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Clinical application of a sequencing-based method for concurrent preimplantation genetic testing for mitochondrial DNA disease and aneuploidy

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KEY WORDS

Preimplantation genetic testing; Mitochondrial DNA disease; Next-generation sequencing; Embryos; Mitochondria

WORD COUNT (MAIN TEXT)

4,422

ABSTRACT

BACKGROUND

Preimplantation genetic testing (PGT) can prevent transmission of heteroplasmic mitochondrial DNA (mtDNA) disease, in which wild-type and mutant mtDNA co-exist, by identifying embryos with subclinical mutant load for transfer to the uterus. Current methods for PGT for mtDNA disease do not reveal the presence of aneuploidy, which is extremely common causing implantation failure and miscarriage. Therefore, a novel PGT method was developed, based on next-generation sequencing, permitting concurrent testing for mtDNA disease and aneuploidy.

METHODS

Following extensive validation, the method was clinically applied to embryos from four patients at risk of transmitting mtDNA disease caused by m.8993T>G (Patients A and B), m.10191T>G (Patient C) and m.3243A>G (Patient D). Only Patients A and D were heteroplasmic for somatic mutant mtDNA.

RESULTS

Aneuploidy affected half of embryos tested (16/33). Mutations were detected in ~40% of specimens from Patients A and D (10/24) but not in specimens from Patients B and C (n=29). Patients B and C gave birth to healthy children following PGT and natural conception, respectively. m.8993T>G displayed skewed segregation (absence of intermediate mutation levels), while m.3243A>G only displayed relatively low mutation levels and exhibited a possible decrease during preimplantation development progression.

CONCLUSION

Considering the high aneuploidy rate, concurrent PGT for mtDNA disease and aneuploidy presents a significant advantage over alternative methods. Heteroplasmic women had a higher recurrence than those with undetectable mutant mtDNA, but were still able to conceive mutation-free embryos. The study supports the existence of mutation-specific segregation mechanisms occurring during oogenesis and possibly during embryogenesis.

MAIN TEXT

INTRODUCTION

Mitochondrial dysfunction caused by pathogenic mutations in the nuclear DNA (nDNA) or mitochondrial DNA (mtDNA) can result in severe multi-systemic diseases[1]. Mitochondrial diseases cannot be cured and treatment is limited to symptom management[2]. Various different reproductive options for reducing disease recurrence are available to women at risk of transmitting mitochondrial disease to children. These include *in vitro* fertilisation (IVF) with donor oocytes, preimplantation genetic testing (PGT) and prenatal diagnosis (PND)[3, 4]. PGT and PND offer the advantage that affected couples can conceive healthy and genetically-related children. However, if an affected fetus is identified by PND, couples face the difficult decision of continuing or terminating the pregnancy. A significant advantage of PGT is the low risk of carrying a fetus with mitochondrial disease, since only embryos predicted to be free of a clinical phenotype are considered for transfer to the uterus[5].

PGT for mitochondrial disease caused by nDNA mutations involves assessment of the presence or absence of the causative mutation. In contrast, PGT for disease resulting from mtDNA mutations also requires a quantitative component to determine the frequencies of wild-type and mutant mtDNA molecules. This is crucial because most mtDNA mutation carriers are heteroplasmic (i.e. their cells contain a mixture of wild-type and mutant mitochondria). Disease only occurs when the proportions of mutant mitochondria exceed critical thresholds, which may differ depending on the specific mutation[6]. Consequently, not only mutation-free embryos but also those with low mutation levels, unlikely to cause disease, may be eligible for uterine transfer[7]. Mutation levels can differ markedly between sibling embryos, presumably a consequence of the mitochondrial bottleneck occurring during female germ cell development[6, 8]. Consequently, women affected by mtDNA disease and asymptomatic women carrying pathogenic mtDNA mutations can potentially benefit from PGT as both are at risk of conceiving clinically affected children[9].

Methods employed for PGT for mtDNA disease are commonly based on restriction fragment length polymorphism analysis or on amplification refractory mutation system techniques, whereby a mtDNA mutation is targeted directly by an allele-specific primer or

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probe or by a restriction endonuclease, respectively, and then quantified using techniques such as electrophoresis or quantitative polymerase chain reaction (qPCR)[10–15]. These methods are technically challenging and difficult to combine with other diagnostic or screening procedures. For instance, combination with testing for numerical chromosome abnormality (aneuploidy) or embryo gender is problematic. Aneuploidy, however, is extremely common in human embryos, causing implantation failure, early pregnancy loss and aneuploid pregnancies[16–20]. Furthermore, selection of a male embryo for transfer, if available, may be considered as a means of eliminating mtDNA disease transmission for future generations[7]. This is particularly relevant if entirely mutation-free embryos cannot be identified and embryos with subclinical mutation levels are transferred.

We designed a novel sequencing-based method for PGT for mtDNA disease, which can easily be combined with PGT for aneuploidy (PGT-A). Because most PGT-A methods require whole genome amplification (WGA) to enrich the genetic starting material, we established a next-generation sequencing method, which utilises WGA products for the detection and quantification of mtDNA mutations in embryo specimens. Here we present extensive validation of the novel PGT approach and its clinical application in embryo specimens from four patients, referred for PGT due to either Leigh Syndrome caused by the m.8993T>G or the m.10191T>G mutation or due to mitochondrial myopathy caused by the m.3243A>G mutation. The results also provide information concerning the segregation patterns of different mtDNA mutations during oogenesis and embryogenesis.

MATERIALS AND METHODS

Patient details

Pedigrees of Patients A, B, C and D are shown in Figure 1. None of the patients’ families had a known history of mtDNA disease. For Patients A, B and C the possibility of disease risk only became apparent after children with Leigh Syndrome were born. Patient A’s daughter and Patient B’s son were effectively homoplasmic (~100%; blood) and heteroplasmic (~96%; blood) for the m.8993T>G mutation, respectively. Patient C’s daughter was heteroplasmic (~78%; muscle) for the m.10191T>C mutation. Patients A, B and C and Patient B’s mother underwent genetic testing. A mutation level of ~56% was identified in Patient A’s blood.

Conversely, blood, urine and cheek swabs of Patient B and her mother and blood and urine of Patient C were free of the mutations observed in their affected children. Patient D had no child at the time of referral and was undergoing PGT due to the presence of ~28% heteroplasmy of the m.3243A>G mutation (muscle), which caused her to suffer from mitochondrial myopathy resulting in ptosis and restricted eye movement. Patient D's mother and brother were not genetically tested. However, both presented with hearing and vision loss, symptoms frequently associated with the m.3243A>G mutation[21, 22].

Patients were provided with descriptions of treatment procedures and information on method limitations, risks and potential benefits and were consented prior to treatment initiation. Patient consent was also obtained for the use of their own or their children's genomic DNA (gDNA) and cell samples for method development and validation.

Utilised techniques

The SurePlex DNA Amplification System (Illumina) was employed for WGA and used as recommended by the supplier.

PGT-A was performed by array comparative genomic hybridisation (aCGH) or next-generation sequencing according to Illumina's 24sure and VeriSeq protocols, respectively. For aCGH, using the 24sure microarray, hybridisation areas were identified and captured using an InnoScan 700 laser scanner (Innopsys). For the sequencing-based protocol, WGA products were processed using the VeriSeq PGS Kit and sequenced on a MiSeq Sequencing System (Illumina). All aCGH and sequencing outputs were analysed using Bluefuse Multi Software (Illumina).

For PGT for mtDNA disease, patient-specific mutation loci were initially enriched by conventional PCR using flanking primers (m.8993: 5'CCCTAGCCCACTTCTTACCA3' and 5'GGTGGCCTGCAGTAATGTTAG3'; m.10191: 5'ATCCACCCCTTACGAGTGC3' and 5'GGGTAAAAGGAGGGCAATTT3'; m.3243: 5'ACCCAAGAACAGGGTTTGT3' and 5'TGGCCATGGGTATGTTGTTA3'). Each PCR consisted of 0.5µl WGA product, 12.49µl nuclease-free PCR grade water (Roche Diagnostics), 1.5µl HotMaster Taq DNA Buffer with Magnesium (5 Prime), 0.12µl forward and reverse primer pool (100µM Eurogentec), 0.3µl dNTP Mix (10mM ThermoFisher) and 0.09µl HotMaster Taq DNA Polymerase (5 Prime).

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PCRs were performed according to following thermal cycler settings: 96.0°C for one minute, followed by 35 cycles of 94.0°C for 15 seconds, 61.0°C (m.8993) or 57.0°C (m.10191 and m.3243) for 15 seconds and 65.0°C for 45 seconds, followed by a final incubation at 65.0°C for two minutes. Reagents, sequencing equipment and data analysis tools required for mtDNA variant detection and quantification were obtained from ThermoFisher and all procedures performed according to the supplier’s recommendations. PCR products were processed using the Ion Plus Fragment Library and Ion PGM Template OT2 200 Kits. Resulting libraries were sequenced on the Ion Personal Genome Machine System using the Ion PGM Sequencing 200 Kit v2. The Torrent Browser and inbuilt Torrent Suit Software were utilised for data processing. The Torrent Variant Caller plugin was used to detect and quantify mtDNA mutations.

Validation of the method

The method for PGT for mtDNA disease was validated using gDNA and cell samples from cheek swabs and/or blood collected from patients, their affected children and control individuals. The DNA Isolation kit DDK-50 (Cell Projects Ltd) and the BuccalAmp DNA Extraction Kit (Epicentre) were used to obtain gDNA and buccal cells from cheek swabs, respectively. The QIAamp DNA Mini Kit (Qiagen) was used to extract gDNA from blood samples. Ficoll-Paque Plus (GE Healthcare Life Sciences) was used to separate lymphocytes. Individual buccal cells and lymphocytes were isolated manually by micromanipulation and washed in Dulbecco's Phosphate-Buffered Saline (ThermoFisher)/0.1% Polyvinyl alcohol (Sigma-Aldrich).

The method was initially tested on gDNA from two individuals, homoplasmic for either the m.8993 wild-type (G) or mutant (T) variant. To test method accuracy and sensitivity and to ensure that WGA and/or amplification of the mutation site did not distort variant ratios, sample mixtures with 5.0%, 10.0%, 20.0%, 40.0%, 60.0% and 80.0% mutant load were created by mixing the homoplasmic gDNA specimens in appropriate amounts. Mutation site amplification was performed directly on the mixed gDNA samples and also on WGA products created from the gDNA samples. Products were sequenced and obtained mutant loads compared to expected loads.

To test protocol accuracy in samples consisting of single or few cells, equivalent to embryo biopsy specimens, minute quantities of gDNA (~100pg) and cell samples (consisting of 1, 3 or 5 cells) from all patients and affected children were analysed. Again, samples were sequenced either after direct amplification of respective mutation loci or after WGA followed by conventional PCR. Of note, gDNA samples were isolated from same cheek swabs or blood samples as were individual buccal cells and lymphocytes. Results from gDNA and respective cell samples were compared to each other and to mutation levels previously established by reference laboratories.

Of note, for the direct amplification of mutation loci in cell samples, an initial lysis step was performed, whereby each sample was combined with 0.75µl nuclease-free PCR grade water, 1.25µl DL-Dithiothreitol solution (0.1M; Sigma) and 0.5µl Sodium hydroxide solution (1.0M; Sigma). Following incubation at 65°C for 10 minutes, 7.99µl nuclease-free PCR grade water and 2.5µl Tricine (0.4M; Roche Diagnostics) were added followed by the PCR components described above.

Clinical application of established method

IVF procedures, such as oocyte retrieval, embryo culture, biopsy and transfer, were performed according to clinics' standard protocols. Oocyte fertilisation was performed by intracytoplasmic sperm injection (ICSI). Biopsies were performed at either the blastocyst-stage (embryos of Patients A, B and D) or the cleavage-stage (embryos of Patient C). Biopsied embryos were vitrified and those predicted to be unaffected were transferred to the uterus in a later cycle.

To maximise the information for patients concerning their mtDNA disease transmission risk, testing was applied to unfertilised and abnormally fertilised (three pronuclei) oocytes, and to non-viable arrested embryos. Arrested embryos were either disaggregated into individual cells (providing information on mutation level uniformity within an embryo), split into halves or tubed whole.

Patient B delivered a child following IVF with PGT. To confirm the original PGT for mtDNA disease result, mutation analysis was performed in gDNA from cord blood and cheek swabs, sampled at two and three weeks of age.

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Statistical data analysis

Data analysis was conducted using GraphPad Prism (GraphPad Software). Linear regression analysis was performed to assess linearity of obtained mutant loads. The Pearson's rank correlation coefficient was calculated to assess consistency between expected and obtained mutant loads. One-way ANOVA was used to compare mutation levels at different stages of preimplantation development. Tests were performed two-tailed with a 95% confidence interval. A p-value <0.05 was considered significant.

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Results of method validation

Figure 2 illustrates m.8993T>G mutant loads obtained from gDNA sample mixtures and pure (homoplasmic) samples after mutation site amplification alone (Figure 2-A) and after WGA combined with mutation site amplification (Figure 2-B). Numerical mutant loads are provided in Supplementary Table 1. Mutant loads obtained after direct amplification of the mutation site were nearly identical to expected ratios [mean difference: 2.4%±2.8%SD; r=0.9972 (p<0.0001)]. A similar correlation (r=0.9987; p<0.0001) was observed when WGA was performed prior to mutation site amplification (mean difference: 1.8%±1.9%SD). Both experiments resulted in nearly perfect linear regressions (r²=0.9944 and 0.9975). Of note, a homoplasmic result was obtained in all samples containing either 0.0% or 100% mutant load. Furthermore, low-level heteroplasmy was confirmed in all samples containing the lowest expected mutant load of ~5.0%.

Figure 3 illustrates mean mutant loads detected in gDNA and cell samples from Patients A and D and affected children of Patients B and C. Mean mutant loads are shown after direct mutation site amplification and after WGA followed by amplification of respective mutation loci. Results from homoplasmic individuals [child of Patient A (mutant mtDNA); Patients B and C (wild-type mtDNA)] are not illustrated as homoplasmy was confirmed in all samples. Numerical mutant loads are provided in Supplementary Table 2. Differences in mean mutant loads between samples subjected to direct mutation site amplification and those subjected to WGA prior to mutation site amplification were minimal (gDNA samples: 0.8%±0.8%SD; cell samples: 3.5%±5.8%SD). Of note, mean differences were slightly

higher in cell samples, which likely reflects intercellular variation, ranging from 0.0%-100%, 39.5%-100%, 75.2%-94.3% and 0.0%-63.8% in Patient A, the children of Patients B and C and Patient D, respectively.

Excellent agreement was also observed between gDNA samples and corresponding cell samples, differing on average by $1.5\% \pm 1.6\%SD$ and $3.2\% \pm 4.9\%SD$ after direct mutation site amplification and after WGA followed by PCR, respectively. Of note, the slightly higher difference in samples subjected to WGA was caused by large variation between cheek swab gDNA and cell samples of Patient D (Figure 3-D).

Mean mutant loads in samples from individuals carrying the m.8993T>G mutation confirmed those previously reported by reference laboratories [Patient A: $54.3\% \pm 1.0\%SD$ (blood) and $55.7\% \pm 0.5\%SD$ (cheek swab) versus ~56% (blood); daughter of Patient A: 100% (blood) versus 100% (blood); son of Patient B: $95.2\% \pm 3.4\%SD$ (blood) versus ~95% (blood)]. Mean mutant loads in samples from Patient C's daughter, who carries the m.10191T>C mutation, were slightly higher than the previously established mutation level [$84.6\% \pm 2.5\%SD$ (cheek swab) versus ~78% (muscle)]. Mean mutant loads measured in samples from Patient D, who carries the m.3243A>G mutation, were lower than the previously established mutation level [$7.3\% \pm 1.1\%SD$ (blood) and $11.4\% \pm 7.3\%SD$ (cheek swab) versus ~28% (muscle)].

Results of clinical application

Results from PGT procedures including predicted embryo cytogenetic status and measured mtDNA mutation levels in oocyte/embryo specimens from all patients are summarised in [Supplementary Table 3](#).

Patient A underwent two consecutive cycles. Two oocytes were collected in each cycle. Three oocytes fertilised. One zygote developed to the blastocyst-stage and was biopsied. The unfertilised oocyte and both arrested embryos were tubed whole. Analysis of the m.8993T>G mutation was performed in all samples. PGT-A by aCGH was performed in the biopsy and arrested embryos. The mutation was not detected in the biopsy and one embryo but present at high levels in the unfertilised oocyte (91.2%) and the other embryo (88.7%). PGT-A revealed that the biopsy was aneuploid, with a predicted karyotype of 48,XXY,+16, and consequently

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the blastocyst was not transferred. PGT-A further revealed that the mutation-free, arrested embryo was euploid, while the arrested embryo carrying the mutation was aneuploid.

Patient B underwent one cycle. Eighteen oocytes were collected of which seventeen fertilised. Fourteen zygotes developed into blastocysts and were biopsied. The unfertilised oocyte and one embryo, which arrested during blastocyst formation, were tubed whole. Blastomeres of two embryos arrested during cleavage divisions were tubed individually. Analysis of the m.8993T>G mutation and PGT-A by aCGH was performed in all samples. The mutation was not detected in any sample. Ten biopsies were euploid. All other samples were aneuploid. The transfer of one male embryo resulted in a pregnancy and delivery of a healthy boy at 40 weeks of gestation. The mutation was not detected in any of the newborn’s samples (Supplementary Table 4).

Patient C underwent two separate cycles. Three oocytes were collected during her first cycle. Two oocytes fertilised. One zygote developed to the cleavage-stage and was biopsied. The unfertilised oocyte and blastomeres from the arrested cleavage-stage embryo were tubed individually. Mutation analysis at position m.10191 was performed in all samples. Sequencing-based PGT-A was performed in the cleavage-stage biopsy. The mutation was not detected in any sample. The biopsy was identified as euploid. The embryo was transferred but failed to implant. In the second cycle, eight oocytes were collected. Seven oocytes fertilised. Three zygotes developed to the cleavage-stage and were biopsied. Unfertilised oocytes and embryos arrested during cleavage divisions were tubed whole. Again, mutation analysis was performed in all samples and sequencing-based PGT-A in biopsies. The mutation was not detected in any sample. PGT-A revealed that one cleavage-stage biopsy was euploid and the corresponding embryo was transferred. Although the embryo implanted, evident by a positive pregnancy test, the pregnancy was lost shortly after and no fetal heartbeat was detectable. Because the mutation was not detected in any of the analysed embryo specimens, the patient’s transmission risk was estimated to be low. Patient C went on to conceive naturally and underwent PND (chorionic villus sampling). No mutant variant at position m.10191 was detected and a healthy boy was delivered at full term.

Patient D underwent one cycle. Twenty oocytes were subjected to ICSI. Nineteen oocytes fertilised and ten zygotes developed to the blastocyst-stage. Of these, nine were

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3 biopsied. One blastocyst, which developed from an abnormally fertilised zygote, and the
4 unfertilised oocyte were tubed whole. Nine embryos, which arrested during cleavage
5 divisions, were split into two parts. Each part was tubed separately. Analysis of the
6 m.3243A>G mutation was performed in all but one sample, which failed to amplify. PGT-A by
7 aCGH was performed in trophectoderm biopsies. The mutation was detected in the
8 unfertilised oocyte, in both parts of four arrested cleavage-stage embryos and in three
9 trophectoderm biopsies of which two also were aneuploid. Of the remaining six
10 trophectoderm biopsies two were euploid and corresponding embryos consecutively
11 transferred. Although both embryos implanted and fetal heartbeats were seen, the patient
12 miscarried in both transfer cycles at eight weeks of gestation.

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22 Of note, this particular case presented with some interesting and potentially important
23 findings. Firstly, almost identical mutation levels were detected in the two halves of arrested
24 cleavage-stage embryos (mean difference: $0.9\% \pm 1.3\%SD$, range: 0.0%-3.2%). Moreover,
25 when wild-type homoplasmy was present, it was always detected in both parts of the embryo.
26 Secondly, approximately two thirds of embryos (12/20) were likely mutation-free. Thirdly,
27 although not significant ($p=0.1570$), detected mutation levels declined steadily during
28 progression through preimplantation development (Figure 4). The highest mutation level was
29 detected in the unfertilised oocyte (37.5%). At the cleavage-stage, a mean mutation level of
30 $13.0\% \pm 18.2\%SD$ was detected. The lowest mutation level was detected in blastocyst-stage
31 specimens with a mean mutation level of $4.4\% \pm 9.4\%SD$. Less than half of the cleavage-stage
32 embryos (4/9) had detectable mtDNA mutation levels, ranging from 5.7%-38.0% (mean:
33 $29.3\% \pm 15.7\%SD$) amongst carrier embryos. Less than one-third of blastocysts (3/10) were
34 identified as carriers (mean mutation level of carrier embryos was $14.6\% \pm 13.3\%SD$, ranging
35 from 3.1%-29.2%).

DISCUSSION

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52 Validation experiments proved that the proposed next-generation sequencing method
53 is highly accurate and sensitive in mtDNA mutation detection and quantification and can
54 safely be applied to minute samples, including embryo biopsy specimens. An excellent
55 correlation was achieved between expected and obtained mutant loads in sample mixing
56 experiments and between mutant loads detected in individuals' gDNA and cell samples.

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Importantly, results were equally good when WGA was performed prior to the amplification of mutation loci, verifying that WGA does not distort variant ratios. Equally important, despite variation in intercellular mutant loads, which is frequent in cells of mtDNA mutation carriers[11, 23–26], mean cell mutant loads were always close to levels seen in corresponding bulk gDNA samples.

Mutation levels established by reference laboratories were confirmed in blood from m.8993T>G carriers. We did not have access to muscle DNA in which m.10191T>C and m.3243A>G reference levels were established and mutant loads were consistently higher or lower in blood, respectively (present study). This was expected because m.3243A>G mutant load varies between tissue types and tends to be lowest in leucocytes followed by buccal cells and then skeletal muscle cells[27, 28]. This probably also occurs in m.10191T>C patients but has not been demonstrated[29, 30]. Of note, m.8993T>G heteroplasmic load is usually relatively uniform across tissue types[31].

Patients A, B and C were referred for PGT for Leigh Syndrome (OMIM: 256000), a progressive neurodegenerative disorder, which often results in childhood death[32]. To date, ≥60 genes implicated in the disease have been identified[33]. Those located in the mtDNA cause maternally inherited Leigh Syndrome (MILS), which accounts for up to one quarter of all Leigh Syndrome cases[34]. Mutations in the MT-ATP6 gene (mitochondrially encoded ATP synthase membrane subunit 6), which encodes for a sub-unit of the respiratory chain complex V, are a frequent cause of MILS. Of these, the base change T>G at position m.8993 is the most frequent[35].

Severity of the m.8993T>G mutation is strongly dependent on mutant mtDNA load[33, 36]. For instance, the load of mutant mtDNA is >56% in Patient A who is asymptomatic, while her severely affected son was homoplasmic for the mutation. Patient A’s single competent blastocyst was likely mutation-free but was affected by trisomy 16, a type of aneuploidy common in miscarriage[37]. This highlights the benefit of combining PGT for mtDNA disease and PGT-A. PGT further revealed that one of Patient A’s arrested embryos was also mutation-free, while the other and the unfertilised oocyte carried extremely high mutation levels (~90%). Extreme segregation of m.8993T>G mtDNA towards homoplasmy for either wild-type or mutant is common in oocytes and embryos of m.8993T>G carriers[10, 38–40].

While mothers tend to be asymptomatic, severely diseased children are often the only family member with MILS[36, 41, 42].

Oftentimes, mothers of affected children are not only asymptomatic but also, at least seemingly, mutation-free[42–44]. In these cases, mtDNA mutations may arise *de novo* during germ cell or early embryo development and their recurrence risk in subsequent pregnancies is low[42]. For instance, Patient B's son carried high m.8993T>G mutation levels but the mutation was undetectable in Patient B, her mother and in Patient B's oocyte/embryo specimens. That said, maternal inheritance cannot entirely be ruled out due to the possibility of gonadal mosaicism and presence of a small subpopulation of affected oocytes[42]. In this instance, PGT provided information suggestive of a low recurrence risk and ultimately identified an unaffected euploid male embryo that led to the birth of a healthy son. Importantly, genetic testing of the baby confirmed the PGT result.

Similarly, no mutant mtDNA was detected either in Patient C or her oocytes/embryos, although high levels of the m.10191T>C mutation were detected in the muscle biopsy of her daughter. The m.10191T>C mutation is a rare cause of mitochondrial disease, the limited data available suggesting that phenotypic severity is driven by heteroplasmic mutant load[29, 45]. The m.10191T>C mutation is located in the MT-ND3 gene (mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 3), which encodes for a sub-unit of the respiratory chain complex I. Cases of *de novo* and maternal origin have been reported[45]. The absence of any detectable mutant mtDNA in any of Patient C's oocyte/embryo specimens supported her initial counselling that the recurrence risk was low[42]. Consequently, after two failed treatment cycles, the patient opted to conceive naturally. PND confirmed absence of the mutation and the patient gave birth to a healthy child.

Unlike the first three patients, who were referred for PGT because of their affected children, Patient D was referred due to the diagnosis of mitochondrial myopathy caused by m.3243A>G. The m.3243A>G mutation, which is located in the MT-TL1 gene [mitochondrially encoded tRNA-Leu (UUA/G) 1], is the most common pathogenic mtDNA mutation[46, 47]. It causes a variety of disorders, including maternally inherited diabetes and deafness (MIDD; OMIM: 520000), mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS Syndrome; OMIM: 540000), chronic progressive external ophthalmoplegia

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(CPEO; OMIM: 590050), myoclonic epilepsy associated with ragged-red fibers (MERRF Syndrome; OMIM: 545000) and other less severe clinical phenotypes[48]. Although Patient D generated a large number of embryos, only two of three euploid blastocysts were identified as likely mutation-free and were transferred. The third euploid blastocyst was not chosen for transfer, although relatively low mutation levels were detected in the biopsy. Unlike many other frequent pathogenic mtDNA mutations, m.3243A>G heteroplasmy levels are poorly predictive of disease expression, onset, type and severity[49]. Mutation levels often increase postnatally and accumulate over time in somatic tissues[50] and it is therefore preferable to transfer embryos which are likely mutation-free. Unfortunately, the patient miscarried twice at eight weeks of gestation. Although reasons were unclear, it should be noted that pregnancy complications, including spontaneous abortion, are not uncommon in women with mitochondrial disease due to m.3243A>G[51, 52].

Analysis of cleavage-stage embryos demonstrated that m.3243A>G mutation levels were uniformly distributed in embryo halves. This finding is particularly important in the context of PGT as it indicates that biopsy mutation levels are likely representative of the entire embryo. This agrees with previous studies demonstrating uniform mutation distribution in embryonic cells, irrespective of mutation type[10–12, 15, 25, 40]. Interestingly, the mutation did not appear to be randomly distributed amongst the patient’s embryos. Not only were nearly two thirds of embryos likely mutation-free but also levels above ~40% were not recorded in any embryo, suggesting that the mutation may not be transmitted via genetic drift alone, but selected against during oogenesis. Additionally, mutation levels and frequencies of mutation-carrying embryos appeared to decrease during preimplantation development progression. While it was not possible to confirm this statistically given the small number of samples available, it raises the possibility that high mutation levels may negatively impact embryo viability. It is highly likely that mechanisms exist, additionally to random genetic drift, to prevent the transmission of pathogenic mtDNA mutations[53–55]. Although exact types and timings of these mechanisms are only partially documented in humans, they have been demonstrated more clearly during mouse oogenesis and embryogenesis[54, 56, 57]. Accumulating evidence suggests that pathogenic mutations, are selected against during human germline development and oocyte maturation[56, 58] and embryo development[59]. There are clear differences between pathogenic mutations, in their transmission patterns[11,

60]. As mentioned before, m.8993T>G often displays skewing towards wild-type or mutant homoplasmy in oocytes and embryos[10, 38–40], which is reflected by major shifts in heteroplasmy levels in pedigrees[41]. In contrast, oocytes and embryos from m.3243A>G carriers display substantially less variation in mutation levels and an absence of very high levels and mutation homoplasmy[11, 15, 40, 61], which is reflected by extreme phenotypic variation in pedigrees[46]. It should also be noted that m.3243A>G, which impairs assembly of several complexes of the ATP-generating machinery, is selected against in rapidly dividing postnatal tissues. In contrast, levels of m.8993T>G, which affects a single complex, remain stable postnatally. Thus, it is highly likely that negative selection also occurs against oocytes and possibly embryos with high m.3243A>G levels, while those carrying m.8993T>G may escape selection[60]. However, data on m.3243A>G distribution in embryos is very limited and a relation with embryo viability has not been reported. Whether the present decline of m.3243A>G during preimplantation development progression was coincidental or truly reflects negative selection should be established in larger cohorts.

Conclusion

Here we report first successful application of a next-generation sequencing-based PGT approach that combines PGT for mtDNA disease and PGT-A. Aneuploidy affected half of the embryos tested and involved chromosomes frequently associated with implantation failure and miscarriage. Thus, identification and avoidance of transfer of aneuploid embryos is predicted to offer a significant advantage over other methods for PGT for mtDNA disease. The study provides useful information for counselling of women at risk of transmitting mtDNA disease. As previously shown[42], the recurrence risk in women, in whom the pathogenic mutant mtDNA is undetectable, is lower than in heteroplasmic women. Furthermore, the chances of identifying mutation-free embryos via PGT are very good. Lastly, the study supports the existence of mutation-specific selective mechanisms influencing mtDNA mutation inheritance.

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ACKNOWLEDGEMENTS

We thank the patients and their families for participation in the study. We thank the staff of collaborating clinics and associated genetic counsellors for patient counselling, consenting and treatment. We thank the former Reprogenetics UK Oxford laboratory for supporting the study.

CONTRIBUTORS

KS, DB and DW designed the study. KS, MK and DW performed the study. KS and DW performed the clinical diagnosis and communicated with referring clinics. JL, TC, JAG and JP counselled patients and oversaw patient treatments. KS drafted the manuscript. JP and DW edited and finalised the manuscript draft. All authors approved the final manuscript.

FUNDING

This work was supported by the National Institute of Health Research (NIHR) Oxford Biomedical Research Centre Programme

COMPETING INTERESTS

None declared.

PATIENT CONSENT FOR PUBLICATION

Required.

ETHICS APPROVAL

Patients were referred by UK- and US-based clinics. PGT of mtDNA disease is licensed by the Human Fertilisation and Embryology Authority (HFEA) in the UK. The study was conducted within an accredited PGT service laboratory (Reprogenetics UK). Design, validation and clinical application followed a set strategy, common to all clinical PGT methods performed at the laboratory. By necessity, PGT methods are often unique, created for individual families/diseases. As such, ethical approval for the current study was not required. The Institutional Review Board of New York University confirmed that the situation in the US is the same as that in the UK and that no approvals are required for new PGT test developments.

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FIGURE LEGENDS

Figure 1: Pedigrees of patients referred for PGT. Pedigrees A, B, C and D refer to Patients A, B, C and D, respectively. Roman numerals indicate family generations. Stars mark patients. DNA sources are given in brackets. The specified ages refer to patients', relatives' and children's ages at the time when genetic testing was performed.

Figure 2: Correlation of obtained and expected mutant loads at position m.8993 (T>G) in gDNA sample mixtures. r^2 indicates r-squared value after linear regression analysis. y indicates equation of the best-fit line. r indicates Pearson's rank correlation coefficient with the corresponding p-value. A: Mutant loads obtained after direct mutation site amplification. B: Mutant loads obtained after WGA followed by mutation site amplification.

Figure 3: Mean mutant loads detected in samples of heteroplasmic mtDNA mutation carriers. Graphs A, B, C and D illustrate mutant loads detected after direct mutation site amplification (PCR) and after WGA followed by mutation site amplification (WGA+PCR) in blood and/or cheek swab samples from Patient A (m.8993T>G), the son of Patient B (m.8993T>G), the daughter of Patient C (m.10191T>C) and Patient D (m.3243A>G), respectively. Error bars depict standard error of the mean. Red dotted lines refer to mutation levels previously measured by reference laboratories.

Figure 4: Mean m.3243A>G mutation levels detected in embryo specimens at different stages of preimplantation development. Grey bars illustrate mean mutation levels at different stages of preimplantation development. Each black dot illustrates the mutation level of a single oocyte/embryo. Dots of cleavage-stage embryos refer to mean levels calculated from both embryo halves (also applicable to the cleavage-stage bar). Error bars depict standard error of the mean. Percentages in bold refer to mean mutation levels. Numerical values and percentages in brackets refer to oocyte/embryo numbers and frequencies.

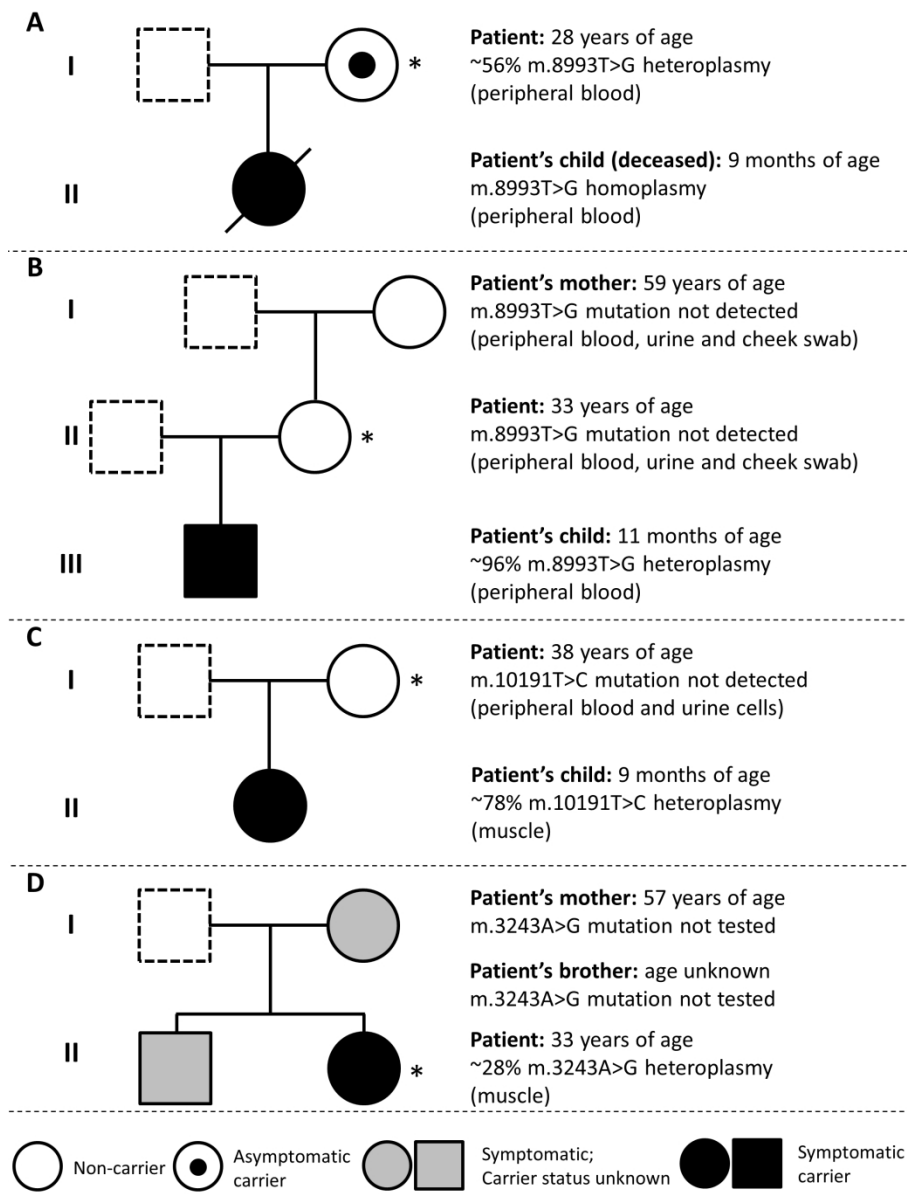


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190x254mm (300 x 300 DPI)

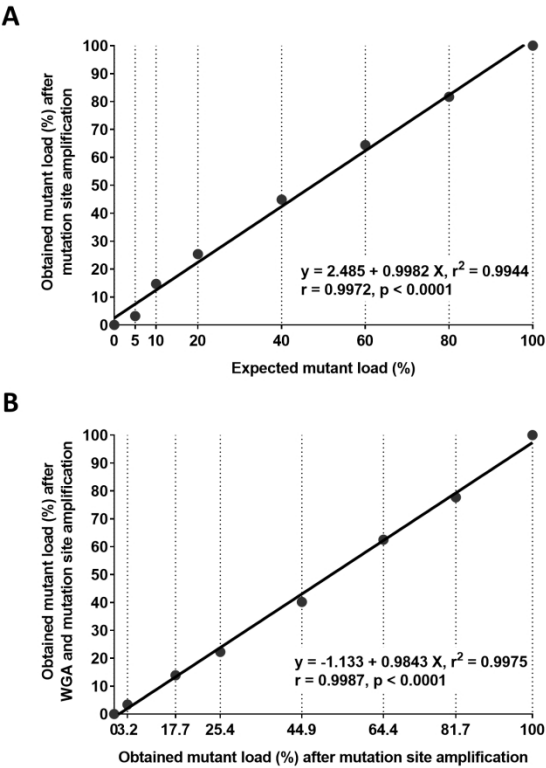


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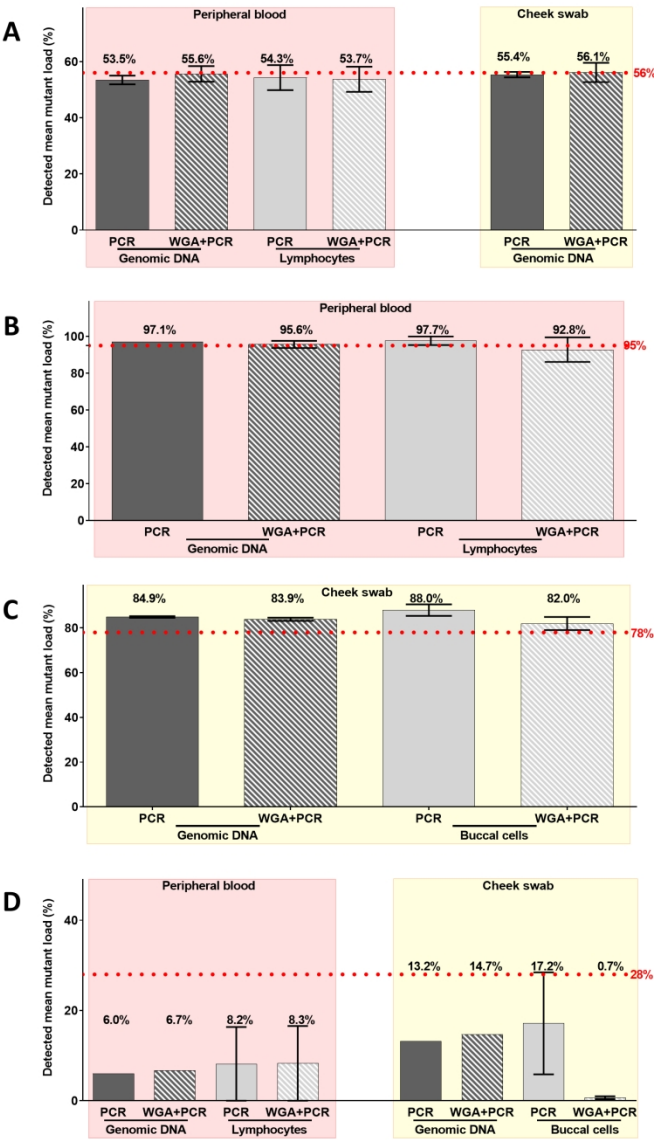


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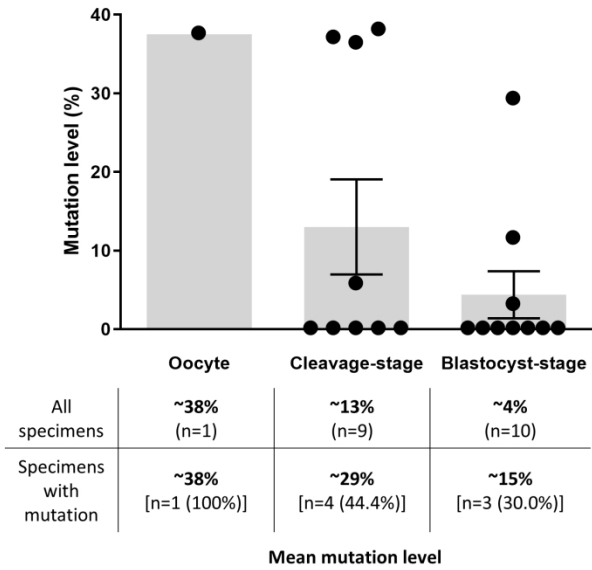


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Supplementary Table 1: Obtained mutant loads at position m.8993 (T>G) in mixed (heteroplasmic) and pure (homoplasmic) gDNA samples after direct mutation site amplification by conventional PCR and after WGA followed by mutation site amplification

Expected mutant load (%)	Obtained mutant load (%)	
	Conventional PCR	WGA and conventional PCR
0.0	ND	ND
5.0	3.2	3.4
10.0	14.7	13.9
20.0	25.4	22.3
40.0	44.9	40.2
60.0	64.4	62.5
80.0	81.7	77.7
100	100	100

Mean coverage of the mutation site was 62,835 reads, ranging from 30,023-96,205. ND=not detected.

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Supplementary Table 2: Detected mutant loads in gDNA and cell samples of patients and their affected children after direct mutation site amplification by conventional PCR and after WGA followed by mutation site amplification

Sample type	Detected mutant load (%)						
	Patient A	Daughter of Patient A	Patient B	Son of Patient B	Patient C	Daughter of Patient C	Patient D
gDNA subjected to conventional PCR	55.0 ¹	100 ³	ND ¹	97.1 ¹	ND ¹	84.2 ²	6.0 ¹
	51.9 ¹	100 ³			ND ¹	85.0 ²	13.2 ²
	56.3 ²				ND ¹	85.0 ²	
	54.4 ²						
gDNA subjected to WGA and conventional PCR	59.6 ¹	100 ³	ND ¹	98.4 ¹	ND ¹	84.5 ²	6.7 ¹
	60.8 ¹	100 ³	ND ¹	91.9 ¹	ND ¹	85.6 ²	14.7 ²
	52.8 ¹			96.6 ¹		82.2 ²	
	49.2 ¹					82.1 ²	
	51.9 ²					84.9 ²	
	50.6 ²						
	56.2 ²						
Cell samples subjected to conventional PCR	97.3 ^{1,4}		ND ¹	93.0 ^{1,4}	ND ¹	83.5 ^{2,4}	ND ^{1,4}
	ND ^{1,4}		ND ¹	100 ^{1,4}	ND ²	83.9 ^{2,4}	49.0 ^{1,4}
	59.8 ^{1,5}		ND ¹	99.9 ^{1,4}		93.9 ^{2,4}	ND ^{1,4}
	91.1 ^{1,5}					90.5 ^{2,4}	ND ^{1,4}
	74.8 ^{1,6}						ND ^{1,4}
	19.6 ^{1,6}						ND ^{1,4}
							ND ^{2,4}
							ND ^{2,4}
							39.1 ^{2,4}
							ND ^{2,4}
Cell samples subjected to WGA and conventional PCR	ND ^{1,4}		ND ¹	100 ^{1,4}	ND ¹	77.3 ^{2,4}	49.7 ^{1,4}
	100 ^{1,4}		ND ¹	100 ^{1,4}	ND ¹	81.7 ^{2,4}	ND ^{1,4}
	33.1 ^{1,5}		ND ¹	99.9 ^{1,4}	ND ²	94.3 ^{2,4}	ND ^{1,4}
	60.2 ^{1,5}		ND ¹	98.8 ^{1,4}	ND ²	75.2 ^{2,4}	ND ^{1,4}
	72.2 ^{1,6}		ND ¹	100 ^{1,4}		85.9 ^{2,4}	ND ^{1,4}
	95.7 ^{1,6}		ND ¹	98.9 ^{1,4}		77.3 ^{2,4}	ND ^{1,4}

				39.5 ^{1,4}			ND ^{2,4}
				100 ^{1,4}			1.2 ^{2,4}
				98.0 ^{1,4}			1.3 ^{2,4}
							ND ^{2,4}
							ND ^{2,4}
							1.6 ^{2,4}

Mean coverage of mutation loci was 75,420 reads, ranging from 12,376-421,777. ¹gDNA or lymphocyte(s) from peripheral blood. ²gDNA or buccal cells from a cheek swab. ³Source unknown. ⁴Single cell. ⁵Three cells. ⁶Five cells. ND=not detected.

Supplementary Table 3: Results from PGT-A and PGT for mtDNA disease of embryo specimens from all patients

Patient ID	Sample ID	Sample type	Mutation level (%)	Predicted karyotype	Interpretation
A	1	Trophectoderm biopsy	ND	48,XXY,+16	Mutation not detected; aneuploid
	2 ¹	Whole embryo	ND	46,XX	Mutation not detected; euploid
	3 ¹	Whole embryo	88.7	47,XXY,5,+6	Mutation detected; aneuploid
	4	Unfertilised oocyte	91.2	Not tested	Mutation detected
B	1	Trophectoderm biopsy	ND	46,XY	Mutation not detected; euploid
	2	Trophectoderm biopsy	ND	46,XX	Mutation not detected; euploid
	3	Trophectoderm biopsy	ND	46,XX	Mutation not detected; euploid
	4	Trophectoderm biopsy	ND	46,XY	Mutation not detected; euploid
	5	Trophectoderm biopsy	ND	45,XY,-16	Mutation not detected; aneuploid
	6	Trophectoderm biopsy	ND	46,XY	Mutation not detected; euploid
	7	Trophectoderm biopsy	ND	45,XY,-11	Mutation not detected; aneuploid
	8	Trophectoderm biopsy	ND	46,XY	Mutation not detected; euploid
	9	Trophectoderm biopsy	ND	46,XX	Mutation not detected; euploid
	10	Trophectoderm biopsy	ND	46,XX	Mutation not detected; euploid
	11 ¹	a Blastomere	ND	45,YYY,-6,-15	Mutation not detected; aneuploid
		b Blastomere	ND	44,YYY,-2,-6,-15	Mutation not detected; aneuploid
		c Blastomere	ND	45,XY,-15	Mutation not detected; aneuploid
		d Blastomere	ND	47,YYY,+3,-6,-15,+22	Mutation not detected; aneuploid
		e Blastomere	ND	47,YYY,-15,+22	Mutation not detected; aneuploid
	12	Trophectoderm biopsy	ND	46,XX	Mutation not detected; euploid
	13 ¹	a Blastomere	ND	Complex abnormal ⁴	Mutation not detected; aneuploid
		b Blastomere	ND	Complex abnormal ⁴	Mutation not detected; aneuploid
		c Blastomere	ND	Complex abnormal ⁴	Mutation not detected; aneuploid
		d Blastomere	ND	Complex abnormal ⁴	Mutation not detected; aneuploid
		e Blastomere	ND	Complex abnormal ⁴	Mutation not detected; aneuploid
		f Blastomere	ND	Complex abnormal ⁴	Mutation not detected; aneuploid
		g Blastomere	ND	Complex abnormal ⁴	Mutation not detected; aneuploid
		h Blastomere	ND	Complex abnormal ⁴	Mutation not detected; aneuploid
		i Blastomere	ND	Complex abnormal ⁴	Mutation not detected; aneuploid
		j Blastomere	ND	Complex abnormal ⁴	Mutation not detected; aneuploid
		k Blastomere	ND	Complex abnormal ⁴	Mutation not detected; aneuploid
	14 ²	Whole embryo	ND	47,XX,+16	Mutation not detected; aneuploid

	15	Trophectoderm biopsy		ND	47,XX,+22	Mutation not detected; aneuploid
	16	Unfertilised oocyte		ND	24,X,+16	Mutation not detected; aneuploid
	17	Trophectoderm biopsy		ND	45,XX,-16	Mutation not detected; aneuploid
	18	Trophectoderm biopsy		ND	46,XX	Mutation not detected; euploid
C 1 st cycle	1 ¹	a	Blastomere	ND	Not tested	Mutation not detected
		b	Blastomere	ND	Not tested	Mutation not detected
		c	Blastomere	ND	Not tested	Mutation not detected
		d	Blastomere	ND	Not tested	Mutation not detected
	2	Unfertilised oocyte		ND	Not tested	Mutation not detected
3	Blastomere biopsy		ND	46,XX	Mutation not detected; euploid	
C 2 nd cycle	4 ¹	Whole embryo		ND	Not tested	Mutation not detected
	5	Blastomere biopsy		ND	46,XY	Mutation not detected; euploid
	6	Blastomere biopsy		ND	43,XX,-6,-15,-20	Mutation not detected; aneuploid
	7 ¹	Whole embryo		ND	Not tested	Mutation not detected
	8 ¹	Whole embryo		ND	Not tested	Mutation not detected
	9	Blastomere biopsy		ND	Complex abnormal ⁴	Mutation not detected; aneuploid
	10	Unfertilised oocyte		ND	Not tested	Mutation not detected
	11	Unfertilised oocyte		ND	Not tested	Mutation not detected
D	1	Trophectoderm biopsy		29.2	47,XX,-4,-6p,+8,+20	Mutation detected; aneuploid
	2 ¹	a	Half embryo	37.0	Not tested	Mutation detected
		b	Half embryo	35.6	Not tested	
	3	Trophectoderm biopsy		11.5	Complex abnormal ⁴	Mutation detected; aneuploid
	4 ¹	a	Half embryo	ND	Not tested	Mutation not detected
		b	Half embryo	ND	Not tested	
	5 ²	Whole embryo		ND	Not tested	Mutation not detected
	6	Unfertilised oocyte		37.5	Not tested	Mutation detected
	7	Trophectoderm biopsy		ND	46,XX	Mutation not detected; euploid
	8 ¹	a	Half embryo	37.1	Not tested	Mutation detected
		b	Half embryo	36.9	Not tested	
	9	Trophectoderm biopsy		3.1	46,XY	Mutation detected; aneuploid
	10 ¹	a	Half embryo	ND	Not tested	Mutation not detected
		b	Half embryo	ND	Not tested	
	11	Trophectoderm biopsy		ND	46,XX	Mutation not detected; euploid
	12	Trophectoderm biopsy		ND	47,XX,+18	Mutation not detected; aneuploid
	13	Trophectoderm biopsy		ND	45,XX,+1,-3,-10	Mutation not detected; aneuploid

14	Trophectoderm biopsy	ND	48,XX,+4,+16	Mutation not detected; aneuploid
a	Half embryo	ND	Not tested	
15 ¹	b	No amplification		Mutation not detected
16	Trophectoderm biopsy	ND	45,XX,-9	Mutation not detected; aneuploid
a	Half embryo	39.6	Not tested	
17 ¹	b	36.4	Not tested	Mutation detected
a	Half embryo	ND	Not tested	
18 ¹	b	ND	Not tested	Mutation not detected
a	Half embryo	4.6	Not tested	
19 ¹	b	6.8	Not tested	Mutation detected
a	Half embryo	ND	Not tested	
20 ¹	b	ND	Not tested	Mutation not detected

Mean coverage of mutation loci was 94,253 reads, ranging from 12,176-525,522. ¹Arrested at cleavage-stage. ²Arrested at blastocyst-stage. ³>4 chromosome errors. ND=not detected.

Supplementary Table 4: Results from mutation analysis of newborn samples

Sample types	Mutation level (%)
Cord blood	ND
Cheek swab (two weeks after birth)	ND
Cheek swab (three weeks after birth)	ND

Mean coverage of the mutation site was 83,617 reads, ranging from 75,560-98,617. ND=not detected.