# Mitochondrial DNA diseases: preimplantation diagnosis and intervention possibilities

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**ABSTRACT**

Mitochondrial DNA mutations are exclusively maternally inherited and can cause severe diseases for which there is no effective treatment. The recurrence risk of mitochondrial diseases is difficult to estimate due to heteroplasmy and the bottleneck effect during oogenesis. Here we review the literature on current options for preimplantation genetic diagnosis and interventions to prevent mitochondrial disease transmission.

Preimplantation genetic diagnosis can be performed in different developmental stages of the oocyte or the zygote. Preimplantation interventions consist of nuclear transfer, a set of techniques in which the patient's nuclear genetic material is placed in an enucleated donor cell, or genomic edition, through which the mitochondrial genome is altered. These methods are associated with technical barriers, such as ensuring the representativeness of the analysed sample when applying preimplantation genetic diagnosis, maintaining the communication between nuclear and mitochondrial genomes when using nuclear transfer, and avoiding off-target modifications when genome edition is the choice.

Although much has already been accomplished, further research is required to reduce the risk to the offspring and to develop more efficient and safer techniques.

**KEYWORDS**

DNA, Mitochondrial; Mitochondrial Diseases; Preimplantation Genetic Diagnosis; Reproductive Techniques, Assisted; Gene Editing.

## Introduction

Mitochondria are the "powerhouses" of the cell because of their role in adenosine triphosphate (ATP) production.[1-4] Although mitochondrial deoxyribonucleic acid (mtDNA) accounts for less than 0.1% of the total cell DNA,[5] mtDNA mutations are responsible for most inborn metabolic diseases,[6-8] affecting preferentially the most energy demanding tissues.[2,9]

MtDNA mutations may be homoplasmic (only mutated mtDNA is present in all tissues) or heteroplasmic (characterised by variable proportions of normal and mutant mtDNA among cells and tissues).[10-12] Clinical manifestations occur only when the mutated mtDNA load exceeds a threshold that is both tissue and mutation specific,[1,7,13] although there is not always an exact genotype-phenotype correlation.[13-16]

The inheritance of mtDNA is exclusively maternal [1,17-19] and is affected by the genetic bottleneck [12,13,17,18,20] through which a few mtDNA molecules become founders of the offspring. [21,22]

Considering the prevalence,[13] the high severity,[13,23] the absence of curative treatment,[11,13,18,24] and the high recurrence risk for the offspring of female carriers of these diseases,[13] prevention of their transmission would be of great importance. The aim of this review is to describe recent developments in this field.

**Article history**

Received 18 Jul 2019 - Accepted 9 Sep 2019

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## Methods

We conducted a review of Pubmed using the MeSH terms "DNA, Mitochondrial" and "Mitochondrial Diseases" combined with "Reproductive Techniques, Assisted" and "Preimplantation Diagnosis" and excluding the term "Infertility", and of Embase using the corresponding Emtree terms. Additional filters used were: Portuguese and English languages, humans and last ten years. "Conference Abstract", "Letter" and "Editorial" typologies were excluded. 133 journal articles were collected, and 77 were included for analysis and, when appropriate, included in the review.

## Methods for preventing the transmission of mtDNA diseases

### 1. Preimplantation genetic diagnosis

The aim of preimplantation genetic diagnosis (PGD) is to transfer embryos after evaluating their mutation loads, which are variable due to the genetic bottleneck and random segregation during oogenesis.[15,25]

Mitochondria may be accessed for evaluation at different stages: the first polar body of the oocyte and the blastomeres of the cleavage stage or blastocyst stage embryos.[15] Analysing mtDNA is easier and less prone to artifacts than analysing nuclear DNA (nDNA) due to the higher number of mtDNA copies per blastomere.[7,14]

The first polar body biopsy is performed before fertilisation, which may be ethically acceptable to those opposed to embryo testing.[15] However, a low correlation between the mutation load of the polar body and the oocyte, probably due to the asymmetric segregation of mitochondria during meiosis, was described.[7,12,14,25]

One of the major challenges of the other available options is to ensure the representativeness of the mutation load of the sample.[10,23,25] There is no consensus on whether one or two blastomeres should be used when performing blastomere biopsy.[7] While one cell may be sufficient in most cases,[1,13,23] being less detrimental to the embryo's viability,[15,23] most authors suggest using two cells and considering the higher percentage of mutated mtDNA when discrepancies are found.[7,14]

In blastocyst biopsies, trophectoderm cells are collected, as they are judged to be representative of the inner cell mass of the embryo.[12,24,25] Several cells can be removed without a negative impact on embryo development,[7,25,26] allowing a more accurate prediction of the mutation load of the embryo.[7] Nevertheless, in this stage, the cell to cell variation is higher than that found with cleavage stage embryos.[23]

A mutation load below the threshold level is considered the criterion for choosing embryos to transfer.[7,13,17,19,21] Ideally only embryos with no mutated mtDNA should be used.[6,14] However, as the threshold is reduced, fewer embryos will be available.[6,11,15,21] Defining an appropriate threshold is still difficult because of the lack of available data.[6,13,15] Recent studies tried to set a threshold to be applied to all mtDNA mutations, 18% being the value obtained with 95% confidence.[6,7,14,19,24,27]

Obtaining embryos with an acceptable mutation load can require multiple ovulation stimulation cycles in order to find the best possible embryo.[7,14]

PGD cannot be used in women with homoplasmic mutations,[11,16,17,19,20,28] and is of limited value for women with a high mutation load [17,19,20] and for those whose mutations have a poor correlation between mutation load and disease severity.[1,17]

### 2. Ooplasmic transfer (Figure 1)

When adequate embryos are not available, different approaches must be considered.

One option is ooplasmic transfer, which consists of injecting ooplasm with normal mitochondria from a healthy donor into an oocyte containing mutated mtDNA.[1,3,20,29–32]

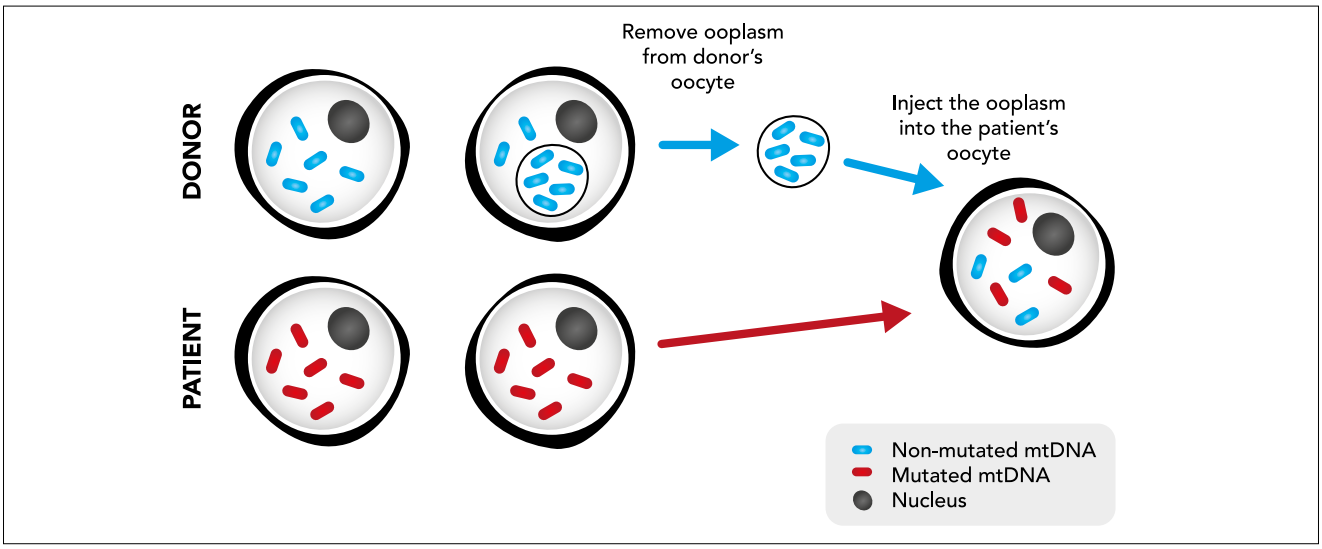
It has been suggested that ooplasmic transfer would lead to a reduction of the effects of mtDNA mutations through a dilution effect,[31] but this is only a theoretical possibility.[17,30]

One of the barriers is that only up to 15% of donor ooplasm can be transferred, whereas a larger amount would be needed.[1,17,30,31] Other suggestions are to use purified mitochondria or to partially remove the mitochondria from the oocyte.[1] Another concern is the lack of information on the long-term effects of introducing a new mtDNA haplotype into the oocyte.[17,22] So far, multiple chromosomal abnormalities and birth defects have been reported, leading to this technique being banned.[1,3,17,32]

### 3. Nuclear transfer

Nuclear transfer (NT) describes a set of techniques involving removal of mutation carrier nDNA, followed by its transfer to an enucleated oocyte from a donor, so as to obtain a new cell with nDNA from the patient and mtDNA from a donor.[4,7,20,22,28,31,33] It can be performed through five different techniques: germinal vesicle transfer (GVT), meiotic spindle trans-

**Figure 1** Schematic representation of ooplasmic transfer.



fer (MST), pronuclear transfer (PNT), polar body transfer (PBT) and blastomere transfer.

MST and PNT have been allowed by the HFEA, which considers them potentially useful for patients whose offspring is at risk of severe mtDNA diseases and have no other option for having their own genetic children.[20,29,34,35]

Possible germline genetic modification associated with NT raises some concerns.[14,20,26,31,35,36] The Nuffield Council on Bioethics concluded that if these techniques are proven to be safe and effective, it would be ethical to use them due to the health and social benefits of a life free from mitochondrial disorders.[2,20,35] These techniques do not lead to a "third parent", either biologically or legally.[2,9,35,36]

A major concern is the risk of co-transference of mutated mtDNA.[7,8,11,20,21,28] Even if this occurs, low levels of mutated mtDNA would be expected, and usually not linked to disease manifestation.[7,20,35] However, there is a slight risk of mutated mtDNA segregation to specific tissues.[3,11,20]

Recent studies showed that the chance of disease recurrence in subsequent generations is dramatically reduced if a mutant load below 5% is achieved.[17,36] There are also concerns over the possibility of one mtDNA haplotype replicating faster than the other,[17,37–39] enabling transformed embryos to "revert" to a damaged condition.[34,40,41] This seems more likely to occur when large DNA sequence differences exist between haplotypes.[22,38]

The consequences of possible mismatch between the patient's nuclear genome and the donor's mitochondrial genome has been studied by comparing differentiation efficiency, mitochondrial enzymatic activity and oxygen consumption rate between cell lines grouped based on single nucleotide polymorphism differences between the patient and the donor's oocyte mtDNA. Similar results were obtained in the different groups, suggesting that compatibility exists.[4,40]

The main drawback for the clinical application of NT is its inefficiency.[1,21]

MST was applied successfully in 2016, resulting in the birth of a male child with reduced levels of pathogenic mtDNA.[33,42]

**a. Germinal vesicle transfer** (Figure 2)

In the germinal vesicle stage, mitochondria are concentrated in the peri-nuclear space, which can lead to co-transfer of a significant amount of mutated mtDNA.[3]

Germinal vesicle removal is less invasive compared with other procedures.[30] A major disadvantage is the requirement of *in vitro* maturation of the oocytes,[1,12,17,30] which is still an inefficient procedure.[1,17,27]

**b. Meiotic spindle transfer** (Figure 3)

The visualisation of the spindle requires the use of polarised light microscopy.[12,17,20,32] Its removal is also difficult [2] and a certain volume of ooplasm has to be co-transferred to prevent chromosome loss.[43] However, as mitochondria are scattered in the ooplasm,[3] this technique is associated with minimal carryover.[12,14,20,44]

Most studies showed that fertilisation rates after this technique were similar to those observed in controls.[35,40] Nevertheless, the spindle is very sensitive to micromanipulation,[43,44] which frequently induces premature activation of oocytes.[16,20] This can lead to abnormal fertilisation due to premature chromatid separation in the absence of the second polar body, resulting in a high incidence of abnormal numbers of pronuclei.[12,14,32] It is also crucial to remove the first polar body from the donor oocyte because it can be reabsorbed, causing polyploidy.[32] Despite all these possible complications, aneuploidy rates seem to be similar to those found in controls.[27,35,40]

**c. Pronuclear transfer** (Figure 4)

This technique requires fertilisation of the donor and the recipient oocytes.[20,28,30,35]

Pronuclei are easier to manage because of the larger volume and membrane, but its removal causes greater cellular trauma.[2]

In the pronuclear stage, mitochondria are concentrated in the peri-nuclear space, which may lead to higher mutated mtDNA carryover.[3,45] Available data on heteroplasmy is not consistent, some studies reporting over 20%,[2,37,43] and others

**Figure 2** Schematic representation of germinal vesicle transfer.

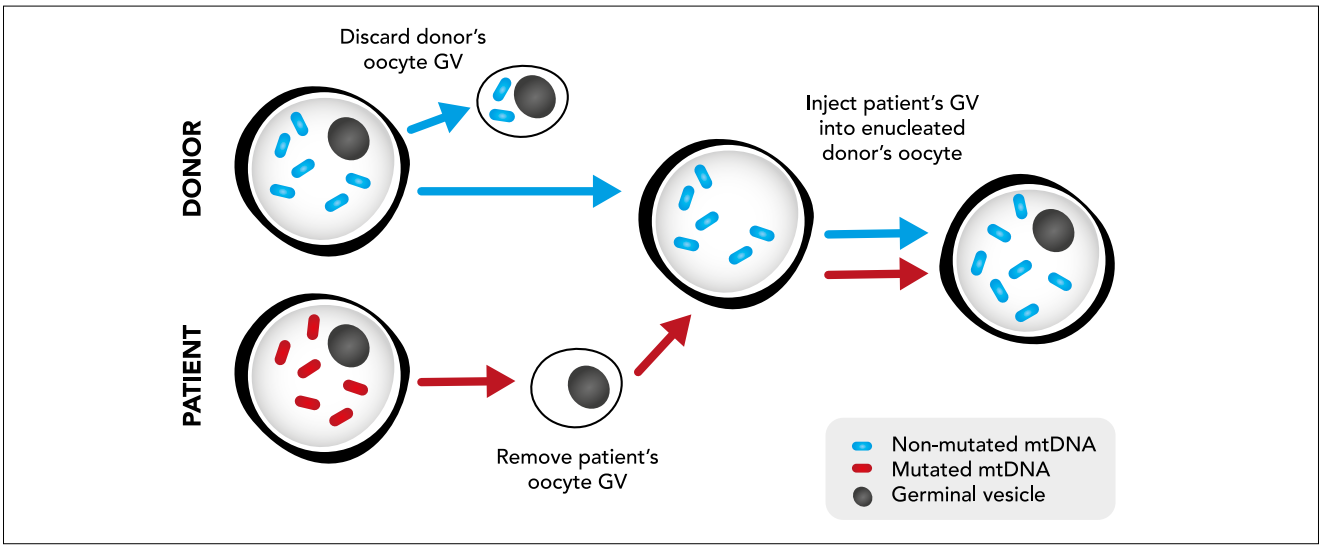


**Figure 3** Schematic representation of meiotic spindle transfer.

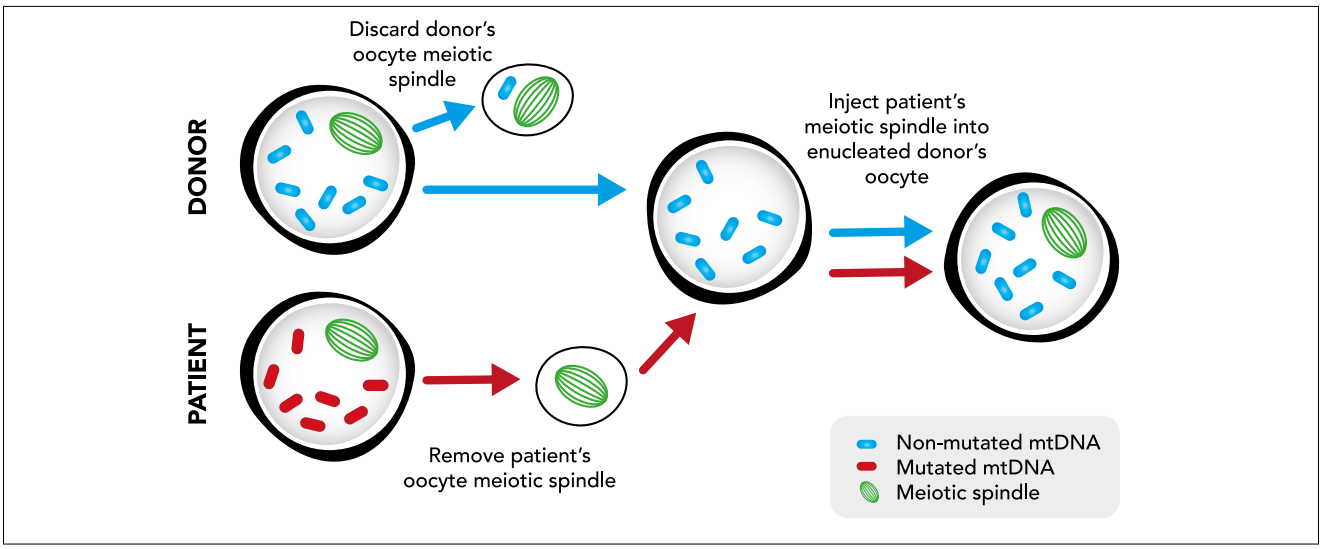
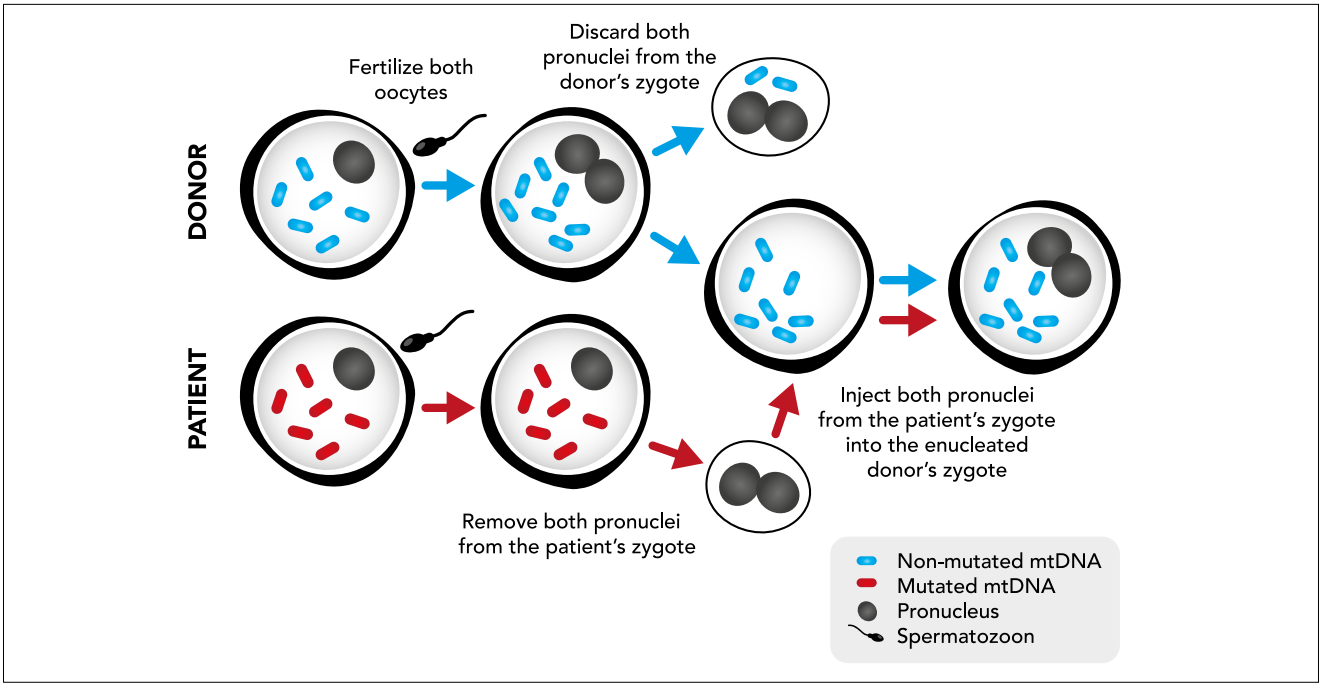



**Figure 4** Schematic representation of pronuclear transfer.




less than 2%.[12,20,32,44] The high percentages observed in some studies are justified by mtDNA amplification around pronuclei induced by zygotic activation.[43]

Studies to date have shown low embryonic development, but further investigation is required in order to clarify whether this is a consequence of the technique or of using abnormally fertilised embryos for testing.[20]

The major disadvantage of this procedure is that half of the embryos created will be discarded.[20,30,43,44]

### d. Polar body transfer

Polar body transfer (PBT) is similar to MST or PNT when performed using the first or the second polar body, respectively.[44]

Polar bodies theoretically share the same genetic information as the oocyte,[43,44] but contain very few cytoplasm and cellular organelles.[2,27,43,44] Thus, minimal mutated mtDNA carryover is expected,[43,44] with several studies showing undetectable mutated mtDNA when first PBT is performed, and around 2% when second PBT is used.[2,37,43] Nevertheless, the reduced amount of cytoplasm can have deleterious consequences.[27]

Another advantage of this technique is the easy visualisation and manipulation, without chromosome loss because of the cellular membrane [2,43] and with minimal damage as polar bodies are separated from the oocyte.[27,44] As the second polar body contains only a haploid genome, removal of the maternal pronucleus of the recipient zygote would be required. Removing only one pronucleus is challenging,[27,43] so the zygote should be enucleated and again fertilised after introducing the second polar body's genome.[43]

If PBT can be successfully performed in parallel with other

NT techniques, the number of donor oocytes required may be reduced by half.[2,43]

Further studies are required to confirm whether the incidence of DNA mutations in polar bodies is identical to that of the sibling oocyte.[44]

**e. Blastomere transfer**

It is still unclear whether the transfer of a blastomere from an affected embryo into an enucleated healthy donor oocyte can successfully prevent mtDNA disease because an entire cell is fused to the recipient oocyte.[32,45] This may result in higher levels of heteroplasmy [32] and in poor developmental competence.[45]

## Genome editing

Genome editing to prevent mtDNA disease transmission consists of removing mutated mtDNA of heteroplasmic cells using site-specific restriction endonucleases, such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 system (CRISPR/Cas-9).[33] With this technique, donor oocytes are not required.[46]

This technique is less invasive than NT.[46] However, the mutation load remaining is higher than that obtained after NT or PGD [7] and the mtDNA copy number may be below the threshold necessary for embryonic implantation and development.[22,46]

There is a risk of cleavage of essential genes due to off-target editing, and therefore careful design of the guiding molecules is required.[47]

Recently, a new approach was proposed in which, instead of removing the mutated DNA, its sequence is altered. This base editing technique converts one base pair to another at a target locus without requiring double-stranded DNA breaks. It has shown a good efficiency and less off-target modifications than CRISPR/Cas-9.[48] This technique has not yet been used in mtDNA, but it may be a potential new method.

## Conclusion

As there is still no treatment for diseases caused by mtDNA mutations, prevention is of major importance.

The techniques here described were tested in substantially different conditions, which makes it difficult to compare their results. There is therefore a need for further research with similar conditions for all the techniques, and also for research into side effects and long-term consequences of these techniques.

As ethical issues remain a limitation, boundaries should be defined to allow further research in this area, possibly allowing more studies on embryos and long-term follow up of children and subsequent generations but still avoiding the "slippery slope" feared by many.

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***Declaration of interest:*** *Authors have nothing to declare*