Associations of sperm mitochondrial DNA copy number and deletion rate with fertilization and embryo development in a clinical setting

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STUDY QUESTION: Are sperm mitochondrial DNA copy number (mtDNA_{cn}) and deletion rate (mtDNA_{del}) associated with odds of fertilization and high embryo quality at Days 3 and 5?

SUMMARY ANSWER: Higher sperm mtDNA_{cn} and mtDNA_{del} were associated with lower odds of high quality Day 3 embryos and transfer quality Day 5 embryos, both of which were primarily driven by lowered odds of fertilization.

WHAT IS KNOWN ALREADY: Sperm mtDNA_{cn} and mtDNA_{del} have been previously associated with poor semen parameters and clinical male infertility. One prior study has shown that mtDNA_{del} is associated with lower fertilization rates. However, it is unknown whether these characteristics are linked with ART outcomes.

STUDY DESIGN, SIZE, DURATION: This prospective observational study included 119 sperm samples collected from men undergoing ART in Western Massachusetts. ART outcomes were observed through to Day 5 post-insemination.

PARTICIPANTS/MATERIALS, SETTINGS, METHODS: As part of the Sperm Environmental Epigenetics and Development Study (SEEDS), 119 sperm samples were collected from men undergoing ART in Western Massachusetts. Sperm mtDNA_{cn} and mtDNA_{del} were measured via triplex probe-based qPCR. Fertilization, Day 3 embryo quality and Day 5 embryo quality measures were fitted with mtDNA_{cn} and mtDNA_{del} using generalized estimating equations.

MAIN RESULTS AND THE ROLE OF CHANCE: After adjusting for male age and measurement batches, higher sperm mtDNA_{cn} and mtDNA_{del} were associated with lower odds of fertilization ($P = 0.01$ and $P < 0.01$), high quality Day 3 embryos ($P = 0.02$ for both) and transfer quality Day 5 embryos ($P = 0.01$ and $P = 0.09$). However, the associations of mtDNA_{cn} and mtDNA_{del} with Day 3 high quality status and Day 5 transfer quality status were attenuated in models restricted to fertilized oocytes. Sperm mtDNA_{cn} and mtDNA_{del} remained statistically significant in models adjusted for both male age and semen parameters, although models including both mtDNA markers generally favoured mtDNA_{del}.

LIMITATIONS, REASONS FOR CAUTION: Our sample only included oocytes and embryos from 119 couples and thus large diverse cohorts are necessary to confirm the association of sperm mtDNA biomarkers with embryo development.

WIDER IMPLICATIONS OF THE FINDINGS: To our knowledge, our study is the first to assess the associations of sperm mtDNA_{cn} and mtDNA_{del} with fertilization and embryo quality. The biological mechanism(s) underlying these associations are unknown. Multivariable

models suggest that sperm mtDNA_{cn} and mtDNA_{del} provide discrimination independent of age and semen parameters; therefore, future investigation of the utility of sperm mtDNA as a biomarker for ART outcomes is warranted.

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Introduction

Infertility treatment using assisted reproductive technologies has had improved success rates over the years. However, 38–49% of couples will not have a child even after undergoing six IVF cycles (Malisia et al., 2009). Biological (Jones et al., 1998; Sabatini et al., 2008), demographic (Klonoff-Cohen and Natarajan, 2004; Rittenberg et al., 2011), and environmental risk factors (Tielemans et al., 2000; Wu et al., 2017) have been associated with poor IVF outcomes.

The mitochondrial genome, which is maternally inherited, comprises ~16.5 kb (Anderson et al., 1981) and encodes 2 rRNAs, 22 tRNAs and 13 subunits of the mitochondrial complexes forming the electron transport chain (Anderson et al., 1981; Rajender et al., 2010). A natural depletion of sperm mtDNA occurs during spermatogenesis, primarily during the spermiogenesis stage when round spermatids take on an elongated form (Hecht et al., 1984; May-Panloup et al., 2003). In order to maintain homoplasmy, the remaining sperm mtDNA are tagged and subsequently degraded in the oocyte cytoplasm shortly after fertilization (Sutovsky et al., 2004). In human embryos, sperm mtDNA can persist until the 4–8 cell stage or even the blastocyst stage in abnormal embryos (St John et al., 2000). In contrast, the replication of oocyte mtDNA increases during oogenesis and is associated with fertilisability (Reynier et al., 2001). The mature human oocyte contains an average of 200 000 copies of mtDNA and, although a reduction by an order of magnitude does not affect fertilization or early embryo development, a critical threshold of 40 000–50 000 mtDNA copies is required to maintain embryo development until the implantation stage when mtDNA replication resumes (Wai et al., 2010).

Sperm mitochondrial DNA copy number (mtDNA_{cn}), defined as the number of mtDNA copies per nuclear DNA copy, has been shown to be a sensitive biomarker of male fertility (Lewis, 2007; Rajender et al., 2010). MtDNA_{cn} varies by cell type, with most mitochondria containing between 1 and 10 copies in somatic cells (Phillips et al., 2014). Epidemiologic evidence show that higher sperm mtDNA_{cn} is associated with abnormal semen parameters both in a clinical setting (May-Panloup et al., 2003) and in the general Chinese (Tian et al., 2014; Zhang et al., 2016) and Spanish (Diez-Sanchez et al., 2003) populations, although one study reported that mtDNA depletion is also associated with sperm motility (Kao et al., 2004). Relevant to ART outcomes, one study demonstrated that a 3-fold reduction in sperm mtDNA_{cn} does not hinder fertilization (Wai et al., 2010), while another reported that some abnormal embryos contained sperm mtDNA at the blastocyst stage and speculated that this may be in part due to poor oocyte quality (St John et al., 2000).

Similar to mtDNA_{cn}, sperm mitochondrial deletion (mtDNA_{del}) is another biomarker that reflects mtDNA integrity and damage. A

number of clinical studies have reported that deletions in sperm mitochondrial DNA are more common among men with poor semen parameters compared to men with normal semen parameters (Kao et al., 1995, 1998; St John et al., 2001; Song and Lewis, 2008; Ieremiadou and Rodakis, 2009; Ambulkar et al., 2016a, 2016b; Bahrehmand Namaghi and Vaziri, 2017; Mughal et al., 2017; Talebi et al., 2017). One study of 67 Greek men seeking fertility treatment additionally reported an inverse correlation between the frequency of sperm mtDNA_{del} and fertilization rates after conventional IVF, but not after intracytoplasmic sperm injection (Ieremiadou and Rodakis, 2009).

To our knowledge, it is unknown whether mtDNA_{cn} and mtDNA_{del} are linked with ART outcomes such as embryo development. Therefore, our primary objective is to examine the association of these sperm biomarkers with fertilization and Days 3 and 5 embryo quality under an ART setting.

Materials and Methods

Study population and sample collection

As part of the Sperm Environmental Epigenetics and Development Study (SEEDS), this study comprised the male partners of 119 couples recruited from 2014 to 2016 at Baystate Reproductive Medicine in Springfield, MA. The inclusion criteria for SEEDS were: (i) male partners between 18 and 55 years old without vasectomy; (ii) female partners between 18 and 44 years old; (iii) expected delivery at Baystate Medical Centre; and (iv) fresh ejaculate sperm used for treatment. Written consent from participants was obtained by attending physicians. This study was approved by the institutional review boards at Baystate Medical Centre and at the University of Massachusetts Amherst.

Sample collection and sperm DNA isolation and measurements

Semen samples were collected as part of the IVF protocol in a sterile plastic specimen cup after a 2–3 days abstinence period. Motile sperm cells were enriched from a two-step gradient fractionation where a 40% gradient solution was layered on top of a 80% gradient solution in a 15 ml centrifuge tube. Semen samples were placed over the density gradient columns at room temperature and centrifuged at 200 g for 20 min. The sperm pellets were washed and re-suspended in sperm wash media. For conventional IVF, 5×10^6 /ml was used. In ICSI treatment, sperm were selected based on its morphology and motility pattern under $\times 400$ magnification using an inverted microscope. For sperm DNA isolation, pellets were homogenized with 0.2 mm steel beads for 5 min at room temperature in RLT buffer (Qiagen, Hilden, Germany) containing 50 mM of tris(2-carboxyethyl)phosphine (TCEP; Pierce, Rockford, IL) and total sperm DNA was extracted via silica-column purification (Wu et al., 2015). As part of the routine protocol, trained embryologists microscopically

examined all semen samples for white blood cell (WBC) contamination. Three samples showed WBC contamination in the crude semen samples, but all samples post gradient fractionation were observed to be WBC free.

Sperm mtDNA_{cn} and mtDNA_{del}

For mtDNA_{cn} and mtDNA_{del} quantification, we used a modified version of a previously published triplex qPCR method (Phillips *et al.*, 2014; Huffman *et al.*, 2018). In brief, a segment of the minor arc of the mtDNA was targeted for mtDNA_{cn} assessment due to its stability within the genome, lack of interaction with other targets and high amplification efficiency. For mtDNA_{del}, the 4977 bp 'common deletion' region within the major arc was used. For genomic DNA reference, we used RNase P (ThermoFisher, cat# 4403326), the standard reference assay for copy number analysis. All reactions were conducted in triplicates. Primer sequences can be found in Supplementary Table S1. The cycling conditions were as follows: activation for 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, 55°C for 15 s, and 60°C for 1 min. The % mtDNA_{del} (MajorArc) was normalized to mtDNA_{cn} using the following formulas: $\text{mtDNA}_{cn} = 2^{(\text{Ct}_{\text{RNAseP}} - \text{Ct}_{\text{MinorArc}})}$ and $\text{mtDNA}_{del}(\%) = 2^{(\text{Ct}_{\text{MinorArc}} - \text{Ct}_{\text{MajorArc}})} * 100$.

Fertilization and embryo quality assessment

Within 24 h post-insemination, fertility centre embryologists checked embryos for fertilization. On Days 3 and 5 post-insemination, each live embryo was graded from 1 (best) to 6 (worst) according to their morphologic characteristics. At the cleavage stage (Day 3), embryos were evaluated morphologically for the presence or absence of blastomere multinucleation, symmetry, cell number and amount of fragmentation using the Veeck system (Veeck, 1988). For example, embryos with eight cells, no multinucleated blastomeres and no fragmentation were considered grade 1 quality while embryos with six cells, no multinucleated blastomeres and 20% fragmentation were grade 4 quality. At the blastocyst stage (Day 5), embryos were evaluated for the developmental stage including the expansion of blastocoel and quality of trophectoderm and inner cell mass. For example, embryos at the blastocyst stage with full expansion, hatching or hatched with a large compact inner cell mass and well defined cohesive trophectoderm with many cells were considered grade 1 quality while blastocysts lacking full expansion or having an inner cell mass comprised of a small number of cells or a small number of non-uniform trophectoderm cells were grade 4 quality.

At both Days 3 and 5, embryos were classified into a binary variable where grades 1–2 were considered high quality embryos. At Day 5, grades 1–4 were additionally classified as transfer quality.

Covariate assessment

Semen parameters were assessed by trained embryologists at the IVF clinic: semen volume (mL), sperm concentration (millions/mL), total sperm count (millions), sperm motility (%) and normal morphology (%) according to the Kruger's strict criteria. Relevant demographics (race, age, height, weight) and lifestyle factors (alcohol and cigarette use) were collected by clinic personnel during the IVF cycle. In addition, medical data, clinical diagnoses of infertility and the procedure type (conventional IVF vs ICSI) were obtained from the clinic.

Statistical analysis

For descriptive analyses, characteristics of the study participants were summarized using means and SD for continuous variables, numbers and percentage for binary or categorical variables, and median and range for mtDNA variables. For statistical analyses, general estimating equations (GEE) were used to model relations between independent variables and embryo quality outcomes in order to address the correlation among data for multiple

embryos from the same couples. These GEE models specified a binomial distribution and exchangeable correlation structure with a logit link function to yield odds ratio estimates. Fertilization, Day 3 high quality, Day 5 high quality and Day 5 transferable quality status were fitted as binary outcomes while mtDNA_{cn} and mtDNA_{del} were analysed as both continuous variables and quartiles, with the first quartile as the reference group.

A priori, male participant age and measurement batch were decided to be included in multivariable models. Other factors including BMI, race (white vs non-white), alcohol use (ever/never), cigarette smoking (current/non-current) and measurement batch (categorical, 1–5) were considered as potential confounders and evaluated in bivariate analyses. For these bivariate analyses, each potential covariate was modelled with the exposures and outcomes of interest. The covariate selection for multivariable models was based on biological plausibility and statistical significance in the bivariate models. In order to achieve parsimonious models to maximize statistical power, address confounding and avoid analytically induced biases, only variables related to, but not affected by, mtDNA and also outcomes of interest were considered for inclusion in final models (Robinson and Jewell, 1991; Greenland and Morgenstern, 2001). Supplementary Table SII shows the correlation between the potential covariates and embryo outcomes. Of the demographic and lifestyle factors, age of the male partner was inversely associated with all embryo quality outcomes ($P < 0.05$). Male race was associated with Day 5 high quality status ($P = 0.02$) while current smoking status was borderline significantly associated with fertilization status ($P = 0.07$). None of the assessed demographic and lifestyle covariates were associated with mtDNA_{cn} or mtDNA_{del} (data not shown) and therefore, apart from age, they were not included in the main statistical models, accordingly.

Physician diagnosed male infertility (i.e. based on semen parameter analyses) and procedure type (e.g. conventional IVF vs ICSI) were both associated with mtDNA biomarkers as well as embryo quality. However, because mtDNA is known to be associated with semen parameters, which in turn influences decisions regarding procedure type and male infertility risk, it is unclear whether these factors represent causal intermediates or confounders. As a result, we did not include these factors in primary analyses of mtDNA measures and embryo quality outcomes. Instead, the possible influence of infertility and procedure type on the associations between mtDNA measures and embryo quality outcomes were investigated in secondary, sensitivity analyses.

For additional sensitivity analyses, we restricted the embryos under analysis to only mature or fertilized oocytes. To cross-validate our GEE model, Poisson models were fitted by aggregating the number of embryos and using the total number of embryos as an offset.

All analyses were conducted using R (v3.3.2, R Foundation for Statistical Computing, Vienna, Austria).

Results

Select demographics and clinical information for the 119 study couples are presented in Table I. The mean age and BMI of the male partners were 36.4 years (SD = 5.5) and 29.3 (SD = 5.8), respectively, with non-Hispanic whites accounting for 75.6% of the study population. About 30% of the male partners reported to having smoked at least 100 cigarettes in their lifetime, but only 8 (6.5%) were found to be current smokers. Based on WHO semen parameter reference values (Cooper *et al.*, 2010), 32 male participants were diagnosed with clinical infertility.

A total of 1626 oocytes were retrieved, yielding a median of 12 oocytes per couple per cycle (Table II). Of the 1626 oocytes, 1238 (mean = 10.40) were mature at the time of insemination and 966 (mean = 8.12) were fertilized. Three days after conventional IVF or

Table I Select characteristics of the study population (*n* = 119)

Mean (SD)		
	Male	Female
Age	36.4 (5.5)	34.2 (3.6)
BMI	29.3 (5.8)	28.7 (7.0)
Number (%)		
	Male	Female
Race		
Non-Hispanic White	90 (75.6%)	59 (49.6%)
Other	13 (10.9%)	3 (2.5%)
Missing	16 (13.4%)	57 (47.9%)
Smoking (current)		
Yes	8 (6.7%)	0 (0%)
No	94 (79.0%)	105 (88.2%)
Missing	17 (14.3%)	14 (11.8%)
Clinical infertility		
Yes	32 (26.8%)	74 (62.2%)
No	87 (73.1%)	45 (37.8%)
Procedure	Couple	
IVF	70 (58.8%)	
ICSI	49 (41.2%)	
Median (range)		
	Male	Female
mtDNAcn	1.9 (0.2–34.7)	NA
mtDNA _{del} (%)	20.1 (3.2–39.0)	NA

mtDNAcn = ratio of mitochondrial DNA copies to genomic DNA copies.

mtDNA_{del} = percent of mitochondrial DNA copies with deletion within the 4977 bp common deletion region.

ICSI, 548 (mean = 4.53) of the oocytes had developed into high-quality Day 3 embryos, while 880 (mean = 7.40) embryos were of sufficient quality to be cultured until Day 5. Of those 880 embryos, 292 (mean = 2.45) were of sufficient quality at Day 5 to be considered for transfer, including 96 (mean = 0.81) high quality embryos.

First, we sought to examine the associations of sperm mtDNAcn and mtDNA_{del} with embryo quality and to assess their dose-response relationships. In adjusted models, mtDNAcn as a linear continuous variable was inversely associated with odds of fertilization ($P = 0.01$), high quality at Day 3 ($P = 0.02$) and transfer quality at Day 5 ($P = 0.01$). This linear relationship was also supported by the quartile analyses where, compared to the lowest quartile, the highest quartile of mtDNAcn was associated with lower odds of fertilization (OR = 0.66, 95% CI: 0.42–1.02), Day 3 high quality embryos (OR = 0.66, 95% CI: 0.42–1.02) and Day 5 transfer quality embryos (OR = 0.77, 95% CI: 0.44–1.34) (Fig. 1, Supplementary Table SIII—Model I).

Similarly, continuous mtDNA_{del} models were inversely associated with odds of fertilization ($P < 0.01$), high quality embryos at Day 3 ($P = 0.02$) and transfer quality embryos at Day 5 ($P = 0.09$), but unlike mtDNAcn, linear dose-response trends were not as apparent.

Table II ART treatment data of the couples in the study population (*n* = 119).

	Median (IQR)
Oocytes retrieved	12 (8–128)
Mature oocytes	10 (5–14)
Fertilized embryos	7 (4–11)
Embryos at Day 3	7 (4–11)
High quality embryos at Day 3	4 (2–6)
Embryos cultured past Day 3*	6 (3–10)
Embryos at Day 5*	1 (3–8)
High quality embryos at Day 5*	0 (0–1)
Transferrable embryos at Day 5*	1 (0–4)

*Excluded two couples with embryo transfers on Day 3.

IQR = interquartile range.

Compared to the lowest quartile of mtDNA_{del}, the highest quartile of mtDNA_{del} was associated with lower odds of fertilization (OR = 0.59, 95% CI: 0.37–0.93), high quality embryos at Day 3 (OR = 0.69, 95% CI: 0.46–1.04) and transfer quality embryos at Day 5 (OR = 0.64, 95% CI: 0.38–1.08), but these did not achieve statistical significance (Fig. 2, Supplementary Table SIV—Model I). In secondary analyses, models were additionally adjusted for smoking, BMI and clinical infertility status, and estimates were not substantively different from those of the primary analyses (Supplementary Tables SIII and SIV). Additional analyses also showed that model estimates were not substantively different after adjustment for the procedure type (conventional IVF vs ICSI) (results not shown).

Next, we were interested in evaluating the relationships between mtDNA biomarkers and embryo quality when analyses were restricted to only fertilized oocytes (Table III). Estimates for the relation between mtDNA markers and embryo quality measures were markedly attenuated when the models were restricted to only fertilized oocytes. This observation suggests that the associations of mtDNAcn and mtDNA_{del} with Day 3 high quality status and Day 5 transfer quality status were primarily driven by the lowered odds of fertilization.

Finally, we assessed the independent contribution of mtDNAcn and mtDNA_{del} in multivariable models with male age and semen parameters. In our study population, semen parameters were not associated with ART outcomes, except that sperm volume was associated with high quality Day 3 embryos ($P = 0.04$) and sperm motility was associated with high quality Day 5 embryos ($P = 0.01$) (Supplementary Table SV). Both mtDNAcn and mtDNA_{del} remained statistically significant in models adjusting for both male age and semen parameters (Supplementary Table SVI). However, when both terms were included in the same model, the models generally favoured mtDNA_{del} over mtDNAcn, i.e. only mtDNA_{del} remained statistically significant in models of fertilization and Day 5 high quality embryos while both were statistically significant in models of Day 5 transfer quality embryos.

Discussion

In this first analysis between sperm mtDNA biomarkers and early ART outcomes, we observed that sperm mtDNAcn and mtDNA_{del} were

Figure 1 Odds ratios and 95% confidence intervals for fertilization and embryo outcomes by quartiles of mitochondrial sperm DNA copy number, adjusted for male age and measurement batches.

Figure 2 Odds ratios and 95% CIs for fertilization and embryo outcomes by quartiles of mitochondrial sperm DNA deletion, adjusted for male age and measurement batches.

associated with lower odds of fertilization, high quality Day 3 embryos and transfer quality Day 5 embryos under an ART setting. Analyses restricted to only fertilized oocytes suggested that the associations at Days 3 and 5 were primarily driven by the lower odds of fertilization. The results did not differ when we adjusted for potentially relevant factors, such as smoking status, and procedure type (IVF vs ICSI). In addition, sperm mtDNA_{cn} and mtDNA_{del} were associated with fertilization and embryo outcomes independent of the male age or semen parameters, suggesting that mtDNA_{cn} and mtDNA_{del} may influence early ART outcomes through different biological mechanisms.

Although inverse associations were observed between mtDNA biomarkers, mtDNA_{cn} and mtDNA_{del}, and three of the four ART outcomes assessed in our study (fertilization, Day 3 high quality embryos and Day 5 transfer embryo quality), it is important to note that when embryo quality analyses were restricted to fertilized oocytes, the associations between mtDNA biomarkers and embryo quality were attenuated. Such results suggest that the lower odds of fertilization was the primary driver between mtDNA biomarkers and high quality embryos at Day 3 and transfer quality embryos at Day 5. Our results are generally in agreement with a study of 67 Greek men seeking fertility treatment, in which an inverse correlation was reported between

Table III Generalized estimating equation results of embryo outcomes by quartiles of sperm mitochondrial DNA copy number (mtDNA_{cn}^a) and mitochondrial DNA deletions (mtDNA_{del}^b), restricted to fertilized oocytes.

	Odds of high quality Day 3			Odds of high quality Day 5*			Odds of transfer quality Day 5*		
	OR	95% CI	P-value	OR	95% CI	P-value	OR	95% CI	P-value
mtDNA _{cn} (Quartile range)									
Q1 (0.2–1.4)		Reference			Reference			Reference	
Q2 (1.4–1.8)	0.80	0.49–1.31	0.37	1.73	0.82–3.68	0.15	1.04	0.62–1.74	0.88
Q3 (1.9–3.5)	0.93	0.53–1.63	0.80	2.21	0.94–5.20	0.07	1.15	0.66–1.97	0.62
Q4 (3.7–34.7)	0.74	0.43–1.27	0.27	1.55	0.64–3.71	0.33	0.93	0.50–1.74	0.82
Linear	0.97	0.93–1.01	0.12	0.97	0.89–1.05	0.40	0.97	0.93–1.00	0.04
mtDNA _{del} (Quartile range)									
Q1 (3.2–13.6)		Reference			Reference			Reference	
Q2 (13.9–20.2)	0.61	0.37–1.02	0.06	1.02	0.52–2.01	0.95	1.00	0.62–1.61	0.99
Q3 (20.3–26.9)	0.53	0.30–0.90	0.02	1.09	0.46–2.57	0.84	0.95	0.54–1.67	0.87
Q4 (27.4–39.0)	1.00	0.58–1.73	0.99	0.74	0.31–1.77	0.50	0.83	0.48–1.43	0.50
Linear	1.00	0.98–1.02	0.89	1.00	0.97–1.03	0.83	1.00	0.98–1.02	0.76

*Excluded two couples who had transfers on Day 3.

Models adjusted for Male Age + Measurement Batches.

^aRatio of mitochondrial DNA copies to genomic DNA copies.

^b% of mitochondrial DNA copies with deletion within the 4977 bp common deletion region.

the frequency of mtDNA_{del} and fertilization rates after conventional IVF; however, they found no such associations after ICSI (Ieremiadou and Rodakis, 2009). The authors speculated that sperm with greater mtDNA_{del} do not produce enough energy for movement, contributing to the observed association among conventional IVF patients, but not among ICSI patients. In contrast, our stratified analyses, based on conventional IVF vs ICSI, did not reveal substantial differences in the associations between mtDNA biomarkers and fertilization by procedure type. Such discrepant findings may be attributable to the small number of participants in our study ($N = 119$) and the previous study ($N = 67$) and/or the lack of multivariate models to adjust for potential confounders in the previous study (Ieremiadou and Rodakis, 2009).

It is interesting to note that of all examined outcomes in this study, fertilization is the only outcome in which the general quality of the semen sample might have a greater influence compared to the contribution of a single sperm. In other words, with respect to male influences on fertilization and embryo development, fertilization in an ART setting likely depends on the quantity and quality of sperm near the oocyte whereas the quality of an embryo post-fertilization is dependent on the characteristics of the single sperm that fertilized the oocyte. In our study, we assessed mtDNA_{cn} and mtDNA_{del} using the same pool of sperm as those used for the fertilization of oocytes via conventional IVF or ICSI. While these mtDNA biomarkers can be used to approximate the general quality of the cohort of sperm used in ART, this is not ideal for studies of embryo development as it cannot accurately characterize mtDNA biomarkers of the single fertilizing sperm. For example, while several studies have shown that mtDNA_{del} are higher in sperm fractions based on motility from gradient centrifugation (Kao et al., 1995, 1998; Ieremiadou and Rodakis, 2009; Gholinezhad Chari et al., 2015; Ambulkar et al., 2016a, 2016b), the degree of heterogeneity of mtDNA biomarker levels among the motile

sperm (i.e. 80% fraction in our study) is unknown. Thus, it is possible that sperm mtDNA_{cn} and mtDNA_{del} are associated with embryo quality post-fertilization, but future studies require either very large sample sizes to overcome the non-differential misclassification induced by the use of pooled mtDNA biomarker measurements or a better approximation of the mtDNA of the fertilizing sperm.

In a natural conception setting, it is plausible that the poor semen parameters associated with high mtDNA_{cn} and mtDNA_{del} may lead to lower odds of fertilization and time-to-pregnancy. However, poor semen parameters do not explain our current observations as conventional IVF normalizes the quality and number of sperm incubated with oocytes while ICSI uses only a single morphologically normal sperm. In our multivariable analyses, mtDNA_{cn} and mtDNA_{del} remained statistically significant in models with age or with semen parameters alone. This suggests that mtDNA_{cn} and mtDNA_{del} provide information independent to that of age and semen parameters; however, it is not clear whether mtDNA_{cn} and mtDNA_{del} are biologically causal of poor odds of fertilization or if this association is a product of some other factor that affects both mtDNA characteristics and fertilization.

MtDNA is maternally inherited and sperm mtDNA content is depleted during spermatogenesis (St John et al., 2010; Luo et al., 2013), and this depletion is important for normal sperm function (Wai et al., 2010). Sperm mitochondrial DNA replication is reliant on the nuclear-encoded polymerase gamma (POLG) and mitochondrial transcription factor A (TFAM) (Amaral et al., 2007); both of which are down-regulated during spermatogenesis, resulting in markedly fewer copies of mtDNA (St John et al., 2010). The evolutionary impetus for the depletion of sperm mtDNA prior to fertilization remains unclear, although it has been hypothesized that such mtDNA depletions reduce ROS-mediated damage to sperm DNA (Tremellen, 2008). Thus, it is possible that high sperm mtDNA_{cn} and mtDNA_{del} are

markers of overall aberrant spermatogenesis, whereby the true effector(s) of poor sperm fertilization capacity may not be mtDNA itself but rather other unmeasured sperm abnormalities. Sperm nuclear DNA fragmentation, another marker of aberrant spermatogenesis, within morphologically normal sperm selected for ICSI has been associated with poor embryo development (Avendano *et al.*, 2010) and may be correlated with sperm mtDNA_{cn} and mtDNA_{del}. Alternatively, it is also possible that the abnormally high mtDNA content, as reflected by a higher mtDNA_{cn}, directly disrupts fertilization and early embryo formation, leading to the observed lower odds of high quality embryos.

To our knowledge, our study was the first to assess the associations of sperm mtDNA characteristics and embryo quality. This is meaningful as the inverse associations of mtDNA_{cn} and mtDNA_{del} with odds of fertilization, Day 3 embryo high quality and Day 5 embryo transfer quality in an IVF setting suggest that paternal mtDNA characteristics may affect fertilization, and consequently, embryo development. We also recognize that our study has two notable limitations. First, our sample size only included 119 participants and it is possible that we could not detect associations of mtDNA_{cn} and mtDNA_{del} with embryo quality measures among fertilized oocytes given the non-differential misclassification of exposure induced by the use of pooled sperm mtDNA. Second, our population, like most other studies examining sperm mtDNA biomarkers, was recruited from an IVF clinic and included patients with both male and female infertility and therefore may not be generalizable to the broader general population. Thus, replication studies using larger cohorts and different study populations are necessary in order to establish the association of sperm mtDNA biomarkers with embryo development *in vitro* and time-to-pregnancy in the general population.

Conclusion

In our study of 119 male partners of couples undergoing IVF, sperm mtDNA_{cn} and mtDNA_{del} were associated with lower odds of oocyte fertilization, which consequently contributed to lower odds of transfer quality embryos at Day 5. In addition, there is evidence that sperm mtDNA_{cn} and mtDNA_{del} provide information independent of male age and semen parameters. Additional studies are needed to assess such relationships in the general population and to determine how mtDNA_{cn} and mtDNA_{del} are related to odds of oocyte fertilization as well as to other ART outcomes such as implantation and delivery rates.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Authors' roles

H.W. was responsible for isolating DNA from sperm samples, analysis of the data, interpretation of the results and the draft and revision of the article. B.W.W. advised on the analysis approach and revised the article. A.H. was responsible for measurement of sperm mtDNA biomarkers. N.B. was responsible for drafting and revision of the article. S.L., K.L. and E.T. were responsible for the recruitment of the participants and IVF-related data collection. T.R. and C.K.S. advised on the

design of the study and the interpretation of the data and revised the article. J.R.P. was responsible for the design of the study, interpretation of the results and the draft and revision of the article.

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Conflict of interest

The authors declare no competing interests.

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