanding of this process we developed and validated a highly sensitive fluorescent assay to measure the changes in heteroplasmy over time, and also measured the absolute amount of mutated and wild-type mtDNA in eleven subjects known to harbor 3243A>G.
METHODS

Patients and control samples
Sequential venous blood samples between 2.0 and 7.8 years apart were collected from eleven subjects known to harbor the 3243A>G mtDNA mutation (5 males and 6 females, Table 1), and single blood samples were taken from 10 healthy controls. For all of the samples, the peripheral blood leukocyte and platelet counts were within the normal range, with a normal proportion of each leukocyte subset. Skeletal muscle biopsies were available for nine of the eleven 3243A>G subjects and six healthy controls for comparison. Total genomic DNA was extracted using a standard procedure.

Establishing an accurate fluorescent 3243A>G heteroplasmy assay
A range of heteroplasmic samples were generated by mixing mutated and wild-type cloned fragments containing nt3243 generated from two siblings, one with high percentage 3243A>G and one with low percentage 3243A>G, but who otherwise had an identical mtDNA sequence. Genomic DNA from each sibling was amplified across the region containing the MTTL1 gene and cloned using the pGEM®-T Easy vector system (Promega), according to manufacturer’s instructions. Plasmids from transformed JM109 competent E. coli cells were purified using the Qiaprep spin mini prep kit (Qiagen) and quantified by UV spectrophotometry (MODEL, Eppendorf). Mutated and wild-type cloned DNA was mixed to generate heteroplasmic samples of 0, 5, 10, 25 and 50%.

The percentage level of mutated mtDNA was determined in triplicate using the gold-standard technique of last cycle hot PCR. A 154-bp region encompassing the MTTL1 gene was amplified using standard cycling conditions and the following primers: nt 3200-3218 forward, nt 3353-3334 reverse. After the addition of 5µCi [α-32P] dCTP (3000Ci/mmol) to the last PCR cycle, products were precipitated and digested at 37°C with HaeIII (10U, New England Biolabs). Restriction fragments were separated through a 12% non-denaturing polyacrylamide gel, dried onto a support and exposed to a PhosphorImager cassette. The level of mtDNA heteroplasmy was quantified using ImageQuant TL software (Amersham Biosciences) 10.

The last cycle hot PCR method was adapted for a fluorescent analyzer (Beckman Coulter CEQ 8000) using modified primers (nt 3155-3171 forward, nt 3353-3334 reverse) and optimized PCR conditions. Fluorescent labeled forward primer was added in the last PCR cycle instead of the radioactive label, followed by HaeIII digestion (10U, New England Biolabs). Serial dilutions (in water) of the last cycle fluorescent-PCR RFLP products were analyzed in triplicate using a Beckman Coulter CEQ 8000 fluorescent DNA fragment analyzer and fragment analysis software (version 8.0).

Real-time PCR
The total amount of mtDNA (referred to as the mtDNA copy number) in homogenate DNA samples from patients and control subjects was determined by real-time PCR using iQ Sybr Green on the BioRad ICycler (BioRad, CA) to a target template spanning from nt 3459 to nt 3569 of the mtDNA ND1 gene. This was normalized using a nuclear-encoded template for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene spanning from nt 804 to nt 903 (NCBI, NM_002046.2). Relative copy number was calculated from the threshold cycle value, ΔCt value, where the mean amount of mtDNA / cell = 2(²ΔCt), to account for the two copies of GAPDH in each cell nucleus. Each reaction was optimized and confirmed linear over an appropriate
concentration range using genomic DNA standards. Samples were analyzed in triplicate for both assays, enabling calculation of the average mtDNA:nDNA ratio. Since the overwhelming majority of blood mtDNA is present in the leukocyte population, this assay provides a good estimation of the average amount of mtDNA within the blood leukocyte population.

**Statistical analysis**
Statistical analyses were carried out using Minitab (v14), including correlations analysis with Spearman’s correlation co-efficient ($R^2$), and two-sample t-tests for the comparison of the amount of mtDNA.
RESULTS

Comparison of the radioactive and fluorescent last cycle assays
The last cycle hot PCR and last cycle fluorescent PCR methods were compared directly by analyzing the mixed cloned DNA. Each assay was performed in triplicate from the mixed clone templates. Fluorescent labeled products can be analyzed in two ways: measuring the area under the curve, or measuring the peak height amplitude. Both measurements are likely to be reliable when the signal to noise ratio is high, but the point at which they become unreliable has not been formally studied. We therefore compared both measurements over a range of dilutions to the current gold standard technique of last cycle hot PCR-RFLP (Fig. 1).

In each case there was a strong correlation between the different measures ($R^2 > 0.95$), but the undiluted and 1:2 dilution of the last cycle fluorescent labeled assay had the strongest correlation with the last cycle hot assay (Fig. 1). For greater dilutions, the last cycle fluorescent assay showed the most variation at lower percentage levels of heteroplasmy. There was no obvious difference between the “area under the curve” or “peak height amplitude” methods.

Sequential blood heteroplasmy quantification
The percentage of mutated mtDNA was determined in each sample from the eleven subjects harboring 3243A>G using the last cycle fluorescent PCR-RFLP. Measurements were made from the original genomic DNA sample on three independent occasions. The last cycle fluorescent PCR labeled Hae III digestion products were not diluted. In each subject the percentage 3243A>G decreased over time (Fig. 2). The overall mean change was a decrease of 0.6% / year (SD = 0.5).

MtDNA copy number in leukocytes.
The mean mtDNA copy number in control blood leukocytes was 370.1 (SD = 61.9, Fig. 3, Table 1). All of the samples from 3243A>G subjects had low levels of mtDNA (mean for sample set 1 = 69.8, SD = 25.4, 2-sample t-test comparison with controls $t = -14.28, P < 0.001$; mean for sample set 2 = 98.0, SD = 52.8, 2-sample t-test comparison with controls $t = -10.79, P < 0.001$). In eight of the 3243A>G subjects, the mean amount of mtDNA per leukocyte increased over time. In three subjects, the mean amount of mtDNA per leukocyte decreased over time. The overall mean change was an increase in the mean amount of mtDNA per leukocyte (4.4 molecules / cell / yr; SD = 7.6, Fig. 4). There was no clear correlation between the total amount of mtDNA and the age of the subjects, the initial percentage level of mutated mtDNA, nor the absolute amounts of mutated and wild-type mtDNA.

Skeletal muscle mtDNA.
In each patient, the percentage 3243A>G was greater in muscle than in blood. The amount of mtDNA / nucleus was greater in the skeletal muscle of 3243A>G subjects than controls (supplementary table 1 on line).
DISCUSSION

A number of conclusions can be drawn from this work. On the technical side, we have shown that the addition of a fluorescent labeled oligonucleotide primer in the last PCR cycle enables the accurate quantification of mtDNA heteroplasmy. Although the strongest correlation was between the undiluted and 1:2 diluted fluorescent products and the last cycle hot-PCR, all of the assays correlated well with an \( R^2 > 0.95 \). We were surprised to find that the serial dilution of the fluorescent PCR products did not have a dramatic effect on the assay. This probably reflects the automated electrostatic injection methods used by most capillary-based DNA analyzers, which load a fixed amount of labeled DNA rather than a fixed volume of solution. It is possible that different results would be obtained for manually-loaded gel-based systems. Arguably, the reproducibility of the fluorescent assay exceeds that of last cycle hot PCR (see the \( \sim 50\% \) 3243A\( \rightarrow \)G data in Fig.1), and given the inherent risks of using radiochemicals, last cycle fluorescent analysis should now be the benchmark for measuring mtDNA heteroplasmy.

Using the last cycle fluorescent PCR we have confirmed that the percentage level of 3243A\( \rightarrow \)G decreases in blood over time. The mean percentage decrease of 0.6% / year corresponds well with the largest previous study (0.69% / year; SD = 0.61).\(^6\) In our study (Fig.2) the greatest rate of decrease in the percentage level of mutated mtDNA was seen in the younger subjects, consistent with an exponential loss of 3243A\( \rightarrow \)G from the peripheral blood. With this in mind, reporting mean values for the percentage decrease could be misleading, and explains why the mean value in our adult study was significantly less than was found in one study of six subjects (1.12% / year; SD = 0.65)\(^7\) which predominantly involved observations made over childhood (2-sample t-test comparing the data in Table 1 with the data in \(^7\): t=3.53, P = 0.004).

The most striking novel finding is the profound depletion of mtDNA in blood leukocytes in patients harboring this mutation. Our control data for the mean mtDNA copy number / leukocyte is remarkably similar to other published values based on real time PCR and other techniques,\(^11,12\) confirming that we were using a reliable assay. How can we therefore explain these observations? Given that the peripheral blood count was normal in each case, it is unlikely that a change in the number of cell nuclei or cell types accounts for the profound depletion we have observed. From first principles, the mtDNA depletion could be due to decreased rates of mtDNA synthesis relative to the intense cellular proliferation in leukocyte precursors. An alternative (and non-exclusive) explanation is an increased loss of mtDNA from the precursors of the mature cells.

The peripheral leukocyte population is continuously recycled. Neutrophils are the largest single component (up to 80% of circulating leukocytes), maturing in the bone marrow for 11-12 days before being released into the circulation where they remain for 6-8 hours.\(^13\) The entire neutrophil population in the peripheral blood (\( \sim 5 \times 10^{10} \) cells) is therefore replenished on a daily basis, requiring a massive amount of mtDNA replication to maintain mtDNA levels within normal limits. This is sustained by a limited number of stem cells (1 in \( 10^4 \) to 1 in \( 10^5 \) marrow cells), which turn over at a low rate and repopulate the blood through a hierarchical cascade of cell divisions.\(^14\) It is therefore far more likely that any effect on mtDNA replication would become manifest during the massive expansion of daughter cells leading to the formation of mature leukocytes, rather than in the stem cell population itself. Even a subtle decrease in the replication rate could lead to the dramatic depletion that we
have observed in the peripheral blood. For example, if the mtDNA replication rate was a fraction $f$ of the normal replication rate, then after a series of $n$ cell divisions the leukocyte precursors would be depleted by a factor $f^n$. Twenty cell divisions (the number required for a million-fold increase in cells) would give our observed depletion to 23\% of the normal level with $f = 0.93$.

Could the bioenergetic defect be responsible for a relative decrease in rate of mtDNA synthesis in the leukocyte precursors? Whilst plausible, this explanation is difficult to reconcile with the increased amount of mtDNA seen in post-mitotic tissues of patients harboring 3243A>G.\textsuperscript{15} In addition, if the loss of mtDNA were directly related to a defect of ATP synthesis, we would expect a closer relationship between the percentage level of mutated mtDNA and the degree of depletion. This was clearly not the case - for example, low amounts of mtDNA were found in a young subject with $\sim 50\%$ 3243A>G in blood and an older subject with $\sim 5\%$ 3243A>G in blood (Figs 2 & 3). Recent \textit{in vitro} studies of human NT2 teratocarcinoma cybrids harboring 3243A>G (NT2.3243 cybrids) cast light on this issue.\textsuperscript{16} High percentage levels of 3243A>G were associated with a profound loss of mtDNA from the NT2.3243 cybrids. This appeared to be mutation specific as the same depletion was not seen in NT2 cybrids homoplasmic for the 1555A>G mtDNA mutation. Moreover, the presence of a known suppressor mutation (12300G>A) which ameliorates the biochemical effects of 3243A>G at high levels, did not prevent the loss of mtDNA from the cybrid cell lines, indicating that the biochemical defect is not the principal factor behind the depletion. Remarkably, not all the cell lines with $\sim 99\%$ 3243A>G lost their mtDNA, proving that the percentage level of mutated mtDNA is not an absolute determinant of the depletion. Further work showed slow rates of segregation of the 3243A>G mutation in NT2.3243 cybrids, providing indirect evidence that functional or physical partitioning of mtDNA molecules contributes to the segregation process. These observations highlight the complex interplay between intercellular and intracellular signals that are involved in the mitotic segregation of the 3243A>G mutation.\textsuperscript{16} Similar mechanisms may be involved in rapidly dividing tissues \textit{in vivo} including the leukocyte population. It is intriguing that, as the percentage of mutated mtDNA in blood decreases in patients harboring 3243A>G (Fig. 2), there is overall increase in the amount of mtDNA in blood, and particularly the wild-type (Fig. 4). This suggests a degree of recovery from the depletion as time passes, although the amount never reaches normal values.

An alternative explanation for the loss of one mtDNA genotype in blood is through immune surveillance mediated through an abnormal cell surface epitope. Support for this hypothesis comes from the description of a complex I (ND) gene variant in mice that forms a maternally transmitted major histocompatibility antigen.\textsuperscript{17} However, recent work in heteroplasmic mice has shown that immune-mediated cell loss does not occur in heteroplasmic mice with the C57B/BALB genotype, and nuclear genes may be important.\textsuperscript{18}

Finally, the depletion may be a secondary phenomenon as in other mitochondrial disorders. Mutations in \textit{OPA1} cause the most frequent form of autosomal dominant optic atrophy. In a recent study, patients with \textit{OPA1} mutations had significantly lower levels of mtDNA in leukocytes than control subjects.\textsuperscript{11} Given the central role of OPA1 in maintaining the structural integrity of mitochondria and mtDNA, it was suggested that the primary genetic defect led to the loss of both mitochondria and mitochondrial genomes. A similar structural disintegration of the mitochondrial network has been seen in patients harboring primary mtDNA defects, potentially explaining the observations we report here. By contrast, one study
described a mild increase in the amount of mtDNA in leukocytes from subjects harboring mtDNA mutations that cause Leber hereditary optic neuropathy (LHON).\textsuperscript{19} However, in contrast to the 3243A>G mutation, the biochemical defect in LHON is mild and sometimes undetectable.\textsuperscript{20,21} Further work will determine whether mtDNA depletion is seen in other mtDNA disorders.

If the 3243A>G mutation is causing depletion through an effect on the structural integrity of mitochondria in leukocytes, why does this not occur in non-dividing tissues such as skeletal muscle, where there tends to be a proliferation of mitochondria and mtDNA (supplementary table 1 on line)? At present we can only speculate, but recent work in our laboratory has shown that, although initially the amount of skeletal muscle mtDNA is high of patients with mtDNA mutations, there is a progressive loss of mtDNA over time.\textsuperscript{22} In part, this could be due to muscle deconditioning, but it may be due to a more general process related to mitochondrial fragility. These findings have important implications for the molecular diagnosis of mitochondrial disorders – the presence of depletion may be a secondary phenomenon and the primary molecular defect may be a mtDNA mutation or a nuclear gene mutation not directly involved in the synthesis of mtDNA.
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REFERENCES


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Figure Legends

Figure 1
Comparison of the last cycle fluorescent PCR-RFLP method with the last cycle hot PCR-RFLP method to quantify the percentage level of 3243A>G mutation in mixed clones (see methods). Y-axis: percentage 3243A>G mutation determined from the relative fluorescence units (+/- SD from 3 independent measurements) of either the peak height amplitude (left hand graphs) or the area under the curve. X-axis: percentage 3243A>G mutation determined by last cycle hot PCR (+/- SD from 3 independent measurements). Each pair of graphs shows the results from the serial dilution (from undiluted to 1 in 32 in water) of the last cycle fluorescent PCR-RFLP products run independently on the fluorescent genetic analyzer in triplicate R^2 = Spearman correlation co-efficient using the individuals data points (i.e. not the mean values).

Figure 2
Relationship between the percentage level of the 3243A>G mtDNA mutation in blood and age in years for eleven patients measured by last cycle fluorescent PCR. Each patient has a different symbol and color, with serial measurements on the same patient connected by a straight line. PCR products were not diluted before loading.

Figure 3
Total number of mtDNA molecules/cell in ten healthy control subjects and eleven patients with the 3243A>G mutation. Serial measurements are represented by timepoint 1 and timepoint 2. Total mtDNA (mtDNA copy number) was determined by real-time PCR using iQ Sybr Green on the BioRad ICycler. Each data point represents the mean of three independent measurements from the original genomic DNA sample. Solid horizontal lines represent the mean value for each data set.

Figure 4
Mean amount of wild-type mtDNA in eleven patients with the 3243A>G mutation on two occasions. Y-axis: Wild-type mtDNA (mtDNA copy number) was determined by real-time PCR in triplicate using iQ Sybr Green on the BioRad ICycler to determine the total amount of mtDNA. The wild-type mtDNA was calculated from the mean percentage level determined by last cycle fluorescent PCR shown in figure 2. X-axis: age of the subject in years at the time of sampling. Each patient has a different symbol and color, with the serial measurements connected by a straight line and using the same color code as Fig. 2.
Table Legends

Table 1 Percentage 3243A>G mutation and the mean amount of mtDNA (copy number) in peripheral blood for eleven patients.

Mean percentage mutated mtDNA was determined by last cycle fluorescent PCR (undiluted, mean of three measurements from the genomic sample). Copy number measurements were determined by real-time PCR using iQ Sybr Green on the BioRad ICycler (mean of three measurements from the genomic sample). mtDNA: using a target template spanning from nt 3459 to nt 3569 of the mtDNA NDI gene. Nuclear DNA (nDNA) using a target template spanning from nt 804 to nt 903 of the single-copy nuclear gene GAPDH. Wild-type and mutated mtDNA copy numbers were determined from the mean total mtDNA copy number per cell, and the mean percentage mutated mtDNA $\Delta$Ct = difference between the threshold cycles for nDNA and mtDNA. Relative copy number was calculated from the $\Delta$Ct value, $2(2^{-\Delta\text{Ct}})$, to account for the two copies of GAPDH in each mononuclear peripheral blood cell. SD = standard deviation.
Table 1 Percentage 3243A>G mutation and the mean amount of mtDNA (copy number) in peripheral blood for eleven patients.

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<th>Age at sample (years)</th>
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Supplementary table 1 on line.
The mean amount of mtDNA (copy number) in peripheral blood for ten control subjects patients.

Copy number measurements were determined by real-time PCR using iQ Sybr Green on the BioRad ICycler (mean of three measurements from the genomic sample). mtDNA: using a target template spanning from nt 3459 to nt 3569 of the mtDNA ND1 gene. Nuclear DNA (nDNA) using a target template spanning from nt 804 to nt 903 of the single-copy nuclear gene GAPDH. The mean percentage mutated mtDNA $\Delta Ct = \text{difference between the threshold cycles for nDNA and mtDNA}$. Relative copy number was calculated from the $\Delta Ct$ value, $2(2^{\Delta Ct})$, to account for the two copies of GAPDH in each mononuclear peripheral blood cell. SD = standard deviation.

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<td>18.50</td>
<td>0.529</td>
<td>26.40</td>
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<td>477.7</td>
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<td>7</td>
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<td>0.015</td>
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<td>388.0</td>
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<td>26.43</td>
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<td>-7.7</td>
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<td>20.70</td>
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<td>27.80</td>
<td>0.007</td>
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<td>25.97</td>
<td>0.013</td>
<td>-7.3</td>
<td>315.2</td>
</tr>
</tbody>
</table>

Supplementary table 2 on line.
The percentage 3243A>G mutation and amount of mtDNA (copy number) relative to nuclear DNA (nDNA) in skeletal muscle for the study subjects.
Numbering corresponds to the patient index in the text. The methods used were identical to those in the text. Muscle tissue was not available for subjects 6 and 8.

<table>
<thead>
<tr>
<th>Subject</th>
<th>3243A&gt;G (%)</th>
<th>mtDNA</th>
<th>SD</th>
<th>nDNA</th>
<th>SD</th>
<th>$\Delta Ct$</th>
<th>Total copy number / diploid nucleus</th>
</tr>
</thead>
</table>

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<p>| | | | | | | |</p>
<table>
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</thead>
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<td>1</td>
<td>64.6</td>
<td>20.1</td>
<td>0.115</td>
<td>30.6</td>
<td>0.208</td>
<td>-10.5</td>
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<td>2</td>
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<td>20.9</td>
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<td>30.5</td>
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<tr>
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</tr>
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<td>-</td>
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<tr>
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<td>33.3</td>
<td>0.351</td>
<td>-11.3</td>
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</table>
Supplementary table 3 on line.
The amount of mtDNA (copy number) relative to nuclear DNA (nDNA) in skeletal muscle from six healthy control subjects. The methods used were identical to those in the text. These samples did not have the 3243A>G mutation using the last fluorescent cycle method described in the text.

<table>
<thead>
<tr>
<th>Subject</th>
<th>mtDNA</th>
<th>SD</th>
<th>nDNA</th>
<th>SD</th>
<th>ΔCt</th>
<th>Total copy Number / diploid nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20.9</td>
<td>0.100</td>
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<td>0.321</td>
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<td>0.153</td>
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<tr>
<td>4</td>
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<td>19.8</td>
<td>0.231</td>
<td>28.2</td>
<td>0.058</td>
<td>-8.4</td>
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<tr>
<td>6</td>
<td>19.8</td>
<td>0.058</td>
<td>27.7</td>
<td>0.208</td>
<td>-7.9</td>
<td>477.7</td>
</tr>
</tbody>
</table>
Figure 1

PEAK HEIGHT

AREA UNDER THE CURVE

Fluor (%) vs. Hot (%)

- **Undiluted**
  - $R^2 = 1.0$

- **1 in 2**
  - $R^2 = 0.99$

- **1 in 4**
  - $R^2 = 0.96$

- **1 in 8**
  - $R^2 = 0.97$

- **1 in 16**
  - $R^2 = 0.99$

- **1 in 32**
  - $R^2 = 0.99$
Figure 4

Wild-type mtDNA molecules/cell vs. Age (Years)
Depletion of mitochondrial DNA in leukocytes harboring the 3243A>G mtDNA mutation

Angela Pyle, Robert W Taylor, Steve E Durham, Marcus Deschauer, Andrew M Schaefer, David C Samuels and Patrick F Chinnery

*J Med Genet* published online September 1, 2006

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