Accurate quantitation of mitochondrial DNA reveals uniform levels in human blastocysts irrespective of ploidy, age, or implantation potential

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Objective: To accurately determine mitochondrial DNA (mtDNA) levels in human blastocysts.
Design: Retrospective analysis.
Setting: IVF clinic.
Patient(s): A total of 1,396 embryos derived from 259 patients.
Intervention(s): Blastocyst-derived trophectoderm biopsies were tested by next-generation sequencing (NGS) and quantitative real-time polymerase chain reaction (qPCR).
Main Outcome Measure(s): For each sample the mtDNA value was divided by the nuclear DNA 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 = 1,396), 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 with unviable blastocysts when analyzed by NGS (implanted n = 101, nonimplanted n = 140) and by qPCR (implanted n = 49, nonimplanted 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. (Fertil Steril® 2017;107:34–42. ©2016 by American Society for Reproductive Medicine.)
Key Words: Blastocyst mtDNA, correction factor, embryonic mitochondria, IVF biomarker, PGS/PGD

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Human cells contain anywhere from 100 to 150,000+ copies of 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

Received August 3, 2016; revised September 7, 2016; accepted September 13, 2016; published online October 25, 2016.
A.R.V. has nothing to disclose. A.J.B. has nothing to disclose. J.C.T. has nothing to disclose. D.K.G. has nothing to disclose. C.G.Z. has nothing to disclose. F.L.B. has nothing to disclose. M.V. has nothing to disclose.
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Fertility and Sterility® Vol. 107, No. 1, January 2017 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2016.09.028

mtDNA levels and by extension increased adenosine triphosphate 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, suboptimal 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 owing to laboriousness and difficulty in interpreting results. More commonly used 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 used in studies of mtDNA levels in embryos.

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. 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 deidentified NGS workflow data and whole genome amplification (WGA) product from patients consenting to preimplantation genetic screening during routine IVF procedures. It is exempt from institutional review board review by the US Department of Health and Human Services under 45 CFR 46.101(b) (4).

Embryo Processing

In vitro fertilization and culturing of embryos took place at Zouves Fertility Center using standard techniques. Briefly, fertilization was accomplished using intracytoplasmic sperm injection, and zygotes were cultured for 5–7 days in either G1/G2 Plus medium (Vitrolife) or GTL (Vitrolife) in 5.5% CO_2, 5.5% O_2 balance N_2 at 37°C in a humidified atmosphere. Five- to ten-cell trophectoderm biopsies were collected from blastocyst stage embryos and stored at −80°C until further processing. Whole genome amplification 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 Bluefuse Multi Software (Illumina). We investigated mtDNA content in a total of 1,396 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 (Illumina) files in the BAM and FASTQ format were uploaded into Geneious R9 (Biomatters) 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 nuclear mitochondrial DNA segments (NUMTs) (11). The number of mtDNA mapped reads was divided by the number of nDNA mapped reads after bioinformatic processing and filtering by MiSeq Reporter Software and displayed in Bluefuse Multi Software. Resulting values were further subjected to a mathematical correction factor described in the next section. Number of reads pertaining to chromosome (Chr) 1 were determined in Geneious R9.

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 equation 1.

$$m_{NGS} = \frac{r_m}{r_n} \times F_{NGS} \quad (1)$$

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 composed of 6,072,607,692 base pairs, whereas the male counterpart is 5,976,710,698 base pairs 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, because the male genome is 98.42% the length of the female's (Table 1).

Similarly, an aneuploid embryo has more or less genetic material per cell compared with a euploid embryo, and without correction it would lead to inflated mtDNA counts in the case of nullisomies and monosomies, and

TABLE 1

Correction factors for accurate mtDNA content determination.

A. NGS correction factor

mtDNA	16,569 bp
Diploid female (reference)	6,072,607,692 bp
Diploid male	5,976,710,698 bp
Female−male	95,896,994 bp
% Male vs. female	98.42%
% Difference	1.58%
NGS correction factor female	1
NGS correction factor male	0.984208268
Universal formula to calculate NGS correction factor	(Reference genome length ± loss or gain)/(reference genome length)
Formula for a loss (Z is the number of base pairs in the lost sequence)	(6072607692−Z)/6072607692
Formula for a gain (Z is the number of base pairs in the lost sequence)	(6072607692+Z)/6072607692

Chromosome	Length (bp)	Correction factor monosomy	Correction factor disomy	Correction factor trisomy
1	249,250,621	0.958954928	1	1.041045072
2	243,199,373	0.95995141	1	1.04004859
3	198,022,430	0.967390874	1	1.032609126
4	191,154,276	0.96852188	1	1.03147812
5	180,915,260	0.970207978	1	1.029792022
6	171,115,067	0.971821814	1	1.028178186
7	159,138,663	0.973794016	1	1.026205984
8	146,364,022	0.975897665	1	1.024102335
9	141,213,431	0.976745833	1	1.023254167
10	135,534,747	0.977680964	1	1.022319036
11	135,006,516	0.97776795	1	1.02223205
12	133,851,895	0.977958086	1	1.022041914
13	115,169,878	0.981034527	1	1.018965473
14	107,349,540	0.982322332	1	1.017677668
15	102,531,392	0.983115756	1	1.016884244
16	90,354,753	0.98512093	1	1.01487907
17	81,195,210	0.986629268	1	1.013370732
18	78,077,248	0.987142715	1	1.012857285
19	59,128,983	0.990263	1	1.009737
20	63,025,520	0.989621342	1	1.010378658
21	48,129,895	0.992074262	1	1.007925738
22	51,304,566	0.991551477	1	1.008448523
X	155,270,560	0.974430991	1	1.025569009
Y	59,373,566	0.990222723	1	1.009777277

B. qPCR correction factor

State of nDNA region probed by reference assay:	Nullisomy	Monosomy	Disomy	Trisomy	Tetrasomy
Corresponding correction factor	Use different reference assay	0.5	1	1.5	2

Note: Data based on reference genome GRCh37 Ensembl release 68. (A), NGS correction factors for complete chromosomal monosomies or trisomies. The indicated universal formula is also applicable for whole chromosome nullisomies and other polysomies, as well as segmental losses or gains. (B) Correction factor for qPCR data when the nuclear DNA reference assay probes a chromosomal region that is aneuploid.

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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 1). 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 qPCR experiments were performed using the Taqman system (Applied Biosystems/Thermo Fisher). Surplus WGA product from the Veriseq workflow was diluted 1/10 in water, vortexed for 30–60 seconds, and heated to 95°C for 10 minutes to ensure inactivation of any residual WGA polymerase activity. Two microliters 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 with several other mtDNA assays tested. An assay

probing 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–1E). 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: 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 reference 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 a second assay is designed probing an 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.

$$m_{qPCR} = \frac{c_m}{c_n} \times F_{qPCR} \quad (2)$$

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 4×10^7 counts for the mtDNA target and 2×10^4 counts to the nDNA reference, with the nDNA reference assay is located on Chr 21, would have the following final m_{qPCR} score:

$$m_{qPCR} = \frac{4 \times 10^7}{2 \times 10^4} \times (1.5) = 3,000$$

When comparing euploid embryos of different genders, if the reference assay probes a region in the sex chromosomes, the corresponding correction factors must be used. If probing a region of Chr X, male embryos should be corrected with a factor of 0.5. Probing for a region of Chr Y is not useful in female embryos. 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.

$$m_{qPCR} = 2^{-(Ct_m - Ct_n)} \times F_{qPCR} \quad (3)$$

The same correction factor (details in Table 1) 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 resequenced WGA products, which yielded consistent results amongst separate runs (Supplemental Fig. 1D). Additionally, 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 five 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 five 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 these 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 (The R Foundation). All graphs were prepared in Prism 6 showing means, with error bars indicating SD.

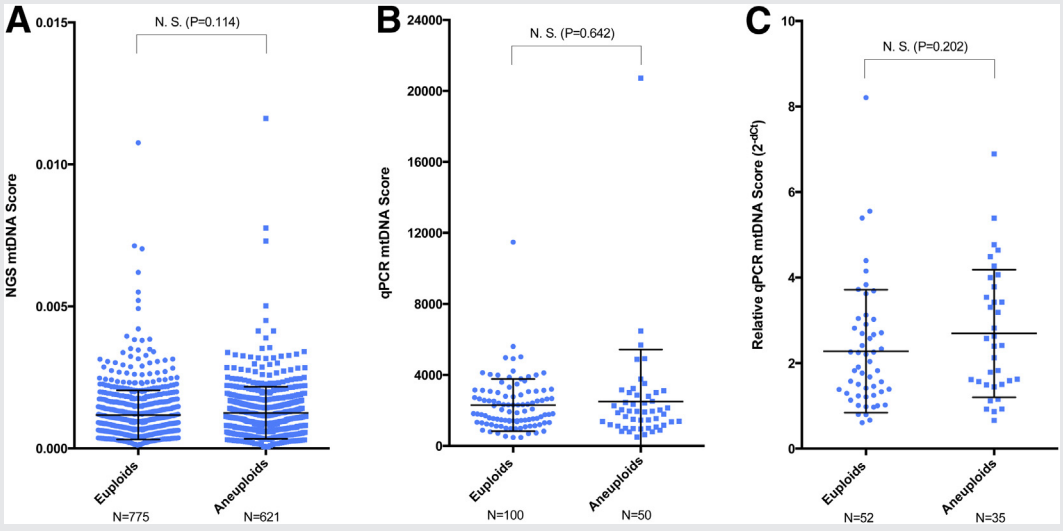
RESULTS

Applying a 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 preimplantation genetic screening 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

FIGURE 1



Mitochondrial DNA scores sorted by euploid and aneuploid blastocysts result in statistically insignificant differences. (**A**) NGS data. (**B**) 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.

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the mathematical correction factors that take the variability of embryonic genomes into account (see Materials and 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 = 1,396). 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=.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=.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=.202) (Fig. 1C). Therefore, regardless of quantitation platform and downstream mathematical calculation used, blastocysts grouped by ploidy never showed statistically significant differences in mtDNA scores. This is in stark contrast with previous reports that did not use a correction factor in their calculations (5, 6).

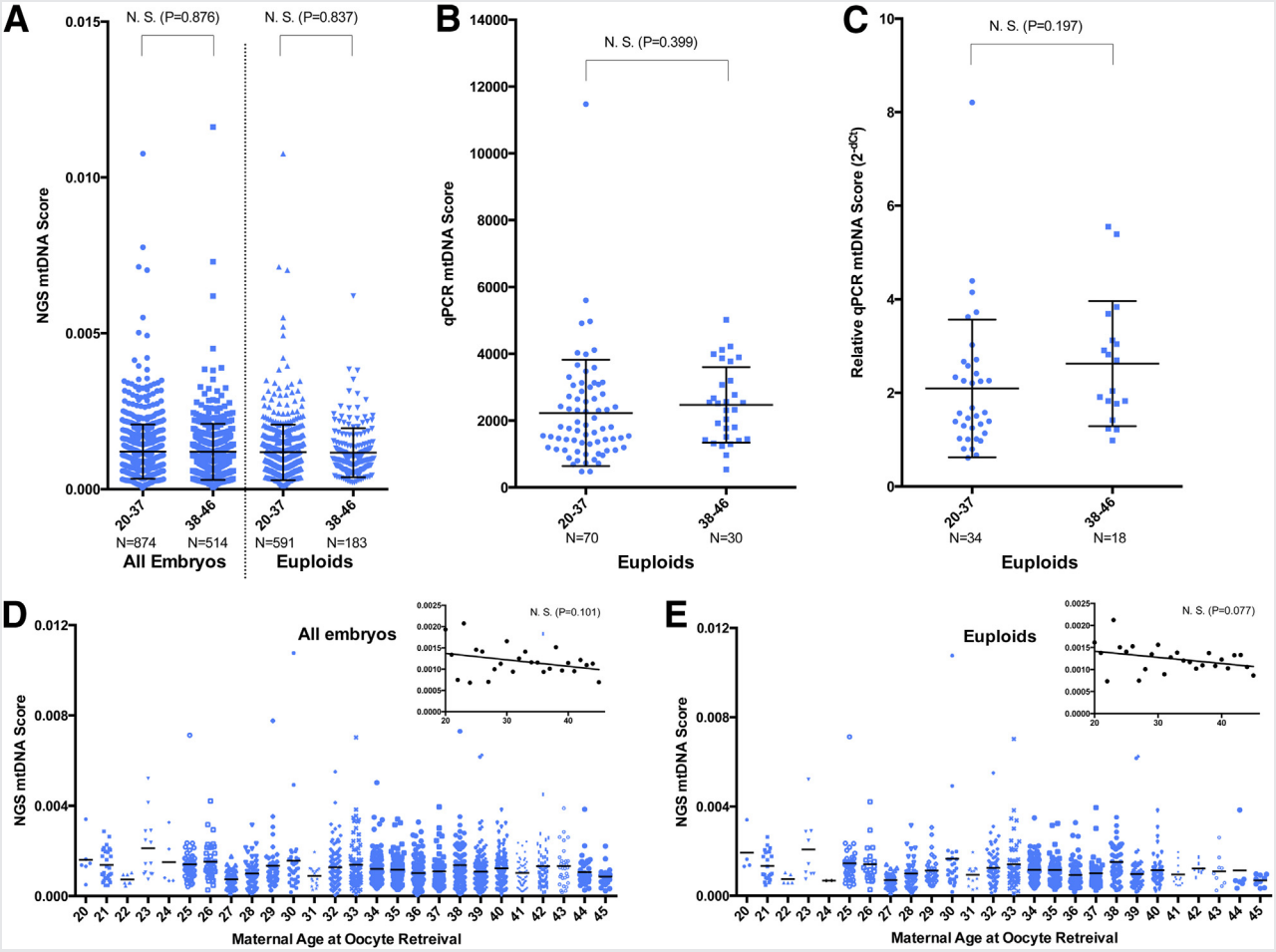
Maternal Age at Oocyte Retrieval Does Not Affect mtDNA Levels of Blastocysts

We divided blastocysts into a younger maternal age group at oocyte retrieval (20–37 years) and an older group (38–46 years). A previous study reported a statistical difference, stating that the older group had higher mtDNA levels (5). We investigated mtDNA scores by NGS among 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 retesting 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 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. The NGS data from 101 implanted and 140 not-implanted blastocysts showed no statistical difference in mtDNA score (P=.510) (Fig. 3A).

FIGURE 2



Mitochondrial DNA scores of blastocysts sorted by maternal age at the time of oocyte retrieval. (**A**) NGS data sorted by two age groups (20–37 years and 38–46 years) 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.

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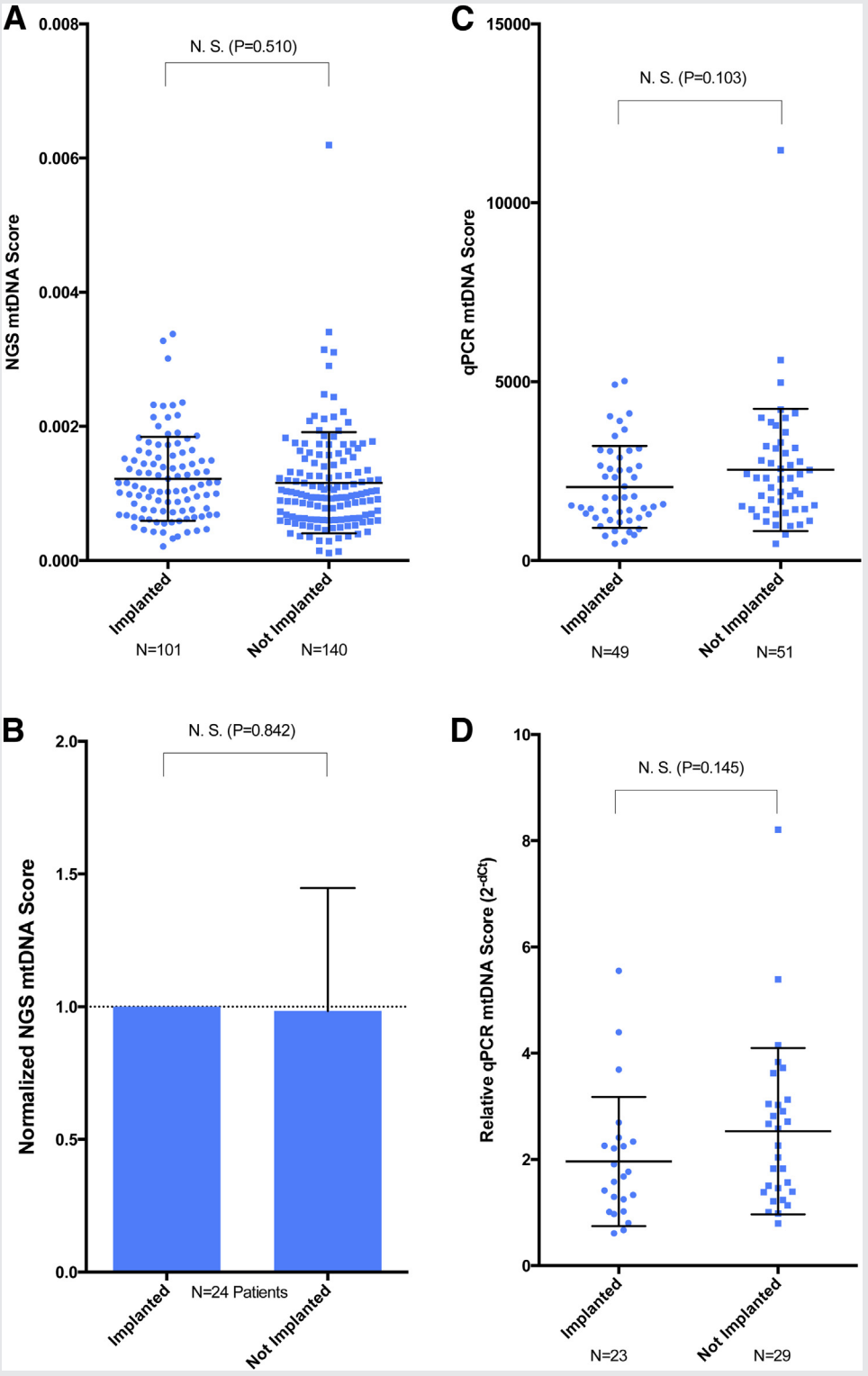
With these combined 241 blastocysts we carried out a logistic regression analysis to investigate whether mtDNA score could function as a predictive tool for clinical outcomes. The test is adjusted for the following confounding factors: cohort size (i.e., how many embryos produced in the cycle), embryo 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 of embryo viability (P=.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 who 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 vs. 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=.842) between their implanted (n = 25) and nonimplanted (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. Because 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).

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=.103)

FIGURE 3



Mitochondrial DNA scores and implantation potential of transferred euploid blastocysts. (**A**) NGS data from all transferred blastocysts shows no statistically significant difference. (**B**) Intracohort 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.

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(Fig. 3C). Finally, out of the latter set of blastocysts we retested 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 ($P=.145$) (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. 3A and 3C, and D), equaling 0.41% of all embryos. Hence, our data indicate that mtDNA content does not represent a statistically relevant or practical method to predict embryo viability among euploid blastocysts.

DISCUSSION

Contrary to previous reports, our study finds no correlation of mtDNA content with blastocyst ploidy, age, or viability. Although we detect a considerable range of mtDNA scores in the tested samples, this range is observed within all populations irrespective of criterion used to subgroup 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. First, 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. Additionally, embryos being tested by qPCR must be corrected when the reference assay lies in a genomic region that is different between embryos being compared, such as in aneuploidies or sex chromosomes. We have developed a mathematical method to accurately determine mtDNA scores of blastocysts that takes the genetic makeup of each sample into account. We propose that 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 appear to have 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 interfacility 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.

Last, it should be noted that previous reports on mtDNA levels, aside from showing interesting concordances, also show several discrepancies among them. For instance Fragouli et al. (5) suggest a maternal age effect, whereas 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. describe a viability effect in cleavage and blastocyst stage embryos, whereas Fragouli et al. only detect it in blastocysts and not at the cleavage stage. These divergences remain to be explained and possibly point toward a technical or laboratory-specific effect.

The qPCR analysis in Diez-Juan et al.'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, in which 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 approximately 10% ADO (17) using the routinely used 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). Although we find no association between mtDNA content and embryonic stress, our data do 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 and 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 adenosine triphosphate 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 whether history repeats itself, this time from the perspective of the human blastocyst.

Acknowledgment: The authors thank all members of the Zouves Fertility Center for support to this study in various forms, including sample preparation, discussions, and manuscript editing.

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SUPPLEMENTAL TABLE 1

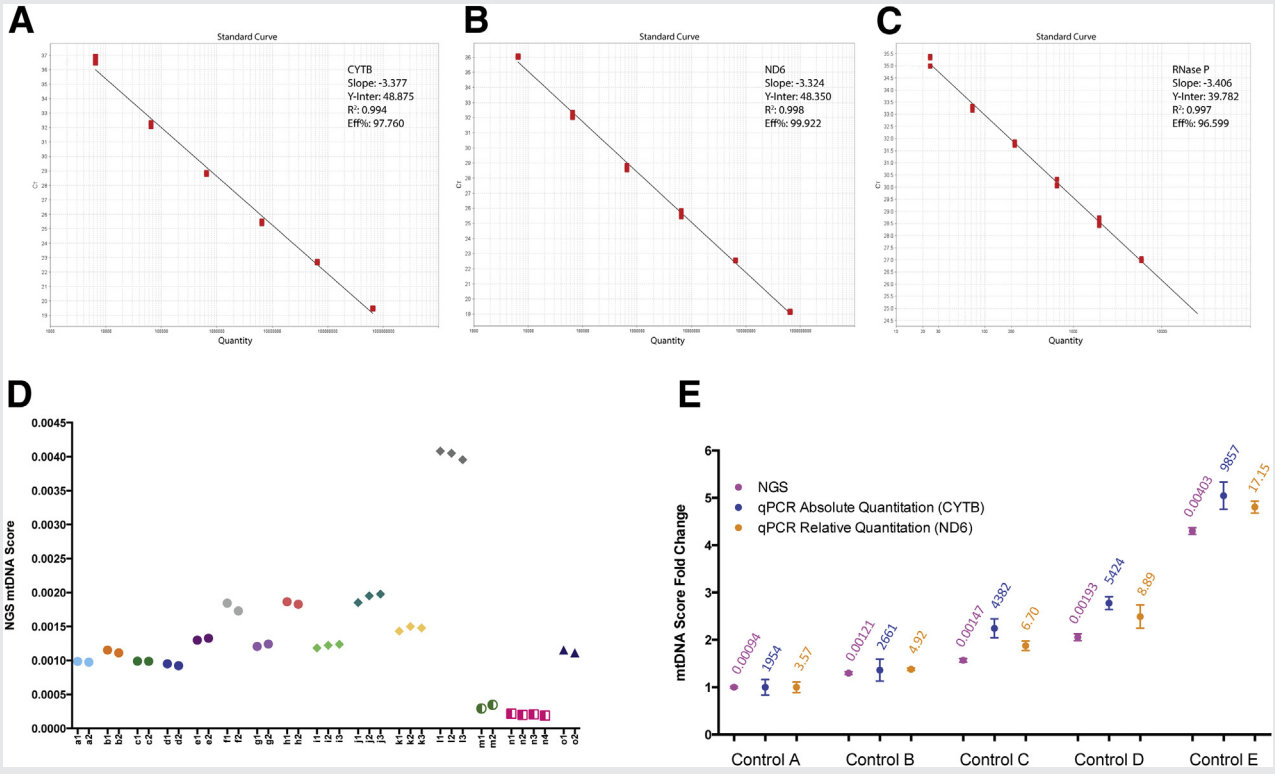
Logistic regression analysis for mtDNA score predicting clinical outcome (implanted or not implanted) upon blastocyst transfer, adjusted for potential confounding factors.

Coefficient	Estimate	SE	*z* value	Pr(> |z|) (*P* value)
(Intercept)	−16.99957	1693.53420	−0.010	.992
mtDNA Score	141.66159	196.88366	0.720	.472
Oocyte_Age	0.02358	0.02760	0.854	.393
Single_Double	0.14068	0.50621	0.278	.781
Age_Recipient	−0.01523	0.02999	−0.508	.612
Embryo_Gender	0.12338	0.28737	0.429	.668
Cohort_Size	0.02848	0.03575	0.797	.426
Embryo_StageGradeBIII	−0.16203	2936.98335	0.000	1.000
Embryo_StageGradeFHBII	16.85901	1693.53392	0.010	.992
Embryo_StageGradeFHBIII	−0.21243	2936.98334	0.000	1.000
Embryo_StageGradeHBI	−0.25392	2385.32511	0.000	1.000
Embryo_StageGradeHBII	16.09310	1693.53370	0.010	.992
Embryo_StageGradeHBIII	16.06627	1693.53382	0.009	.992
Embryo_StageGradeXBI	16.80955	1693.53369	0.010	.992
Embryo_StageGradeXBII	16.11707	1693.53365	0.010	.992
Embryo_StageGradeXBIII	14.86599	1693.53372	0.009	.993

Note: Factors included in the analysis are oocyte age at retrieval, single or paired sibling transfer, patient age at transfer, embryo gender, cohort size (i.e., how many embryos produced in the cycle), embryo stage, and grade.
Deviance residuals: Min: −1.468; 1Q: −1.079; Median: −0.632; 3Q: 1.229; Max: 1.799.
(Dispersion parameter for binomial family taken to be 1.)
Null deviance: 326.67 on 239 degrees of freedom.
Residual deviance: 306.18 on 224 degrees of freedom.
(538 observations deleted because of missingness.)
Akaike Information Criterion: 338.18.
Number of Fisher scoring iterations: 15.

Victor. Uniform levels of mtDNA in blastocysts. Fertil Steril 2016.

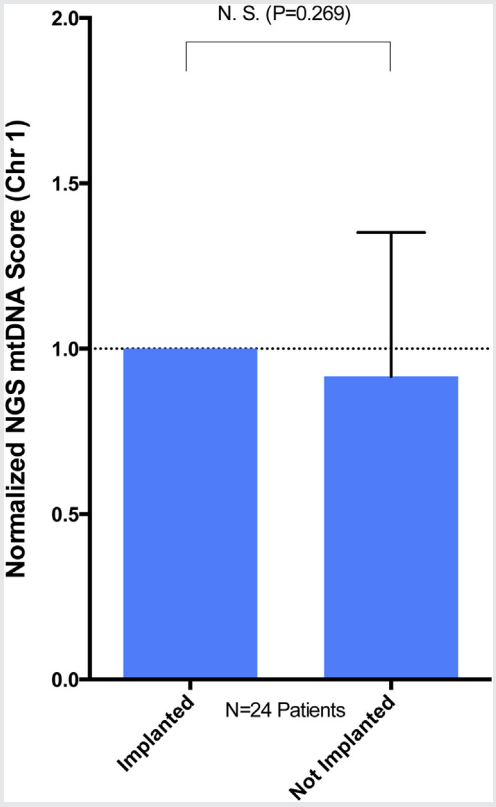
SUPPLEMENTAL FIGURE 1



(**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 duplicate (*full circles*) or triplicate (*full diamonds*) showing consistent mtDNA scores in repeated runs. Each sample from a to 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.

Victor. Uniform levels of mtDNA in blastocysts. Fertil Steril 2016.

SUPPLEMENTAL FIGURE 2



Intracohort 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.

Victor. Uniform levels of mtDNA in blastocysts. Fertil Steril 2016.