High relative deoxyribonucleic acid content of trophectoderm biopsy adversely affects pregnancy outcomes

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Objective: To evaluate the association between relative DNA content of the trophectoderm biopsy and pregnancy outcomes.
Design: Retrospective cohort study.
Setting: Academic-affiliated private practice.
Patient(s): This study included patients undergoing their first single embryo transfer after trophectoderm biopsy and comprehensive chromosome screening (CCS) at a single center between January 2010 and February 2014.
Intervention(s): In phase 1 of the study, a standard curve was developed to estimate the relative DNA content of trophectoderm biopsies. Phase 2 of the study examined reproductive outcomes in patients undergoing single embryo transfer after trophectoderm biopsy and CCS. Samples were divided into quartiles according to their relative DNA content, and clinical outcomes were compared.
Main Outcome Measure(s): Chemical pregnancy rate, clinical implantation rate, ongoing pregnancy rate, live birth rate.
Result(s): The quartile of highest relative DNA content had a significantly lower live birth rate when compared with the other three quartiles (relative risk 0.84, 95% confidence interval 0.75–0.95). There was no difference between the quartiles regarding age, body mass index, ovarian response, or endometrial thickness. Among those patients who had a live birth, there was no difference in hCG levels, gestational age at delivery, or birth weight with respect to biopsy DNA content.
Conclusion(s): Trophectoderm biopsies with the highest relative DNA content are associated with lower live birth rates after single embryo transfer. Possible explanations for this phenomenon include diminished accuracy of the euploid diagnosis vs. a mechanical impact of the biopsy. Regardless of the cause, the outcomes emphasize the importance of obtaining appropriately sized trophectoderm biopsies for CCS. (Fertil Steril® 2017;107:731–6. ©2016 by American Society for Reproductive Medicine.)
Key Words: Comprehensive chromosome screening, IVF, preimplantation genetic diagnosis, trophectoderm biopsy

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In light of recent data, comprehensive chromosome screening (CCS) has become more frequently used to aid in embryo selection and enhance live birth rates. Numerous randomized, controlled trials have shown that trophectoderm biopsy and subsequent CCS improves implantation and delivery rates per ET (1–4). A recent meta-analysis of randomized, controlled trials and observational studies looking at infertile couples undergoing embryo biopsy with CCS confirmed that the use of CCS increases clinical and sustained (beyond 20 weeks) implantation rates when compared with routine care without CCS (5).

Received August 13, 2016; revised November 1, 2016; accepted November 14, 2016; published online December 8, 2016.
S.A.N. has nothing to disclose. J.M.F. has nothing to disclose. E.J.F. has nothing to disclose. M.D.W. has nothing to disclose. S.J.M. has nothing to disclose. X.T. has nothing to disclose. N.R.T. has nothing to disclose. R.T.S. has nothing to disclose.
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Fertility and Sterility® Vol. 107, No. 3, March 2017 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2016.11.013

Early studies regarding the impact of biopsy have inconsistent findings. A frequently cited study demonstrated that blastomere biopsy at the eight-cell stage did not affect preimplantation development, although it is important to note that the investigators did find a decrease in cell mass of the blastocyst in those who underwent biopsy (6). A mouse study examining blastomere biopsy showed similar results (7).

Randomized, controlled trials have provided more information regarding the impact of biopsy. A pilot study seeking to evaluate blastocyst biopsy vs. cleavage stage biopsy for preimplantation genetic diagnosis of

β-thalassemia found that embryos undergoing blastocyst biopsy had a higher implantation rate (47.6%) than those undergoing cleavage stage biopsy (26.7%) (8). A more recent paired sibling embryo trial demonstrated a nearly 40% relative reduction in sustained implantation for cleavage stage biopsy when compared with trophectoderm biopsy (9). These data indicate that the timing of biopsy during embryonic development is important with respect to reproductive outcomes.

Before establishing the importance of biopsy timing, reports that compared clinical outcomes after ET found a reduction in reproductive potential when two blastomeres instead of only one were removed (6, 10, 11). In another study, a 59% reduction in reproductive potential was observed in biopsied embryos when compared with nonbiopsied controls (12). Taken together, these data suggest that the reproductive potential of the embryo may also be impacted by the size of the biopsy obtained.

This study seeks to further characterize the impact of embryo biopsy on reproductive outcomes. As the data discussed suggest, both biopsy timing and biopsy size may be impactful. Thus, we seek to characterize biopsy size while controlling for timing by analyzing only trophectoderm biopsies. Here, an assay measuring relative DNA content as a surrogate for biopsy size is established, and reproductive outcomes are examined.

MATERIALS AND METHODS

Study Population

This is a retrospective cohort study of all patients undergoing their first single embryo transfer after trophectoderm biopsy and CCS at a single infertility center between January 2010 and February 2014. Only data from the first ET for each patient during this time period were included. All stimulation and embryology techniques were performed as per routine practice standards. Embryos were cultured to the blastocyst stage before undergoing trophectoderm biopsy on day 5 or day 6 of embryo development. Biopsies were then used for CCS via quantitative real-time polymerase chain reaction (qPCR)-based assay (13). Single embryo transfer of the morphologically best, euploid embryo was performed on day 6 of the fresh cycle in the event the blastocyst had expanded for trophectoderm biopsy the day prior or in a subsequent frozen embryo transfer cycle if the embryo was biopsied on day 6. In all cases, patients who underwent transfer had an endometrial thickness of at least 6 mm.

Methods

Phase 1: DNA quantification assay. To characterize relative DNA content of trophectoderm biopsies, additional analysis of qPCR-based CCS (14) data was performed. When performing qPCR, the number of PCR cycles required to reach an arbitrary signal threshold (threshold cycle or CT) for a given genomic locus is inversely related to the amount of DNA at that locus. Therefore, samples observed reaching the threshold at a lower cycle number than others contain more DNA. Quantitative PCR-based CCS involves testing 96 genomic loci in parallel. Eight of these loci are on the sex chromosomes and therefore excluded from analysis of DNA content, given that some embryos will be male and some female. Therefore, the mean CT values for the remaining autosomal chromosomes (88 loci) were evaluated as a surrogate marker of DNA content. Mean CT values were analyzed from samples with known numbers of cells (1, 2, 3, 4, 5, 10, 15, and 20) from a euploid fibroblast cell line (Coriell Cell Repository, ID# GM00323). Twelve replicates of each cell number were performed. A standard curve was then established, based on the cell number and mean CT value. This assay provides a method of relative DNA content in a specimen but is not a method that is capable of counting cells in a particular sample. Given that more cells in a sample will have more DNA content, it is possible to determine relative size of an embryo biopsy, although not the exact cellularity. Thus, this curve was used to estimate the DNA content of trophectoderm biopsies, and these values were stratified into quartiles from least (1) to most (4) DNA content.

Phase 2: Outcome measures. Human chorionic gonadotropin levels were measured 9 days after ET and repeated 48 hours later if positive, as per routine in this program. The rise in serum hCG levels was calculated by dividing the repeat hCG value at 48 hours by the initial hCG value. To eliminate variation in hCG levels based on timing of blood draw, only those cycles in which the hCG was measured at the specified time points (namely, 9 days after ET and 48 hours later) were included in this analysis. This resulted in the exclusion of 608 subjects from this subanalysis. These subjects were, however, included in the main analysis of pregnancy outcomes that follows.

The following pregnancy outcomes were measured for each quartile: chemical pregnancy rate, clinical implantation rate, ongoing pregnancy rate, and live birth rate. A chemical pregnancy was defined as a positive initial hCG at 4 weeks' gestation. Implantation was defined as the presence of an intrauterine gestational sac on ultrasound. Ongoing pregnancy was defined as fetal cardiac activity at the time of discharge to an obstetrician, typically at 9 weeks' gestation. Live birth was defined as the birth of a living infant at $\geq$23 weeks' gestation. For all live births, gestational age at delivery and birth weight were recorded. Given the known association of both males and neonates born after frozen embryo transfer with higher birth weight (15), these results were reported by gender and by fresh vs. frozen transfer. Pregnancy outcomes were adjusted for embryo quality, which was assessed as good, fair or poor according to the simplified Society for Assisted Reproductive Technology embryo scoring system (16).

Statistical Analysis

Categorical data were analyzed using χ^2 to compare outcomes between groups. Analysis of variance was used to evaluate continuous variables. Multivariate analysis was conducted to account for possible confounders, including oocyte age, fresh vs. frozen embryo transfer, embryo quality, and DNA content. Statistical significance was determined according to a P value $\leq$.05. All retrospective data analysis was performed under institutional review board–approved protocols.

RESULTS

In phase 1 of the study, the standard curve was developed according to the described method (Fig. 1). This curve was then used to estimate the relative DNA content of the trophectoderm biopsies for the ETs that were studied in phase 2. Although this method is unable to precisely quantify cellularity, knowing the relative DNA content enabled an approximation using our model. The biopsies in this study were estimated to range in cellularity from 1 to 20 cells.

Phase 2 of this study includes a total of 1,147 patients and ETs. The average age at the time of oocyte retrieval was 36.1 years (range, 22–44.8 years), body mass index 24.9 kg/m^2 (range, 16.1–61.4 kg/m^2), and endometrial thickness 9.6 mm (range, 6.0–18.0 mm). Approximately two-thirds of patients (67.7%) underwent frozen embryo transfers. Demographics of the patient population stratified by trophectoderm biopsy relative DNA content can be found in Table 1. There were no differences among the four quartiles with respect to age at the time of oocyte retrieval, body mass index, or maximal endometrial thickness at time of hCG administration. The percentage of ETs done in the frozen cycle was similar (63%–66%) in quartiles 1–3. In the quartile of highest DNA content (quartile 4), frozen embryo transfers were increased compared with fresh transfers ($P<.01$).

Figure 2 displays pregnancy outcomes by quartile of biopsy relative DNA content. The quartile of highest relative DNA content (quartile 4) had a lower chemical pregnancy rate and implantation rate when compared with the other three quartiles; however, this difference was not statistically significant. The quartile of highest relative DNA content did have a significantly lower ongoing pregnancy rate and live birth rate when compared with the other three quartiles (Table 1).

Among those patients who had a live birth, there was no difference in the initial hCG or rate of rise in hCG over 48 hours with respect to biopsy DNA content. There were no differences in gestational age at delivery or birth weight with respect to biopsy DNA content (Table 1). As expected, males and neonates born after frozen embryo transfer had higher birth weights than females and neonates born after fresh embryo transfer. Of note, there were seven sets of monozygotic twins. These pregnancies were excluded when calculating mean hCG levels, gestational age at delivery, and birth weight.

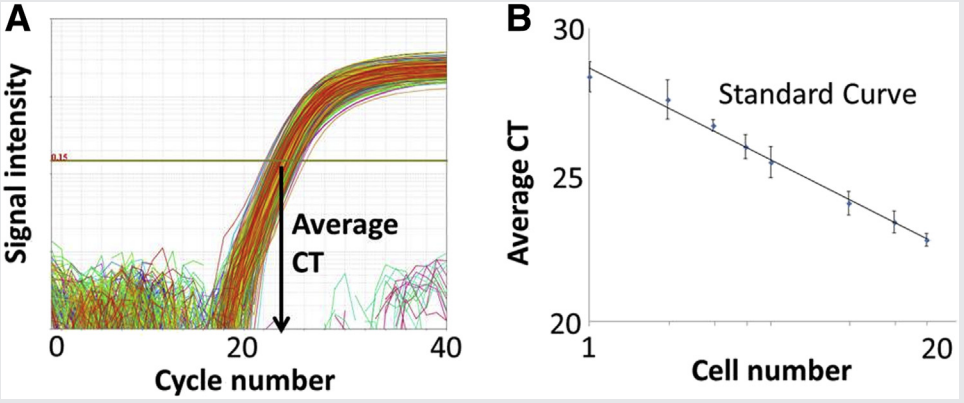
Given that quartile 4 had a lower ongoing pregnancy rate and also a higher percentage of frozen transfers when compared with the other quartiles, the data were reanalyzed after stratifying by fresh vs. frozen transfer, as shown in Table 2. Interestingly, the mean initial hCG level was significantly higher for frozen transfers when compared with that for fresh transfers (155.2 mIU/mL vs. 108.1 mIU/mL, $P<.001$). There was no difference in the rate of rise in hCG between fresh and frozen transfers. With respect to pregnancy outcomes, there were no statistically significant differences between fresh and frozen transfers.

Multivariate analysis was conducted to account for embryo quality in addition to oocyte age, fresh vs. frozen transfer, and biopsy DNA content. The addition of this potential confounder did not affect the observed pregnancy outcomes; namely, quartile 4 had lower ongoing pregnancy and live birth rates. Data from this analysis can be found in Supplemental Table 1 (available online).

DISCUSSION

The data presented here indicate that the relative DNA content of trophectoderm biopsy is an important factor that can influence reproductive competence of embryos. Although trophectoderm biopsy has been shown to be safer and more accurate than blastomere biopsy at the cleavage stage, the trophectoderm biopsies with the relatively highest DNA content were correlated with a lower chance for delivery. These data, taken with previously published data, establish that both timing of biopsy and size of biopsy are important variables when implementing CCS.

FIGURE 1



Method for determination of biopsy DNA content as a surrogate for biopsy size. (**A**) Quantitative PCR-based mean threshold cycles (CT) from a 96-plex reaction were analyzed on samples with known numbers of cells (1, 2, 3, 4, 5, 10, 15, and 20) from a cultured cell line. (**B**) A standard curve was established, based on the cell number and average CT value. This curve was used to estimate the relative DNA content of each biopsy.

Neal. Embryo biopsy size and pregnancy outcome. Fertil Steril 2016.

TABLE 1

Demographic information, cycle characteristics, pregnancy outcomes, and neonatal outcomes by quartile (Q).

	DNA content			
Variable	**Q1 (n = 264)**	**Q2 (n = 290)**	**Q3 (n = 282)**	**Q4 (n = 311)**
Oocyte age (y), mean (range)	35.9 (23.2–44.2)	35.7 (25.6–45.8)	36.2 (22.6–44.9)	36.4 (22.0–44.5)
Body mass index (kg/m^2), mean (range)	25.5 (17.5–46.2)	24.5 (16.7–46.5)	24.9 (16.8–50.4)	24.7 (16.1–61.4)
Endometrial thickness (mm), mean (range)	9.5 (6–16)	9.7 (6–17)	9.8 (6–18)	9.6 (6–18)
Frozen embryo transfers, n (%)	173 (65.5)	192 (66.2)	180 (63.8)	232 (74.6)
Embryo quality, n (%)				
Good	49 (18.6)	61 (21.0)	85 (30.1)	125 (40.2)
Fair	195 (73.9)	217 (74.8)	189 (67.0)	171 (55.0)
Poor	20 (7.6)	12 (4.1)	7 (2.5)	13 (4.2)
Mean initial hCG (mIU/mL)	158.3	160.0	153.9	158.3
Relative rise hCG	2.7	2.6	2.6	2.9
Chemical pregnancy, n (%)	209 (79.2)[a]	225 (77.6)[b]	217 (77.0)[b]	226 (72.7)[c]
Implantation, n (%)	188 (71.2)[d]	199 (68.6)[e]	201 (70.9)[d]	195 (62.7)[f]
Ongoing pregnancy, n (%)	168 (63.6)[g]	179 (61.7)[g]	175 (62.1)[g]	165 (53.1)[h]
Live birth, n (%)	163 (61.7)[i]	171 (59.0)[j]	172 (61.0)[i]	159 (51.1)[k]
Mean gestation age at delivery (wk)	37.7	38.0	38.0	38.1
Mean birth weight (g)	3,335.6	3,341.7	3,397.7	3,437.5
Fresh transfer (n = 226)	3,295.6	3,244.1	3,361.2	3,294.5
Frozen transfer (n = 433)	3,356.6	3,402.2	3,421.8	3,483.2
Female (n = 310)	3,348.5	3,294.0	3,170.8	3,325.3
Male (n = 349)	3,326.2	3,387.3	3,601.7	3,548.4

Note: Population numbers (n) for hCG parameters are lower owing to exclusion of subjects who did not have hCG drawn at the specified time points (Q1, n = 142; Q2, n = 143; Q3, n = 153; Q4, n = 134). Population numbers (n) for neonatal outcomes are lower owing to exclusion of subjects who did not have a singleton live birth (Q1, n = 163; Q2, n = 170; Q3, n = 169; Q4, n = 157). There were no differences in age, body mass index, or endometrial thickness across quartiles. Frozen embryo transfer rates were higher in the quartile of highest DNA content (Q4) ($P < .01$). This quartile also had a higher proportion of good-quality embryos. There was no difference in pregnancy outcomes among quartiles 1–3. Pregnancy rates were lower for the biopsy group with highest DNA content (Q4). There were no differences seen in initial hCG, rise of hCG, or neonatal outcomes across quartiles.
Superscript letters a vs. c, d vs. f, g vs. h, i vs. k: $P < .05$.

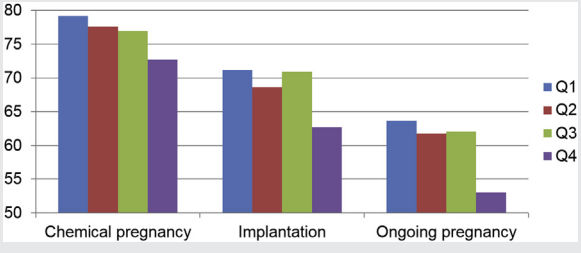
Neal. Embryo biopsy size and pregnancy outcome. Fertil Steril 2016.

With level 1 evidence of the benefit of euploid embryo transfer to enhance pregnancy rates in IVF (1, 2, 5), focus has turned to how to further enhance selection because 30%–40% of euploid blastocysts fail to implant. Previous data demonstrated that blastomere biopsy is detrimental to an embryo's reproductive potential, resulting in an approximately 40% relative decrement in the chance of delivery (9). Although trophectoderm biopsy at the blastocyst stage was found to be safe, not all biopsies are the same.

Embryologists are trained to obtain biopsies that contain approximately five trophectoderm cells, although in highly cellular blastocysts it is difficult to precisely quantify cellularity at the time of laser-assisted biopsy. Therefore, a qPCR-based standard curve was established to ascertain relative DNA content, which was used as a surrogate marker for relative cellularity. We elected to focus on relative DNA content rather than cellularity because our model was based on a fibroblast cell line, and there may not be an exact correlation with human embryos. To better estimate the cellularity of a trophectoderm biopsy, the same standard curve would need to be created with biopsies of varying sizes from discarded embryos.

These data demonstrate that, when using a cell line–validated measure of relative DNA content, biopsies with increased relative DNA content impact clinical outcomes. Possible explanations for this phenomenon include diminished accuracy of the euploid diagnosis, resulting from either technical error or biologic error vs. a mechanical impact of the biopsy itself. Although qPCR-based CCS is highly accurate, technical error may be more likely to occur in the setting of a very cellular biopsy, which could result in assay saturation (17). Given that the assay is calibrated to accommodate the average trophectoderm biopsy, assay saturation should generally not occur. However, the assay performance may be diminished in the setting of a relatively larger biopsy. Increased DNA content may also result in higher biologic error by dampening the detection of mosaicism. Furthermore, because polyploidy cannot be detected using qPCR-based CCS, it is plausible that polyploid embryos may have been included in this study. Our method of estimating biopsy DNA content, which relies on a qPCR-based assay, would likely estimate biopsies of these embryos to be highly cellular, when in fact the higher signal would be due to increased DNA

FIGURE 2



Pregnancy outcomes across quartiles of trophectoderm biopsy DNA content. The biopsy quartile with highest DNA content (Q4) demonstrated poorer outcomes across all measured outcomes.

Neal. Embryo biopsy size and pregnancy outcome. Fertil Steril 2016.

TABLE 2

Data stratified by fresh vs. frozen embryo transfer.

	DNA content				
Variable	**Q1 (n = 264)**	**Q2 (n = 290)**	**Q3 (n = 282)**	**Q4 (n = 311)**	**All (n = 1,147)**
Mean initial hCG (mIU/mL)					
Fresh	129.8	133.0	115.2	91.1	120.1
Frozen	171.9	178.4	179.5	178.5	177.0
P value	.02	<.01	<.01	<.01	<.01
Relative rise hCG					
Fresh	2.6	2.6	2.5	3.0	2.6
Frozen	2.7	2.6	2.7	2.8	2.7
P value	.34	>.99	.06	.16	.06
Chemical pregnancy, n (%)					
Fresh	73 (80.2)	75 (76.5)	78 (76.5)	58 (73.4)	284 (76.8)
Frozen	136 (78.6)	150 (78.1)	139 (77.2)	168 (72.4)	593 (76.3)
P value	.76	.76	.89	.86	.87
Implantation, n (%)					
Fresh	61 (67.0)	68 (69.4)	75 (73.5)	50 (63.3)	254 (68.6)
Frozen	127 (73.4)	131 (68.2)	126 (69.4)	145 (62.5)	529 (68.0)
P value	.28	.84	.47	.90	.81
Ongoing pregnancy, n (%)					
Fresh	58 (63.7)	67 (68.4)	70 (68.6)	39 (49.4)	234 (63.2)
Frozen	110 (63.6)	112 (58.3)	105 (58.3)	126 (54.3)	453 (58.3)
P value	.98	.10	.09	.45	.11
Live birth, n (%)					
Fresh	56 (62.6)	65 (66.3)	68 (66.7)	39 (49.4)	228 (61.9)
Frozen	107 (61.3)	106 (55.2)	104 (57.8)	120 (51.7)	437 (56.1)
P value	.83	.07	.14	.72	.06

Note: Population numbers (n) for hCG parameters are lower owing to exclusion of subjects who did not have hCG drawn at the specified time points (Q1, n = 142; Q2, n = 143; Q3, n = 153; Q4, n = 134). Mean initial hCG following frozen embryo transfer was significantly higher for all when compared with mean initial hCG following fresh embryo transfer. There was no difference in relative rise over 48 hours. There was no difference in pregnancy outcomes between the fresh and frozen embryo transfer groups.

Neal. Embryo biopsy size and pregnancy outcome. Fertil Steril 2016.

content rather than increased relative cellularity. Last, one must consider that a large or very cellular biopsy may have an adverse mechanical impact on the remaining blastocyst, perhaps resulting in lower pregnancy rates.

Although the quartile with the lowest pregnancy rates also had the highest percentage of frozen embryo transfers, this difference does not account for the lower pregnancy rates. Further analysis revealed that the initial hCG levels for frozen embryo transfers were significantly higher than those for fresh embryo transfers. However, the chemical pregnancy, implantation, ongoing pregnancy, and live birth rates did not differ between fresh and frozen embryo transfers. This finding is consistent with previously published data regarding reproductive outcomes following frozen embryo transfer at the study center and elsewhere (2, 18).

Although blastocyst morphology has been shown to be predictive of pregnancy outcomes in unscreened embryos (19, 20), additional studies have demonstrated conflicting results regarding the impact of morphology on pregnancy outcomes after transfer of euploid embryos (21, 22). Our study failed to find an association between morphology and pregnancy outcome; however, this lack of association may be a byproduct of limited sample size. Multivariate analysis confirmed that even when adjusting for embryo quality, ongoing pregnancy and live birth rates remained lower in the quartile of highest DNA content. In addition, implantation rate was lower in this quartile.

This study only examined pregnancy outcomes following ETs for which the trophectoderm biopsy yielded a (euploid) result. We did not examine the relative DNA content of biopsies that had uninterpretable results; however, it is possible that uninterpretable results are more likely to arise from a biopsy that is too small, with poor amplification and impaired fidelity as a consequence. Therefore, simply minimizing the size of the trophectoderm biopsy may not be the best solution.

It is difficult to determine the exact number of cells when performing a trophectoderm biopsy, and our analysis does not intend to provide guidelines regarding the optimal number of cells to obtain. Rather the purpose is to demonstrate that not all biopsies are the same, and ones with relatively higher DNA content, likely correlating with a relatively more cellular biopsy, may have an adverse impact on the implantation potential of the transferred embryo. More research is needed to elucidate the causes of this decrement in pregnancy outcomes and to continue to optimize the safety and efficacy of trophectoderm biopsy.

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SUPPLEMENTAL TABLE 1

Multivariate analysis.

Variable	Chemical pregnancy	Implantation	Ongoing pregnancy	Live birth
Oocyte age	0.94 (0.91–0.97)	0.96 (0.93–0.99)	0.97 (0.94–1.00)	0.96 (0.93–0.99)
Embryo score: fair (ref: poor)	1.2 (0.62–2.24)	1.35 (0.74–2.41)	1.36 (0.76–2.41)	1.23 (0.69–2.18)
Embryo score: good (ref: poor)	1.29 (0.64–2.48)	1.55 (0.83–2.88)	1.63 (0.89–3.00)	1.47 (0.80–2.70)
Frozen embryo transfer (ref: fresh)	1.06 (0.79–1.44)	1.07 (0.82–1.41)	0.89 (0.68–1.15)	0.87 (0.67–1.13)
Q2 (ref: Q1)	0.89 (0.59–1.34)	0.86 (0.59–1.24)	0.90 (0.64–1.27)	0.87 (0.62–1.23)
Q3 (ref: Q1)	0.89 (0.59–1.35)	1.00 (0.68–1.45)	0.92 (0.64–1.30)	0.96 (0.67–1.36)
Q4 (ref: Q1)	0.70 (0.47–1.05)	0.67 (0.47–0.96)	0.64 (0.45–0.90)	0.64 (0.46–0.90)

Note: Pregnancy outcomes are expressed as odds ratio (95% confidence interval). After adjusting for oocyte age, embryo quality, and fresh vs. frozen embryo transfer, the biopsy quartile with highest DNA content (Q4) remained a predictor of significantly lower ongoing pregnancy and live birth rates. It also emerged as a predictor of lower implantation rate. ref = reference value.

Neal. Embryo biopsy size and pregnancy outcome. Fertil Steril 2016.