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ORIGINAL ARTICLE ***Reproductive genetics***

Clinical implications of mitochondrial DNA quantification on pregnancy outcomes: a blinded prospective non-selection study

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STUDY QUESTION: Can quantification of mitochondrial DNA (mtDNA) in trophectoderm (TE) biopsy samples provide information concerning the viability of a blastocyst, potentially enhancing embryo selection and improving IVF treatment outcomes?

SUMMARY ANSWER: This study demonstrated that euploid blastocysts of good morphology, but with high mtDNA levels had a greatly reduced implantation potential.

WHAT IS KNOWN ALREADY: Better methods of embryo selection leading to IVF outcome improvement are necessary, as the transfer of chromosomally normal embryos of high morphological grade cannot guarantee the establishment of an ongoing pregnancy. The quantity of mtDNA in embryonic cells has been proposed as a new biomarker of viability—higher levels of mtDNA associated with reduced implantation potential.

STUDY DESIGN, SIZE, DURATION: mtDNA was quantified in 199 blastocysts, previously biopsied and shown to be chromosomally normal using preimplantation genetic testing for aneuploidy (PGT-A). These were generated by 174 couples (average female age 37.06 years). All patients underwent IVF in a single clinic. The study took place in a blinded, non-selection manner—i.e. mtDNA quantity was not known at the time of single embryo transfer. The fate of the embryos transferred was subsequently compared to the mtDNA levels measured.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Embryos were biopsied at the blastocyst stage. The TE samples obtained were subjected to whole genome amplification followed by comprehensive chromosome analysis via next generation sequencing. The same biopsy specimens were also tested using quantitative PCR, allowing highly accurate mtDNA quantification. After blastocyst transfer, the code used for blinding was broken and analysis undertaken to reveal whether the amount of mtDNA had any association with embryo implantation.

MAIN RESULTS AND THE ROLE OF CHANCE: mtDNA analysis of the 199 blastocysts revealed that 9 (5%) contained unusually high levels of mtDNA. All embryo transfers involved a single chromosomally normal blastocyst of good morphology. Of these, 121 (60%) led to ongoing pregnancies, 11(6%) led to biochemical pregnancies, and 10 (5%) spontaneously miscarried. All (100%) of these blastocysts had mtDNA levels considered to be normal/low. The remaining 57 (29%) blastocysts failed to implant. Among these non-viable embryos there were 9 (16%) with unusually high levels of mtDNA. This meant that the ongoing pregnancy rate for morphologically good, euploid blastocysts, with normal/low levels of mtDNA was 64% (121/190). In contrast, the ongoing pregnancy rate for the same type of embryos, but with elevated mtDNA levels, was 0/9 (0%). This difference was highly statistically significant ($P < 0.0001$).

LIMITATIONS REASONS FOR CAUTION: To determine the true extent of any clinical benefits a randomized clinical trial will be necessary. Research is needed to improve understanding of the biology of mtDNA expansion.

WIDER IMPLICATIONS OF THE FINDINGS: This is the first investigation to evaluate the clinical impact of increased mtDNA in a prospective blinded manner. Results confirm that embryos with elevated mtDNA rarely implant, supporting its use as a viability biomarker. A total of 64% of euploid blastocysts with normal/low mtDNA implanted versus 60% for the cohort as a whole.

STUDY FUNDING/COMPETING INTEREST(S): This study was supported by institutional funding (Reprogenetics UK and Reprogenetics). DW is supported by the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre Programme. None of the authors have any competing interests.

Key words: Mitochondrion / mitochondrial genome / mtDNA quantification / blastocyst / viability biomarker

Introduction

Data obtained from the Society of Assisted Reproductive Technology (SART) indicates that infertility is very common among couples attempting to reproduce. Specifically, one out of every eight North American couples attempting to reproduce will seek some sort of medical intervention due to infertility, many ultimately undergoing IVF treatment. Assisted reproductive procedures have shown substantial evolution over the past 30 years and the likelihood of successful treatment has slowly increased. However, despite these improvements the live birth rate following IVF remains relatively low, averaging between ~20 and 30% per fresh embryo transfer in most clinics (Ishihara *et al.*, 2015). While some aspects of IVF have seen considerable change, the methods used to assess viability and prioritize embryos for transfer to the uterus have altered little over the last decade, and are still typically based on the assessment of morphological characteristics (Gardner and Schoolcraft, 1999). Unfortunately, embryos assigned the highest morphological grades often fail to produce a birth, while those of poorer morphology, although having lower potential for successful implantation on average, nevertheless give rise to viable pregnancies at an appreciable rate.

One of the main reasons for implantation failure and pregnancy loss is the presence of chromosome abnormalities (aneuploidy) in embryonic cells. Preimplantation genetic testing for aneuploidy (PGT-A) was developed in order to detect such abnormalities in embryo biopsy samples, enabling the identification and prioritization for transfer of chromosomally normal embryos (Munné *et al.*, 1993). Several randomized controlled trials (RCTs) have provided evidence supporting the clinical utility of modern PGT-A strategies, which involve examination of the entire chromosomal complement in small numbers of cells sampled from the trophectoderm (TE) of blastocyst stage embryos (Yang *et al.*, 2012; Forman *et al.*, 2013; Scott *et al.*, 2013; Rubio *et al.*, 2017). In each of these RCTs, pregnancy rates were higher for embryos characterized euploid using PGT-A than for those that were transferred untested and of uncertain cytogenetic status. However, it remained the case that at least 30% of the chromosomally normal embryos failed to produce an ongoing pregnancy. These findings indicate that the transfer of an embryo, which succeeds in developing to the blastocyst stage, and is characterized as chromosomally normal, still cannot guarantee the establishment of an ongoing pregnancy. It is clear that additional factors, maternal and/or embryonic, also affect IVF treatment outcome.

Energy requirements during preimplantation development have been the focus of several investigations. Metabolic rates and ATP content of human oocytes and embryos have been shown to vary significantly (e.g. Van Blerkom *et al.*, 1995; May-Panloup *et al.*, 2005). It has also been suggested that the fertilization ability of an oocyte, as well as its capacity to sustain the resulting embryo is dependent on its ATP content (Van Blerkom *et al.*, 1995). Mitochondria are the cellular organelles responsible for ATP production, via the biochemical reaction of oxidative phosphorylation (OXPHOS), which takes place in their electron transport chain. They are also involved in the regulation of other important cellular processes, including signal transduction, apoptosis, calcium homeostasis and redox potential via the generation of reactive oxygen species (ROS) (Dumollard *et al.*, 2009; Circu and Aw, 2010; Giorgi *et al.*, 2012).

A feature unique to mitochondria, compared to other cellular organelles, is the fact that they contain one or more copies of their own genome, the mitochondrial DNA (mtDNA). The human mtDNA encodes for 13 polypeptides, involved in the construction of four of the electron transport chain complexes (I, III, IV and V). The transmission of mitochondria and their genome is also unusual, in that they are exclusively inherited from the mother. Any sperm mitochondria that enter the oocyte undergo degradation and removal by autophagosomes (Zhou *et al.*, 2016).

Once fertilization is complete and mitosis begins, oocyte-derived mitochondria are distributed to the newly formed blastomeres. Mitochondrial replication is not thought to begin until fetal development. This means that the key cellular processes carried out by mitochondria, including ATP production, are entirely supported by the population of organelles derived from the oocyte (Van Blerkom, 2011). Replication of the mtDNA, on the other hand, is thought to start earlier, during preimplantation development, and after the embryo has differentiated to form a blastocyst (Van Blerkom, 1993; Sathananthan and Trounson, 2000; St John *et al.*, 2010). Embryonic mtDNA replication is initially observed in the TE of the blastocyst, after the tissue has undergone differentiation and is committed to formation of the placenta (Pikó and Taylor, 1987; Thundathil *et al.*, 2005; Spikings *et al.*, 2007; St John *et al.*, 2010). mtDNA replication in the inner cell mass does not occur until later in development, likely coinciding with loss of pluripotency (St John *et al.*, 2010).

We recently identified a relationship between the implantation potential of chromosomally normal blastocysts and their mtDNA

content. Specifically, we described a threshold of mtDNA quantity above which implantation of a euploid embryo rarely if ever occurs (Fragouli et al., 2015). Based upon these results, we postulated that the quantity of mtDNA might represent a new biomarker of embryo viability, which could be deployed in order to assist embryo selection. Our initial findings were subsequently confirmed in a large blinded retrospective study carried out by our group (Ravichandran et al., 2017), as well as by other investigations that sought to measure mtDNA quantity in embryo biopsy specimens (Diez-Juan et al., 2015; Spinella et al., 2016).

The current study involves the first application of mtDNA quantification in a prospective, blinded non-selection setting. We aimed to further validate our methodology, determine the positive and negative predictive values of the test, and consider the potential benefit of mtDNA quantification on the clinical outcomes of IVF cycles in which PGT-A has been utilized.

Materials and Methods

Study design

This non-selection study was the result of a collaboration involving a medical laboratory specializing in PGD and testing for aneuploidy (PGT-A) (Reprogenetics) and a single large IVF clinic (New York University Langone Medical Center, New York University). The mtDNA content of TE samples biopsied from 199 blastocysts, characterized chromosomally normal during the course of routine PGT-A, was quantified. All embryos were vitrified post-biopsy, and were subsequently warmed and underwent single embryo transfers (SETs). Transfers took place without knowledge of the results of mtDNA quantification, with embryos selected based primarily on a chromosomally normal PGT-A result and secondly on morphological criteria. Once all transfers for patients who participated in the study were completed and clinical outcomes became available, mtDNA quantity data were decoded, and negative and positive predictive values were calculated.

Ethical approval

The study was reviewed and approved through the Aspire Independent Review Board. Samples were analyzed only after participating patients provided informed consent.

Participating patients and embryo samples

The embryos investigated during this study were produced by 174 couples receiving IVF treatment in combination with PGT-A due to various indications at NYU's Langone Medical Center IVF clinic. The average female age was 37.06 years (age range 25–47 years). The samples were derived from blastocyst stage embryos that had undergone biopsy and PGT-A using next generation sequencing (NGS). All of the embryos included in the study had received a chromosomally normal result and had been transferred to the uterus.

Embryo sample processing and comprehensive cytogenetic analysis

Patient ovarian stimulation, oocyte collection, embryo micromanipulation, biopsy and preparation of TE samples for cytogenetic analysis all occurred as described in Maxwell et al. (2016). TE sample whole genome amplification (WGA) was performed using the SurePlex method as described by the manufacturer (Illumina, USA). This produced sufficient amplified DNA for chromosomal analysis and also mtDNA quantification. Comprehensive cytogenetic analysis was achieved with the use of the VeriSeq NGS strategy (Illumina, USA), according to the manufacturer's instructions. This NGS strategy was previously validated for the purposes of PGT-A (Fiorentino et al., 2014a,b; Maxwell et al., 2016; Fragouli et al., 2017). The results obtained were analyzed using the BlueFuse Multi v4.1 software (Illumina, USA).

Relative quantification of mtDNA copy number

mtDNA was assessed in WGA products of blastocysts, which were classified as euploid with NGS, using a modified version of our previously published methodology (Fragouli et al., 2015). The modification and the protocol validation were described in a recent publication by our group (Ravichandran et al., 2017). Briefly, two separate custom-designed TaqMan assays (ThermoFisher, USA) targeting distinct mtDNA sites were employed, to increase test accuracy. The first was as described previously (16S rRNA; Fragouli et al., 2015), whereas the second targeted an mtDNA sequence on the opposite side of the circular mitochondrial genome (MajArc, Ravichandran et al., 2017). A separate threshold was established for each of the two TaqMan assays. These were 0.0004 (relative mtDNA quantity threshold) for the mitochondrial 16 s ribosomal RNA assay (Fragouli et al., 2015) and 0.000335 (relative mtDNA quantity threshold) for the MajArc assay. Details on mitochondrial sequence selection/TaqMan assay design and threshold establishment are described in Ravichandran et al. (2017). It should be noted that the thresholds were established empirically. In order for a sample to be classified as having 'elevated' mtDNA quantity, both assays needed to give results above the established thresholds. Samples were analyzed in triplicate using the Viia7 real-time PCR machine (ThermoFisher, USA). As previously described (Fragouli et al., 2015; Ravichandran et al., 2017), a nuclear multicopy Alu sequence was used as an endogenous standard, allowing normalization of data within samples, controlling for differences due to PCR efficiency and number of biopsied cells. Additionally, every amplification experiment included previously quantified DNA samples of known mtDNA quantity, acting as a reference for the purposes of maintaining consistency across different runs.

Statistical analysis

Statistical analysis of data was conducted using the Fisher's exact test, the Student's *t*-test and the Shapiro–Wilks test. A *P*-value <0.05 was considered to be statistically significant with $P < 0.001$ indicating high significance.

Results

mtDNA copy number in biopsied TE samples

mtDNA was quantified from TE biopsy specimens derived from a total of 199 blastocyst stage embryos. All 199 blastocysts were classified as being chromosomally normal, based upon the NGS analysis of their corresponding TE samples, and were considered to be of good morphology. All of the embryos had been cryopreserved following biopsy and were transferred to the uterus one at a time (i.e. SET). Most (153/174, 88%) of the participating patients underwent one SET, 17/174 (10%) underwent two separate SETs, and 4/174 (2%) underwent three SETs. The choice of which embryo to transfer was based solely upon the confirmation of euploidy in the TE sample and a morphological evaluation of the blastocyst. The embryologist selecting which embryo to transfer was not aware of the results of mtDNA evaluation.

Quantification showed that the great majority (190/199, 95%) of analyzed blastocysts had mtDNA levels in the normal range (i.e. below established thresholds for the two mtDNA sequences assessed). Only

9 (9/199, 5%) of the examined blastocysts were classified as having elevated mtDNA quantities (above the established thresholds for the two mtDNA sequences). The distribution of mtDNA quantities in TE samples biopsied from the blastocysts examined during the course of this study is shown in Supplementary Figure S1.

The average female age of the participating patients was 37.06 years (age range 25–47 years). The mtDNA quantity appeared to increase with advancing female age for both targets on the mitochondrial genome, echoing findings in previous studies (Fragouli *et al.*, 2015; Ravichandran *et al.*, 2017), although the difference was not statistically significant. A summary of mtDNA quantities according to female age for each of the two mtDNA sequences assessed is shown in Table I.

mtDNA quantity and clinical outcomes

Of the 199 blastocysts transferred 142 implanted and 57 did not. Hence, the resulting implantation rate was 71%. Eleven of the embryos that implanted (6% of the total cohort) produced biochemical pregnancies, but proceeded no further, and another 10 (5%) ended with a miscarriage. Thus, the ongoing pregnancy rate was 60% (121/199).

Quantities of mtDNA in the normal range were scored for all 142 blastocysts that implanted (100%). This group included 121 blastocysts associated with ongoing pregnancies and 21 embryos that initially implanted but ultimately resulted in either biochemical pregnancy or miscarriage. In contrast, in the non-implanting group of embryos 9/57 (16%) were classified as having elevated mtDNA quantities, while the remaining 48/57 (84%) having levels in the normal range. No blastocysts associated with high levels of mtDNA led to a pregnancy.

The results obtained during this study indicate that the ongoing pregnancy rate for a group of euploid blastocysts, which are of good morphology and have mtDNA levels in the normal range, is 64% (121/190). Conversely, the ongoing pregnancy rate for a similar group of blastocysts with mtDNA levels above previously established thresholds (elevated) is 0%. The difference in implantation potential between the two groups is highly statistically significant ($P < 0.0001$). These results are outlined in Table II and are shown in Fig. 1.

The predictive value of mtDNA quantification

A total of 190 blastocysts were identified to have normal mtDNA levels. Of these, 21 (21/190, 11%) led to either biochemical pregnancies or miscarriages, 48 (48/190, 25%) failed to implant, and the remaining 121 resulted to ongoing pregnancies. This meant that the positive predictive value of mtDNA quantification (ongoing pregnancy/delivery per embryo with normal mtDNA levels transferred) so as to determine a blastocyst with the potential for forming a viable pregnancy was 64%. Moreover, nine of the examined blastocysts were identified to have elevated mtDNA levels, and none of these implanted after transfer. The negative predictive value of mtDNA quantification (failure of ongoing pregnancy per embryo with elevated mtDNA levels transferred) for the group of investigated blastocysts was therefore 100%.

Discussion

Our group initially identified a relationship between mtDNA quantity and the implantation potential of euploid blastocysts during a retrospective study of a total of 131 transferred embryos (Fragouli *et al.*, 2015). The data suggested that blastocysts with mtDNA quantities above a certain threshold have a reduced ability to implant (Fragouli *et al.*, 2015). Similar observations concerning blastocyst implantation ability and mtDNA quantity were subsequently reported by Diez-Juan *et al.* (2015) and Spinella *et al.* (2016). Additionally, evidence has been presented suggesting that levels of mtDNA in embryo culture medium

Table I The average relative quantities of mtDNA scored in euploid blastocysts generated by women of different age ranges.

Female age range	No. of euploid blastocysts assessed	Average mtDNA quantity/assay 1	Average mtDNA quantity/assay 2	Blastocysts with low mtDNA levels (%)	Blastocysts with elevated mtDNA levels (%)
25–29	8	0.00017503	0.000186096	8 (100%)	0 (0%)
30–41	164	0.000188175	0.000259261	157 (96%)	7 (4%)
42–47	27	0.0002155195	0.000552472	25 (93%)	2 (7%)

The total number of blastocysts with low (normal) and elevated mtDNA quantities is shown.

Table II Number of euploid blastocysts with low (normal) mtDNA levels and euploid blastocysts with elevated mtDNA levels in relation to clinical outcome after SET.

Clinical outcome after frozen SET	No. of euploid blastocysts transferred	No. of euploid blastocysts with low mtDNA levels (%)	No. of euploid blastocysts with elevated mtDNA levels (%)
Ongoing pregnancies	121	121 (100%)	0 (0%)
Biochemical pregnancies	11	11 (100%)	0 (0%)
Spontaneous miscarriages	10	10 (100%)	0 (0%)
Failure of implantation	57	48 (84%)	9 (16%)

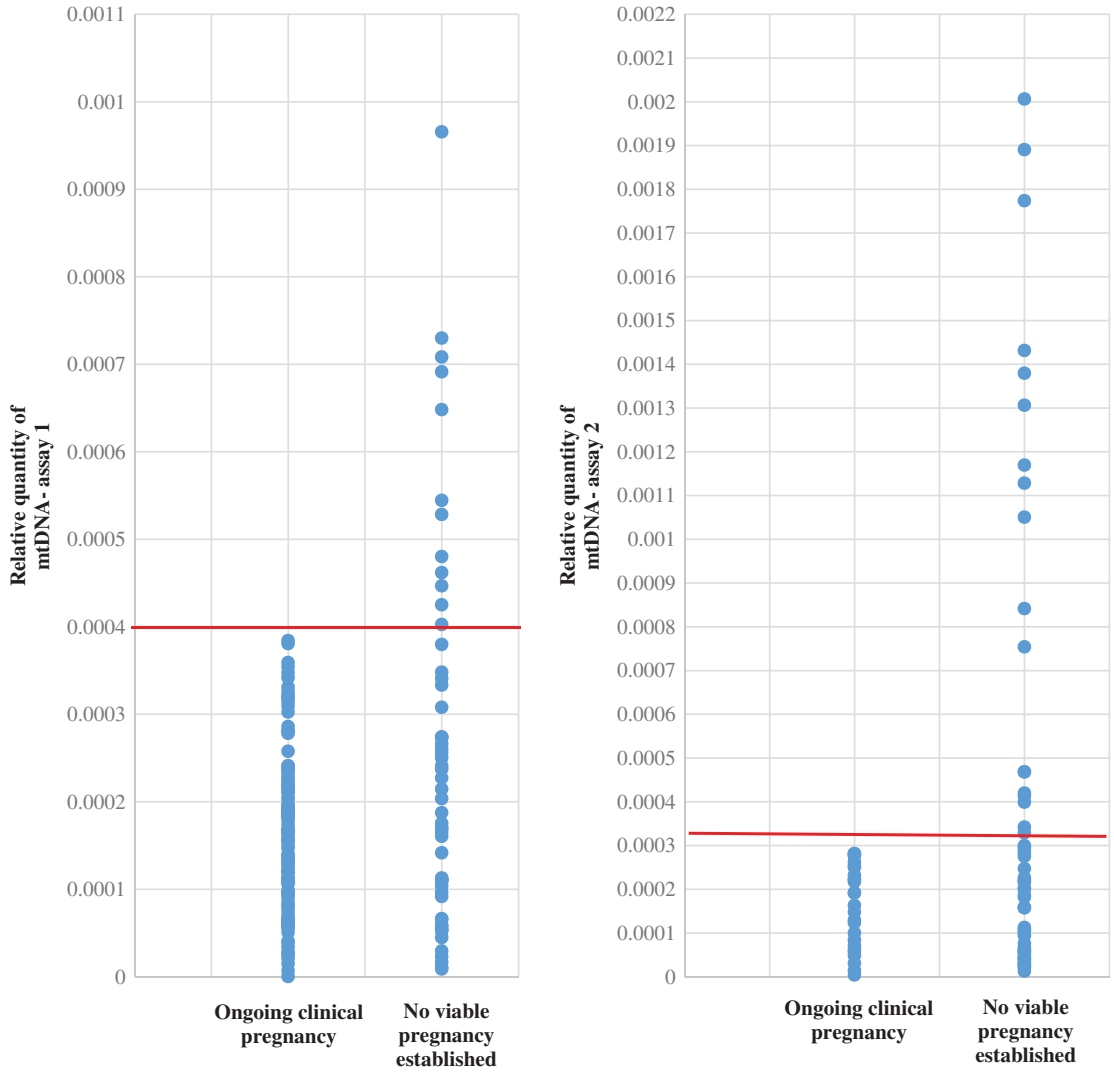

Figure 1 A total of 121 of the transferred blastocysts led to ongoing pregnancies, and all had low/normal mtDNA levels. A total of 57 embryos failed to implant, and 9 (16%) of these were identified to have elevated mtDNA levels, based on results of two assays targeting the mitochondrial genome. Hence, the ongoing pregnancy rate for a group of chromosomally normal blastocysts of good morphology and low/normal mtDNA levels was 64%, whereas the ongoing pregnancy rate for a similar group of good quality euploid blastocysts with elevated mtDNA levels was 0% ($P < 0.0001$). The negative predictive value of mtDNA quantification (failure of ongoing pregnancy per embryo with elevated mtDNA levels transferred) for the group of investigated blastocysts was 100%.

and cumulus cells could also serve as potential biomarkers of viability (Stigliani *et al.*, 2013, 2014; Desquiret-Dumas *et al.*, 2017). Most recently, our preliminary findings were confirmed in a much larger blinded retrospective study of TE biopsies (Ravichandran *et al.*, 2017).

The methodology used for mtDNA quantification is critical for obtaining accurate results. Our initial study involved measurement of mtDNA levels using a real-time PCR approach, with results validated using an independent NGS protocol (Fragouli *et al.*, 2015). Subsequent iterations of this methodology employed a slightly modified technique, in which an additional target on the mitochondrial genome was assessed with the intention of increasing the robustness of the test. The validation of this modified protocol, which was also employed in the current study, has been described in full by Ravichandran *et al.* (2017). Briefly, the mtDNA was quantified in TE biopsy specimens from a total of 1505 euploid blastocysts. These were generated in 35 different North American clinics, which had not been included in our earlier investigation. About 9% of the blastocysts were identified as having mtDNA quantities above the established thresholds, none of which implanted after transfer. The combination of the outcome data from this large retrospective investigation with those obtained previously, suggested that in a non-implanting group of euploid blastocysts, approximately one-third typically have mtDNA quantities above the established thresholds.

While most published data supports the hypothesis that unusually high levels of mtDNA in TE biopsy specimens are associated with reduced blastocyst implantation rates, not all studies are in agreement. A recent investigation carried out by Victor and colleagues (2017),

failed to detect a relationship between quantities of mtDNA and embryo implantation in a large group of blastocysts. However, the conclusions of that research have been questioned due to perceived technical issues that could invalidate conclusions from the data collected (discussed in Ravichandran *et al.*, 2017). Another recent study, which was also unable to detect a link between the amount of mtDNA and embryo viability, may also have suffered from technical deficiencies (Treff *et al.*, 2017). The protocol used in that investigation involved normalization of mtDNA against a multicopy nuclear DNA sequence (Alu), which is an acceptable strategy. However, prior to mtDNA analysis the samples were processed for aneuploidy detection. This involved subjecting the minute amount of DNA in the sample to multiple rounds of heating and cooling, a process that inevitably caused some of the DNA to degrade. More importantly, some of the samples tested were several years old, which again increased the likelihood of sample deterioration. This is a major problem as the amplified mtDNA and the Alu sequences they are normalized against differ significantly in length. Hence, they undergo PCR-refractory degradation at different rates (the longer Alu fragments experience more degradation affecting their amplification than the shorter mtDNA amplicons), potentially leading to distortions in the data.

Given that there is some controversy over the use of mtDNA measurement to assist the prioritization of embryos for uterine transfer, it is clear that further studies are needed in order to provide clarity. The research described in this paper represents the first blinded non-selection study to investigate the ability of mtDNA quantification to predict embryo implantation potential. Of the 199 blastocysts examined in the current study, only 9 (5%) were identified as having elevated mtDNA quantities (i.e. amounts above our previously established thresholds). This contrasts with Ravichandran's larger data set, including embryos from 35 distinct clinics, which indicated that 9% of euploid blastocysts contained elevated mtDNA levels (Ravichandran *et al.*, 2017). The difference between the studies may reflect clinic-to-clinic variation (0–27% in Ravichandran *et al.*, 2017) in the incidence of affected embryos. Some clinics produce few (if any) embryos with elevated mtDNA levels, whereas others generate such embryos at a much higher frequency. Variation among clinics could be due to factors associated with embryo culture and/or stress, such as the choice of medium, incubator type, gas composition, or extent of embryo handling during the time in culture. It could also be related to patient selection (age, hormonal stimulation, etc.), although thus far we have been unable to identify any patient-specific factors affecting the rate of embryos having mtDNA levels above the established threshold. Variation in the frequency of blastocysts with elevated mtDNA means that embryo selection based upon detection of this phenomenon will be more useful in some clinics than others.

Embryo transfers during this study took place blindly with respect to mtDNA quantity. The single blastocyst transferred was primarily chosen on the basis that it was chromosomally normal, with morphological assessment used as a secondary selection tool in cases where more than one euploid embryo was available. The ongoing pregnancy rate of euploid blastocysts with normal levels of mtDNA was 64%, while for chromosomally normal embryos with elevated mtDNA, which were morphologically indistinguishable from those with lower levels the rate was 0% ($P < 0.0001$). In the current study, the relatively low frequency of affected blastocysts meant that the difference in implantation rate for the subset of embryos with mtDNA levels in the normal range was only slightly higher than for the entire cohort of embryos (60% versus 64%). Nonetheless, the high negative predictive value of the assay (100% in this data set) suggests that analysis of this biomarker might have value, especially in clinics where affected embryos are more common. We are in the process of further examining the ability of mtDNA quantification to predict blastocyst implantation potential, in the context of a double transfer study (ClinicalTrials.gov study ♯ NCT02673125). This study is currently ongoing, and involves the transfer of a euploid blastocyst with low/normal mtDNA levels, and another euploid blastocyst with elevated mtDNA levels (i.e. above the set thresholds for the examined assays).

The results discussed here represent a direct clinical evaluation of the ability of mtDNA to provide an insight into the viability of euploid blastocysts. The data confirms that mtDNA quantification has the capacity to identify a subset of blastocyst stage embryos with highly impaired implantation potential. As previously demonstrated, mtDNA quantities are independent of any of the patient and embryo characteristics examined thus far, including morphology and sex (Ravichandran *et al.*, 2017). The very different clinical outcomes for embryos with/without elevated mtDNA suggest that mtDNA quantification may have the capacity to enhance embryo selection, improving the likelihood of transferring a viable blastocyst in IVF cycles where PGT-A has been carried out. Following this logic, the use of mtDNA as an additional biomarker of embryo viability could be especially valuable in the context of elective SETs, especially for patients who have produced several euploid blastocysts. Advances in the ability to detect viable embryos should, theoretically, reduce the number of transfers required to achieve a live birth and therefore also reduce the time to pregnancy.

The reason why some non-viable blastocysts exhibit elevated mtDNA levels remains to be determined. One possibility is that elevated mtDNA quantities are related to increased energetic requirements that accompany raised metabolic activity, which may in turn be associated with an embryo's attempts to resolve some form of stress. Alternatively, it could be that some embryos increase their levels of mtDNA in an effort to compensate for some sort of mitochondrial deficiency. Poor organelle function could be the result of mutations in the mitochondrial or even the nuclear genome. At this point, functional experiments are essential in order to clarify the underlying cause of this phenomenon.

As already discussed, the proportion of embryos with elevated mtDNA levels varies between clinics. This variation does not appear to be related to differences in patient populations and seems more likely to be a consequence of clinic-specific factors (e.g. differences in laboratory conditions/procedures). If increases in mtDNA are confirmed to be related to embryo stress, it is possible that quantification of mtDNA could become a valuable quality control measurement for IVF laboratories, alerting clinics when their procedures are suboptimal.

To conclude, the results obtained during this blinded prospective non-selection study provide further evidence that mtDNA quantification has the potential to serve as a new biomarker of embryo viability. Increased mtDNA levels were found to be relatively uncommon among euploid blastocysts at the single clinic involved in the study. Nonetheless, for affected blastocysts the clinical implications were dramatic—elevated mtDNA was accompanied by implantation failure in all cases in which it was detected. The clinical value of mtDNA quantification, in terms of the extent to which it could enhance embryo selection and improve outcomes in IVF/PGT-A cycles, is likely to be clinic-specific, influenced by the frequency of embryos with elevated

mtDNA levels in each embryology laboratory. Ultimately, a multicentre RCT will likely be required in order for the clinical utility of mtDNA quantification to be accurately defined.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

Conception and design of the study: E.F., J.A.G., S.M. and D.W. Data acquisition: E.F., C.MC., K.R., K.S., J.A.G. Data analysis and interpretation: E.F., K.R., D.W. and S.M. Drafting of the manuscript: E.F. Supervision and critical revision of the manuscript for important intellectual content: E.F., D.W., S.M. and J.A.G. Final approval of the version to be published: E.F., C.MC., K.R., K.S., J.A.G., S.M. and D.W. All authors have read, and confirm that they meet, the authorship criteria.

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Conflict of interest

None declared. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

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