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► To cite this version:

Sophie Monnot, Nadine Gigarel, David C Samuels, Philippe Burret, Laetitia Hesters, et al.. Segregation of mtDNA Throughout Human Embryofetal Development: m.3243A>G as a Model System. Human Mutation, 2010, 32 (1), pp.116. 10.1002/humu.21417 . hal-00602304

HAL Id: hal-00602304

<https://hal.science/hal-00602304>

Submitted on 22 Jun 2011

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Segregation of mtDNA Throughout Human Embryofetal Development: m.3243A>G as a Model System

Journal:	<i>Human Mutation</i>
Manuscript ID:	humu-2010-0326.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	28-Oct-2010
Complete List of Authors:	<p>Monnot, Sophie; Paris Descartes University; INSERM, Unit U781; Necker Hospital, Medical Genetics Gigarel, Nadine; Necker Hospital, Medical Genetics Samuels, David; Vanderbilt University Medical Center, Molecular Physiology and Biophysics Burllet, Philippe; Necker Hospital, Medical Genetics Hesters, Laetitia; Antoine Beclere Hospital, Reproductive Medicine and Obstetrics Frydman, Nelly; Antoine Beclere Hospital, Reproductive Medicine and Obstetrics Frydman, René; Antoine Beclere Hospital, Reproductive Medicine and Obstetrics Kerbrat, Violaine; Antoine Beclere Hospital, Reproductive Medicine and Obstetrics Funalot, Benoit; Necker Hospital, Medical Genetics MARTINOVIC, Jéléna; Assistance Publique - Hôpitaux de Paris, APHP, Genetics Benachi, Alexandra; Necker Hospital, Obstetrics Feingold, Josué; Necker Hospital, Medical Genetics Munnich, Arnold; Paris Descartes University; INSERM, Unit U781; Necker Hospital, Medical Genetics Bonfont, Jean-Paul; Paris Descartes University; INSERM, Unit U781; Necker Hospital, Medical Genetics Steffann, Julie; Paris Descartes University; INSERM, Unit U781; Necker Hospital, Medical Genetics</p>
Key Words:	mitochondrial DNA, MELAS, m.3243A>G, embryo, mitochondria

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For Peer Review

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3 1 **Segregation of mtDNA Throughout Human Embryofetal Development:**
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5 2 **m.3243A>G as a Model System**
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12 5 Sophie Monnot,¹ Nadine Gigarel,¹ David C. Samuels,² Philippe Bulet,¹ Laetitia
13 6 Hesters,³ Nelly Frydman,³ René Frydman,³ Violaine Kerbrat,³ Benoit Funalot,¹ Jelena
14 7 Martinovic,⁴ Alexandra Benachi,⁵ Josué Feingold,¹ Arnold Munnich,¹ Jean-Paul
15 8 Bonnefont,^{1*} Julie Steffann^{1¶}
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25
26 10 ¹Université Paris-Descartes, Unité INSERM U781, and Hopital Necker-Enfants
27 11 Malades (Assistance Publique-Hopitaux de Paris), Paris, 75015, France ; ²Center for
28 12 Human Genetics Research, Department of Molecular Physiology and Biophysics,
29 13 Vanderbilt University Medical Center, Nashville, TN 37232, USA. ³Service de
30 14 gynécologie et médecine de la reproduction, Hôpital Antoine Bécère (Assistance
31 15 Publique-Hopitaux de Paris), Clamart, 92140, France ; ⁴Service d'histo-embryologie
32 16 cytogénétique, Hopital Necker-Enfants Malades (Assistance Publique-Hopitaux de
33 17 Paris), Paris, 75015, France ; ⁵Maternité, Hôpital Necker-Enfants Malades
34 18 (Assistance Publique-Hopitaux de Paris), Paris, 75015, France
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51 20 ¶ Both authors equally contributed to the work
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55 21 Correspondence: *jean-paul.bonnefont@inserm.fr
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1 Abstract

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9 Mitochondrial DNA (mtDNA) mutations cause a wide range of serious diseases with
10 high transmission risk and maternal inheritance. Tissue heterogeneity of the
11 heteroplasmy rate (“mutant load”) accounts for the wide phenotypic spectrum
12 observed in carriers. Owing to the absence of therapy, couples at risk to transmit
13 such disorders commonly ask for prenatal (PND) or preimplantation diagnosis (PGD).
14 The lack of data regarding heteroplasmy distribution throughout intra uterine
15 development however hampers the implementation of such procedures. We tracked
16 the segregation of the m.3243A>G mutation (*MT-TL1* gene) responsible for the
17 MELAS syndrome in the developing embryo/fetus, using tissues and cells from 8
18 carrier females, their 38 embryos and 12 fetuses. Mutant mtDNA segregation was
19 found to be governed by random genetic drift, during oogenesis and somatic tissue
20 development. The size of the bottleneck operating for m.3243A>G during oogenesis
21 was shown to be individual-dependent. Comparison with data we achieved for the
22 m.8993T>G mutation (*MT-ATP6* gene), responsible for the NARP/Leigh syndrome,
23 indicates that these mutations differentially influence mtDNA segregation during
24 oogenesis, while their impact is similar in developing somatic tissues. These data
25 have major consequences for PND and PGD procedures in mtDNA inherited
26 disorders.

27 Key words

28 Mitochondria, mitochondrial DNA, MELAS, NARP, respiratory chain deficiency,
29 embryo, preimplantation genetic diagnosis

1 Introduction

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Mitochondrial DNA (mtDNA, Genbank NC_001807.4) disorders are common diseases with maternal inheritance. Their high clinical variability is explained, at least in part, by variation of the mutant load (heteroplasmy) among individuals, and also across organs and tissues within an individual, so that mutant loads will surpass the tissue specific threshold, leading to the manifestation of symptom (Chinnery et al., 1997; Jeppesen et al., 2006). The usual severity of these diseases, the recurrence risk, usually high though being difficult to predict at the individual level, in offspring from heteroplasmic carriers (Chinnery et al., 1998), and the absence of efficient therapy, commonly result in a request from at-risk couples for prenatal (PND) or preimplantation diagnosis (PGD). Such procedures are however hampered by the lack of data regarding mtDNA segregation during embryofetal development.

In this respect, most available data come from animal studies. Dramatic shifts of mtDNA populations (variant/wild-type mtDNA ratio) between the mother and her offspring in cattle have suggested that a tight bottleneck might account for such a rapid segregation (Laipis et al., 1988). One important function of this bottleneck could be to ensure that new mutations will either be lost or fixed rapidly within individuals, and thus exposed to selection at the population level (Bergstrom and Pritchard, 1998; Roze et al., 2005). Studies in mice have identified the bottleneck during oogenesis (Jenuth et al., 1996), through either a reduction of mtDNA content in primordial germ cells (PGC, Cree et al., 2008), or selection of a small effective number of segregation units for mtDNA (Cao et al., 2007; Cao et al., 2009; Wai et al., 2008). There are however no available data so far regarding mutant mtDNA segregation in somatic tissues during embryofetal development in animal models.

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3 1 In humans, a small number of studies of primary oocytes carrying mtDNA
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5 2 deletions (Marchington et al., 1998) or point mutations (Blok et al., 1997; Brown et
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7 3 al., 2001; Marchington et al., 2010) have suggested that a bottleneck operates during
8
9 4 oogenesis. Random genetic drift appeared as the principal mechanism determining
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11 5 the level of mutant mtDNA within primary oocytes from a carrier of the most common
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13 6 mtDNA mutation, m.3243A>G (Brown et al., 2001). This mutation affects the *MT-TL1*
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15 7 gene [mitochondrially encoded tRNA leucine 1 (UUA/G), MIM 590050], and is
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17 8 responsible for the MELAS (Mitochondrial myopathy, encephalopathy, lactic acidosis,
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19 9 and stroke-like episodes, MIM 540000, Goto et al., 1992) and diabetes-deafness
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21 10 syndromes (MIM 520000, van den Ouweland et al., 1992; Manouvrier et al., 1995).

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27 11 It is so far not known whether m.3243A>G segregation is governed by random
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29 12 drift in somatic tissues during human embryofetogenesis. The few available studies
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31 13 on m.3243A>G carrier fetuses reported a uniform distribution of mutation load among
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33 14 different tissues from two heteroplasmic 24 and 25 week-old human fetuses
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35 15 (Matthews et al., 1994; Cardaioli et al., 2000). Our group found similar mutant loads
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37 16 in chorionic villi and amniocytes from 3 heteroplasmic fetuses (Bouchet et al., 2006).
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39 17 All these data suggest that MELAS mutant molecules do not segregate much
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41 18 **between** 10 to 25 weeks of gestation.

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46 19 However, we have so far lacked data on the transmission of m.3243A>G by a
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48 20 carrier to early embryos, and subsequently no information on segregation of this
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50 21 mutation throughout the entire period of human embryofetal development has
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52 22 existed. Thanks to our PGD and PND programs, we collected various tissues and
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54 23 cells at various stages of development in embryos and fetuses from m.3243A>G
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56 24 carriers, and investigated local and temporal variation of the mutant load, using a
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3 1 quantification test designed to assess m.3243A>G mutant load at the single cell
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5 2 level.
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8 3 Aggregation of our data in early embryos and fetuses, with those from a
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10 4 primary oocyte study in a m.3243A>G carrier (Brown et al., 2001) indicates that
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12 5 mtDNA segregation is governed by random genetic drift, in germ- and somatic cell
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14 6 lines, throughout the entire period of human intra-uterine development. The size of
15
16 7 the bottleneck operating during oogenesis for m.3243A>G is additionally suggested
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18 8 to vary between individuals. Finally, comparison of the current data with those we
19
20 9 previously reported for another common mtDNA mutation, m.8993T>G (*MT-ATP6*,
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22 10 ATP synthase subunit 6 gene, MIM 516060) responsible for the NARP syndrome
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24 11 (Neuropathy, Ataxia, Retinitis Pigmentosa, MIM 551500; Steffann et al., 2006;
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26 12 Steffann et al., 2007) indicates that these two mutations differentially influence
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28 13 mtDNA segregation during oogenesis, while their putative impact on mtDNA
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30 14 segregation appears quite similar in somatic cells during embryofetal development.
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39 16 **Patients, Materials and Methods**

40 41 42 43 18 **Patients**

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46 19 This study was approved by the National Ethics Committee from l'Agence de
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48 20 Biomedecine and all couples gave informed consent for DNA analyses in themselves
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50 21 and their embryos/fetuses.
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53 22 PND and/or PGD analyses were carried out in 8 individuals from 7 unrelated
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55 23 families, heteroplasmic for the MELAS m.3243A>G mutation. The questionable
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57 24 predictive value of a fetal mutant load for the postnatal outcome was emphasized to
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59 25 all of the couples. In such situations, counseling necessarily referred to postnatal
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3 1 data, attempting to correlate clinical outcome and mutant load (Chinnery et al., 1997;
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5 2 Uusimaa et al., 2007; Whittaker et al., 2009), and to our personal experience with
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7 3 PGD/PND for another mtDNA disorder, *ie* NARP (Gigarel et al., 2005; Steffann et al.,
8
9 4 2006; Steffann et al., 2007) and with PND for MELAS (Bouchet et al., 2006).
10
11 5 Accordingly, we postulated that mutant loads over 60% during prenatal life might be
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13 6 predictive of a serious disorder, while a mutant load below 30% might hopefully be
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15 7 associated with absence or low severity of the disease. The most complex situation
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17 8 was a fetal heteroplasmy value in the intermediate range (30 to 60%), a common
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19 9 finding for the MELAS m.3243A>G mutation in the post-natal period. There is indeed
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21 10 no available data correlating intermediate mutant load in the prenatal period with the
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23 11 postnatal outcome.
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29 12 Seven of the 8 couples (couples 2 to 8) elected to resort to a conventional
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31 13 PND approach at first. Couple 1 declined the option of pregnancy termination (TOP),
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33 14 and thus directly went to PGD, that was also offered to couple 2, who had previously
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35 15 experienced 2 TOP after PND. Both couples were informed that, at this early
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37 16 embryonic stage (Day 3), a number of issues remained unsolved, precluding any
38
39 17 conclusive prediction on the pre/postnatal outcome of a carrier embryo. These
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41 18 couples were offered a PND at 16 gestation weeks (GW) to confirm the PGD result in
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43 19 case of pregnancy.
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50 21 **Materials**

51 22 ***Postnatal period***

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53 23 Mutant loads from carrier females were assessed from blood, and when available,
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55 24 urine and oral mucosa samples. Single lymphocytes were isolated from patients 1, 2,
56
57 25 3, 5, and 14 as already described (Gigarel et al., 2005).
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1 **Early embryos**

2 Patients 1 and 2 were subjected to one and three separate PGD cycles, respectively.

3 A standard in vitro fertilization protocol was performed and oocytes were collected
4 and fertilized by intracytoplasmic sperm injection (ICSI).

5 A total of 38 embryos were analyzed. Twenty-three of these embryos were of
6 appropriate quality, and were therefore submitted to a 2-blastomere biopsy at post-
7 ICSI day 3 for mutant load assessment, as previously described (Gigarel et al.,
8 2004). Briefly, under control by a binocular microscope, the blastomeres were rinsed
9 twice in a drop of PBS supplemented with 0.1%polyvinyl alcohol (Sigma Aldrich,
10 France), using a mouth-controlled, finely pulled glass pipette before being transferred
11 into a transparent microcentrifuge tube containing 3 μ l of lysis buffer (Cui et al.,
12 1989). A small volume of biopsy medium was used as a PCR negative control for
13 each embryo in order to detect any contamination by exogenous DNA. In the 15
14 remaining embryos, assessment of the mutant load was performed on the embryo *in*
15 *toto*. All blastomeres were separately analyzed, when possible (couple 1: embryos 1
16 to 3, and couple 2: embryo 13). Embryo 10 from couple 2 was cultured up to the
17 blastocyst stage (Day 5), enabling a trophectoderm biopsy as described elsewhere
18 (McArthur et al., 2005). The trophectoderm and inner cell mass were analyzed
19 separately.

21 **Late embryos and fetuses**

22 Prenatal diagnosis was performed in 12 fetuses from 7 carrier women. Fetus 2c
23 resulted from embryo 7 and 11 transfer after PGD (patient 2), while the remaining
24 ones were conceived naturally. Chorionic villi (CV, n=8), amniotic fluids 1 (AF1, n=6),
25 and 2 (AF2, n=1), were sampled at 10, 14, and 30 weeks of gestation (GW),

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2
3 1 respectively. Individual fetal cells were isolated from CV and AF samples from 6
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5 2 fetuses, as already described (Steffann et al., 2007), and analyzed separately, to
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7 3 investigate the intercellular variation of heteroplasmy. Cord blood was collected from
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9 4 3 fetuses at birth (fetuses 2c, 3, and 4). Postmortem analyses of various tissues were
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11 5 carried out in two 12 week-old fetuses (fetuses 2a and 2b) and one 19 week-old fetus
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13 6 (5b) after TOP, and in a 20 week-old miscarriage product (fetus 7a). Parental
14
15 7 informed consent was obtained for these postmortem analyses. Fetal tissues were
16
17 8 carefully dissected, and small tissue biopsies were analyzed separately. Multiple
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19 9 samples were taken from two term placentas (fetuses 2c and 3), and two 12GW
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21 10 placentas (fetuses 2a and 2b).
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29 12 **Methods**

30 13 ***DNA extraction***

31 14 DNA was extracted from blood and tissues, using the Nucleon Bacc3 kit (Amersham
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33 15 Biosciences, UK), and a classical phenol extraction method, respectively.
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41 17 ***Quantitative analysis of the m.3243A>G mutant load***

42 18 MELAS mutant load was quantified using a semi-quantitative fluorescent PCR-*HaeIII*
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44 19 restriction test.
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48 20 Since PCR products analysis using a restriction enzyme is thought to allow the
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50 21 possibility of errors in mutant load assessments, secondary to heteroduplex
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52 22 formation during the PCR process (Tanno et al., 1991), we first validated our method
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54 23 of heteroplasmy assessment using mixing experiments and standard curve analysis.
55
56 24 Wild-type and mutant plasmids were mixed to generate 10 samples, each of them
57
58 25 comprising 10^6 mtDNA copies, with the m.3243G>A (mutant) target at concentrations
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1 ranging from 1 to 100%, which were also used to determine the lowest rate of
2 mutation detection. Each sample was amplified in triplicate, using both “single cell”
3 and “tissue” methods, and PCR products were submitted to *HaeIII* digestion.

4 We furthermore ascertained the reliability of our test over a wide range of
5 mtDNA copies, from 10^2 to 10^6 , encompassing the mtDNA copy number usually
6 present in isolated cells such as lymphocytes, blastomeres and oocytes.

7 Single cells and embryos were transferred to 3 μ l alkaline buffer (Cui et al.,
8 1989) and lysed by 10 min heating at 65°C. PCR amplification was carried out using
9 the forward primer (5'-TGAGTTCAGACCGGAGTAATC-3') and the reverse
10 fluorescent primer (*) (5'-(6-Fam) CTTAACAACATACCCATGGC-3'). Separate
11 experimental conditions were devised for whole tissue sample and single cell/early
12 embryo analyses.

13 Tissue sample DNA (1ng) was amplified in a 30 μ l reaction volume containing
14 1.5U Expand Taq DNA Polymerase, 10X PCR buffer 2 (3 μ l, Roche Diagnostics,
15 Mannheim, Germany), 0.5 μ M of each primer (Proligo, Paris, France), and 2mM
16 dNTP mix (Roche Diagnostics, Germany). Initial denaturation was carried out at 97°C
17 for 7 min 20s followed by 20 cycles (97°C for 20s, 60°C for 30s, 68°C for 1min 15s)
18 and final extension of 7 min at 68°C.

19 For a single cell or embryo analysis, the PCR reaction contained 3 μ l of lysis
20 buffer (lysed cell or negative control), each primer at 0.5 μ M, master mix 2X (12.5 μ l,
21 QIAGEN Multiplex PCR kit, QIAGEN S.A, Courtaboeuf, France), and double-distilled
22 water up to a 25- μ l final volume. PCR programs were 15 min of denaturation at 95°C,
23 followed by 25, 26, 27, and 30 PCR cycles for whole embryos, trophoblastic cells,
24 blastomeres, and amniocytes, respectively, consisting of 30s at 94°C, 90s at 60°C
25 and 60s at 72°C, with a 30-min final extension at 60°C.

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3 1 The resulting 251-bp fluorescent PCR products (1 μ l) were digested for 3 hours
4
5 2 using 10 units of *HaeIII* and subsequently submitted to electrophoresis using an
6
7 3 automated genetic analyser ABI3130 (Applied Biosystems). Digestion generated 73-
8
9 4 bp and 170-bp fluorescent fragments for the mutant and wild-type species,
10
11 5 respectively. Results were analysed with the Genescan and Genotyper software
12
13 6 (Applied Biosystems). The mutant load was calculated by dividing the mutant peak
14
15 7 area (73bp) by the sum of normal (171bp) and mutant (73bp) peak areas. Cross
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17 8 hybridization of oligonucleotide primers to nuclear DNA was ruled out by PCR
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19 9 amplification on mtDNA-less *Rho0* cells (Parfait et al., 1998).
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28 ***Ascertainment of the fetal origin of the CV/AF cells***

29 12 The fetal origin of single cells collected from CV, AF1, and AF2, was ascertained by
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31 13 simultaneous analysis of parental and embryofetal DNA using nuclear (CA)_n
32
33 14 microsatellite markers (D6S436, D19S559, D19S559, D16S3395, D6S436, and
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35 15 DXS1073 for fetus 2a, 5a, 5b, 6, 8, and 12b, respectively), enabling to check for
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37 16 biparental contribution to the cell genotype, and the absence of fetal cell
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39 17 contamination by maternal DNA (Gigarel et al., 2004).
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43 18 Amplification of microsatellites markers in single cells required nested PCR.
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45 19 The first “outer” PCR enabled simultaneous amplification of the MELAS mutation and
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47 20 an appropriate microsatellite marker. Briefly, cells were amplified using “single-cell”
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49 21 PCR conditions described above, as a duplex PCR reaction, using 0.5 μ M of each
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51 22 primer. 3 μ l of the first amplification product were subsequently mixed with 22 μ l of
52
53 23 “inner” amplification mix, which contained inner fluorescent microsatellite primers at
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55 24 0.5 μ M, master mix 2X (12.5 μ l, QIAGEN Multiplex PCR kit, QIAGEN S.A,
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3 1 Courtaboeuf, France), and double-distilled water up to a 25 μ l final volume. PCR
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5 2 program was as described above (20 PCR cycles).
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10 4 **Statistical analyses**

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12 5 Variance analysis and calculation of inter or intra class correlation coefficients were
13 6 used to analyze quantitative traits. Qualitative traits were analyzed using a Chi² test.
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15 7 The 95% confidence intervals of the measurements from Patient 2 were made by
16 8 fitting a Kimura distribution (Wonnapijitte et al., 2008) to the 35 mutation level
17 9 measurements from this patient, and then drawing 10,000 simulated sets of 35
18 10 measurements from this Kimura distribution to estimate the confidence intervals due
19 11 to sample size effects (Wonnapijitte et al., 2010).
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33 13 **Results**

34 14 **Sensitivity, linearity, and reproducibility of mutant load quantification**

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36 15 Mixing various proportions of wild type and mutant plasmids enabled us to establish
37 16 that the measured level of heteroplasmy was a linear function of the expected ratio
38 17 (Figure 1). The correlation coefficient for the observed vs expected proportion of
39 18 mutant was 0.99 for both “single cell” and “tissue” conditions. Triplicate experiments
40 19 yielded to similar results (SD<1.7%), and mutant molecules could be detected in a
41 20 proportion as low as 2% of the total mtDNA amount, thus validating reproducibility
42 21 and sensitivity of the assay. These results were got with a 10⁶ mtDNA copy number
43 22
44 23 In order to demonstrate an insensitivity to template input amounts for this particular
45 24 method, we ascertained the reliability of our test over a wide range of mtDNA copies,
46 25 from 10² to 10⁶. Results are displayed in Supp. Figure S1.

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6 2 **Analysis in carrier women**
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8 3 Mutant loads from the 8 carriers applying for MELAS PND/PGD (family 1 to 8, Table
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10 4 1) ranged from 5 to 40%, 8 to 50%, and 15 to 80%, in leucocytes, oral mucosa cells,
11
12 5 and urinary tract cells, respectively. The lowest and highest values were consistently
13
14 6 found in white blood cells and urinary tract cells, respectively, as expected from
15
16 7 previous reports (Chinnery et al., 1997; Chinnery et al., 1999; Frederiksen et al.,
17
18 8 2006; Whittaker et al., 2009).

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22 9 Mutant load assessment in single lymphocytes from 5 carriers showed large
23
24 10 intercellular variations, ranging from 0 to 81%, 0 to 81%, 0 to 95%, 0 to 21%, and 0 to
25
26 11 100 in patients 1, 2, 3, 5, and 14, respectively. When pooling values from all cells
27
28 12 analyzed for a given individual, average mutant load was very close to that achieved
29
30 13 from the overall lymphocyte extract recovered from a 10ml blood sample.

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34 14 Comparison of the mean heteroplasmy rate vs one standard deviation of the
35
36 15 heteroplasmy distribution at the single-cell level for each of the five lymphocyte pools
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38 16 ($16.2 \pm 8.6\%$) showed a 0.92 correlation coefficient ($p < 0.05$) with a positive slope
39
40 17 (1.14 ± 0.3) (Table 2 and **Supp. Figure S2**).

41 42 43 44 45 19 **Analysis in **Day 3-Day 5** embryos**

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48 20 Thirty-eight embryos from 2 unrelated heteroplasmic women were analyzed. The
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50 21 m.3243A>G mutant load was assessed in 31 whole embryos and 70 single
51
52 22 blastomeres (Table 3). Exogenous DNA contamination of the embryonic sample was
53
54 23 never detected in any experiment.

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56
57 24 Within the whole cohort of analyzed embryos, six had no detectable mutation
58
59 25 (detection threshold: 2%). Taking into account Patient **2** only (35 embryos), the

1 transmission rate was 83%. None of the embryos were homoplasmic mutant.
2 Heteroplasmy levels ranged from 5 to 77%, with an apparently homogeneous
3 dispersion of the mutant rates among embryos. The mutant load value was similar in
4 all blastomeres derived from a single embryo (n = 2 to 11) with a 6% maximal
5 interblastomere variability. The intraclass correlation coefficient (ICC) was equal to
6 0.994, consistent with the high level of agreement of intra-embryo measurements,
7 and contrasting with the inter-embryo variability. When possible, comparison of
8 heteroplasmy levels between the whole embryo (collected at Day 5) and two of
9 his/her blastomeres (collected at Day 3) showed a variation rate <7% in 13/16
10 embryos, and between 10 and 15% in the remaining ones.

11 When considering all embryos from patient 2, the distribution of the mutation
12 level values was consistent with the distribution predicted from neutral drift theory (p-
13 value of 0.53 by KS test) comparing the data against the null hypothesis of the
14 Kimura distribution (Wonnapijit et al., 2008). When pooling mutant loads measured
15 in each embryo from a given woman, mean values were 30±15% and 32±23% for
16 patients 1 and 2, respectively. Each of these values was close to the mutant load
17 measured in oral mucosa cell DNA from the corresponding woman (30% and 27% for
18 patients 1 and 2, respectively).

19 Embryo 10 from family 2, harbouring a 13% mutant load at Day 3, was
20 cultured up to the blastocyst stage (Day 5) and separated in 2 parts, namely, the
21 inner cell mass and the trophoctoderm. PCR failed to amplify the inner cell mass
22 DNA, while the heteroplasmy level of the trophoctoderm was consistent with the level
23 measured in Day 3 blastomeres (15% vs 13%).

24 25 **Analysis in fetuses over 10GW**

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3 1 Mutant loads were assessed in extraembryonic or embryonic tissues collected
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5 2 between 10GW to term, in 12 fetuses from 7 carriers (families 2 to 8, Table 4).
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8 3 One fetus did not carry the mutation in his two tested tissues (amniocytes at
9
10 4 16 GW, and cord blood at birth, fetus 4). As observed in early embryos, no fetus was
11
12 5 homoplasmic for the mutant. The mutation load ranged from 4% to 79% in the 11
13
14 6 carriers. There was no substantial variation in mutant loads assessed at various
15
16 7 stages of pregnancies until birth (Table 4). When excluding data from extra-
17
18 8 embryonic tissues (10GW trophoblast or at term placenta), m.3243A>G mutant loads
19
20 9 were identical in all tested tissues from a given fetus [mean±SD: 74.6±0.7% (3
21
22 10 tissues), 42±0.8% (5 tissues), 71±2% (5 tissues), and 78±0.9% (7 tissues) for fetuses
23
24 11 2a, 2b, 5b and 7a, respectively]. When placenta and other tissues were available at
25
26 12 the same term of gestation (3 fetuses), values were similar in two fetuses (74.6 vs
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28 13 74% and 78 vs 78% for fetus 2a and 7a, respectively), while fetus' 2b mutant load
29
30 14 was higher in placenta than in other tissues (57±2% vs 42±2%, respectively). Multiple
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32 15 samples in various parts of placenta, aimed at testing the mutant load distribution,
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34 16 did not show any substantial variation in 2, 6, 10, and 18 various loci of fetuses' 2a,
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36 17 2b, 2c, and 3 placentas, respectively (Table 4), irrespective of the gestation stage (12
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38 18 GW or at birth).
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45 19 Single trophoblastic cells (n=83) and amniocytes (n=64) were isolated from 6
46
47 20 fetuses to investigate the intercellular mutant load variation (Figure 2). Simultaneous
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49 21 analyses of parents' and fetal cell DNAs using a polymorphic marker proved the fetal
50
51 22 origin and the absence of contamination by maternal DNA of the analyzed cells (not
52
53 23 shown). Large intercellular variations were found both in trophoblastic cells (range: 7-
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55 24 21%, 6-38%, 23-55%, 23-70%, and 64-100%, for fetuses 5a, 6, 12b, 8, and 2a,
56
57 25 respectively), and amniocytes, either at 14 GW (range : 10-23%, 16-42%, and 52-
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3 1 94%, for fetuses 12b, 6 and 5b, respectively), or at 30 GW (range : 9-17% and 5-28%
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5
6 2 in fetuses 5a and 12b, respectively). When pooling values from all cells analyzed for
7
8 3 a given cell type in a given fetus, the average mutant loads were very close to those
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10 4 achieved from overall chorionic villi sampling (CVS) and amniotic fluid sample (AFS)
11
12 5 mutant loads (variation<10%, Figure 2).

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15 6 Comparison of the mean heteroplasmy rate vs one standard deviation of the
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17 7 heteroplasmy distribution at the single-cell level for each of the ten fetal cell pools
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19 8 (34.3±23%) showed a 0.81 correlation coefficient ($p<0.01$) with a positive slope
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21 9 (0.13±0.03) (Table 2 and Supp. Figure S2).

10 11 **Predictive value of a prenatal mutant load for the postnatal outcome**

12 Assessment of the predictive value of a prenatal mutant load for the disease severity
13
14 13 in the postnatal period is of crucial importance. A follow-up of our small cohort of
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16 14 patients born after PGD (n=1) or PND (n=9) provided a preliminary indication, even
17
18 15 though for ethical reasons we could not check for the presence of the mutation in
19
20 16 these asymptomatic children. All these children, harbouring mutant loads between 0
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22 17 and 35% in prenatal period, are indeed healthy at 1 month to 5 years of age (Table
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24 18 4).

19 20 **Discussion**

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22 22 Analysis of tissues and cells from human embryos and fetuses is currently the only
23
24 23 approach to get some insight into processes that govern maternofetal transmission of
25
26 24 common mtDNA mutations in humans, owing to the numerous technological
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28 25 difficulties met in attempts at creating animal models of mitochondrial disorders

1 (Inoue et al., 2000; Fan et al., 2008). We used this approach to study the segregation
2 of m.3243A>G mtDNA molecules during human *in utero* development, thanks to our
3 PND/PGD activities, offered to carriers of this mutation.

4 These data rely on a robust, specific and sensitive assay we devised for the
5 quantification of the m.3243A>G MELAS mutation in tissues and single cells.

6 While last-cycle labeling PCR indeed is a well-established and agreed-upon
7 standard protocol for the quantification of mtDNA heteroplasmy, a major concern with
8 this approach is the need to add the labeled primer prior to the last PCR cycle. This
9 process indeed runs a significant risk of contamination of both the endogenous PCR
10 product by exogenous DNA, and of the laboratory dedicated to single cell analyses
11 by the endogenous PCR product. Such a risk, being a minor one when amplifying a
12 large amount of DNA, becomes a major hurdle to the use of this method, when
13 applied to single cell analysis.

14 We ascertained the reliability of our test over a wide range of mtDNA copies, from
15 10^2 to 10^6 . This range encompassed the mean value of mtDNA copies in
16 lymphocytes [around 150 copies, Urata et al 2008; Maagaard et al. 2006]
17 blastomeres [11 000 – 449 000 copies, Lin et al 2004] and oocytes [200 000 –
18 800 000 copies, Craven et al. 2010]. Our results demonstrated an insensitivity to
19 template input amounts for this particular method, thus validating the results of the
20 experiments carried out on various cells and tissues.

21 22 **Analysis in Day 3-Day 5 embryos**

23 To our knowledge, data on the presence of the m.3243A>G mutation at the Day 3
24 stage of embryonic development have not been reported so far. This mutation was
25 found in 32 of 38 embryos (84%) from 2 unrelated carriers. This transmission rate is

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2
3 1 in agreement with a previous report on the presence of m.3243A>G in adult carrier's
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5 2 primary oocytes (Brown et al., 2001), whose 74 of 82 oocytes (90%) were
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7 3 heteroplasmic. A woman carrying the pathogenic mutation may thus have mutation-
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9 4 free embryos, advocating PGD as an appropriate procedure for preventing
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11 5 recurrence of affected offspring in carriers, **at least in the mutant load range reported**
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13 **in this study.**
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19 7 Mutant DNA molecules were constantly found to be equally distributed among
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21 8 the various blastomeres of a carrier embryo until **Day 5**, in agreement with data on
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23 9 mtDNA polymorphisms achieved in mouse (Dean et al., 2003) and human embryos
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25 10 (Marchington et al., 2010), as well as on the m.8993T>G (NARP) mutation in human
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27 11 (Steffann et al., 2006). Single-cell analysis thus reflects the whole embryo mutant
28
29 12 load, supporting relevance of the usual PGD procedure for assessment of the overall
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31 13 embryo genotypic status. It is however worth noting some occasional variability (up to
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33 14 15%) in mutant load between single cell measurements and the whole embryo. This
34
35 15 discrepancy could have arisen from either a technical artifact (such as partial loss of
36
37 16 mitochondrion during whole embryo recovery) or physiological distortion in
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39 17 m.3243A>G segregation from **Day 3** (blastomere sampling stage) to **Day 5** (whole
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41 18 embryo recovery stage). This last hypothesis is unlikely since mutant load values
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43 19 were very close in **Day 5** trophectoderm and **Day 3** single-blastomeres in the only
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45 20 embryo where both tissues were available (Table 3), additionally indicating that the
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47 21 mtDNA molecule segregation is not skewed across embryonic and extraembryonic
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49 22 cells (trophectoderm) at this developmental stage. Finally, inter blastomere stability of
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51 23 the mutant load suggests that embryonic heteroplasmy determined from a single cell
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53 24 analysis provides an accurate estimate of the whole embryo mutant load, **as**
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3 1 previously reported for other mtDNA variants (Steffann et al., 2006; Craven et al.,
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5 2 2010)
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8 3 The m.3243A>G mutant load was found to be highly heterogeneous among
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10 4 various embryos from a given individual, ranging from 18 to 50% and from 0 to 77%
11
12 5 in patients' 1 and 2 embryos, respectively. These values on "early" embryos are in
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14 6 agreement again with those on primary oocytes from a m.3243A>G carrier, the
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16 7 mutant load of which ranged from 1 to 50% (Brown et al., 2001). Two conclusions
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18 8 can be derived from these observations.
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22 9 The first conclusion concerns the mode of transmission of m.3243A>G from
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24 10 the mother to her progeny. The distribution of the mutation level values among
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26 11 Patient 2's embryos was consistent with the Kimura distribution calculated from the
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28 12 neutral drift theory. Furthermore, an equal number of her embryos were found to
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30 13 harbour a mutation level greater than the mean value (18 embryos) and less than the
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32 14 mean (17 embryos). Finally, the mean level of heteroplasmy amongst all embryos
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34 15 was very close to the heteroplasmy level seen in the mother (oral mucosal cells and
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36 16 blood, patients 1 and 2). These data argue for random genetic drift as the
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38 17 mechanism governing mtDNA segregation during oogenesis. This observation in
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40 18 humans is in full agreement with the results of a study of heteroplasmy distribution in
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42 19 the progeny of mice carrying apparently neutral BALB/c and NZB mtDNA sequence
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44 20 variants (Jenuth et al., 1996). Assuming that blastomere analysis provides a direct
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46 21 insight on mature oocyte mutant load, our data, gathered with those of Brown et al.,
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48 22 suggest that mtDNA segregation is governed by a random genetic drift mechanism
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50 23 over the whole oogenesis period.
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58 24 The second conclusion regards the size of the bottleneck operating for
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60 25 m.3243A>G during oogenesis (table 5). The embryos from patient 2 had a larger

1 normalized variance (Wonnapijitte et al., 2010), and therefore a smaller bottleneck
2 parameter, than the oocytes from the individual in Brown's study (Brown et al., 2001).
3 The confidence intervals did not quite overlap, indicating that there is a real
4 difference between the bottleneck sizes in these two individuals, though this
5 difference is not large. These data therefore suggest that some individual-dependent
6 parameter modulates the bottleneck size for a given mtDNA mutation.

8 **Analysis in 10 – 37 GW fetuses**

9 Very few data are available on m.3243A>G segregation during fetal development so
10 far. We looked for this mutation in 12 fetuses from 8 carriers, and aggregated these
11 data with those that we (patients 12 and 13, Table 4, Bouchet et al., 2006) and other
12 groups (patients 9 to 11, Table 4, Matthews et al., 1994; Cardaioli et al., 2000; Chou
13 et al., 2004) had previously published. Maternal m.3243A>G was passed on to 17 of
14 20 fetuses (85% transmission rate). Taking embryonic and fetal data together,
15 carriers passed on the mutation to 49 of their 58 offspring (84%, Tables 3 and 4).
16 This transmission risk fits with the MELAS transmission rate calculated from the
17 mutant load determined in buccal mucosa samples from carriers' offspring in the
18 postnatal period (Uusimaa et al., 2007). It can not be ascertained whether the 3
19 fetuses shown to be mutation-free at 10GW in the current study were mutation-free
20 embryos initially or alternatively resulted from subsequent loss of mutant mtDNA
21 molecules they carried at early embryonic stage. All fetuses but one (fetus 2c) were
22 indeed conceived out of a PGD procedure, and mutant load values at the **Day 3**
23 stage were therefore not available. Our data however argue for mutant load stability
24 throughout the entire period of embryofetal development. In fetus 2c, loads in 14GW
25 amniocytes and in term cord blood were indeed 4 and 5%, respectively, in agreement

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3 1 with those assessed at early embryonic stage (embryos 7 and 11 from patient 2,
4 carrying 5 and 14%, respectively, only one of whom, -fetus 2c-, developed). We
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9 similarly did not find any temporal variation of the MELAS mutant load in multiple
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11 4
12 samples taken within 10 to 39 GW (Table 4 A and B).

13 5 Study of various tissues from six 12-25GW fetuses (2a, 2b, 5b, 7a, 9, 10)
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16 showed a tight intertissue stability in each of them. Mutant load assessed in extra-
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19 embryonic (placenta) and embryonic tissues collected at the same gestation term
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22 from 4 fetuses, was found to be similar in 3 of them (2a, 7a, and 9), being higher in
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25 trophoblast (57 vs 42%) from the remaining one (fetus 2b). Discrepancy between
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27 10
28 trophoblast and non trophoblastic fetal tissues did not seem to result from an
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30 11
31 ascertainment bias secondary to loco-regional mutant load heterogeneity within
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34 placenta, recently shown to exist (Marchington et al., 2006). The heteroplasmy level
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37 was indeed highly similar in six separate regions of fetus 2b placenta (Table 4). Thus,
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40 this placento-fetal discrepancy might be accounted for by selection of a few cells
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43 which will form the first source for the placental membranes in the partitioning
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46 blastocyst. This observation additionally emphasizes that analysis of single CVS
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49 sample, carried out in a PND frame, may fail to assess the fetal mutant load reliably,
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52 as recently reported (Marchington et al., 2010).

53 19 The observation of tight mutant load stability, both across tissues and with
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56 time, in all tested fetuses, suggests that mtDNA segregation is mainly governed by
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59 random genetic drift in somatic tissues throughout the entire period of human
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23 embryofetal development. Furthermore, as already noted in embryos, none of the 20
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25 fetuses analyzed so far (Table 4) was found to carry a heteroplasmy rate exceeding
80%, over a wide range of maternal mutant loads (from 5% in WBC to 80% in urinary tract cells). Whether such a 80 % value has a biological significance (i.e., a

1 hypothetical selection against highly mutated embryos/fetuses) remains speculative.
2 Since mutations such as m3243A>G exhibit a relatively normal pattern of distribution
3 around the maternal mean, it would be unlikely, given the relatively small sample size
4 and the mean maternal mutant load value in majority below 40 % (in blood) in our
5 series (table 1) to find embryos with greater than 80 % heteroplasmy, even in the
6 absence of selection.

7 We attempted to establish at the single cell level the basis of the mutant load
8 stability observed across fetal tissues. We thus analyzed 147 cells distributed into ten
9 pools of trophoblastic or amniotic cells (7-30 cells per pool) collected in six unrelated
10 fetuses within the 10-30 GW period (Figure 2). Except for one homoplasmic mutant
11 cell (fetus 2a), all cells were heteroplasmic with a mutant load ranging from 5 to 95%.
12 This observation is in line with the few studies available on MELAS single cells
13 collected during the postnatal life, that indeed failed to detect mutant loads over 92%
14 and 98% in lymphocytes (Saitoh et al., 1999) and muscle fibers (Silvestri et al.,
15 2000), respectively. These data argue that, over a critical threshold of heteroplasmy,
16 the resulting impact on respiratory chain function promotes fetal cell death
17 (Sasarman et al., 2008).

18 When pooling values from all cells belonging to a given pool, mutant load
19 dispersion ranged from 10 to 47% (Figure 2). The frequency distribution of single cell
20 mutant load within each of the ten cell pools corresponded to a binomial distribution,
21 with a median value identical or very close to the mean value of heteroplasmy, in
22 keeping with the random genetic drift mechanism. We subsequently looked for an
23 optional relationship between a mutation rate at the tissue (chorionic villi or
24 amniocytes) level and the amplitude of mutant load dispersion at the single cell level
25 in this tissue (Supp. Figure S2). A previous study using adult carrier lymphocytes had

1 indeed suggested that, the higher the proportion of mutated mtDNA molecules, the
2 wider the dispersion of heteroplasmy level (Saitoh et al., 1999). Though the number
3 of single cells analyzed was low in each pool, we however assumed that each of the
4 ten cell pools was fairly representative of the whole tissue sample, based on mean
5 mutant load variance consistently less than 10% between the cell pool and the whole
6 sample. It clearly appeared that, the higher the tissue mutant load, the larger the
7 heteroplasmy dispersion in trophoblastic and amniotic cells, even if the low number
8 of analyzed cells precluded any firm conclusion on the biological relevance of this
9 observation. We then attempted to evaluate whether such a hypothetical relationship
10 can be considered in various tissues from m.3243A>G carriers, collected in prenatal
11 and postnatal periods as well. We considered that Saitoh's study was exposed to
12 some bias in heteroplasmy assessment, due to the complex experimental procedure
13 of this study (first PCR, electrophoresis gel extraction of the PCR products, second
14 PCR, PCR products enzymatic digestion, southern blotting, and bioimager analysis).
15 Using DNA from five adult carrier females, we therefore recapitulated Saitoh's study
16 with our own method of mutant load assessment, in an effort to homogenize our
17 data. We thus found a statistically significant correlation between the mean mutant
18 load in each of the five lymphocyte pools and the heteroplasmy distribution at the
19 single cell level. Interestingly, comparison of our data in lymphocytes and fetal cells
20 showed a marked difference between curve slopes for a same range of
21 heteroplasmy, arguing for some tissue-dependence of mtDNA segregation at the cell
22 level (Supp Figure S2). These data contrast with the few available data of the
23 literature, regarding single muscle fibers in adult carriers. The latter indeed showed
24 that, the higher the mean mutant load of a cell pool, the lower the heteroplasmy
25 distribution at the cell level (Petruzzella et al., 1994; Tokunaga et al., 1994; Silvestri

1 et al., 2000). However, the range of mutant loads does not overlap between skeletal
2 muscle (60-100%) and the other tissues (5-60%). Thus, assuming that the apparent
3 relationship between mean mutant loads at a whole sample level and mutant load
4 dispersion at the single cell level makes biological sense in all these tissues, it would
5 remain to be seen whether the proposed tissue-dependance of such correlations is
6 valid throughout the 0-100% range of heteroplasmy.

7

8 **Comparison of prenatal and postnatal data**

9 It is difficult to reconcile the prenatal inter-tissue stability observed in this study, and
10 the tissue-dependance of m.3243A>G mutant loads in adult carriers, who harbour
11 heteroplasmy rates almost constantly higher in skeletal muscle, urinary epithelial
12 tract cells, and hair follicles, than in white blood cells (Chinnery et al., 1999;
13 Frederiksen et al., 2006; Whittaker et al., 2009).

14 This tissue dependance has been suggested to result from a replicative
15 disadvantage of cells harbouring a high mutant load, thus promoting “mutation
16 epuration” from fast regenerating tissues. Should such a biological process operate
17 prenatally, it could be missed out within the “short” course of a pregnancy, by
18 reference to the very slow decrease of mean mutation level in adult white blood cells
19 (Rajasimha et al., 2008), thus reflecting the apparent inter-tissue mutant load stability
20 throughout the prenatal period. Alternatively, cell proliferation rate would be identical
21 among all tissues throughout intra-uterine life, and would become tissue dependant
22 from a fairly late postnatal period. A similar value of mutant load (65 to 70%) has
23 indeed been reported in amniocytes at 21 GW, and in peripheral blood and hair
24 follicles sampled in a four-year old carrier (Chou et al., 2004).

25

1 Comparison of mtDNA metabolism in m.3243A>G (MELAS) and m.8993T>G 2 (NARP) mutations throughout human embryofetal development

3 Comparing mtDNA segregation data throughout human embryofetal development for
4 two different mtDNA mutations, namely, m.3243A>G/ MELAS and m.8993T>G/
5 NARP, highlights striking differences (Figure 3).

6 While all MELAS embryos herein reported, originating from 20 %-mutant load
7 carriers, are mutation-free or heteroplasmic up to 75% with an apparently random
8 distribution of heteroplasmy, the only two reports on m.8993T>G at Day 3-Day 5
9 embryonic stage indicated that the 3 NARP embryos from a 18-% mutant load carrier
10 were either wild-type or mutant homoplasmic (Steffann et al., 2006), the 5 remaining
11 ones carrying low levels of heteroplasmy (4-22%, Tajima et al., 2007). Though the
12 low number of embryos analyzed so far precludes drawing firm conclusion on a
13 putative difference of segregation between m.8993T>G and m.3243A>G molecules
14 during oogenesis, these apparently different patterns of segregation are however
15 substantiated by the few available analyses of primary oocytes from carriers of these
16 mutations (Blok et al., 1997; Brown et al., 2001). A wild-type (1/7) or mutant
17 homoplasmic state (6/7) was indeed the rule in the 7 NARP primary oocytes from a
18 50%-mutant load carrier, contrasting with the presence of heteroplasmy in 90% of 82
19 primary oocytes in a m.3243A>G carrier with a 8 %- mutant load in WBC. It can be
20 speculated from these data that bottleneck would be of different size between
21 m.3243A>G and m.8993T>G.

22 Whether a purifying selection operates in the human germline against
23 m.8993T>G and m.3243A>G is a matter of debate. Our data in early NARP embryos
24 (Steffann et al., 2006), those from the analysis of primary oocytes in a m8993T>G
25 carrier (Blok et al 1997), as well as our previous report on 2 fetuses with a mutant

1 load over 85 % from 2 m8993T>G carriers with a 30% mutant load in WBC (Steffann
2 et al. 2007), clearly argue against such a negative selection for this mutation in
3 human. As mentioned above, no conclusion can be drawn at this point on the
4 occurrence of a purifying selection against m.3243A>G in the human germ line,
5 owing to the limited number of data we achieved, all drawn from low (20 %) mutant
6 load carriers. It has however to be emphasized that we never observed any fetus
7 with a mutant load over 80% among the 8 pregnancies from 6 m.3243A>G carriers
8 with a WBC mutant load equal or even higher than 30 % (patients 6-9, 11, 12, table
9 1), These data are difficult to reconcile with those from murine germline segregation
10 studies indicating that mutations in protein coding genes of mtDNA are strongly
11 selected against, whereas mutations affecting tRNA genes (such as m.3243A>G) are
12 largely refractory from such a process (Fan et al, 2008, Stewart et al, 2008).

13 Regarding the somatic tissue development, we previously showed the
14 existence of fetuses carrying m.8993T>G with various degrees of mutant loads, from
15 0% to 100 %, over the 10-GW stage of pregnancy until delivery (Steffann et al.,
16 2007). m.8993T>G and m.3243A>G mutant loads remain at a steady-state level with
17 time or across various tissues, thus supporting a random segregation of mutant
18 mtDNA molecules throughout somatic embryo-fetogenesis irrespective of the
19 mutation type.

20 **In conclusion**, one can speculate that the bottleneck size, **and possibly**, the
21 mutant load threshold critical for embryo/fetus survival, varies among mtDNA
22 mutations, thus hampering genetic counselling and PND/PGD procedures in
23 mitochondriopathies resulting from “private” mtDNA mutations. To test this
24 speculation, we will need to collect data on mtDNA mutant loads for other rarer
25 mtDNA mutations.

Acknowledgments

We gratefully acknowledge Dr C Elie and Dr JP Jais for their assistance with the statistics. We also thanks the families for their cooperation. This research was supported, in part, by grants from l'Association Française contre les Myopathies (AFM), l'Agence de Biomédecine, l'INSERM, and the European network Mitocircle. S.M was funded by l'AFM

Web resources

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim>

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FIGURES LEGENDS

Figure 1. Standardization of m.3243A>G load assessment

The rate of m.3243A>G heteroplasmy was assessed by semi-quantitative fluorescent PCR using 10 samples containing various proportions of mutant plasmid. Each sample comprised 10^6 mtDNA copies. Each value is the mean of three independent experiments. Bars depict standard deviations. WT: wild-type DNA.

Figure 2. Distribution of m.3243A>G mutation levels across isolated cells

MELAS mutant load was assessed in trophoblastic cells sampled at 10GW (open boxes) and amniocytes sampled at 14 (hatched boxes) or 30 (filled boxes) GW in 6 fetuses (numbering refers to Table 4). n: number of analyzed cells. Each box depicts the mean mutant load plus (top bar), and minus 1 standard deviation (bottom bar) for a given cell pool. Whiskers depict maximal (top) and minimal (bottom) values for each sample. Central bar: median. * mean mutant load assessed on the whole chorionic villi, or amniocyte sample.

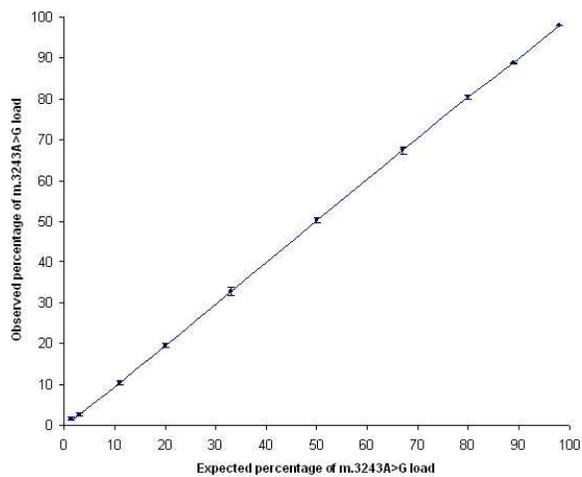
Figure 3. Comparative distribution of m.3243A>G and m.8993T>G/C mutant loads in mature oocytes, blastomeres, and fetal tissues

◇ m.3243A>G (MELAS syndrome), ○ m.8993T>G/C (NARP syndrome). Scatter plots are drawn from personal (black symbols) and literature data (white symbols: Harding et al.1992; Matthews et al., 1994; Blok et al., 1997; Ferlin et al., 1997; White et al., 1999; Cardaioli et al., 2000; Brown et al., 2001; Leshinsky-Silver et al., 2003; Chou et al., 2004 ; Pettman et al., 2007; Tajima et al., 2007). Each point depicts one oocyte or one individual (embryo or fetus). All oocyte values were from a single

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3 1 carrier of m.3243A>G (Brown et al., 2001) or m.8993T>G (Blok et al., 1997). When
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5 2 several values were available for one individual, the average heteroplasmy value was
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8 3 selected. Both Day 3 and 14GW values were available (and therefore depicted) for
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10 4 one "NARP" (0% and 0%) and one "MELAS" individual (5% and 5%) only. All other
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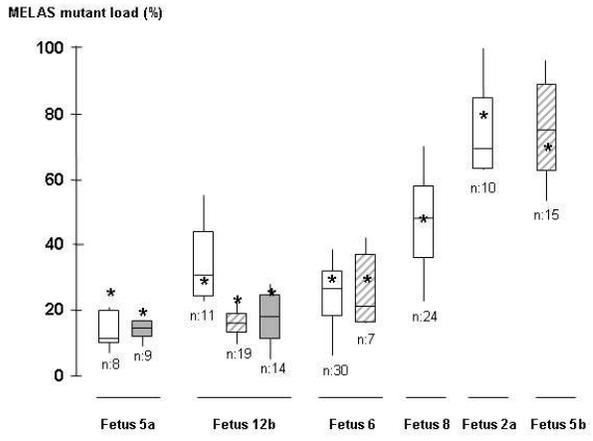
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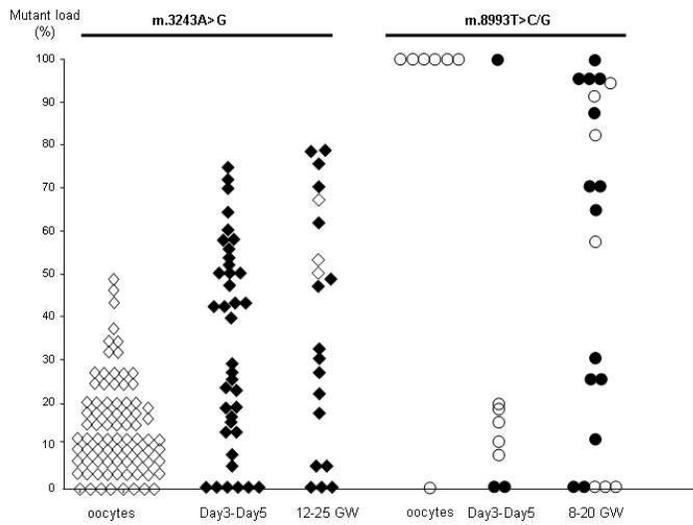
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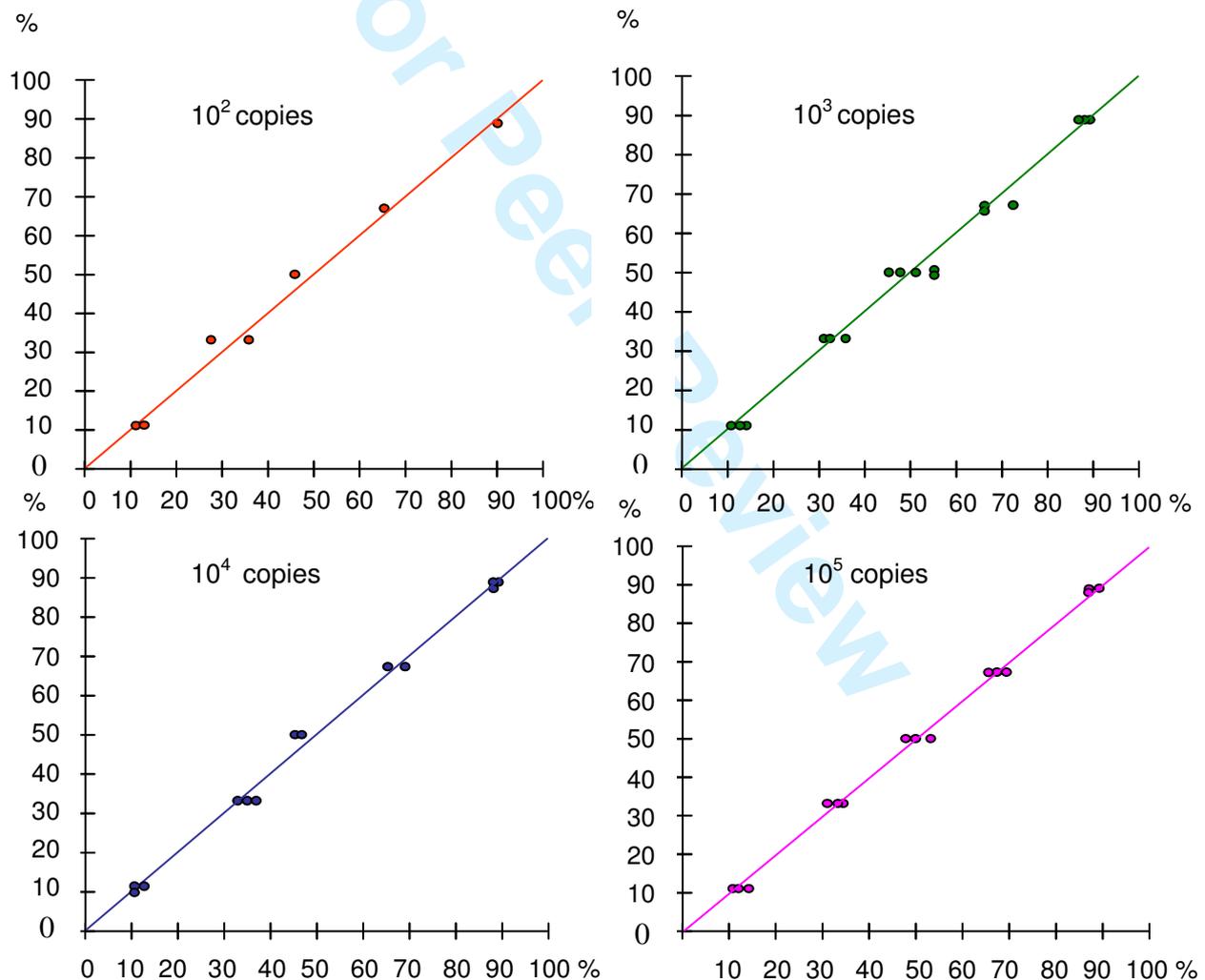


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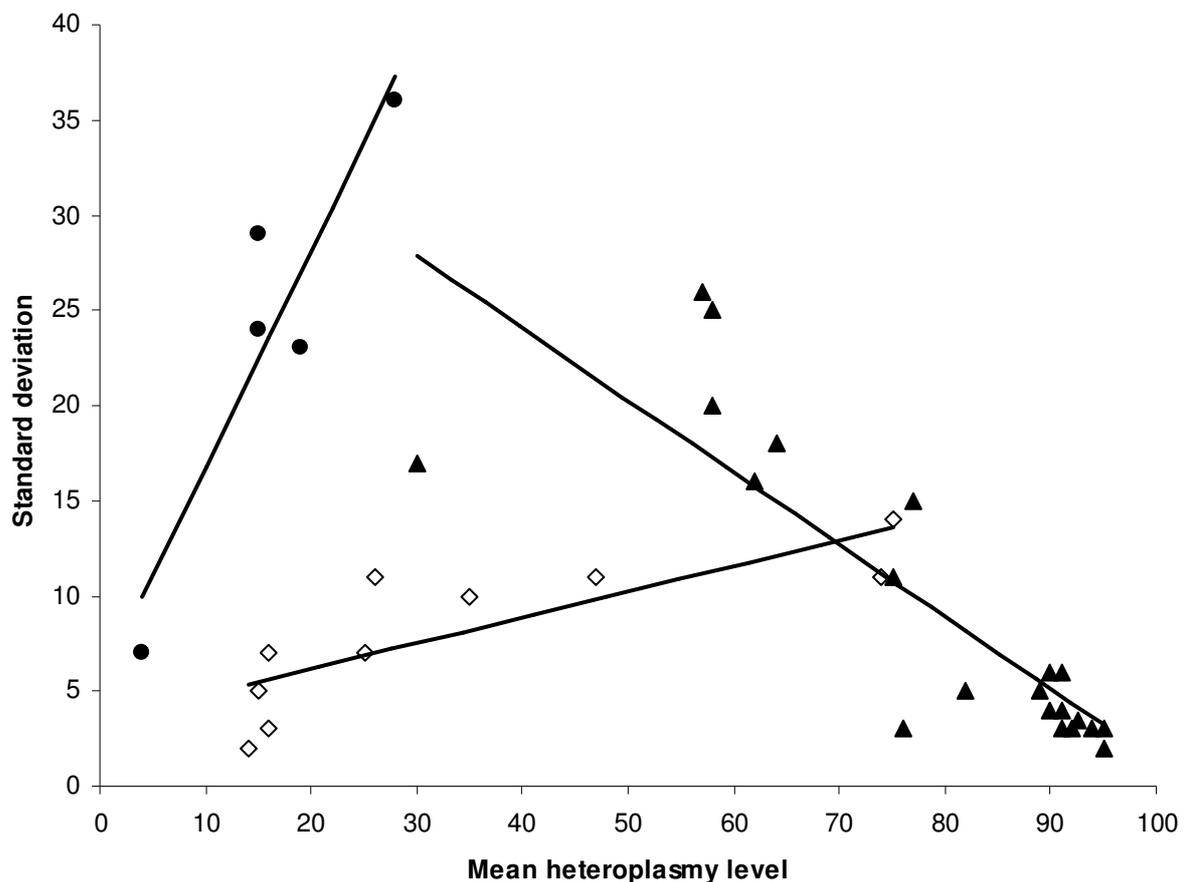
Supplementary Figure 1. Standardization of m.3243A>G load assessment over a 10^2 - 10^5 mtDNA copy range

The rate of m.3243A>G heteroplasmy was assessed by semi-quantitative fluorescent PCR using 5 samples containing various proportions of mutant plasmid (11%, 33%, 50%, 67%, 89%) and 5 different template amounts (10^2 , 10^3 , 10^4 , 10^5 , 10^6). Values achieved with 10^6 copies are displayed as Fig 1. Each dot depicts the result of one experiment. Y axis: expected percentage of m.3243A>G mutant load. X axis: observed percentage of m.3243A>G mutant load.



Supplementary Figure 2. Distribution of inter cell dispersion of m.3243A>G mutant load according to the heteroplasmy rate of overall cell pools

Data are represented as standard deviation of the mean mutant load vs mean heteroplasmy rate for each pool. \diamond prenatal cell pools: syncytiotrophoblasts and amniocytes (data from Table 2 and Figure 2); Black symbols: postnatal cell pools: \bullet lymphocytes (data from Table 2), \blacktriangle muscle fibers (data from Petruzella et al., 1994; Tokunaga et al., 1994; Silvestri et al., 2000). Each symbol reflects the analysis of one cell pool for one individual.



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Table 1. Maternal m.3243A>G mutant load

	Patient	Mutant load (%)		
		WBC	OMC	UTC
A	1	20	30	65
	2	20	27	30
	3	20	40	50
	4	7	8	15
	5	5	10	35
	6	30	50	80
	7	30		70
	8	40		55
B	9	39	48	
	10	20		
	11	63		
	12	34	45	80
	13	21		

A, Patients analyzed in this study. **B**, Patients reported elsewhere: (9) Matthews et al., 1994, (10) Cardaioli et al., 2000, (11) Chou et al., 2004, (12, 13) our group (Bouchet et al., 2006). WBC, white blood cells; OMC, oral mucosa cells; UTC, urinary tract cells.

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Table 2. m.3243A>G mutant load in single cells of various origins

	Lymphocytes	Syncytiotrophoblasts/amniocytes	Muscle fibers^a
Number of patients	5	5	8
Number of cell pools	5	10	21
Total cell number	84	147	341
Heteroplasmy level (mean \pm SD)	16.2 \pm 8 %	34.3 \pm 13 %	78.5 \pm 17 %
Correlation coefficient (p)	0.92 (< 0.05)	0.81 (<0.05)	0.84 (<0.01)
Slope \pm SD	1.14 \pm 0.28	0.13 \pm 0.03	-0.38 \pm 0.05

^a calculation from data by Tokunaga et al., 1994; Petruzella et al., 1994; Silvestri et al., 2000

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Table 3. m3243A>G mutant load in preimplantation embryos

Patient	Embryo	Single blastomere		Overall embryo Median	Mean (±SD)
		Blastomere 1	Blastomere 2		
1	1			18±1 (n=6)	↑
	2			23±2 (n=5)	23%
	3			50±3 (n=3)	↓
2	1	0 ^a	0		↑
	2	0	0		
	3	0	0	0	
	4			0	
	5			0	
	6			0	
	7	5	5		
	8	7	8		
	9	12	14		
	10	13	13	I ^b T: 15	
	11	13	16		
	12	17	22	15	
	13	14	15	16±2 (n=11)	
	14	^a	25	23	
	15	27	27	23	
	16			28	
	17	15	21	30	
	18	40	41	37	37%
	19			42	
	20			42	
	21			43	
	22			47	
	23	51	55	48	
	24			50	
	25			50	
	26	39	40	50	
	27	54	55	50	
	28			55	
	29			60	
	30	55	55	62	
	31	63	66	63	
	32	51	54	66	
	33	67	68	74	
	34	72	77		
	35	69	71	75	↓

Patient numbering refers to Table 1. Individual values *i*) represent the heteroplasmy level of 2 single blastomeres or of embryos in toto, *ii*) are expressed as mean ± SD when more than two blastomeres from one embryo were analyzed (number of analyzed blastomeres in brackets). Embryo 10 from family 2 was separated in two parts before PCR analysis, ie, the inner cell mass (I), and the trophectoderm (T). Calculation of "overall mean value" and "overall median value" took into account either the mean of the values achieved through analysis of all blastomeres when only blastomeres were available, or values from embryos only in other cases. ^adetection threshold:2%; ^b PCR failure.

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Table 4. m.3243A>G load in extra-embryonic and embryo-fetal tissues

Patient	Fetus	CVS	AF1	AF2	Muscle	Liver	Heart	Skin	Kidney	Lung	Gut	Brain	Optic nerve	Thymus	Umbilical cord	Placenta	Cord blood	
A	2	2a	77±3		75±1.5		74±3.2			74±0						73, 75 ^a		
		2b	59±6		42±1		41±2	42±2	43±2	43±0.3							57±2 ^b	
		2c			4±0												15±5 ^c	5±1
		3	3	17±3	16±0.5												22±6 ^d	16±0
		4	4		0													0
		5	5a	20±0.5	15±3	15±2												
			5b		70±1		72±2		73±0	68±2					72±1			
			5c	4±0.6														
		6	6	32±1	28±1													
		7	7a			79±0	78±0.6	77±0.9	78±0.3	79±0	79±0.3					78±1.7	78±1.7	
		7b	79±2															
	8	8	49±0.7															
B	9	9			55±1	52±4	54±3	51±3	56±5		53±4	52±3	51±3			55±3		
		10	10		50	50							50					
		11	11			67												
		12	12a	35	33	29												
			12b	31	23	26												
		13	13a	60	63													
			13b	0	0													
		13c	0	0														

A, Fetuses analyzed in this study. Chorionic villi (CVS) were sampled at 10 GW, and amniotic fluids (AF) at 14 (AF1) and 30 (AF2) GW, respectively. The other tissues were sampled at 12 GW (fetuses 2a and 2b), or at 19, 20, 24, and 25 GW for fetuses 5b, 7, 9, and 10, respectively, and at term for fetuses 2c and 3. Cord blood was sampled at birth for fetuses 2c (38GW), 3 (37GW) and 4 (39 GW). All assays were carried out in triplicate. Values are expressed as mean ±SD. Placenta values measured on ^a2, ^b6, ^c10, and ^d18 separate biopsies, respectively (SD: intersample variation). **B**, Fetuses reported elsewhere: (9) Matthews et al., 1995, (10) Cardaioli et al., 2000, (11) Chou et al., 2004, (12, 13) our group (Bouchet et al., 2006). In boldface: patients born after PGD or PND.

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For Peer Review

Table 5. Comparative analysis of mutation levels in preimplantation embryos from patient 2 and primary oocytes from a published report

	Patient 2		Brown et al., 2001	
Sample size (n)	35		82	
Mean mutation level (p_0)	33.7%	[25.8% – 42.1%]	12.6%	[10% - 15%]
Mutation level variance (V)	0.06	[0.037 – 0.086]	0.014	[0.009 – 0.020]
Normalized variance ($V/(p_0(1-p_0))$)	0.27	[0.18 – 0.38]	0.13	[0.09 – 0.17]
Bottleneck parameter (b)	0.72	[0.62 – 0.82]	0.87	[0.83 – 0.91]
Probability of fixing on wild-type	8.4%	[1.5% - 19%]	14%	[6% - 24%]
Probability of fixing on the mutant	0.4%	[0 – 2.0%]	2×10^{-7}	[0 – 1×10^{-6}]
Probability of having > 60% mutant	17%	[7% - 27%]	0.3%	[0.02% - 0.8%]

Values are given as mean [95% confidence intervals].

The distribution of the mutation level values from Patient 2 was consistent with the Kimura distribution (p-value of 0.53 by KS test).