

Preventing the transmission of pathogenic mitochondrial DNA mutations: can we achieve long-term benefits from germ-line gene transfer?

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Mitochondrial medicine is one of the few areas of genetic disease where germ-line transfer is being actively pursued as a treatment option. All of the germ-line transfer methods currently under development involve some carry-over of the maternal mitochondrial DNA (mtDNA) heteroplasmy, potentially delivering the pathogenic mutation to the offspring. Rapid changes in mtDNA heteroplasmy have been observed within a single generation, and so any 'leakage' of mutant mtDNA could lead to mtDNA disease in future generations, compromising the reproductive health of the first generation, and leading to repeated interventions in subsequent generations. To determine whether this is a real concern, we developed a model of mtDNA heteroplasmy inheritance by studying 87 mother–child pairs, and predicted the likely outcome of different levels of 'mutant mtDNA leakage' on subsequent maternal generations. This showed that, for a clinical threshold of 60%, reducing the proportion of mutant mtDNA to <5% dramatically reduces the chance of disease recurrence in subsequent generations, but transmitting >5% mutant mtDNA was associated with a significant chance of disease recurrence. Mutations with a lower clinical threshold were associated with a higher risk of recurrence. Our findings provide reassurance that, at least from an mtDNA perspective, methods currently under development have the potential to effectively eradicate pathogenic mtDNA mutations from subsequent generations.

Key words: mitochondria / mtDNA / gene therapy / prenatal diagnosis / germ line

The importance of preventing mitochondrial DNA disease

Pathogenic mitochondrial DNA (mtDNA) mutations are found in 0.5% of the population (Elliott *et al.*, 2008), and are a frequent cause of maternally inherited human disease affecting at least 1 in 6500 of the population (Schaefer *et al.*, 2008). Many of these mutations are heteroplasmic, with a mixture of mutated and wild-type mtDNA present in varying proportions within cells of the same individual. High percentage levels of mutated mtDNA are associated with severe multi-system diseases that often affect the nervous system. In addition, some homoplasmic mutations can also cause disease (DiMauro and Schon, 2003). There are currently no treatments for these diseases (Pfeffer *et al.*, 2012), so preventing maternal transmission is a high priority (Brown *et al.*, 2006; Poulton *et al.*, 2009). The medical ethics of modifying the germ-line mitochondrial genome have been discussed in detail (Bredenoord *et al.*, 2011) and the UK

Human Fertilisation and Embryology Authority is moving forwards with developing a legal framework for these methods (Callaway, 2012). Concerns include unexpected genetic and epigenetic consequences of the procedure itself. Although animal studies may provide some reassurance, including work in non-human primates (Tachibana *et al.*, 2009), any complications of the procedure may be subtle and take time to emerge. Thus, despite extensive pre-clinical evaluation, there will inevitably be concerns about safety when the procedure is first used in humans, coupled to the ethical issue of generating a child harbouring genetic material from 'three parents'.

Preventing mtDNA diseases: the challenges

Several unique features of mitochondrial genetics, including the physical separation of the mitochondrial and nuclear genome in the cell,

heteroplasmy of some pathogenic mtDNA mutations and the generally high level of mutant mtDNA required to cause serious pathogenic effects, all contribute to the practicality of developing effective germ-line transfer in diseases due to pathogenic mtDNA mutations. Several techniques are currently under development to prevent the transmission of pathogenic mtDNA mutations. Prenatal diagnosis (PND) with amniocentesis or chorionic villus biopsy, and preimplantation genetic diagnosis (PGD) are currently offered in a few centres, based on the accurate measurement of mtDNA heteroplasmy in tissue samples from pre- or post-implantation embryos (Thorburn and Dahl, 2001; Jacobs *et al.*, 2005; Steffann *et al.*, 2007). However, these approaches cannot be used to prevent the transmission of homoplasmic mutations. For heteroplasmic mutations, interpreting the measured level of heteroplasmy is challenging, particularly if there is an intermediate level (20–80%), if the mutation is rare or unique to that particular family, or if the mutation level is known to change over time (as is the case for the most common pathogenic heteroplasmic mtDNA mutation, m.3243A>G) (Craven *et al.*, 2011). Often both the clinician and the prospective heteroplasmic mother will use PND, or more so PGD, to identify embryos harbouring absolutely none of the pathogenic mtDNA mutation. It is also possible to select for fetuses and embryos, and thus prevent disease in the next generation and subsequent transmission. However, these approaches can result in termination in the context of PND, and will also reduce the number of embryos available for implantation in the context of PGD. As a result, several new techniques are in pre-clinical development, which can reduce the risk of transmission still further, and be broadly applicable to all mtDNA diseases. Early approaches involved cytoplasmic transfer (Meirelles and Smith, 1998), but more effective recent developments include spindle transfer (Tachibana *et al.*, 2009; Tachibana *et al.*, 2012) and pronuclear transfer (Brown *et al.*, 2006; Craven *et al.*, 2010). However, none of the current and proposed approaches assure the complete removal of mutant mtDNA. This raises concerns about the long-term consequences of ‘mutant mtDNA carry-over’ on future generations down the female line (Brown *et al.*, 2006; Poulton *et al.*, 2009). Although low levels (<20%) are unlikely to cause disease in the immediate offspring, the amount of the pathogenic mtDNA mutation could change on future transmission down the female line, limiting the effectiveness of the treatment in the longer term. Likewise, for mothers with a homoplasmic mutation, any approach that reduces the mutation load, but does not completely eliminate the mutation, will also introduce the possibility of recurrence in future generations, even if the level of mutation in the next generation is <20%. Why should this be the case?

Large changes in the percentage level of mutated mtDNA are observed in small human pedigrees transmitting heteroplasmic mtDNA mutations (Chinnery *et al.*, 2000), and so even low levels of mutant mtDNA carry-over could lead to maternal descendants with high mutation levels, causing severe multi-system disease (DiMauro and Schon, 2003). The shifts in mutation level that have been observed are sufficiently large to cause disease recurrence even within a single generation after producing effectively treated offspring. Should this be a cause for concern, and how low must the maternal mtDNA carry-over be in order to consider these therapies successful? These questions will be difficult to address experimentally because current animal models of mtDNA disease do not closely

resemble human disorders (Nakada and Hayashi, 2011) and there may be significant differences in the mechanism of mtDNA transmission (i.e. the mtDNA bottleneck) between humans and mice (Wonnapijit *et al.*, 2010). This means that the results of animal studies could be either falsely reassuring, or overly pessimistic. Primate work may provide further reassurance that there are no catastrophic unwanted side effects of the treatment (Tachibana *et al.*, 2009), but short-term experiments will not detect late-onset complications related to epigenetic reprogramming. Given the theoretical risks and uncertainties, experimental treatment in humans transmitting non-pathogenic, polymorphic variants are likely to be considered unethical. It is therefore likely that the first-in-human studies will actually be to offer mitochondrial gene replacement as a potential treatment. A means of estimating the risks is essential to enable patients to make informed decisions at that stage.

Modelling the inheritance of mtDNA heteroplasmy

mtDNA heteroplasmy levels are well described by the Kimura distribution, which is based on neutral genetic drift theory, although at this time rigorous testing of this theory in humans is limited to the common pathogenic variation m.3243A>G (Wonnapijit *et al.*, 2008). This model has two parameters, p_0 and b . The parameter p_0 was set as the amount of mutation carry-over from the mother. The parameter b is the bottleneck parameter determining the width of the heteroplasmy distribution in the offspring. The bottleneck parameter can be set from the heteroplasmy variance of a number of offspring from a single mother, or by pooling the heteroplasmy values from offspring from mothers with similar heteroplasmy levels. For human data, only the latter choice is practical. Mothers with heteroplasmy in the range of 40–60% were used, since in this range the variation in offspring heteroplasmy from the mother’s heteroplasmy level is minimal. Siblings were not included.

The key parameters of the Kimura distribution during transmission were determined by studying 87 human mother–offspring pairs transmitting a known pathogenic mtDNA mutation (Wonnapijit *et al.*, 2010). This parameter value was set at $b = 0.66$ based on 87 human mother–offspring pairs (Lott *et al.*, 1990; Ciafaloni *et al.*, 1992; Larsson *et al.*, 1992; Martinuzzi *et al.*, 1992; Tatush *et al.*, 1992; Zhu *et al.*, 1992; Hammans *et al.*, 1993, 1995; Piccolo *et al.*, 1993; Howell *et al.*, 1994; Santorelli *et al.*, 1994; Harding *et al.*, 1995; Houstek *et al.*, 1995; Makelabengs *et al.*, 1995; Black *et al.*, 1996; Mak *et al.*, 1996; Carelli *et al.*, 1997; Uziel *et al.*, 1997; Olsson *et al.*, 1998; Onishi *et al.*, 1998; Tanaka *et al.*, 1998; Chinnery *et al.*, 1999; White *et al.*, 1999; Lien *et al.*, 2001; Porto *et al.*, 2001; Hurvitz *et al.*, 2002; Wong *et al.*, 2002; Kaplanova *et al.*, 2004; Enns *et al.*, 2006; Phasukkijwatana *et al.*, 2006), including the following mutations: m.3243A>G (15 pairs), m.83446A>G (10 pairs), m.11778G>A (23 pairs), m.3460G>A (15 pairs), m.9883T>C (10 pairs) and m.8993T>G (14 pairs). Data from the A3243G mutation taken from blood samples were adjusted to correct for the known decrease in the A3243G mutation level in blood with age (Rajasimha *et al.*, 2008). Of course, these pathogenic variants cause a range of different phenotypes, which could in principle affect the inheritance of that variant. With the limited data currently available, the best that can be done is to average the data from all of

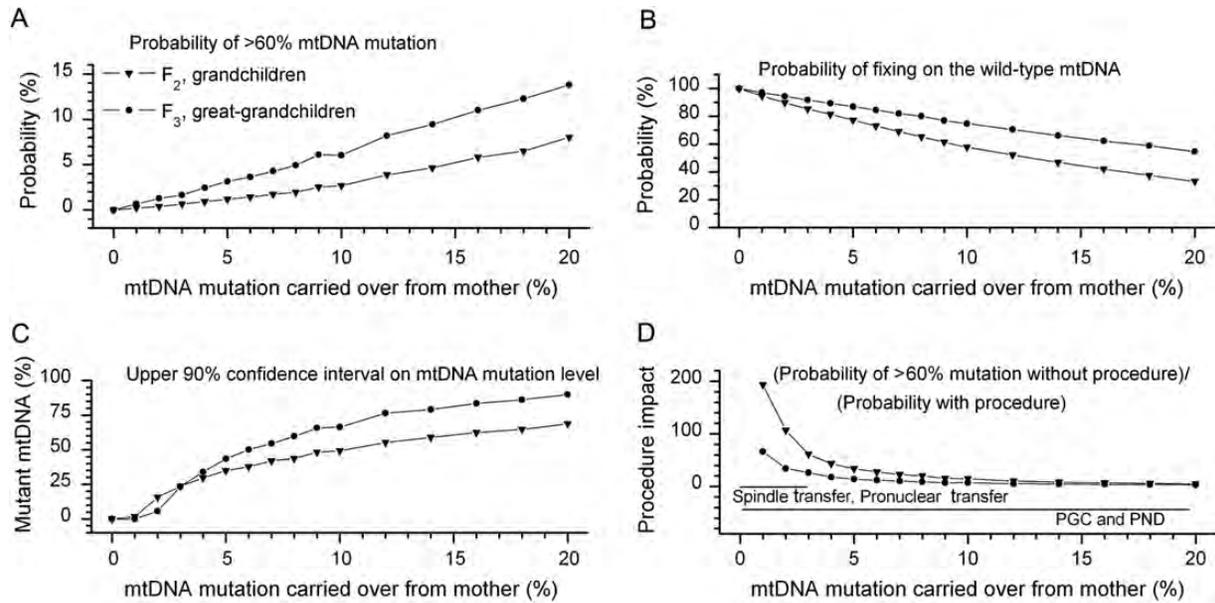


Figure 1 The effects of a carry-over of mutated mtDNA from the mother into the embryo on later generations. Triangles = grandchildren of the original mother seeking treatment (F₂), and circles = the great-grandchildren (F₃). This graph shows the recurrence risk in subsequent generations down the maternal line. **(A)** The probability that a descendant along the female line would develop an mtDNA mutation heteroplasmy level above 60% as a function of the amount of mutant mtDNA remaining in the treated embryo. This was chosen as a conservative threshold since levels below 60% are unlikely to cause disease. **(B)** The probability of fixing on the wild type for descendants along the female line. Having had more time to segregate, the chance of inheriting only wild-type mtDNA was greater in the great-grandchildren. **(C)** The upper 90% confidence interval on the mutant mtDNA level in the immediate descendants along the female line. **(D)** A measure of the impact of the transfer on later generations calculated by dividing the probability of a descendant having >60% mutation after the transfer by the probability without the transfer, for a mother with 60% mtDNA mutation threshold. Similar trends were seen for mutations with a lower clinical threshold, although the recurrence risk was greater and the impact of the gene transfer techniques was less. Conversely, for mutations with a higher percentage clinical threshold, the recurrence risk for subsequent generations was lower, and the impact of the gene transfer procedure was greater (Fig. 3). Horizontal arrows denoting the reported range of mtDNA carry-over for the spindle transfer and pronuclear transfer techniques, as well as for PGD and PND are given at the bottom of the figure. It should be noted that the higher range of levels of carry-over with PND and PGD are based on a decision made by the clinician and the prospective mother, and could be zero if so required. The range indicated in the figure shows the heteroplasmy values determined by PND and PGD in pregnancies that were allowed to continue because the perceived risk of a child being affected with this level of mutation is low.

the different pathogenic variants, to provide a general inheritance model. As more data become available in the future, specific inheritance models for each pathogenic mutation could be developed.

From this data-based model of heteroplasmy transmission in humans, we calculated the effect of low levels of mutant mtDNA carry-over on subsequent maternal generations for a range of different clinical heteroplasmy threshold values. Our analysis began with the initial percentage level of mutated mtDNA 'carried over' in the treated embryo that formed the F₁ generation. We then calculated heteroplasmy levels based on a Kimura distribution (Wonnapijit et al., 2008) in 20 000 offspring in the next generation (F₂, or 'grandchildren' of the original mother) and used these values to calculate heteroplasmy levels in the subsequent generation (F₃, or 'great-grandchildren'). Although there is still the theoretical possibility that low levels of heteroplasmy might segregate to higher levels in different tissues as the embryo develops, the heteroplasmy level appears to be uniformly distributed in both pre- and early post-implantation human embryos (Harding et al., 1992; Matthews et al., 1995; Steffann et al., 2007), and extreme differences have not been observed within neonates following the transmission of mtDNA heteroplasmy. Thus,

we estimated the recurrence risks using a range of clinical thresholds of mutant mtDNA, showing a disease threshold of >60% mutant as an example (Fig. 1), and other clinical threshold values (Fig. 2).

The fate of low-level heteroplasmy on future generations

As expected, a higher mutant mtDNA carry-over from the mother increased the chance that later maternal descendants would inherit high levels of the mutation, and thus be at risk for recurrence of the mitochondrial disease (Fig. 1A) (Poulton and Turnbull, 2000). Likewise, a higher mutant mtDNA carry-over reduced the chance of grandchildren and great-grandchildren fixing on the wild-type mtDNA (Fig. 1B). The same trends were observed for other clinical threshold heteroplasmy levels (Fig. 2). Fixation on wild-type mtDNA would protect all subsequent maternal generations from developing the disease, which is the ultimate goal of germ-line therapies. For the >60% clinical threshold, decreasing the mutation carry-over

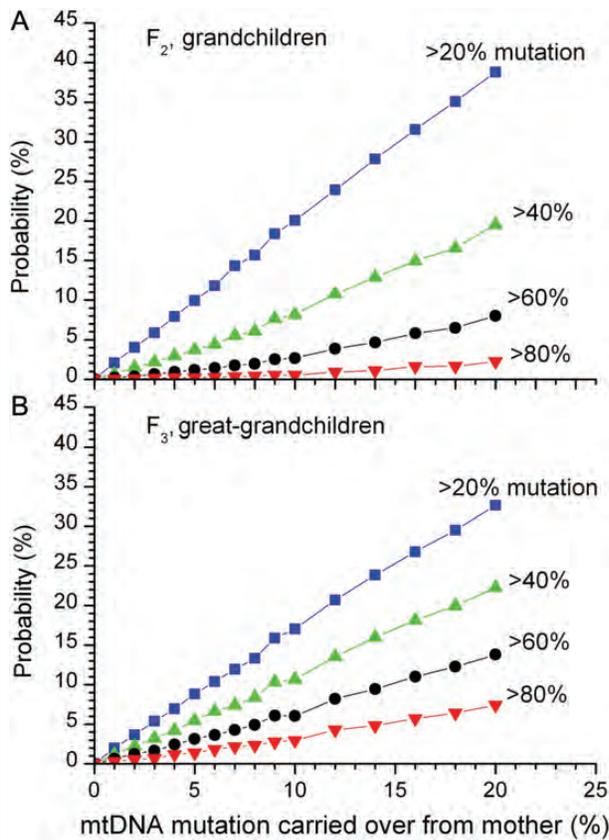


Figure 2 Simulation results for the probabilities that grandchildren (A) or great-grandchildren (B) would have mtDNA mutation levels greater than given thresholds as a function of the mutation level carried over from the mother. Results were calculated for 20% mutation, 40% mutation, 60% mutation (the same as in Fig. 1A and B) and 80% mutation thresholds.

below 3% dramatically reduced the probability of subsequent generations inheriting high levels of mutant mtDNA (Fig. 1C), and thus the impact of the treatment was greatly increased (Fig. 1D). In contrast, even relatively modest levels of maternal mtDNA carry-over of >5% were associated with a strong possibility of inheriting high levels of mutant mtDNA in later generations (Fig. 1C), leading to the re-emergence of the disease within the family, and thus a poor outcome (Fig. 1D). These calculations illustrate the importance of limiting the carry-over of the mutant mtDNA to very low levels of <3% for the success of the germ-line transfer in future generations.

Similar trends were observed for other clinical threshold heteroplasmy levels (Fig. 3), with lower clinical thresholds associated with a greater risk of recurrence in subsequent generations, and less impact of the gene transfer procedure. Conversely, a higher clinical threshold was associated with a reduced risk of recurrence in subsequent generations, and a greater impact of the gene transfer procedure.

Conclusions and future prospects

The development of germ-line therapies for mtDNA diseases is now focused on limiting the carry-over of the mutant mtDNA as much as is

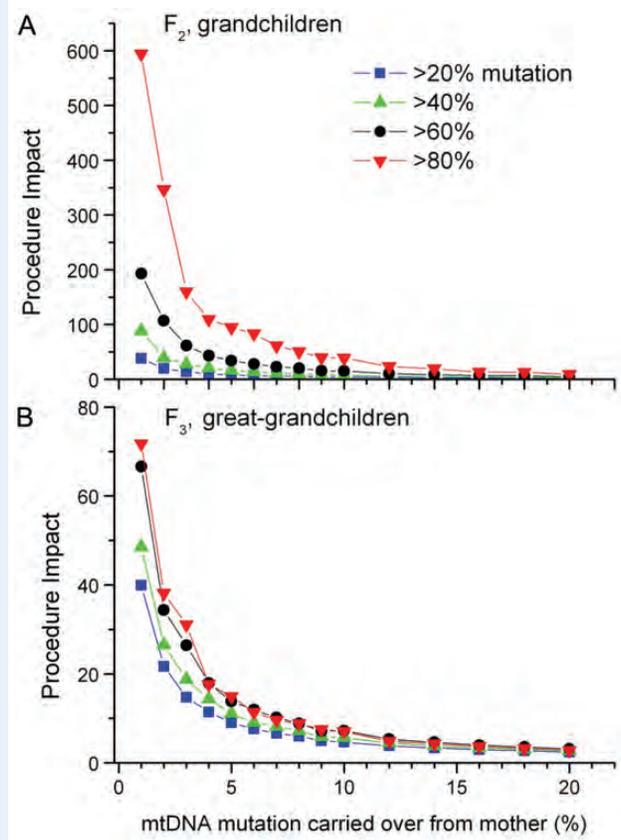


Figure 3 Simulation results for a measure of the impact of the procedure on grandchildren (A) or great-grandchildren (B). Impact is defined as in Fig. 1D, as the ratio of the probability maternal line descendants have greater than the given threshold without the procedure divided by the probability with the procedure, for a mother with 50% mtDNA mutation. A value of 1 indicates no impact of the procedure while higher values represent a larger positive impact. Results were calculated for 20% mutation, 40% mutation, 60% mutation (the same as in Fig. 1D) and 80% mutation thresholds.

practically possible. It has been shown that it is technically possible to transmit <3% maternal mtDNA with spindle–chromosomal complex transfer in non-human primates (Tachibana *et al.*, 2009, 2012), and with pronuclear transfer in preimplantation human embryos (Craven *et al.*, 2010). Our observations indicate that this level of mutant mtDNA carry-over is highly unlikely to cause mitochondrial disease in any maternal descendants, effectively (and quite likely completely) eradicating the disease for good. These predictions not only apply to mtDNA gene transfer techniques, but also for embryos screened by PGD and PND. At present, both PND and PGD remain the first port-of-call for the prevention of mtDNA disease. These techniques can also be used in subsequent generations if there is an ongoing perceived risk of recurrence. However, our findings support the clinical development of gene transfer techniques as a definitive approach to prevent mtDNA disease in these families for all subsequent generations.

Further pre-clinical work in animal models is required to provide further reassurance that these new approaches do not have

catastrophic consequences in higher mammalian species, and the ethical and legal debate will need to progress in parallel. However, ultimately, the first successful mtDNA gene transfer in humans will probably be offered to a patient with severe, highly penetrant mtDNA disease, for whom the benefits would outweigh the perceived risks. The work we present here will hopefully assist the prospective mother in making this bold decision.

Authors' roles

The study was conceived by P.F.C. and D.C.S. D.C.S. supervised the modelling, which was carried out by P.W. P.F.C. and D.C.S. wrote the manuscript.

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Conflict of interest

The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health.

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