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A 10-year Prospective Cohort

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Lecocytes Mutation load Declines with Age in Carriers of the m.3243A>G Mutation. A 10-year Prospective Cohort.

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Abstract

Carriers of the mitochondrial mutation m.3243A>G presents highly variable phenotypes including mitochondrial encephalomyopathy, lactoacidosis and stroke-like episodes (MELAS). We conducted a follow-up study to evaluate changes in leucocyte heteroplasmy and the clinical phenotypes in m.3243A>G carriers. Leucocyte heteroplasmy was determined by next generation sequencing covered by 100,000 X reads in 32 individuals with a median follow-up of 10.2 years. Ten-year clinical follow-up is reported on 46 individuals. The annual leucocyte mutation level declined by -0.7 (±0.4) percentage points/year (p<0.0001), and correlated with the level of the initial sample (ρ =-0.92, p<0.0001). Eleven of 46 m.3243A>G carriers died, and clinical symptoms progressed. This longitudinal study demonstrates the decline in leucocyte m.3243A>G heteroplasmy associates with the level of the initial sample. Further, there was a high mortality among carriers.

Keywords: m.3243A>G, heteroplasmy, mitochondria, MELAS.
Introduction

Carriers of mitochondrial point mutation m.3243A>G present highly variable clinical phenotypes including mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS). In addition, carriers may develop diabetes, hearing impairment, cardiomyopathy, migraine, epilepsy, ptosis, and nephropathy. However, the phenotypic heterogeneity is only partially understood.

The levels of heteroplasmy i.e. the mixture of m.3243A>G and wildtype m.DNA, contribute to the severity of mitochondrial dysfunction. Cross-sectional studies show negative associations between the heteroplasmy level in leucocytes and the age of onset of diabetes or hearing impairment as well as with the Newcastle Mitochondrial Disease Adult Scale (NMDAS) measures, and a few longitudinal studies report an annual decline in leucocyte heteroplasmy of -0.69 to -1.4 percentage points (pp). The leucocyte mutation burden is lower or undetectable compared to urinary epithelial cell (UCE) and skeletal muscle. Nevertheless, m.3243A>G carriers with low heteroplasmy levels in leucocytes and no previous symptoms are still at risk of developing MELAS or sudden death while others may remain asymptomatic carriers. Consequently, at present it is not possible to predict the individual phenotype or prognosis based solely on mutation load in leucocytes.

Traditionally, m.3243A>G heteroplasmy is quantified by different methods including real-time PCR and pyro-sequencing. The introduction of massive parallel sequencing i.e. next generation sequencing (NGS), provides a method for simultaneous detection and quantification of mitochondrial genome-wide heteroplasmies resulting in a higher sensitivity compared to other methods.

To extend the knowledge on heteroplasmy and the clinical phenotypes in the m.3243A>G carriers, we conducted a 10-year follow-up study on changes in leucocyte mutation burden and on development of symptoms during aging.

Materials and Methods
Heteroplasmy

The participants were recruited from a Danish cohort of 102 individuals identified as carriers of m.3243A>G between March 1995 and July 2014. Among these individuals, a total of 152 blood, 37 muscle, 66 buccal mucosa, and 68 urine samples were collected during this period. Matched pairs of samples included: leucocytes and UEC (n=44), leucocytes and buccal mucosa (n=44), and UEC and buccal mucosa (n=42). Association of heteroplasmy between skeletal muscle and blood was allowed for with a maximal interval of 3 years between the collections of samples (n=15). This cohort included 32 subjects from whom paired blood samples were collected at two different time-points.

Clinical presentations

Eight families comprising 46 individuals were eligible for a 10-year clinical follow-up. The initial diagnosis and investigations were performed in 2003-2006. Clinical data was obtained by the attending physicians or from systematic review of electronic medical records.

All participants provided signed informed consent. The study was approved by the Regional Scientific Ethical Committee for Southern Denmark (S-20100112) and Danish Data Inspection Board (J.nr. 2013-41-2173).

Determination of heteroplasmy level

Total DNA was isolated from peripheral blood leucocytes, buccal mucosa, UEC or skeletal muscle performed with MaxWell®16 (Promega, WI, USA) according to manufactures protocol. Level of heteroplasmy was determined using Next Generation Sequencing with amplicons adapted from Illuminas 16S Metagenomic protocol.
Primers were designed to amplify the m.3243A>G mutation using forward primer

TCGTCGGCGAGTCATGTGATAAGAGACAGGTAATGATATCATCTCAACTTAG and reverse primer

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGATTAGAATGGGTACAATGAGG manually designed according to Illumina's guidelines and designed to avoid amplification of homologue regions in the genome using BLAST.

The library was prepared as follows: First stage PCR was performed with above mentioned primers according to Illumina's protocol. A second stage PCR with dual indexing primers was performed using Illumina seq Ilumina Nexttera XT index Kit. Quantification was done on Quibit Fluorometer, Invitrogen Turner BioSystems, (Thermo Fischer Scientific, MA, USA).

Concentration was then normalized and samples were pooled. The PhiX174 bacteriophage genome was added to ensure sufficient complexity in sequencing cycles. A maximum of 96 samples were loaded in a HiSeq Rapid lane. Sequencing was performed using 100 cycles single reads.

Data analysis

Raw (Single-end, 1x100bp) reads were aligned using BWA-MEM alignment software to the human reference genome (GRCh37). The variant allele frequency and total coverage of the m.3243A>G variant were determined using the bam-readcount tool. Counting the number of C and T nucleotides in the m.3243 position assessed sequencing error-rates. Quality parameters included that samples covered by > 100,000 X were included. Eight samples with read coverage <100,000 X reads were excluded.

Statistics

Differences in annual leucocyte heteroplasmy level pp were tested with a one-side Student’s t-test.

Regression analysis was applied for investigating the relation between the annual change in heteroplasmy

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and the heteroplasmy level in the initial sample, and the age of the participant at the initial blood sample. The distribution of data was examined using the Shapiro-Wilks normality test, and data are presented as mean ± SD or median [IQR] according to their distribution. All statistical calculations were performed using STATA statistical package version 14 (StataCorp LP, College Station, Texas, US).

Results

Methods for analysis of heteroplasmy

The analysis for heteroplasmy levels was validated with two standard curves (Figure S1, Appendix), both showing strong correlation with $R^2 = 0.9903$ and 0.9911, respectively. For evaluation of the precision, eight samples were run twice presenting a strong correlation ($R^2=0.9996$) (Figure S2, Appendix). In addition, two null samples were tested with heteroplasmy levels comparable to background noise. Heteroplasmy in two null samples and in two negative controls (i.e. the error rate) ranged from 0.61-0.93%. The detection limit was set at 1.5%.

Heteroplasmy – longitudinal changes in leucocytes

Longitudinal changes in leucocyte heteroplasmy levels were analyzed in 32 individuals. (Figure 1.A) The median [IQR] between sampling was 10.2 [9.2-11.6] years. The mean change (± SD) in heteroplasmy level per year was ($\Delta = -0.7 (± 0.4)$ pp/year, $p<0.0001$). (Data on leucocyte, heteroplasmy, Table S1, Appendix). Regression analysis of the annual change in heteroplasmy level with the heteroplasmy level in the initial sample and the age of each individual at the initial sample as independent variables showed a significant negative association with the heteroplasmy level in the initial sample ($\rho = -0.92$, $p<0.0001$) (Figure 1.B).
but not with the age. (Data on associations between the heteroplasmy levels of the paired samples, Figure S3, Appendix)

**10-years clinical follow-up**

During the 10-year follow-up of 46 m.3243A>G mutation carriers from eight families, there were 11 deaths at a median age of 51.7 years (45-83 years). (Table 1) The causes of death included stroke-like episodes (n=4), congestive heart failure (n=2), sudden cardiac arrest (n=1), pulmonary embolism (n=1), kidney failure (n=1), and unknown cause of death (n=2). Patients who developed MELAS, cardiomyopathy or nephropathy presented with UEC m.3243A>G mutation loads higher than 45%. (Table S2, Appendix). In one family, two sisters developed severe cardiomyopathy. The eldest sister died at the age of 45 from congestive heart failure, while the 6 years younger sister had a successful cardiac transplantation at the age of 45 years. In a second family, the brother and his 5 years younger sister developed kidney failure and received kidney transplantation at the age of 49 and 43, respectively. (Table S2, Appendix).

**Discussion**

The present study represents the largest longitudinal study of change in mutation load of the m.3243A>G mutation in leucocytes, and the first report of the annual rate of decline of mutation burden strongly associates to the level of mutation burden of the initial sample. The annual decline in leucocyte heteroplasmy is in accordance with previous reports. 8-10 In addition, there was a high mortality rate and progression in the symptoms.

Heteroplasmy in UEC correlate with the levels in skeletal muscle as well as to the clinical phenotype evaluated by the NMDAS. 11, 12 In a recent study by Fayssoil *et al* reporting 5.5-year follow-up on forty-three m.3243A>G mutation carriers, UEC mutation loads >45% predicted a high risk for major adverse events including stroke. 21 Although our study was not designed to evaluate UEC as a prediction tool, all
patients developing MELAS, cardiomyopathy or nephropathy during the follow-up presented with UEC m.3243A>G mutation loads higher than 45% while their leucocyte heteroplasmy levels ranged between 7.2% and 45%. (Table S2, Appendix). Consequently, as suggested by the authors’ measurement of UEC heteroplasmy may contribute to the identification of individuals at high risk for major adverse events, who may be recommended extended surveillance programs. Still, quantification of UEC mutation burden is not at strong prediction tool for the development of the individual m.3243A>G phenotype. Therefore, it is still difficult to provide the m.3243A>G carriers including women who plan to conceive precise counseling about disease manifestations.

In two families, we noticed strikingly similar, but less frequently reported phenotypes among siblings who developed cardiomyopathy and nephropathy, respectively. A previous study reported on post-mortem findings in two monozygotic twins both presenting with MELAS phenotypes as well as similar levels of heteroplasmy in all tissues. 22 Albeit speculative and based on only few observations, this may suggest some degree of clustering of symptoms within families. However, larger follow-up studies are needed to clarify whether some m.3243A>G families present with a “family specific” phenotype.

The clinical information was assessed from all 46 individuals, but may be mitigated by the systematic review of the medical records, which represent a limitation. The strength includes measurement of leucocyte mutation burden with NGS. Compared to the conventional 30 X in NGS based genetic analyses the deep sequencing offers a high confidence on the read counts, and thus heteroplasmy estimations. The n.DNA contains several areas with high homology to the m.DNA. However, the present method reduces the bias from sequencing homologous n.DNA areas.

In conclusion, the longitudinal study on m.3243A>G leucocyte mutation burden assessed by NGS shows declining levels, and the annual change associates with the level of the initial sample. In addition, in the 10-year follow-up there was a high mortality.
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18. *16S Metagenomic Sequencing Library Preparation - Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System.* Illumina, Inc. San Diego, California, USA.


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**Figures legends**

Figure 1 A. Leucocyte heteroplasmy level in relations to age at sample.

Relation between the heteroplasmy level of the m.3243A>G mutation in blood (%) and age (years) in 32 subjects. Each line represents one subject with a straight line connecting the first and last sample.

Figure 1 B. Change in leucocyte heteroplasmy level per year in relation to leucocyte heteroplasmy level in initial sample.

Relation between the annual change in heteroplasmy level [pp/year] and heteroplasmy level in the intial sample (%) in 32 individuals ($\rho = 0.92$, $p<0.0001$).
Figure S1. Standard curve for dilution-rows. Standard curve with 1:3 dilution-rows (1 to 1/3) from two independent samples (leucocyte, DNA). $R^2 = 0.9903$ and 0.9911 for curve 1 (navy) and curve 2 (green), respectively.

Figure S2. Evaluation of precision. Leucocyte DNA from m.3243A>G carriers (n=8) were run twice. Correlation ($R^2=0.9996$).

Figure S3. Relation between heteroplasmy levels (%) in different tissues.

Table 1. Clinical and phenotypical presentation of m.3243A G mutation-positive subjects.

<table>
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<th>2006</th>
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<tr>
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<td>No. (%)</td>
<td>Age at diagnosis (years)</td>
<td>No. (%)</td>
<td>Age at diagnosis (years)</td>
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<td>46 (100) / 0 (0)</td>
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<td>Children (&lt;18 yr.)</td>
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<td>Males</td>
<td>19 (41)</td>
<td></td>
<td>21 (46)</td>
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<tr>
<td>Deceased</td>
<td>-</td>
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<td>11 (24)</td>
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<tr>
<td>Age (years)</td>
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<td></td>
<td>51.9 [21-88]</td>
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</tr>
<tr>
<td>Age at death (years)</td>
<td>51.7 [45-83]</td>
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<td>Symptoms</td>
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<td>MELAS</td>
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<td>4 (9)</td>
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<td>Epilepsy</td>
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<table>
<thead>
<tr>
<th>Condition</th>
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<th>No.</th>
<th>Median [Range]</th>
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</thead>
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<td>4 (9)</td>
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<tr>
<td>Cardiomyopathy</td>
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<td>4 (9)</td>
<td></td>
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<tr>
<td>Non-diabetic nephropathy</td>
<td>1 (2)</td>
<td></td>
<td>4 (9)</td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>19 (41)</td>
<td>41 [22-72]</td>
<td>23 (50)</td>
<td>48 [14-76]</td>
</tr>
<tr>
<td>Impaired glucose tolerance</td>
<td>10 (22)</td>
<td></td>
<td>10 (22)</td>
<td></td>
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<tr>
<td>Hearing impairment</td>
<td>30 (65)</td>
<td>27 [10-72]</td>
<td>33 (72)</td>
<td>28 [10-62]</td>
</tr>
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</table>

Values are numbers with percentages in parenthesis or medians [range].