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Running Title: Uniform Levels of mtDNA in Blastocysts

ACCURATE QUANTITATION OF MITOCHONDRIAL DNA REVEALS UNIFORM LEVELS IN HUMAN BLASTOCYSTS IRRESPECTIVE OF PLOIDY, AGE, OR IMPLANTATION POTENTIAL

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Abstract Capsule:

Accurate quantitation of mitochondrial DNA content in human blastocysts reveals unvarying levels regardless of embryo ploidy, age, and implantation potential.

ABSTRACT

Objective: To accurately determine mitochondrial DNA (mtDNA) levels in human blastocysts.

Design: Retrospective analysis.

Setting: PGS laboratory of IVF clinic.

Patient(s): 1396 embryos derived from 259 patients.

Intervention(s): Blastocyst-derived trophectoderm biopsies were tested by NGS and qPCR.

Main Outcome Measure(s): For each sample the mtDNA value was divided by the nuclear DNA (nDNA) value and the result was further subjected to mathematical analysis tailored to the genetic makeup of the source embryo.

Result(s): On average the mathematical correction factor changed the conventionally determined mtDNA score of a given blastocyst via NGS by 1.43% +/-1.59% (N=1396), with maximal adjustments of 17.42%, and via qPCR by 1.33% +/-8.08% (N=150), with maximal adjustments of 50.00%. Levels of mtDNA in euploid and aneuploid embryos showed a statistically insignificant difference by NGS (euploids N=775, aneuploids N=621) and by qPCR (euploids N=100, aneuploids N=50). Blastocysts derived from younger or older patients had comparable mtDNA levels by NGS ('young' age group N=874, 'advanced' age group N=514) and by qPCR ('young' age group N=92, 'advanced' age group N=58). Viable blastocysts did not contain significantly different mtDNA levels compared to unviable blastocysts when analyzed by NGS (implanted N=101, non-implanted N=140) and by qPCR (implanted N=49, non-implanted N=51).

Conclusion(s): We recommend implementation of the correction factor calculation to laboratories evaluating mtDNA levels in embryos by NGS or qPCR. When applied to our in-house data, the calculation reveals that overall levels of mtDNA are largely equal between blastocysts stratified by ploidy, age, or implantation potential.

Key Words: embryonic mitochondria; blastocyst mtDNA; correction factor; PGS/PGD; IVF biomarker

INTRODUCTION

Human cells contain anywhere from 100 to 150,000+ copies of the mitochondrial DNA (mtDNA) molecules depending on cell type (1, 2), and several conditions correlate with changes in mtDNA copy number including aging, myopathies, neuropathies, diabetes, and cancer (3). In human embryos, recent studies have investigated amounts of mtDNA, generally describing a correlation of high mtDNA levels with a 'stressed' state (4-6). Conditions that deviate from the steady state such as aneuploidy, advanced maternal age, or chemically induced-stress tend to associate with higher mtDNA content. Of clinical relevance, such reports suggested that embryos with mtDNA levels above the norm showed significantly poor frequency of pregnancy when transferred (4, 5).

The described observations are in line with the 'Quiet Embryo Hypothesis', which postulates that under ideal circumstances embryos are engaged in low metabolic activity (7). Elevated mtDNA levels and by extension increased ATP production could be a compensatory mechanism providing distressed embryos with more chemical energy to overcome adverse conditions. Therefore, in the case of euploid embryos being chosen for transfer, sub-optimal intrinsic or environmental factors could elicit higher levels of mtDNA. Consequently, mtDNA content has been proposed as a biomarker for embryo viability (4, 5).

Crucial to such investigations are methods that permit precise measurements of mtDNA levels. In situ hybridization has previously been used (8), probing for a region of the mtDNA sequence, but this method has not been widely adopted possibly due to laboriousness and difficulty in interpreting results. More commonly employed technologies are quantitative real-time polymerase chain reaction (qPCR) and sequencing platforms, especially next generation sequencing (NGS). Both methods yield one value for mtDNA and one value for nuclear DNA (nDNA) quantity, and the ratio of mtDNA to nDNA is the principal mode to assess mtDNA quantity per cell (9). Crucially,

using nDNA values for normalization assumes that the composition of nDNA is equal across samples.

When nDNA composition varies across samples, such as in cancer, the disparities must be accounted for. For such a purpose the use of a mathematical correction factor has been proposed in mtDNA studies of cancer (10). The correction factor takes into account characteristics of tumor biology that affect nDNA values such as genetic abnormalities of cancerous cells and tumor cell heterogeneity due to stroma and immune infiltrates (10). Only after such a mathematical adjustment are side-by-side comparisons of mtDNA levels across tumor samples appropriate. To our knowledge, such a correction factor has not been employed in studies of mtDNA levels in embryos.

In order to determine mtDNA levels in blastocysts accurately, we developed a mathematical formula adapted to the nuances of human embryology, and derived a correction factor that accounts for genomic variation due to embryo gender and ploidy. We then undertook an analysis of mtDNA levels in biopsies from human blastocysts derived from patients with infertility at the Zouves Fertility Center (ZFC). In contrast to previous reports, our corrected values show no statistically significant differences in blastocysts grouped by ploidy, maternal age, or implantation potential.

MATERIALS AND METHODS

Study Design

This study was a retrospective analysis of de-identified NGS workflow data and WGA product from patients consenting to preimplantation genetic screening during routine IVF procedures. It is exempt from IRB review by the U.S Department if Health & Human Services under 45 CFR 46.101(b)(4).

Embryo Processing

IVF and culturing of embryos took place at ZFC using standard techniques. Briefly, fertilization was accomplished using intracytoplasmic sperm injection and zygotes were cultured for 5 to 7 days in either G1/G2 Plus medium (Vitrolife) or GTL (Vitrolife) in 5.5% CO2, 5.5% O2 balance N2 at 37 C in a humidified atmosphere. Five to ten-cell trophectoderm biopsies were collected from blastocyst stage embryos and stored at -80 degrees Celsius until further processing. WGA on each biopsy was performed using the Sureplex system (Rubicon) and followed by NGS with the MiSeq sequencer (Illumina), as per the standard Veriseq protocol (Illumina). Embryo ploidy was assessed with the Bluefuse Multi Software (BMS) (Illumina). We investigated mtDNA content in a total of 1396 embryos, 241 of which were selected for frozen embryo transfer (225 as single embryos and 16 as paired siblings).

Determination of mtDNA content by NGS

For each sample, MiSeq Reporter Software (MCS) (Illumina) files in the BAM and FASTQ format were uploaded into Geneious R9 (Biomatters Ltd) to determine number of reads aligning to the mtDNA reference genome as per Genome Reference Consortium (GRC)h37. For FASTQ files, reads were aligned under maximal stringency to avoid potential multi-mapping to NUMTs (11). The number of mtDNA mapped reads was divided by the number of nDNA mapped reads after bioinformatic processing and filtering by MCS and displayed in BMS. For Supplemental Fig. 2, number reads aligning to Chromosome (Chr) 1 were determined in Geneious R9. Resulting values were further subjected to a mathematical correction factor described below.

Precise calculation of mtDNA Score from NGS Data

To calculate the mtDNA score (m_{NGS}) for each sample using NGS, the number of reads mapping to the mitochondrial genome (r_m) is divided by the number of reads mapping to the nuclear genome (r_n) to normalize for technical batch-to-batch variability during WGA and NGS as well as number of cells collected during biopsy. The resulting value is multiplied by the correction factor F_{NGS} as per formula 1.

(1)

$$m_{NGS} = \frac{r_m}{r_n} \times F_{NGS}$$

F_{NGS} takes into account two parameters necessary to correctly normalize for number of cells probed: embryo gender and ploidy. Without the correction factor, the formula assumes that nuclear genomes across all samples are equal in length. According to GRCh37 (Ensembl Release 68) the diploid female human genome is comprised of 6,072,607,692 base pairs (bp), while the male counterpart is 5,976,710,698 bp long, a difference of 1.58%. Without correction, all results from male embryos are inflated by 1.58% because the denominator r_n is artificially small. To correct for this, F_{NGS} for all

male embryos contains a multiplier of 0.9842, since the male genome is 98.42% the length of the female's (see Table 1A).

Similarly, an aneuploid embryo has more or less genetic material per cell compared to a euploid embryo, and without correction it would lead to inflated mtDNA counts in the case of nullisomies and monosomies, and deflated mtDNA counts in trisomies and other polysomies. To correct for this, the mtDNA value for each embryo is multiplied by a correction factor tailored to its chromosomal composition (Table 1A). The correction factors for different chromosomes should be multiplied to each other when embryos have aneuploidies in more than one chromosome. For example a male embryo with a Chr 1 monosomy and Chr 21 trisomy with 3,000 reads mapping to mtDNA and 900,000 reads mapping to nDNA would have the following final m_{NGS} score:

$$m_{NGS} = \frac{3{,}000}{900{,}000} \times (0.9842 \times 0.9590 \times 1.0079) = 0.003171$$

Determination of mtDNA content by qPCR

All quantitative real time PCR (qPCR) experiments were performed using the Taqman system (Applied Biosystmems/Thermo Fisher). Surplus WGA product from the Veriseq workflow was diluted 1/10 in water, vortexed for 30-60 seconds and heated to 95 degrees Celsius for ten minutes to insure inactivation of any residual WGA polymerase activity. Two microliter of the resulting solution were used in the Taqman Fast Advance Master Mix reaction, and run with a Taqman 7500 Real-Time PCR instrument (Applied Biosystems/Thermo Fisher). All samples were run in three technical replicates. Taqman assays CYTB (Hs02596867_s1) and ND6 (Hs02596879_g1) were used for mitochondrial genes, based on consistently high qPCR experimental efficiencies compared to several other mtDNA assays tested. An assay targeting the RNase P component RPPH1 (Hs03297761_s1) was used for the nuclear gene; this is a routinely used copy number reference assay known to be present once on Chr 14 per haploid human genome (12).

The qPCR efficiency was experimentally determined to exceed 95% for all three assays used in this study (Supplemental Fig. 1C-E). Using standard curves for CYTB and RNase P the exact number of copies of mtDNA and nDNA was established in each sample as per the absolute quantitation methods described before: http://www6.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf. The ratio of the two values was determined and was further subjected to a correction factor outlined below.

Relative quantitation of mtDNA scores was performed using the qPCR Ct values from ND6 as the target assay and Ct values from RNase P as the control assay, followed by a log-to-linear conversion (2^{-dCt}).

Precise Calculation of mtDNA Score from qPCR Data

When using a qPCR platform, an assay is designed that probes an mtDNA region (the target), and second assay is designed probing a nDNA region (the reference). The mtDNA score (m_{qPCR}) may be determined by absolute quantitation with a standard curve or relative quantitation.

The absolute quantitation method calculates values of unknown samples by interpolating their quantity from a previously determined standard curve. This establishes an exact count of mtDNA molecules (c_m) and nDNA molecules (c_n) for each sample. Dividing the former by the latter normalizes for technical batch-to-batch variability as well as cell numbers collected in each biopsy. A correction factor (F_{qPCR}) must be applied to account for the nuclear genomic composition of the tested embryo, resulting in formula 2.

(2)

$$m_{qPCR} = \frac{c_m}{c_n} \times F_{qPCR}$$

F_{qPCR} equals 1 for euploid embryos, as well as for aneuploid embryos with chromosomal aberrations in genetic regions other than the reference sequence. If an embryo is monosomic for the reference region, that embryo's cells only contain one copy of the nDNA region being quantified, instead of the normal two copies. Consequently, this leads to c_n being artificially small, which in turn inflates the m_{qPCR} value. To correct this, F_{qPCR} must include the multiplier 0.5. By extension, a trisomy of the reference sequence must include F_{qPCR} with the multiplier 1.5. For example an embryo with a Chr 1 monosomy and Chr 21 trisomy with $4x10^7$ counts for the mtDNA target and $2x10^4$ counts to the nDNA reference, and the nDNA reference assay is located on Chr 21, would have the following final m_{qPCR} score:

$$m_{qPCR} = \frac{4 \times 10^7}{2 \times 10^4} \times (1.5) = 3000$$

The m_{qPCR} value represents the precise number of mtDNA molecules per nDNA molecules, or how many mtDNA copies there are per haploid genome. Doubling the value results in the number of mtDNA copies per diploid cell.

Similarly, when using a relative quantitation qPCR mode such as 2^{-dCt} the value must be adjusted if the reference assay is located in an aneuploid region, as per formula 3.

(3)

$$m_{qPCR} = 2^{-(Ct_m - Ct_n)} \times F_{qPCR}$$

The same correction factor (see Table 1B for details) should be applied when executing a fold change calculation such as the 2^{-ddCt} method (13).

Validation of Detection Platforms

Reproducibility of the NGS platform in determining mtDNA scores was tested by re-sequenced WGA products, which yielded consistent results amongst separate runs (Supplemental Fig. 1D). Also, there were unvarying mtDNA scores between multiple samples stemming from the same cell line with separate WGA and NGS runs. The starting amount of DNA for each cell line experiment was 33 pg, the equivalent DNA from a biopsy of 5 cells (assuming 6.6 pg per diploid genome). Furthermore, two separate blood samples from a single patient yielded equivalent mtDNA scores with individual DNA isolation, WGA and NGS procedures.

To attain cross-platform validation, we compared the mtDNA scores of 5 embryo biopsy WGA samples by NGS and the two different qPCR methods described above. For each case the level of mtDNA score obtained by all three platforms was comparable (Supplemental Fig. 1E). From this data we deduce that mtDNA scores are highly correlative across detection platforms.

Statistics and Graphs

Group analyses were performed using Welch's parametric two-tailed unpaired t-test in Prism 6 (GraphPad Software). The logistic regression analysis was performed in R Statistical Software. All graphs were prepared in Prism 6 showing means with error bars indicating standard deviation.

RESULTS

Applying Correction Factor Substantially Changes mtDNA Scores in Blastocyst Samples

We analyzed mtDNA scores in blastocyst embryos used in our clinic for IVF, all of which had undergone routine PGS for chromosomal abnormalities. We used three distinct platforms to determine mtDNA and nDNA levels in a biopsy sample: NGS, qPCR by absolute quantitation testing for the mitochondrial gene CYTB, and qPCR by relative quantitation assaying for the mitochondrial gene ND6. Both qPCR methods also probed for the nuclear gene RNase P, which is routinely used to quantify nuclear DNA for normalization purposes.

For each sample the mtDNA score was obtained by dividing the mtDNA value by the nDNA value to normalize for technical batch-to-batch variation and number of cells collected at biopsy. All resulting values were subjected to the mathematical correction factors that take the variability of embryonic genomes into account (See Methods for full explanation of the rationale and formulas).

The NGS correction factor changed the mtDNA score on average by 1.43% +/- 1.59% (N=1396). The largest change in our samples was 17.42%. Applying the correction factor for qPCR changed the values by 1.33% +/-8.08% (N=150) on average when using the absolute quantitation method, with changes ranging up to 50.00%. When using the relative quantitation method, the qPCR correction factor changed mtDNA scores on average by 0.575% +/-5.36% (N=87), with changes ranging up to 50.00%.

Euploid and Aneuploid Blastocysts Have Equal mtDNA Score Distributions

When stratified by euploid and aneuploid blastocysts, the mtDNA scores by NGS did not result in a statistically significant difference (P=0.114) (Fig. 1A). Samples were randomly

selected out of these groups and tested by qPCR absolute quantitation assaying the mitochondrial CYTB gene. Again, euploid and aneuploid cohorts were not statistically different (P=0.642) (Fig. 1B). To probe these observations by a third method, a subset of embryos was further assayed with different mitochondrial gene (ND6) and compared by a relative quantitation method (2^{-dCt}). Once more we observed insignificant differences between euploids and aneuploids (P=0.202) (Fig. 1C). Therefore, regardless of quantitation platform and downstream mathematical calculation employed, blastocysts grouped by ploidy never showed statistically significant differences in mtDNA scores. This is in stark contrast with previous reports that did not employ a correction factor in their calculations (5, 6).

Maternal Age at Oocyte Retrieval Does Not Affect mtDNA Levels of Blastocysts

We divided blastocysts into a younger maternal age group at oocyte retrieval (20-37 years) and an older group (38-46). A previous study reported a statistical difference stating that the older group had higher mtDNA levels (5). We investigated mtDNA scores by NGS amongst all embryos regardless of ploidy, and observed no difference between age groups (Fig. 2A). When analyzing only euploid embryos, again there was no significant variance (Fig. 2A). We confirmed this observation by re-testing a number of embryos by the two described qPCR methods, using two different mitochondrial genes CYTB and ND6 (Fig. 2B and 2C). We further subdivided all embryos tested by NGS by individual numerical maternal age at oocyte retrieval in an effort to reveal any trends, but linear regression analysis failed to show statistically significant tendencies (Fig. 2D and 2E). Hence, blastocysts derived from oocytes of advanced maternal age do not contain higher mtDNA levels according to our results.

mtDNA Levels Show No Correlation with Viability and Do Not Predict Blastocyst Transfer Clinical Outcome

We determined the mtDNA score of embryos that had undergone frozen embryo transfer (FET) and had either implanted or had failed to do so, as determined by the presence or absence of a fetal sac at 6 weeks. Biopsies from all embryos had been collected at the blastocysts stage. NGS data from 101 implanted and 140 not implanted blastocysts showed no statistical difference in mtDNA score (P=0.510) (Fig 3A).

With these combined 241 blastocysts we carried out a logistic regression analysis to investigate whether mtDNA score could function as a predictive tool for clinical outcomes. The test is adjusted for the following confounding factors: cohort size (i.e. how many embryos produced in the cycle), embryo gender, single or paired sibling transfer, oocyte age at retrieval, patient age at transfer, embryo stage and grade. The results indicate that mtDNA score is a statistically insignificant predictor embryo viability (P=0.472) even when adjusted for confounding factors (Supplemental Table 1).

In another effort to correct for confounding factors in our study, we proceeded to compare embryos from individual cycle cohorts. We focused on patients that had undergone two or more embryo transfers in our clinic within at most 12 months. For each patient we compared mtDNA levels between embryos that resulted in pregnancy versus those that failed implantation. The NGS mtDNA score of each implanting embryo was set to 1, and the relative mtDNA score for the non-implanted embryos was calculated. This normalization step allowed us to pool data from several patients into a single graph. Results from 24 patients show a statistically insignificant difference (P=0.842) between their implanted (N=25) and non-implanted (N=34) embryos (Fig. 3B). We repeated this analysis but in the second iteration we used uncorrected number of reads aligning to Chr 1 as a standardization factor. Since this particular evaluation only tests euploid embryos, the latter should be a valid alternative calculation method. Indeed, both analyses yield virtually equal results (Fig. 3B and Supplemental Fig. 2).

Out of all the transferred embryos tested by NGS, 49 implanted and 51 not implanted samples were re-examined by qPCR absolute quantitation assaying for CYTB, again showing no statistically significant difference (P=0.103) (Fig. 3C). Finally, out of the latter set of blastocysts we re-tested 23 implanted and 29 not implanted embryos by relative quantitation (2^{-dCt}) probing the ND6 gene, once more observing no statistical difference in mtDNA score between groups (P=0.145) (Fig. 3D). Two previous studies described an mtDNA value threshold that when surpassed served as a biomarker for embryos that would fail implantation (4, 5). In our data across all three detection systems we only observed a single sample in the 'not implanted' group that repeatedly showed mtDNA scores substantially above the bulk distribution (see outlier in Fig. 3 A, C, and D), equaling 0.41% of all embryos. Hence, our data indicates that mtDNA content does not represent a statistically relevant or practical method to predict embryo viability amongst euploid blastocysts.

DISCUSSION

Contrary to previous reports, our study finds no correlation of mtDNA content with blastocyst ploidy, age, or viability. While we detect a considerable range of mtDNA scores in the tested samples, this range is observed within all populations irrespective of criterion used to sub-group the blastocysts. The absolute quantitation method by qPCR reveals the exact mtDNA copy number in each cell of our analyzed blastocysts. This value ranges from 945 to 41,427, with a mean of 4,740, falling within the previously estimated range of mtDNA copies per human cell (2, 14-16).

We propose several explanations why our findings deviate from previous reports. Firstly, accurate determination of mtDNA levels must take the composition of the sample's nuclear genome into account. If ignored, the embryo's gender and ploidy can substantially skew the calculated mtDNA score for a given sample. For instance when comparing euploids and aneuploids by NGS, if the aneuploid group randomly contains more monosomic than trisomic cases the overall mean of the group will be artificially shifted to larger mtDNA values. Also, embryos being tested by qPCR must be corrected when the reference assay falls in a genomic region that is aneuploid, as each extra or missing copy will deflate or inflate the calculated mtDNA score by a factor of 50%. We have developed a mathematical method to accurately determine mtDNA scores of blastocysts that takes the genetic make-up of each sample into account. We propose the outlined correction factors be utilized by all laboratories investigating mtDNA levels whenever applicable. In addition, the outlined formulas can be used for mtDNA quantitation at any stage of mammalian embryology and studies of adult patients with aneuploidies.

A further possible confounding factor between studies is that both previously published reports on embryo viability originate from reference laboratories that

collected data from numerous centers, agglomerating all their numbers (4, 5). As a result, it is unclear whether their findings hold true individually within all the different clinics. Ours is the first investigation stemming from a single center, thereby correcting for several potential inter-facility variables such as culture media, temperature, biopsy technique, or equipment. Furthermore, we have amassed a number of samples unprecedented to date for this type of study in the published literature.

Lastly, It should be noted that previous reports on mtDNA levels, aside from showing interesting concordances, also show several discrepancies amongst them. For instance Fragouli et al (5) suggest a maternal age effect, while Diez-Juan et al (4) did not see a maternal age correlation. Secondary analysis of the data in Tan et al (6) also shows no age effect. Furthermore, Diez-Juan et al describes a viability effect in cleavage and blastocyst stage embryos, while Fragouli et al only detects it in blastocysts and not at the cleavage stage. These divergences remain to be explained and possibly point towards a technical or laboratory-specific effect.

The qPCR analysis in Diez-Juan's report relies on a single copy locus to normalize for nDNA, like our study. Fragouli et al utilize a multicopy Alu sequence, with the rationale that allele-drop-out (ADO) effects during WGA might be mitigated. In the context of blastocysts, where a 5-10 cell biopsy yields 10-20 initial copies of a single copy locus (in euploids), use of a multicopy sequence is unlikely to confer an advantage. The maximal estimated ~10% ADO (17) using the routinely employed Sureplex WGA system (Rubicon) would affect the initial 10-20 single locus copies or a multicopy sequence in a similar manner. Furthermore, there is well-documented variability in Alu sequence frequencies and compositions within the population (18-20), leading us to believe that a known single-copy locus such as RNAseP (12) is a superior method of standardization between embryos. Nevertheless, the potential for ADO error persists in our study as well. Another important limitation is that our NGS protocol does not permit differentiation between embryos with homogenously haploid, diploid, triploid, etc. genomes.

The Quiet Embryo Hypothesis postulates that an embryo with a calm metabolic state is more viable than another with an overactive metabolism (7). While we find no association between mtDNA content and embryonic stress, our data does not refute the proposed concept that embryos actively increase mitochondrial function and energy output as a compensatory response to overcome strained conditions. The number of mtDNA copies per mitochondrion in human cells can vary widely between 0 to 15 (21-23), meaning that the number of mtDNA copies per cell does not necessarily correlate to number of mitochondrial organelles. At least one report has demonstrated that mtDNA copy is a poor biomarker for mitochondrial content (24). Different techniques such as immunofluorescence for direct organelle quantitation or chemical ATP detection would yield a clearer picture of mitochondrial number and function in embryos, which in turn could prove to be valid biomarkers for embryo viability.

We find an interesting historical parallel to this narrative. Within the context of the mammalian oocyte, initial studies reported that mtDNA levels might serve as a predictive biomarker for implantation (25-28), but later reports rebutted these findings (2, 29, 30). Time and further studies will tell if history repeats itself, this time from the perspective of the human blastocyst.

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FIGURE LEGENDS

Figure 1. mtDNA scores sorted by euploid and aneuploid blastocysts result in statistically insignificant differences. (**A**) Next Generation Sequencing (NGS) data. (**B**) Quantitative RT-PCR (qPCR) data probing for a locus in the CYTB mitochondrial gene analyzed by absolute quantitation. (**C**) qPCR data probing for a locus in the ND6 mitochondrial gene analyzed by relative quantitation. N. S. = not significant.

Figure 2. mtDNA scores of blastocysts sorted by maternal age at the time of oocyte retrieval. (**A**) NGS data sorted by two age groups (20-37 and 38-46) analyzing all embryos and euploids alone, indicating no relevant differences. (**B-C**) mtDNA scores derived by the two described alternative qPCR methods showing no significant differences. (**D-E**) NGS values sorted by individual age, evaluating all embryos and euploids alone. Insets show linear regression through the means, resulting in statistically insignificant P values. N. S. = not significant.

Figure 3. mtDNA scores and implantation potential of transferred euploid blastocysts. (**A**) NGS data from all transferred blastocysts shows no statistically significant difference. (**B**) Intra-cohort analysis for 24 patients with repeat transfers results in insignificant differences. (**C-D**) Statistically insignificant differences resulting from absolute and relative quantitation qPCR probing two different mitochondrial genes CYTB and ND6, respectively. Only one sample in the 'not implanted' group consistently shows an mtDNA score above a possible threshold across platforms. N. S. = not significant.

Supplemental Figure 1. (**A-C**) Standard curves for the three qPCR assays used, showing high experimental efficiency. (**D**) NGS validation for mtDNA score reproducibility. Blastocyst biopsy-derived WGA samples were sequenced in duplicates (full circles) or triplicates (full diamonds) showing consistent mtDNA scores in repeated runs. Each sample from a-l represents one embryo WGA biopsy and numbers represent number of replicate NGS runs. Cell samples from two different cell lines (m and n) underwent repeated WGA and subsequent NGS. Half circles represent two replicates for cell line m, and half squares represent four replicates for cell line n. The consistent values for each cell line indicate that neither WGA nor NGS introduce variability in the mtDNA score determination. Full triangles (o) depict a duplicate run of blood isolated from a patient, for which DNA isolation, WGA, and NGS were run separately, and resulted in consistent mtDNA scores. (**E**) WGA samples from five blastocysts (Controls A-E) were used to determine mtDNA scores by NGS (purple circles), qPCR assaying the mitochondrial CYTB gene by absolute quantitation (blue circles), and qPCR assaying the mitochondrial ND6 gene by relative quantitation (orange circles). Raw values for each technique (indicated above the circles) were normalized to Control A. Fold changes of mtDNA scores A<B<C<D<E for all three techniques, indicating cross-platform consistency of mtDNA score ranking.

Supplemental Figure 2. Intra-cohort analysis of mtDNA scores for 24 patients with repeat transfers sorted by blastocyst viability, showing a statistically insignificant difference. For this NGS analysis only reads aligning to Chr 1 were used for standardization. N. S. = not significant.