INTRODUCTION

In 1990, the Adenine to Guanine transition at position 3,243 of mitochondrial DNA (m.3243A>G) in the MT-TL1 encoding tRNA^{LEU(UUR)} was found as the molecular basis for MELAS (Goto, Nonaka, & Horai, 1990; Kobayashi et al., 1990). The acronym MELAS was first used in 1984 by Pavlakis, Phillips, DiMauro, De Vivo, and Rowland (1984) to describe a group of patients with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes. As the m.3243A>G mutation is the most common cause of MELAS syndrome (MIM 540000), it is also reported as the MELAS mutation although other phenotypic expressions have been described. These include maternally inherited diabetes and deafness.
(MIDD, MIM 520000) (Ouweland et al., 1992), hypertrophic cardiomyopathy (Lev et al., 2004), macular dystrophy (Laat, Smeitink, Janssen, Keunen, & Boon, 2013), gastrointestinal involvement (Laat et al., 2015), and oligosymptomatic variants of the acronym MELAS (Dvorakova et al., 2016). The m.3243A>G mutation is one of the most prevalent pathogenic mutation of the mitochondrial DNA, prevalence being reported in the range of 7.59–236/100,000 persons (Chinnery et al., 2000; Majamaa et al., 1998; Manwaring et al., 2007).

Since mitochondria and mitochondrial (mt) DNA are present in all tissues except red blood cells, heteroplasmy percentages can theoretically be assessed in virtually every tissue. Two problems arise when testing heteroplasmy: Most human tissues are practically not accessible and differences in heteroplasmy levels between samples might exist. For example, invasively obtained skeletal muscle tissue DNA usually gives higher and more consistent heteroplasmy levels than DNA extracted from a less invasively obtained blood sample (Rahman, Poulton, Marchington, & Suomalainen, 2001). The lower levels in blood might even lead to false-negative results (Laat et al., 2012). Previous studies showed a superiority of urine over blood as preferred noninvasive tissue for mutation analysis in patients at risk of carrying the m.3243A>G mutation (Frederiksen et al., 2006; Laat et al., 2012; Ma et al., 2009; Marotta et al., 2009). However, the relationship between mutation load and clinical phenotype has been a subject of research for many years (Chinnery, Howell, Lightowlers, & Turnbull, 1997; Grady et al., 2018; Liu et al., 2012; Nesbitt et al., 2013).

Surprisingly in several studies, including one of ourselves, a relationship between heteroplasmy levels in urinary epithelial cells (UEC) and clinical symptoms was suggested. In these small sample sizes, the reported correlation coefficients were however rather low (Laat et al., 2012; Ma et al., 2009; Nesbitt et al., 2013; Whittaker et al., 2009). However, the relationship between mutation load and clinical phenotype has been a subject of research for many years (Chinnery, Howell, Lightowlers, & Turnbull, 1997; Grady et al., 2018; Liu et al., 2012; Nesbitt et al., 2013). They all participate in our natural history cohort study (Laat et al., 2012). The ethics committee of the Nijmegen-Arnhem region approved this study. Written informed consent according to the Helsinki agreement was obtained from all patients.

Patient characteristics regarding age, sex, and clinical expression of the m.3243A>G were extracted from the data of the national cohort study, including Newcastle Mitochondrial Disease Adult Scale (NMDAS)-scores, and mtDNA heteroplasmy levels in other tissues. All patients were asked to report symptoms of urinary tract infections, fever, smoking, and alcohol use.

2.2 Urine sample collection and mutation analysis

All patients received an isolation box with 5 urine containers. They were instructed to collect five urine samples in a 14-day window. The urine samples were to be collected in the morning of days 1, 4, 7, 10, and 13. The samples were stored at 3–6°C and send with regular postal service to the laboratory in the provided isolation box after collection of the fifth sample. DNA was isolated from the urine samples, after centrifugation of the urine for 10 min at 3,000 rpm, and the pellet was washed with phosphate-buffered saline. DNA was extracted using a commercially available DNA isolation kit (PuregeneTM DNA isolation kit; Gentra Systems, MN).

Heteroplasmy levels were determined in all urine samples using PyrosequencingTM technology (Pyrosequencing, Uppsala, Sweden) as earlier described by Lowik, Hol, Steenbergen, Wetzels, and van den Heuvel (2005). The pyrosequencing reaction of the m.3234A>G mutation had a precision of 1.5%, and the lowest limit of detection was 5%. The detection limit for the m.3243A>G mutation was determined by serial dilutions of a sample containing this mutation with wild-type mtDNA.

2.3 Statistics

We used descriptive statistics in analyzing the data.

3 RESULTS

3.1 General patient characteristics

Fifteen carriers were included in the study (Table 1). Four carriers (27%) were male. Median age was 39 years (range: 20–69 years). Patients had different phenotypic expressions of the m.3243A>G mutation: MELAS syndrome (one patient), MIDD (seven patients), isolated myopathy and fatigue (four patients), and cardiomyopathy (one patient). The remaining two patients were clinically asymptomatic and should be categorized as dormant carriers. The median NMDAS score was 8 (range: 1–56 with 1 being the least severe disease expression).

2 | METHODS

2.1 Patients

All subjects were genetically diagnosed with the m.3243A>G mutation in DNA extracted from skeletal muscle and/or blood.
| No. | Sex/age (years) | Clinical diagnosis | NMDAS | Intra-patient variability (UEC; %) | Previous samples (%) | Urinary epithelial cells | Blood | Saliva |
|-----|----------------|-------------------|-------|----------------------------------|----------------------|--------------------------|-------|--------|
| 1   | M/33           | MELAS             | 56    | 98 97 97 97 97 97 97            | 96 49 63            |
| 2   | M/23           | Myopathy          | 8     | 96 95 na 96 95               | 96 49 68            |
| 3   | M/69           | MIDD              | 21    | 87 86 84 83 82            | 86 11 41            |
| 4   | M/50           | MIDD              | 7     | 74 na 75 76 77            | 75 19 33            |
| 5   | F/35           | Cardiomyopathy    | 16    | 72 88 85 64 66            | 72 42 47            |
| 6   | F/20           | Dormant carrier   | 1     | 60 56 74 54 72            | 74 39 55            |
| 7   | F/42           | MIDD              | 11    | 75 51 60 56 72            | 73 29 50            |
| 8   | F/38           | Myopathy          | 3     | 55 63 62 69 58            | 55 11 na            |
| 9   | F/39           | MIDD              | 7     | 62 42 56 53 54            | 61 21 40            |
| 10  | F/48           | MIDD              | 11    | 43 42 47 40 45            | 40 23 42            |
| 11  | F/61           | Dormant carrier   | 3     | 43 36 39 29 40            | 38 7 16             |
| 12  | F/34           | MIDD              | 8     | 35 34 34 40 41            | 40 27 45            |
| 13  | F/67           | Myopathy          | 11    | 31 20 44 17 21            | 15 5 25             |
| 14  | F/36           | Myopathy          | 2     | 15 13 15 15 13            | 22 8 10             |
| 15  | F/65           | MIDD              | 18    | 6 6 4 2 6              | 6 5 23             |

Notes: MIDD, maternally inherited diabetes and deafness; na: not available; NMDAS: Newcastle Mitochondrial Disease Adult Scale.
3.2  |  Heteroplasmy levels

A total of 75 urine samples were collected. In two samples, it was not possible to extract sufficient amounts of DNA for heteroplasmy analysis. Heteroplasmy level measurement was successful in the remaining 73 samples (Table 1). In the samples of six patients (Patients: 1, 2, 3, 4, 14, and 15), heteroplasmy levels were within 5% margin of each other (Figure 1a). In the samples of five patients (Patients: 5, 6, 7, 9, and 13), the margin was >20% (Figure 1b). In the remaining four patients (patients: 8, 10, 11, and 12), the variation between the heteroplasmy levels in the different samples was between 5% and 20%.

Patients 9 and 12 reported some complaints of a viral upper airway infection during the first days. Patient 14 reported to have smoked during all days. There was no alcohol usage among the patients.

4  |  DISCUSSION

Urinary epithelial cells have been identified as the most optimal noninvasive tissue for measurement of heteroplasmy of mtDNA mutations (Laat et al., 2012). Heteroplasmy levels in UEC have also been correlated with disease severity (Whittaker et al., 2009). In this study, we show that UEC m.3243A>G heteroplasmy levels within one patient might show important day-to-day variations. Based on these results, we conclude that the level of UEC heteroplasmy should be cautiously interpreted in predicting disease severity and the results of intervention studies in carriers of the m.3243A>G mutation.

We hypothesize that the difference in heteroplasmy levels between the different urine samples from individual patients is caused by a variation in different types of (epithelial) cells in the urine samples. Previous studies extensively studied the different epithelial cells in random urine samples and report a broad variation in different kinds of epithelial cells between samples (Schumann, 1981). Due to a genetic bottleneck, there is variation between the heteroplasmy levels of cells from different organs or tissues in one patient (Cree, Samuels, & Chinnery, 2009). If there is a larger proportion of the cells in a urine sample with a higher or lower heteroplasmy level compared to another sample, this could explain the differences reported in this study.

Patients with a very high or very low heteroplasmy level in UEC have a smaller range of heteroplasmy levels compared to patients with heteroplasmy levels in the middle range (Figure 1a,b). This is consistent with our hypothesis that there is a variation in cell types in the different urine samples, a normal distribution of the variance can be expected.

As several cohort studies (Laat et al., 2012; Nesbitt et al., 2013), including one of our own, have used the heteroplasmy levels in UEC to correlate the genotype of the patients to the clinical severity, it is essential to have studied the intra-patient variability of the heteroplasmy levels in UEC. Overall, the disease progression in carriers of the m.3243A>G mutation has no significant day-to-day variation. The interpretation of a correlation between heteroplasmy levels in urine and disease severity is therefore not reliable.

5  |  CONCLUSION

In this study, we demonstrated that the measurement of m.3243A>G heteroplasmy levels in UEC might have a substantial intra-patient day-to-day variability. The use of UEC m.3243A>G heteroplasmy levels as a prognostic biomarker should be interpreted in light of these findings.

ACKNOWLEDGMENTS

Parts of this work are supported by the ZonMW Priority Medicines Grant (113302003) and the Stichting Energy4All.

DISCLOSURE

Jan Smeitink is the founding CEO of Khondrion BV.
AUTHORS’ CONTRIBUTIONS

Conception and design: PdL, MJ, JS; Analysis and interpretation of data: PdL, RR, MJ; Drafting the article: PdL; Critically revising the manuscript: RR, MJ, JS.

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REFERENCES

Chinnery, P. F., Howell, N., Lightowler, R. N., & Turnbull, D. M. (1997). Molecular pathology of MELAS and MERRF. The relationship between mutation load and clinical phenotypes. Brain, 120(Pt 10), 1713–1721. https://doi.org/10.1093/brain/120.10.1713

Chinnery, P. F., Johnson, M. A., Wardell, T. M., Singh-Kler, R., Hayes, C., Brown, D. T., … Turnball, D. M. (2000). The epidemiology of pathogenic mitochondrial DNA mutations. Annals of Neurology, 48(2), 188–193. https://doi.org/10.1002/1531-8249(200008)48:2<188:AID-ANA3>3.0.CO;2-P

Cree, L. M., Samuels, D. C., & Chinnery, P. F. (2009). The inheritance of pathogenic mitochondrial DNA mutations. Biochimica Et Biophysica Acta, 1792(12), 1097–1102. https://doi.org/10.1016/j.bbadis.2009.03.002

de Laat, P., Koene, S., van de Heuvel, L. P. W. J., Rodenburg, R. J. T., Janssen, M. C. H., & SMEITINK, J. A. M. (2012). Clinical features and heteroplasmy in blood, urine and saliva in 34 Dutch families carrying the m.3243A>G mutation. Journal of Inherited Metabolic Disease, 35(6), 1059–1069. https://doi.org/10.1007/s10545-012-9465-2

de Laat, P., SMEITINK, J. A. M., JANSSEN, M. C. H., KEUNEN, J. E., & Boon, C. J. (2013). Mitochondrial retinal dysrophy associated with the m.3243A>G mutation. Ophthalmology, 120(12), 2684–2696. https://doi.org/10.1016/j.ophtha.2013.05.013

de Laat, P., Zweers, H. E., Knuijt, S., SMEITINK, J. A. M., WANTEN, G. J., & JANSSEN, M. C. H. (2015). Dysphagia, malnutrition and gastrointestinal problems in patients with mitochondrial disease caused by the m.3243A%3eG mutation. Netherlands Journal of Medicine, 76(4), 30–36.

Dvorakova, V., Kolarova, H., Mager, M., TESAROVA, M., Hansikova, H., Zeman, J., & Honzik, T. (2016). The phenotypic spectrum of fifty Czech m.3243A>G carriers. Molecular Genetics and Metabolism, 118(4), 288–295. https://doi.org/10.1016/j.ymgme.2016.06.003

Frederiksen, A. L., Andersen, P. H., Kyyvik, K. O., Jeppesen, T. D., Vissing, J., & Schwartz, M. (2006). Tissue specific distribution of the 3243A>G mtDNA mutation. Journal of Medical Genetics, 43(8), 671–677. https://doi.org/10.1136/jmg.2005.039339

Goto, Y., Nonaka, I., & Horai, S. (1990). A mutation in the tRNA(Leu)(UUR) gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. Nature, 348(6302), 651–653. https://doi.org/10.1038/348651a0

Grady, J. P., Pickett, S. J., Ng, Y. S., Alston, C. L., Blakey, E. L., Hardy, S. A., … McFarland, R. (2018). mtDNA heteroplasmy level and copy number indicate disease burden in m.3243A>G mitochondrial disease. EMBO Molecular Medicine, 10(6), e8262. https://doi.org/10.15252/emmm.201708262

Kobayashi, Y., Momoi, M. Y., Tominaga, K., Momoi, T., Nihei, K., Yanagisawa, M., … Ohita, S. (1990). A point mutation in the mitochondrial tRNA(Leu)(UUR) gene in MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes). Biochemical and Biophysical Research Communications, 173(3), 816–822. https://doi.org/10.1016/S0006-291X(05)80860-5

Lev, D., Nissenkorn, A., Leshinsky-Silver, E., Sadeh, M., Zeharia, A., Garty, B. Z., … Lerman-Sagie, T. (2004). Clinical presentations of mitochondrial cardiomyopathies. Pediatric Cardiology, 25(5), 443–450. https://doi.org/10.1007/s00246-003-0490-7

Liu, C. H., Chang, C. H., Kuo, H. C., Ro, L. S., Liou, C. W., & Wei, Y. H. (2012). Huang CC. Prognosis of symptomatic patients with the A3243G mutation of mitochondrial DNA. Journal of the Formosan Medical Association, 111(9), 489–494. https://doi.org/10.1016/j.jfma.2011.06.014

Lowik, M. M., Hol, F. A., Steenbergen, E. J., Wetzel, J. F., & van den Heuvel, L. P. (2005). Mitochondrial tRNA(Leu)(UUR) mutation in a patient with steroid-resistant nephrotic syndrome and focal segmental glomerulosclerosis. Nephrology, Dialysis, Transplantation, 20(2), 336–341. https://doi.org/10.1093/ndt/gfh546

Ma, Y., Fang, F., Yang, Y., Zou, L., Zhang, Y., Wang, S., … Qi, Y. (2009). The study of mitochondrial A3243G mutation in different samples. Mitochondrion, 9(2), 139–143. https://doi.org/10.1016/j.mito.2009.01.004

Majumara, K., Moilanen, J. S., Uimonen, S., Remes, A. M., Salmela, P. I., Kärpät, M., … Hassinen, I. E. (1998). Epidemiology of A3243G, the mutation for mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes: Prevalence of the mutation in an adult population. American Journal of Human Genetics, 63(2), 447–454. https://doi.org/10.1086/301959

Manwaring, N., Jones, M. M., Wang, J. J., Rochtchina, E., Howard, C., Mitchell, P., & Sue, C. M. (2007). Population prevalence of the MELAS A3243G mutation. Mitochondrion, 7(3), 230–233. https://doi.org/10.1016/j.mito.2006.12.004

Marotta, R., Reardon, K., McKelvie, P. A., Chiotis, M., Chin, J., Cook, M., & Collins, S. J. (2009). Association of the MELAS m.3243A>G mutation with myositis and the superiority of urine over muscle, blood and hair for mutation detection. Journal of Clinical Neuroscience, 16(9), 1223–1225.

Nesbitt, V., Pitcaethly, R. D., Turnbull, D. M., Taylor, R. W., Sweeney, M. G., Mudanohwo, E. E., … McFarland, R. (2013). The UK MRC Mitochondrial Disease Patient Cohort Study: Clinical phenotypes associated with the m.3243A>G mutation–implications for diagnosis and management. Journal of Neurology, Neurosurgery, and Psychiatry, 84(8), 936–938. https://doi.org/10.1136/jnnp-2012-303528

Pavlakis, S. G., Phillips, P. C., DiMauro, S., De Vivo, D. C., & Rowland, L. P. (1984). Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes: A distinctive clinical syndrome. Annals of Neurology, 16(4), 481–488. https://doi.org/10.1002/ana.410160409

Rahman, S., Poulton, J., Marchington, D., & Suomalainen, A. (2001). Decrease of 3243 A→G mtDNA mutation from blood in MELAS syndrome: A longitudinal study. American Journal of Human Genetics, 68(1), 238–240. https://doi.org/10.1086/316930

Schumann, G. B. G. (1981). Renal epithelial fragments in urine sediment. Acta Cytologica, 25(2), 147–152.

van den Ouweland, J. M., Lemkes, H. H., Ruitenbeek, W., Sandkuijl, L. A., de Vrijder, M. F., Struyvenberg, P. A., … Maassen, J. A. (1992).
Mutation in mitochondrial tRNA(Leu)(UUR) gene in a large pedigree with maternally transmitted type II diabetes mellitus and deafness. *Nature Genetics*, 1(5), 368–371. https://doi.org/10.1038/ng0892-368

Whittaker, R. G., Blackwood, J. K., Alston, C. L., Blakely, E. L., Elson, J. L., McFarland, R., ... Taylor, R. W. (2009). Urine heteroplasmy is the best predictor of clinical outcome in the m.3243A>G mtDNA mutation. *Neurology*, 72(6), 568–569. https://doi.org/10.1212/01.wnl.0000342121.91336.4d

**How to cite this article:** de Laat P, Rodenburg RJ, Smeitink JAM, Janssen MCH. Intra-patient variability of heteroplasmy levels in urinary epithelial cells in carriers of the m.3243A>G mutation. *Mol Genet Genomic Med*. 2019;7:e523. https://doi.org/10.1002/mgg3.523