ORIGINAL ARTICLE: ASSISTED REPRODUCTION

# First pilot study of maternal spindle transfer for the treatment of repeated in vitro fertilization failures in couples with idiopathic infertility

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**Objectives:** To gain insights into the technical feasibility of maternal spindle transfer (MST) applied in the context of repeated in vitro fertilization (IVF) failures for the treatment of idiopathic infertility.
**Design:** A prospective pilot study.
**Setting:** IVF center.
**Patient(s):** Twenty-five infertile couples with multiple previous unsuccessful IVF cycles (range, 3–11), no previous pregnancy, and no history of mitochondrial DNA (mtDNA) disease participated. The study focused on women <40 years, with previous IVF attempts characterized by a pattern of low fertilization rates and/or impaired embryo development. Couples with severe male-factor infertility were not eligible. Oocyte donors with previous successful IVF outcomes were matched with patients according to standard practice.
**Intervention(s):** We performed MST by transferring metaphase II spindles from the patients' oocytes into the previously enucleated donor oocytes, followed by intracytoplasmic sperm injection, in vitro embryo culture, blastocyst biopsy, and vitrification. Only euploid blastocysts were considered for embryo transfer.
**Main Outcome Measure(s):** Outcome measures included oocyte fertilization, blastocyst development, clinical pregnancy and live birth, incidence of mitochondrial carryover and potential mtDNA reversal, as well as general health of the children born.
**Result(s):** Twenty-eight MST cycles produced 6 children (19 embryo transfers, 7 clinical pregnancies). Pediatric follow-up of the children, performed at intervals from birth to 12–24 months of age, revealed their development to be unremarkable. DNA fingerprinting confirmed that the nuclear DNA of MST children was inherited from both parents, without any contribution from the oocyte donor. For 5 of the children, mtDNA was derived almost exclusively (>99%) from the donor. However, 1 child, who had similarly low mtDNA carryover (0.8%) at the blastocyst stage, showed an increase in the maternal mtDNA haplotype, accounting for 30% to 60% of the total at birth.
**Conclusion(s):** This pilot study provides the first insights into the feasibility of applying MST for patients with idiopathic infertility and repeated IVF failures. Reconstructed oocytes produced embryos capable of implanting, developing to term and producing apparently healthy newborns/children. However, claims concerning the efficacy of MST with respect to infertility treatment would be premature considering the limitations of this study. Importantly, mtDNA reversal was detected in one child born after MST, a finding with possible implications for mitochondrial replacement therapies.

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**Key Words:** Maternal spindle transfer, mitochondrial replacement therapies, infertility, mtDNA reversal

A subset of women with infertility undergoing in vitro fertilization (IVF) treatments present with poor oocyte quality. Cytoplasmic deficiencies, including, but not limited to mitochondrial dysfunction, have the potential to cause oocyte fertilization failure and/or early embryo developmental arrest (1–7). In theory, mitochondrial replacement therapy (MRT) strategies, which aim at replacing the oocyte's entire non–nuclear cytoplasmic content, might serve as treatments to restore oocyte developmental competence. One technique involves the transfer of metaphase II (MII) spindles from poor quality oocytes into enucleated donor oocytes of presumed greater developmental potential, a process known as 'maternal' or 'meiotic' spindle transfer (MST). The proof of concept studies, conducted in animal models, have confirmed the technical feasibility of MST and provided reassurance concerning the safety in transgenerational studies (3, 8, 9).

Procedures for MRT were initially developed to avoid the transmission of maternally inherited mitochondrial DNA (mtDNA) disorders to children (10, 11). However, the inheritance of mutant mtDNA cannot be entirely eliminated because of the concomitant transfer of small numbers of potentially affected maternal mitochondria along with the nuclear DNA of the patient. This mitochondrial carryover generally accounts for <1% of the total mtDNA (1) in the resulting preimplantation embryos. Such low proportions of mutant mtDNA would not be expected to produce a disease phenotype, but evidence from in vitro studies using human embryonic stem cells (ESCs) derived from MRT embryos has indicated the possibility that this relatively low quantity of maternal mtDNA may increase dramatically, leading to a reversal of the mitochondrial haplotype (detected in approximately 15% of the cell lines studied) (11–13). Although 'reversal' has not previously been reported in humans *in vivo*, the theoretical possibility that it might occur has led to concerns that the use of MRTs for the avoidance of mtDNA disease transmission could be less reliable than initially assumed (13, 14).

To shed more light on the dynamics of mitochondrial populations after MRTs, as well as to gather preliminary data on the clinical feasibility of MST in the context of fertility treatment (i.e., restoring oocyte competence), we conducted a pilot study involving a cohort of 25 patients with infertility who presented with multiple unsuccessful IVF treatments without known mtDNA disease. The outcomes monitored included the usual measures of IVF success (oocyte fertilization, blastocyst development, and clinical pregnancy and live birth rates), incidence of mitochondrial carryover and the possibility of mtDNA reversal, as well as the general health of children born after MST. Results described herein represent the first clinical experience using MST for infertility treatment. The data additionally provide important insights that can be extrapolated to the potential use of MST to reduce the risk of disease transmission in patients carrying pathogenic mtDNA mutations.

## MATERIAL AND METHODS

### Trial Oversight

Study design and protocols were approved and monitored by the *Greek Authority of Assisted Reproduction* (license 437/23.9.2016) and the *IASO maternity Hospital institutional review board with periodic renewals* (2017-001/25.05.2017-2022). Written informed consents were obtained from all couples and oocyte donors to conduct the MST and IVF procedures and to observe the health of MST children. All patients were informed about the experimental nature of the MST technique and the potential for risks resulting from the use of this procedure, some of which may be unknown at this time. Periodic progress reports were reviewed by *the Greek Authority of Assisted Reproduction*. The clinical pilot study was registered in the ISRCTN registry under the accession number ISRCTN11455145. Considering the experimental nature of this pilot study, the entire treatment and follow-up of the MST children were offered free of cost (including, consultations, genetic and blood tests undertaken for patients and donors, medication for patients and donors, oocyte retrievals, IVF+MST procedures, preimplantation genetic testing for aneuploidy (PGT-A), genomic and mtDNA analysis, embryo transfers, prenatal tests, delivery, hospital and medical expenses, children's pediatric neuropathology, and regular health check-ups). Donors were compensated for their time, effort, discomfort, and inconvenience associated with the donation process, in accordance with the *Greek Law* 3305/2005.

### Cohort of Patients

Twenty-five infertile couples with multiple previous unsuccessful IVF cycles, no previous pregnancy, and no history of mtDNA disease were recruited. Inclusion criteria included women under the age of 40 years with several failed IVF attempts and a consistent pattern of low fertilization rates and/or impaired embryo development attributed to poor oocyte quality. Couples diagnosed with severe male-factor infertility were excluded. Healthy oocyte donors with previous successful IVF outcomes were matched with patients according to the clinic's standard oocyte donation practice, without considering their mtDNA haplotype.

### Trial Procedures

Baseline screening was conducted in patients and donors, including medical history and physical examination,

assessment of body mass index and antral follicle count. Patients and donors underwent ovarian stimulation and oocyte retrieval that employed standard IVF protocols and procedures. All patient oocytes were vitrified until donor oocytes were available, except for one patient for whom both vitrified and fresh oocytes from 2 different stimulation cycles were used. Meiotic MII spindles were transferred from the patients' mature oocytes into the enucleated donor oocytes, as previously described (1), followed by conventional IVF procedures (intracytoplasmic sperm injection [ICSI], in vitro embryo culture, blastocyst biopsy, and vitrification). Briefly, MII spindles were visualized under polarized light and isolated ensuring minimal cytoplasm carryover (1). The resulting karyoplast was then placed in the perivitelline space of a previously enucleated donor oocyte (cytoplast, containing donor mtDNA) and fused using the hemagglutinating virus of Japan-envelope (Supplemental Fig. 1, available online). Embryos successfully developing to the blastocyst stage with good morphological grading were biopsied and tested for aneuploidy (Supplemental Methods in Supplemental Material). Only euploid blastocysts were considered for uterine transfer.

### Mitochondrial and Nuclear Genome Analysis

Whole mtDNA sequencing was performed to reveal the sites of sequence variation between patients and their respective donors. Maternal mtDNA carryover was determined in embryo biopsies, amniotic fluids, and tissues from MST children through targeted deep sequencing of the mtDNA sequence variants identified. These results were complemented by sequencing of the entire mitochondrial genome in the umbilical cord samples collected after births (see Supplemental Methods in Supplemental Material). Short tandem repeat markers and 1 sex typing marker were used for DNA fingerprinting to confirm the nuclear DNA parentage of the children born (see Supplemental Methods in Supplemental Material).

### Trial Outcomes

Oocyte fertilization, blastocyst development, and clinical pregnancy and live birth rates were assessed to gain an insight into the various aspects of the MST technique when applied to patients with infertility. All gestations were observed closely until the completion of pregnancy. The pediatricians closely monitored the development and general health of MST children through regular health check-ups (Supplemental Material).

### Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 7.0 software. Data were analyzed using Pearson nonparametric test for correlation, independent group t-test, or chi-square test for pairwise comparison. A $P$ value $<.05$ was considered significant.

## RESULTS

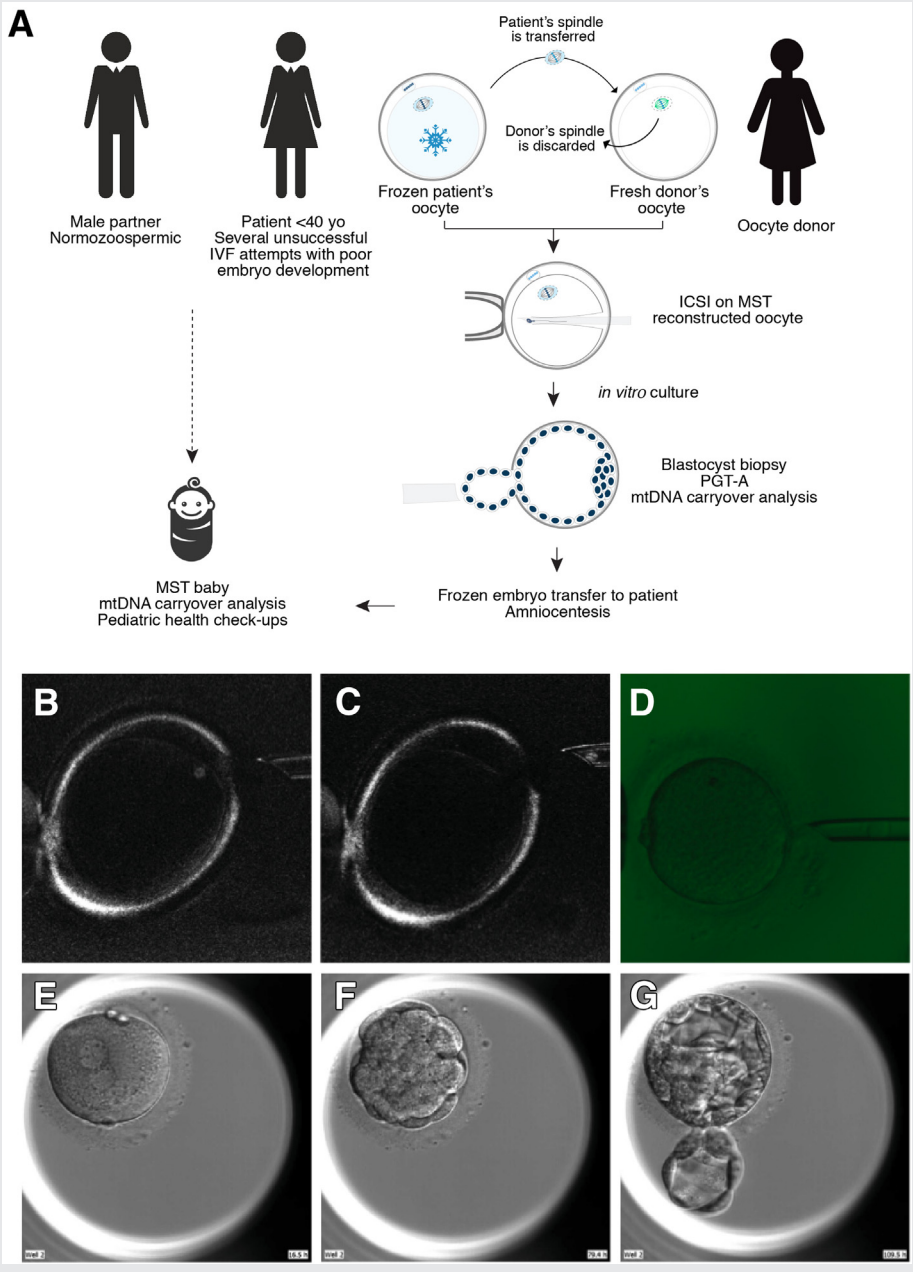
### Previous IVF History and Demographics of Patients Enrolled in the Trial

After the registration of the pilot study on February 20, 2018, a total of 327 patients expressed an interest in participation and were assessed for eligibility. Of these, 289 were excluded as they did not meet the stated inclusion criteria, whereas a further 10 were not eligible because it was not possible to obtain the records of their previous IVF attempts. From the 28 eligible patients, 3 ultimately decided against participation, and 25 were recruited (Supplemental Table 1, available online). The average age of the female patients was 37.2 years. Of the 25 recruited women, 15 were diagnosed with idiopathic infertility, 3 with polycystic ovarian syndrome, and 7 with poor ovarian response to stimulation, which was associated with severe endometriosis in 2 patients. In all cases, poor oocyte quality was indicated as a principal factor contributing to infertility based on repeated low fertilization or complete fertilization failure and poor embryo development and/or embryonic developmental arrest within the first days of in vitro culture (failure to produce any blastocysts) (Supplemental Table 1). Overall, before trial enrolment, patients had undergone a total of 159 stimulation cycles without any pregnancy (mean previous IVF cycles per couple: $6.4 \pm 2.1$; range, 3–11), during which 423 oocytes were inseminated either by ICSI or conventional IVF. Of these, 225 were successfully fertilized (53.2%). Nine patients had never received an embryo transfer owing to the lack of embryos of adequate quality in all previous cycles (average of 7.3 previous cycles in these patients). In the remaining 16 patients (average of 5.6 previous unsuccessful cycles), 58 embryos had been transferred at various developmental stages, but none had succeeded in producing a biochemical or clinical pregnancy (Supplemental Table 1).

### MST pilot trial outcomes

From March 2018 to December 2019, the 25 recruited couples underwent a total of 28 MST cycles. The first embryo transfer was performed in August 2018 and resulted in a pregnancy. The second embryo transfer took place in February 2019 when there was clinical evidence to conclude that the first pregnancy was proceeding normally. As a result, the first child was born in April 2019 and the second not until Nov 2019. As the project progressed, MST cycles were performed gradually during 2019, with the corresponding embryos transferred after Nov 2019. On oocyte retrieval, patients' mature MII oocytes were cryopreserved by vitrification and used for MST later, whereas donor oocytes were always used fresh (Fig. 1). This strategy was followed because our previous experience, during pre-clinical validation studies, indicated superior results for fresh donor cytoplasts (1, 3). In one case (cycle ID PTMST-11a), none of the patient's 18 vitrified oocytes survived after warming, and therefore the patient (PT-11) underwent an additional stimulation and MST cycle with fresh rather than cryopreserved oocytes (PTMST-11b, Supplemental Table 2, available online). Two other patients (PT-01 and PT-18) were offered a second MST cycle because

**FIGURE 1**




Maternal spindle transfer (MST) procedure overview.
(**A**) Schematic representation of the MST process. (**B–G**) Representative images of the MST procedure from PTMST-02 cycle, resulting in the trial's first born MST baby (BBMST-01). The MST process involved transfer of the patient's oocyte spindle, visualized by polarized light (**B, C**), into a donor's fresh enucleated oocyte (**D**). Reconstructed oocytes were inseminated with the patient's partner's sperm by intracytoplasmic sperm injection and cultured in vitro. (**E**) A fertilized oocyte with 2 pronuclei developed into (**F**) a compacted morula and (**G**) a blastocyst 5 days after insemination. Good morphology blastocysts were biopsied, tested for chromosomal normalcy, and mtDNA carryover.

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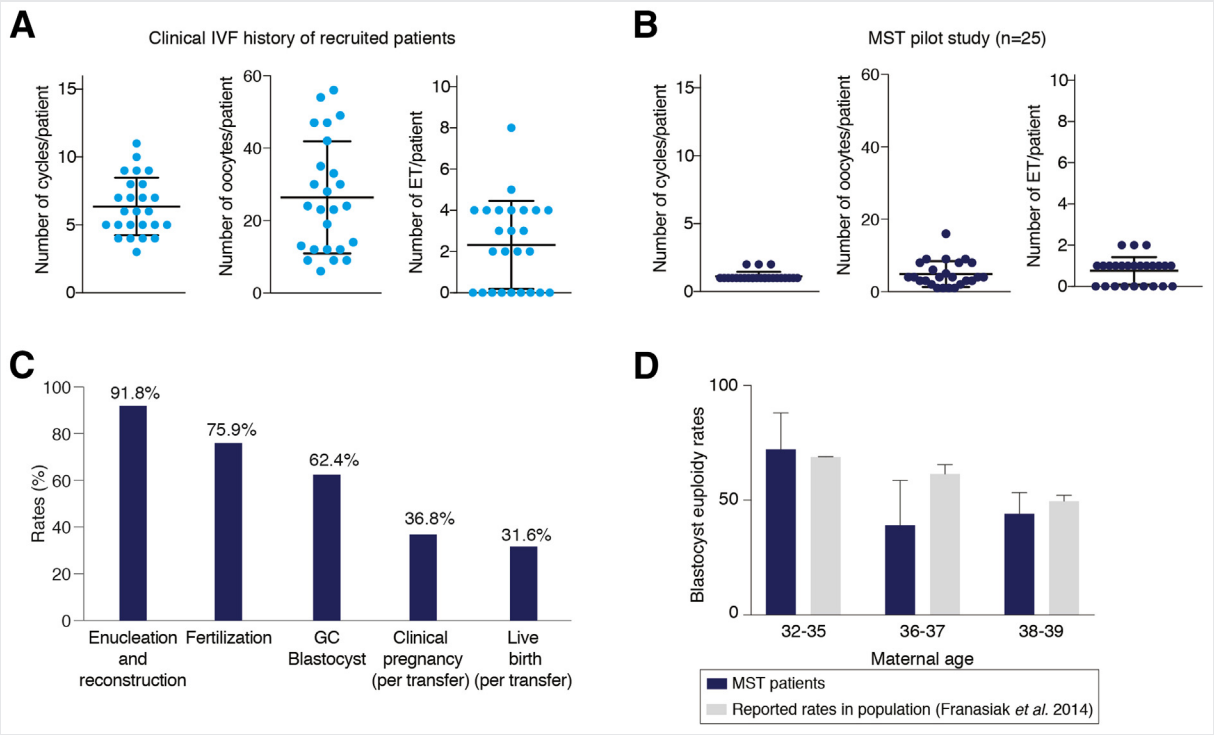
although they succeeded in producing blastocysts, the embryos were either chromosomally abnormal (PTMST-18a), in which case no embryo transfer took place, or a pregnancy could not be established after the transfer of one MST euploid blastocyst (PTMST-01a, Supplemental Table 2). The remaining 22 patients underwent only one MST cycle (Fig. 2B).

From a total of 28 MST cycles performed, 122 MII patient/donor oocytes were used (Fig. 2C), of which 112 were successfully reconstructed (91.8% survival rate after microsurgical procedures and fusion, Supplemental Table 2). All reconstructed oocytes were inseminated by ICSI using the partner's sperm resulting in 85 zygotes (75.9% fertilization rate). Of

## FIGURE 2




Summary of maternal spindle transfer (MST) pilot study outcomes.
(**A**) Summary of clinical history of recruited patients (average 6.4 cycles/patient, a total of 423 oocytes used for standard in vitro fertilization and a total of 58 embryo transfers at different developmental stages. (**B**) Summary of MST pilot trial in terms of number of cycles (average of 1.1 cycles/patient), oocytes used (a total of 122 enucleated oocytes), and a total of 19 embryo transfers of good morphology and euploid blastocysts. (**C**) Embryo development and clinical outcomes of MST study. Out of the 7 clinical pregnancies there was one miscarriage at 9 weeks. (**D**) Euploid rates for blastocysts produced during the study compared with the reported rates in Franasiak et al., 2014. Error bars indicate SEM.

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these zygotes, 53 (62.4%; from 21 couples) reached the blastocyst stage and were graded as good quality, according to the inner cell mass and trophectoderm morphology, as well as the degree of blastocoel cavity expansion. Good quality blastocysts were biopsied and assessed for aneuploidy. A total of 24 MST blastocysts, derived from 16 couples, were euploid (45.2%, Supplemental Table 2). Five couples (PTMST-04, PTMST-09, PTMST-10, PTMST-12, and PTMST-25) produced only aneuploid blastocysts. However, the average number of oocytes available for MST in these cases was very low (2.4 ± 0.8), out of which an average of 1.4 ± 0.5 blastocysts per patient were produced. In the remaining 4 couples, embryos did not develop to the blastocyst stage owing to fertilization failure (PTMST-07 and PTMST-19) or early embryonic arrest (PTMST-20 and PTMST-24). Again, the number of MII oocytes available for MST in these 4 cycles was lower than the average number of oocytes used in the remaining MST cases (2 ± 0.8 versus 4.5 ± 2.7, Supplemental Table 2, Fig. 2B).

Overall, the euploidy rate after MST was within the expected references described in the literature for the corresponding maternal age group (Fig. 2D). Owing to the desire of the recruited patients to have a genetically related child, a control group with non-manipulated donor oocytes could not be performed. Similarly, because of the limited number of patient's oocytes available (average 4.5 ± 2.7, Fig. 2B), all oocytes were used for MST, except for one case (PTMST-11b) where the patient had a relatively large number of oocytes available (16 MII, Supplemental Table 2). In this case, oocytes were randomly assigned to MST and to a control group. Nine oocytes were reconstructed by MST, 7 of which fertilized normally, ultimately yielding 4 blastocysts of good morphological grade. In contrast, no blastocysts were produced from the 7 oocytes that were assigned to the control group and did not undergo any manipulation (Supplemental Table 2). In total, 19 transfers of single euploid blastocysts in 16 women were performed, resulting in 7 clinical pregnancies (Fig. 2C). One patient miscarried at week 9 (PT-13), whereas 6 had live births.

Overall, the MST trial resulted in a 36.8% clinical pregnancy rate per transfer (7 pregnancies from 19 single embryo transfers), a live birth rate per transfer of 31.6% (6 live births). The live birth rate per patient was 24% (25 patients) (Fig. 2C).

## Gestations and Live Births after MST

All gestations resulting from the transfer of a single euploid MST blastocyst were observed closely, carefully monitoring the fetal growth and development. Considering the

experimental nature of the trial, amniocentesis was recommended, and consent was obtained from 5 pregnant women. In these cases, amniotic fluid was collected between 17 and 18 weeks of gestation. The cytogenetic results obtained from amniotic fluids were in agreement with those from PGT-A, examining cells biopsied from blastocyst stage embryos, confirming 2 euploid male (46,XY) and 3 euploid female (46,XX) gestations (Supplemental Table 3, available online). One patient (PT-11) did not consent to amniocentesis and was offered a noninvasive prenatal test, which indicated a normal male karyotype (MST cycle reference: PTMST-11b; MST baby reference: BBMST-3), consistent with the PGT-A result (Supplemental Table 2). Fetal growth parameters were examined using periodic scans and were normal throughout all pregnancies. All patients delivered through cesarean section, as per maternal desire and obstetricians' decision. The mean gestational age for the 6 MST deliveries was 37.8 ± 0.9 weeks, which is within the WHO references for naturally conceived children (Supplemental Table 3). Similarly, the mean body length and weight were 51.3 ± 2.2 cm and 2988.3 ± 244 g, respectively, and the mean weight of the placentae was 531 ± 105.9 g, which were also within normal ranges (Supplemental Table 3). In all cases, the mothers recovered from cesarean sections without complications, and all the MST children had uneventful courses and were discharged from the hospital within 1 week of birth.

## Nuclear DNA and mtDNA Inheritance in MST Children

To verify the nuclear DNA parentage in embryos and children resulting from MST, genetic fingerprinting was performed in DNA isolated from blastocyst biopsies, amniotic fluids, and tissue samples from 5 MST children. Comparison of fingerprinting profiles from MST embryos and children with those from their parents and oocyte donors confirmed that the nuclear DNA was inherited from both parents, and there was no evidence of nuclear DNA contribution from the oocyte donors (Supplemental Tables 4–8, available online).

Mitochondrial genome inheritance was determined by whole mtDNA sequencing. For each MST pregnancy, the maternal and paternal mtDNA sequences were compared with that of the corresponding oocyte donor, revealing single-nucleotide variants (SNVs) suitable for distinguishing the 3 potential sources of mtDNA. For the children born, the total number of SNVs that differed between mothers and donors ranged from 25 to 40, which represents 0.15%–0.24% sequence mismatch across the entire mitochondrial genome (Supplemental Fig. 2, available online). The greatest number of SNVs was located in the protein coding genes, including non-synonymous substitutions leading to amino acid changes (Supplemental Fig. 2A and Supplemental Tables 9–13, available online).

## mtDNA Carryover in MST Blastocysts and Children

To assess the presence of mtDNA carryover (i.e., inheritance of both donor and patient mtDNA), mtDNA heteroplasmy levels were analyzed by targeted deep variant sequencing in trophectoderm biopsies as well as in amniotic fluids and tissue samples collected from MST children after birth and during postnatal medical examinations for up to 24 months after birth. Sequencing results confirmed minimal maternal mtDNA carryover (≤1% heteroplasmy) in all blastocysts from MST cycles that resulted in a live birth (Fig. 3A). Similarly, in 5 of 6 established pregnancies, heteroplasmy levels in the amniotic fluids and children's tissue samples (cord blood, cord, urine, peripheral blood, and saliva) remained below 1% and were maintained through the follow-up period (up to 24 months) (Fig. 3B–F) (Supplemental Tables 14, 15, 17–19, available online). However, mtDNA analysis of one child (BBMST-3, MST cycle ID PTMST-11b) revealed the presence of a mixture of donor and maternal mtDNA haplotypes (Fig. 3D and Supplemental Table 16, available online). For this child, heteroplasmy levels for maternal mtDNA at birth were measured at 36.0 ± 0.7% in cord blood, 42.4 ± 0.6% in cord, 51.1 ± 1.5% in urine, and 37.7 ± 0.5% in peripheral blood, with the remaining mtDNA having the haplotype of the donor. The analyses of saliva, blood, and urine samples, collected at 6 months of age, confirmed the previous findings and showed that the level of maternal mtDNA levels had remained constant after birth (Supplemental Table 16). Heteroplasmy for maternal and donor mtDNA haplotypes in all MST children born were independently corroborated in 2 laboratories and confirmed both by targeted Sanger sequencing (Supplemental Fig. 2B) and next-generation sequencing of the whole mtDNA genome in cord blood DNA (Supplemental Tables 20–24, available online). No paternal mtDNA was detected in any MST children (Supplemental Fig. 2B).

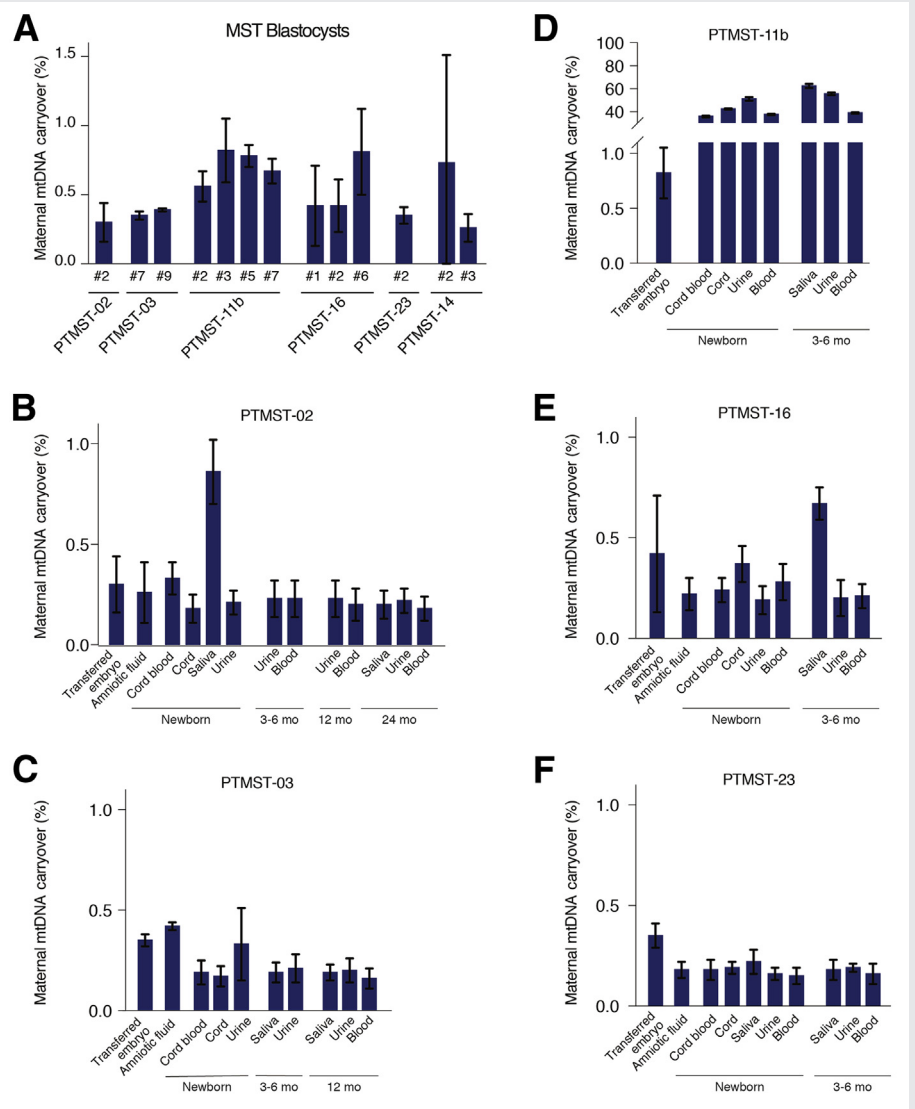
## Postnatal Development of MST Children

Postnatal periodical physical and neurological examinations were performed in all MST children, according to the *Hammersmith Infant Neurological Examination (HINE) scale* (Supplemental Table 3). All children presented with normal HINE scores (Supplemental Table 3), as well as normal development and growth during all routine follow-up tests, suggesting that their general health status was unremarkable.

# DISCUSSION

Poor oocyte quality leading to fertilization failure and/or impaired embryo development is a factor contributing to female infertility. Several previous findings suggest that compromised oocyte cytoplasm is one of the main causes of this form of infertility (2, 3, 15–20). Currently, IVF treatments for patients affected by this problem are limited to the use of donor oocytes or embryos, excluding the female patient from a genetic contribution to the child. Maternal spindle transfer (MST) offers the possibility of replacing the mitochondria and other cytoplasmic components while retaining the female partner's nuclear DNA. We have recently shown that the replacement of the entire cytoplasm in mature murine oocytes can overcome a phenotype of early embryonic arrest, which is characteristic of some mouse strains (3, 6, 21). Similarly, other groups have shown in mice that spindle transfer is capable of

## FIGURE 3




Maternal mitochondrial DNA (mtDNA) carryover analysis in maternal spindle transfer (MST) blastocysts, amniotic fluids, and samples from MST children.

(**A**) mtDNA carryover levels in blastocysts from MST cycles with a successful outcome (newborns). (**B–F**) Maternal mtDNA carryover levels in transferred blastocysts, amniotic fluid from amniocentesis, embryonic and extraembryonic tissue samples from MST newborns, as well as urine, blood, and saliva samples collected from children (aged up to 2 years) during follow-up assessments (when available). The transferred embryos that resulted in a newborn are included in each graph for reference (Supplemental Tables 14–19).

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rescuing poor embryo development and embryo arrest associated with advanced maternal age (6, 21). Here, we conducted a pilot study to explore the feasibility of using MST to treat infertility in humans. Oocytes reconstructed by MST were shown to have the potential to implant and develop to term, producing children who were of unremarkable health and developmental status up to 2 years of age. The results obtained are encouraging, but given the obvious limitations of this pilot study, conclusions concerning the efficacy of the method would be premature, and we make no claims in this regard. Studies with stricter inclusion/exclusion criteria, involving larger numbers of patients, ideally in the context of a randomized clinical trial, will be needed to evaluate whether spindle transfer is of value for the treatment of female infertility associated with repeated IVF failures secondary to poor oocyte quality.

The results of this clinical research indicate that in most cases the maternal mtDNA can be effectively replaced with mtDNA from a donor, providing a potential intervention for women at risk of transmitting mtDNA disease. It is well known that both mtDNA and nuclear DNA co-encode OXPHOS proteins, and nuclear DNA also provides the necessary replication, transcription, and translation machinery for

mtDNA function (8). For this reason, safety concerns have been raised over the potential for incompatibility between "unmatched" donor mtDNA and the maternal nuclear genome in MST children (9, 10). In the current study, genetic differences between donor and maternal mtDNA for the MST children born so far have ranged from 25 to 40 SNVs, whereas the most diverged human populations are estimated to differ from each other at approximately 130 sites in the mitochondrial genome (11). Results indicate that the birth weights, early postnatal development, and general health of all of the children born from this study are within typical ranges. Thus, data from the current trial suggest that MST using randomly selected "unmatched" donor mtDNA haplotypes is not detrimental during gestation or the first few months/years of life. However, additional longitudinal investigations of the growth and development of MST children are clearly required, and are being undertaken, to fully evaluate the impact of donor/patient mtDNA sequence variations and heteroplasmy.

A difficulty for most MRT strategies employed to prevent mtDNA disease transmission is that a small amount of pathogenic maternal mtDNA is transferred along with the patient's nuclear DNA, resulting in a persistence of small quantities in the resulting offspring (1, 2). Moreover, initially low levels of heteroplasmy for maternal mtDNA have been observed to increase during extended culture in some human ESCs derived from embryos produced using MRT as well as in some MST-derived nonhuman primate offspring. These findings suggest that selective and/or replicative advantages of maternal mtDNA over donor mtDNA may sometimes exist (12–14, 22, 23). Importantly, our investigation confirms for the first time that this phenomenon is not restricted to human ESCs and can also occur in some MST children, with an expansion of maternal mtDNA occurring after the preimplantation stage but before birth. In the single case identified in this study, maternal mtDNA carryover levels, initially measured at $<1\%$ at the blastocyst stage, showed a dramatic increase, reaching 30% to 60% (depending on the tissue analyzed) at birth. It should be noted, however, that in contrast to the observations in human ESCs, this increase in the relative proportion of maternal mtDNA falls well short of a complete reversal, with donor mtDNA continuing to account for at least 40% of the total. A recent long-term follow-up study conducted in nonhuman primates revealed that heteroplasmy levels for the maternal mtDNA in monkeys born after MST are not uniform across all tissues and organs, but can show reversal in some organs, but not in others (23). This suggests that sampling of peripheral tissues such as blood or urine may not be fully representative of heteroplasmy levels throughout the body. It is possible that vital organs and tissues of MST children could have significantly high or low levels of maternal mtDNA.

Our results indicate that if MST is used for women carrying mtDNA disease, it should be possible to consistently reduce the levels of mutant mtDNA in the resulting preimplantation embryos to $<1\%$ of the total. However, there is a possibility that mutant maternal mtDNA may increase significantly in the cells of some children before birth. Based on the present study and its small sample size, the actual risk of mtDNA reversal in the offspring after MST cannot be accurately determined. Additionally, this study did not involve heteroplasmic situations in which the maternal mtDNA contained disease-associated mutations. It is conceivable that mutant mitochondria might have different rates of replication or turnover, potentially increasing or decreasing the likelihood of reversal.

More extensive clinical studies are needed to evaluate the safety and efficacy of MST applied to prevent mtDNA disease transmission. For patient counseling, it is important to note evidence that the procedure may not be 100% effective and that a residual risk remains. This is of great importance, especially given that several trials of MRT technologies are either underway or shortly to be initiated, and that a number of countries are in the process of creating new laws or policies with respect to the clinical utilization of MRTs. If both the maternal and donor mtDNA are nonpathogenic, similar to the women with infertility selected for this study, the reversal of mtDNA in MST children may not produce a phenotype. However, the possibility of adverse effects associated with heteroplasmy for genetically different (but otherwise normal) mitochondria, cannot be entirely excluded at this time. Of note, one study conducted in mice has shown that heteroplasmy for nonpathogenic mtDNA variants can be associated with cognitive and behavioral abnormalities in that species (22). Future clinical studies should counsel prospective subjects about this possibility and provide sufficient follow-up, and be of adequate sample size, to evaluate this important potential complication of MST. Further research will also be required to definitively conclude whether MST is associated with any negative reproductive consequences, especially with respect to heteroplasmy and its impact, if any, on implantation and placental function. Whatever future work is undertaken involving any forms of MRT, the long-term follow-up of children remains imperative.

## CONCLUSIONS

This exploratory pilot study is the first to provide insights into the clinical feasibility of MST when used in a context of infertility treatment. Although the data obtained is encouraging, with successful pregnancies achieved by patients with a long history of extremely poor IVF treatment outcomes, it must be acknowledged that the study was only a pilot and as such was limited in size and scope. Claims of therapeutic efficacy and safety of MST for the treatment of infertility, or extrapolations related to the potential of MRTs to reduce the risk of disease transmission in patients carrying mtDNA disorders, cannot be made at this time. The results obtained are novel and hint at a clinical potential for MST, especially as a treatment for poor oocyte quality, but a definitive assessment of therapeutic value must await well-controlled phase II or phase III trials, adequately sized, carefully designed and appropriately regulated.

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