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Workshop report

174th ENMC International Workshop: Applying pre-implantation genetic diagnosis to mtDNA diseases: Implications of scientific advances 19–21 March 2010, Naarden, The Netherlands

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1. Introduction

Ten years and 100 workshops after the first ENMC International Workshop on genetic management for mitochondrial DNA (mtDNA) disorders [4], 18 participants, including clinicians, basic scientists, an ethicist and a patient representative from 9 countries gathered on 19–21 March, 2010, in Naarden, The Netherlands. This was in order to (1) provide a consensus statement on the role and application of pre-implantation genetic diagnosis (PGD) to mtDNA disease and (2) review new data on the biology of mtDNA in the germline and assess its implications for genetic management.

As there are no cures for maternally inherited mtDNA disorders, families increasingly look to genetic intervention to prevent transmission of the disorder, or to ameliorate its effects in their offspring [6,7]. Of the genetic options currently available, the most promising recent development is PGD, where embryos created *in vitro* are tested and low risk embryos selected to start a pregnancy. Although promising, PGD for mtDNA disorders faces both technical and ethical challenges. In this workshop report, we will propose consensus guidelines for offering PGD for mtDNA mutations.

2. Background

PGD may be a valuable option for families carrying heteroplasmic mtDNA mutations (where mutant and normal mtDNA co-exist in an affected individual). In these applications, embryos are sampled to assess the proportion of mutant mtDNA in order to estimate their genetic risk. However, in families with homoplasmic mtDNA mutations, where 100% of the mtDNA is the pathogenic mutant, PGD based on mtDNA mutant load (the ratio of mutant to normal mtDNA) is unhelpful. It does, however, have a place in those disorders where penetrance depends on gender (see below).

For heteroplasmic mtDNA mutations, the risk of recurrence in future children is difficult to estimate, partly because it is difficult to predict the degree of heteroplasmy transmitted. In many heteroplasmic disorders there are no symptoms unless the mutant load (proportion of mtDNA that is mutant) exceeds a specific threshold, but in others phenotype and mutant load correlate poorly. While not established for all mutations, symptoms frequently progress as the distribution of mutant mtDNA evolves, accumulating in post-mitotic tissues over time. In addition, the mtDNA bottleneck during oogenesis may cause dramatic and unpredictable inter-generational fluctuations in the proportions of mutant and normal mtDNA. As a result many families reluctantly decide not to have (further) children. Genetic management is simplest in well characterised pathogenic mtDNA mutations with a relatively uniform distribution in all tissues, a stable mutant load, threshold to disease expression and a reasonably predictable outcome, such as m.8993T>G and m.8993T>C (associated with MILS for maternally inherited Leigh syndrome and NARP for neurogenic weakness, ataxia and retinitis pigmentosa). For the purposes of our discussion we termed this category 1. Category 2 mutations such as m.3243A>G (associated with MELAS for mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes and MIDD for maternally inherited diabetes and deafness) have a variable tissue distribution after birth, and blood levels fall with time [8]. The mutant level in accessible tissues is often a poor reflection of the phenotype. In category 3 mutations there is insufficient information to determine a threshold, which is often the case in private or family-specific mutations.

The primary aim of PGD for mtDNA disorders would be to eliminate the risk of a child being affected by the disease, by selecting embryos in which no mutant mtDNA can be detected. However, this criterion may be so stringent that no embryo qualifies for transfer, and hence no pregnancy is achieved. In order to balance the availability of embryos against the likelihood of the child being affected, an intermediate dose may have to be chosen. This risk-reduction strategy is based on the premise of treatment that a high risk of serious harm for the child conceived by means of PGD should be avoided [9]. The exact mutant level which may be acceptable will depend on the load regarded as likely to be subsymptomatic for that specific mutation, the manifestation of the disease, and the family circumstances. Deriving an exact figure is difficult and will need to be calculated from available data as it accumulates. Once settled upon, the figure should be discussed

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with the family before PGD is carried out, and a strategy agreed as to which embryos will be suitable or not for transfer.

There are three possible stages before implantation at which mitochondria may be accessible for analysis.

(a) Before fertilisation, by analysis of the first polar body
The first polar body, containing nuclear and cytoplasmic material constricted off from the oocyte during maturation, can be removed without damage to the egg. While historical data suggested that the mitochondrial dose in the first polar body reflects the mutant load in the egg accurately [10], data presented at this workshop shows that this is highly questionable. However, for those who oppose the destruction of embryos, this approach has an ethical advantage.

(b) Blastomere biopsy
The most frequent sample used for PGD is a cell removed at the 8-cell cleavage stage (usually on day 3). The zona pellucida is breached using acidified Tyrode solution, or a laser, and one or two cells removed for analysis. This is the PGD technique used for mtDNA disease hitherto, and the starting point of this meeting.

(c) Blastocyst biopsy
This is perhaps the most reliable sample for testing nuclear DNA mutations, because multiple trophectodermal cells are available. However, this technique usually requires that embryos are frozen after sampling, because the time needed for the analysis exceeds the window available for transfer of the embryo to the uterus. This approach has not yet been used for mtDNA diseases.

Intracytoplasmic sperm injection (ICSI) is routinely used to achieve fertilisation for PGD, specifically to ensure that the biopsied sample is not contaminated with extraneous DNA-containing sperm. During this process the oocyte membrane is breached in order to deposit the sperm and a small amount of ooplasm is aspirated to confirm entry into the oocyte. Although this ooplasm is replaced on depositing the sperm, a small amount of cytoplasm could be removed without further invasion of the oocyte/embryo. However, there is as yet no data to support the reliability of this method to predict oocyte mutant load.

3. Prerequisites for any centre starting or considering offering PGD for mtDNA

Mitochondrial PGD is clearly a specialist procedure because of the unique transmission genetics of mtDNA disease. In addition to the regular professional requirements and embryology skills for PGD (for example the standards as articulated in the European Union Tissue and Cells Directive and in the ESHRE PGD Consortium Guidelines), specific expertise in genetic counselling for mtDNA disease is likely to be required. In the molecular work up before commencement of treatment the level of mutant mtDNA in representative tissues of the mother must be determined and quantitative single cell protocols established. Quantitative protocols for mtDNA rearrangements are technically more difficult, but women with recurrence risks of <5% rarely choose PGD. We believe that the current protocols for PGD for mtDNA mutations are sufficiently experimental that submission to a Research Ethics Committee may be advisable, also to ensure that patients are sufficiently aware that they are enrolled in an experimental treatment.

4. Entering the treatment program

Once a couple presents at the clinic and asks for PGD for an mtDNA mutation, the following factors should be considered. First, adequate (pre-test) counselling is indispensable to discuss whether PGD is a suitable reproductive option for this couple. Due to the uncertainties inherently linked to mitochondrial genetics, it may be helpful to discuss the pros and cons of the available reproductive strategies for the couple. This discussion must cover the range of possible PGD outcomes, from the worst case scenarios to more favourable outcomes [11]. A second step would be to examine whether PGD is feasible for the particular couple. This includes an assessment of patient-specific characteristics, including their general health, age and body mass index (as these influence success rate). This also includes an assessment of mutation-specific characteristics. Some estimate of ovarian reserve (serum anti-mullerian hormone (AMH), FSH/estradiol ratio, or antral follicle count) should be undertaken to determine whether sufficient oocytes are likely to be obtained for sampling after fertilisation. It may also be acceptable to undertake a stimulation cycle to obtain oocytes in order to estimate whether embryos with a favourable mutant load are likely to be generated [12]. Further, one should ascertain whether a clear threshold dosage relationship between the level of a specific mutant and its effect has been established using techniques such as cybrids [13] or single muscle fibre PCR. In most cases such a threshold is not directly applicable to the expected effects on the embryo.

Before starting the procedure, a decision tree has to be worked out by the couple and the clinicians. In each case, details such as the maximum threshold should be decided (based on its merits and published literature, see below) before initiating treatment. For the ingredients supporting informed consent we refer to [14].

Text box 1 Experiences with PGD for mtDNA disorders

Workshop attendees described data on PGD studies in a total of 9 families, including 6 from Paris [1,2], 2 from Maastricht [3] and 1 from Melbourne [5]. Five different mtDNA mutations were studied, at nucleotide positions 3243, 8344, 8993, 9185 and 10197. Heteroplasmic mutant load in transferred embryos ranged from 0% to 50%. Many of the embryos that were unsuitable for transfer were disaggregated to test heteroplasmic mutant load in approximately 200 individual blastomeres. These data showed only minor differences in mutant load between individual blastomeres derived from a single embryo. Each centre tested 2 blastomeres from day 3 embryos and in each of the completed pregnancies, amniocytes or cord blood was tested and gave results concordant with the blastomere mutant loads. The studies comprised 15 cycles of ovarian hyperstimulation, generating a total of 103 embryos for testing and leading to 7 embryo transfers and birth of 3 children, all of whom were reported to be healthy at 1–5 years of age. An additional pregnancy is ongoing.

5. What sampling protocol should be followed?

Currently it is uncertain that the mutant load is identical in all blastomeres within an embryo for many mtDNA mutations. Such uncertainty can be overcome by sampling two cells per embryo or a polar body plus blastomere. However, this may impair the embryo's viability [15].

A further consideration is whether and when a cut-off point should be established. A cut-off point is a threshold of mutant load above which no embryos are considered eligible for transfer. When choosing the cut-off point a high risk of serious harm for the resulting child should be avoided. The cut-off point should at least be compatible with a reasonable healthy phenotype. These levels must be based, case by case, on emerging data rather than being

set universally. To this end we present examples here of current practice in some centres (see Text box 1).

Studies have shown that phenotype reflects the mutant load in the m.8993T>G and m.8993T>C mutations [16]; for these mutations, well-documented and predictive ranges of mutation load exists. For the m.3243A>G and m.8344A>G mutations a more heterogeneous but large dataset exists [17]. Some of the older published data suffers from technical limitations and a recent well characterised but smaller data set exists for m.3243A>G [18]. The threshold chosen for the m.3243A>G mutation needs to be conservative because of the uncertainties. Some evidence suggests that mutant load below 30% m.3243A>G results in healthy children [1–3]. Increasing the level will increase the risk to the child and decisions should be specific to the couple. In private mutations there may be insufficient information to determine a threshold.

In cases where there is very little information about the relationship between the load of a private mutation and phenotype, clinicians may be reluctant to perform PGD, even to simply reduce the genetic risk. In such cases, families may wish to suggest carrying out a "dummy" run, creating embryos simply for the purpose of determining whether the blastomere sample is representative because of intra-embryo variation in mutant load.

Techniques hitherto used have been the standard Assisted Reproduction Technology (ART) protocols for ICSI and biopsy. Subsequent steps may include the following:

(1) Rinse the biopsied blastomeres because of possible contamination with fragmented blastomeres.
(2) Take as a first PCR step either a multiplex or whole genome amplification in order to use PCR of at least one nuclear probe as a means of detecting any contamination.
(3) Use a robust protocol with restriction digestion after last cycle labelling (fluorescent probe) with additional restriction site to confirm digest is complete.
(4) Check for contamination in a non-template control and detection of parental nuclear markers.

6. Embryo selection and transfer

When deciding which embryo to transfer, several factors should be considered.

The first consideration is the mutant load of the embryo. At one extreme, one might search only for embryos with zero mutant load. However, this approach may be too stringent, as many if not all embryos might be consequentially discarded, seriously reducing the chance of a pregnancy. One could also decide to transfer the embryo with the lowest mutant load. This, however, may be an approach too permissive, as the lowest mutant load embryo may still carry a high mutant load, resulting in a serious affected child. We favour a middle course where we first determine a cut-off point below which embryos are eligible for transfer and then to search for the most suitable embryo. Suitability includes both embryo quality and is not simply the embryo with the lowest mutant load. Hence one has to choose between the futility of transferring an embryo of poor morphology and the lowest mutant load embryo available.

The gender of the embryo is another factor that might influence the decision whether to transfer an embryo with a certain mutant load. As males will not pass their mutant mtDNA to the next generation, preferentially transferring male embryos could be considered as an additional selection criterion, but the proportionality should be taken into account [19]. However, the penetrance of some disorders, particularly of the m.11778G>A mutation causing Leber's Hereditary Optic Neuropathy, is much lower in females than males. In this context, selecting female embryos would reduce the risk. But in homoplasmic families the residual risk to these embryos will be higher than to mutant-free embryos that may be obtained in heteroplasmic disorders. Although morally acceptable, other preventive strategies may eventually seem more suitable. Obviously, choosing which embryo to transfer is highly complex. One has to balance embryo quality, mutant load and possibly gender as an additional selection criterion. Further, this is with the caveat that there have to be a sufficient number of embryos for a choice. It may be well possible that no or just one or two low mutant load embryos are available or that embryos have poor morphology. This leads to the question whether higher numbers of embryos should be generated, for example by using higher stimulation protocols or by starting additional cycles. It may also be relevant to freeze embryos after sampling, with the twofold purpose of building in some extra time to decide which embryo to transfer or to be used in a next treatment cycle (or to be used for research afterwards).

7. Follow up

If a pregnancy is established, the reliability of the PGD test could be checked by means of prenatal diagnosis (chorionic villus sampling or amniocentesis). When considering prenatal diagnosis one should first ensure that testing at this stage adds value (for example, that the information generated is relevant and new). For instance, informing a couple who would not terminate pregnancy that the mutant load had increased would be equivalent to predictive (and possibly pre-symptomatic) testing of the child. One should also bear in mind that prenatal diagnosis confers a small risk of miscarriage.

Further, the PGD procedure can usefully be validated by means of genetically testing offspring, as this would clarify whether the percentage of mutation is constant from the embryo throughout pregnancy until birth. However, genetic testing of minors has many pitfalls, but could be done without disclosing the result to the parents. Because of these difficulties, no consensus regarding testing newborns was reached.

8. Review new data on the biology of mtDNA in the germline and assessing its implications for genetic management

New mouse data [20] support earlier studies on humans (with and without disease) and mice [21], suggesting that a major component of the bottleneck occurs during oocyte development. However, there are two major controversies in this field.

Firstly, even with state of the art mathematical modelling, estimates of the bottleneck size will remain inaccurate until much more human data are collected. Hence there are currently insufficient data to substantiate suggestions that the size of the bottleneck depends on the type of mtDNA mutation or the specific family. Nevertheless, unpredictable inter-generational fluctuations appear to be smaller and less frequent in the mouse than humans. Current data suggests that this is not sampling bias, but a real biological difference [22]. However, the biological basis of the bottleneck is still not clear. Some reports suggest that the physiological basis of the mtDNA bottleneck may be the number of mtDNAs per cell at a specific point in oocyte development. The data from three groups suggest that the minimum number of mtDNAs in mouse models of mtDNA transmission is about 200 mtDNAs at the very earliest stages of primordial germ cell differentiation [20,23,24]. Another group suggests that this figure is nearer to 1500 and that copy number is lower in somatic than in germline cells [25,26]. There are substantial technical differences and different mouse strains utilized at the centres developing these models which might explain the differences. Furthermore, segregation or replication of a specific sub-population of mtDNA mutants at a la-

ter stage of oocyte development may underlie a substantial component of the bottleneck [24].

Secondly, other groups show that there is strong selection against amino acid substitutions in mutant mtDNA during transmission [27,28] in animal models of mtDNA disease. In common with nuclear DNA, the mutation frequency in protein coding genes is highest at positions representing synonymous changes. Moreover, mutant mtDNA load falls with time in oocytes in two different mouse models of mtDNA disease [28,29]. Despite this selection against severe pathological mtDNA mutations, in humans there is a high prevalence of both *de novo* and transmitted mutations of around 1 in 250 [30,31] [32,33]. Studies of the segregation behaviour of mtDNA in somatic tissues of a heteroplasmic mouse model [34] have identified the first nuclear genetic variants that influence the rate of mtDNA segregation. The mitochondrial protein encoded by one of these is currently being characterised.

The meeting also discussed techniques for replacing mutant mtDNA with normal mtDNA, which may become available in future years. Transfer of nuclei from mito-mouse zygotes (single cell embryos) to enucleated normal zygotes reduced the mutant load from 64% to 10%, resulting in improved phenotype and lifespan in a mouse model of mtDNA rearrangements [35]. If mitochondria from the recipient zygote are removed prior to injection, ooplasm transfer may also dilute the mtDNA load to lower than those reported in the past for this technique [36,37]. This method requires centrifugation of the recipient zygote to concentrate mitochondria into a cytoplasmic fraction followed by removal of mitochondria by micromanipulation. On average, ~65% of the host mtDNA is removed by this technique [37]. However, it is not yet clear whether the dilution of recipient mtDNA to a moderate level by the donor mtDNA can be maintained. Transfer of spindle–chromosome complexes, removed from oocytes whose nuclei are undergoing the second division of meiosis, is technically feasible and promising for dramatic reduction in mtDNA mutant load in macaque [38]. Similarly, pronuclear transfer from the unfertilised oocyte to an enucleated oocyte from an unrelated donor has resulted in a dramatic change in mtDNA type in human embryos that survived to day 8 [39]. However, all of these techniques need further ethical and scientific attention before they can be offered to patients.

9. Conclusion

The workshop thus examined and absorbed new data with very important implications for the genetic management of mtDNA disease. Despite technical and ethical challenges we feel optimistic that genetic management of heteroplasmic mtDNA diseases has much to offer the affected families.

10. Participants

- Catherine Aiken, Cambridge, United Kingdom
- Brendan Battersby, Helsinki, Finland
- Jean-Paul Bonnefont, Paris, France
- Peter Braude, London, United Kingdom
- Annelien Bredenoord, Utrecht, The Netherlands
- Marcos Roberto Chiaratti, Pirassununga, Brazil
- Patrick Chinnery, Newcastle upon Tyne, United Kingdom
- Kari Majamaa, Oulu, Finland
- Shoukhrat Mitalipov, Oregon, USA
- Joanna Poulton, Oxford, United Kingdom
- David Samuels, Nashville, USA
- Hiroshi Shitara, Tokyo, Japan
- Hubert Smeets, Maastricht, The Netherlands
- Jim Stewart, Cologne, Germany
- David Thorburn, Melbourne, Australia
- Timothy Wai, Cologne, Germany
- Dagan Wells, Oxford (via Skype)

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