

Sperm mitochondrial DNA biomarkers and couple fecundity

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STUDY QUESTION: Do sperm mitochondrial DNA measures predict probability of pregnancy among couples in the general population?

SUMMARY ANSWER: Those with high sperm mitochondrial DNA copy number (mtDNA_{cn}) had as much as 50% lower odds of cycle-specific pregnancy, and 18% lower probability of pregnancy within 12 months.

WHAT IS KNOWN ALREADY: Semen parameters have been found to poorly predict reproductive success yet are the most prevalent diagnostic tool for male infertility. Increased sperm mtDNA_{cn} and mitochondrial DNA deletions (mtDNA_{del}) have been associated with decreased semen quality and lower odds of fertilization in men seeking fertility treatment.

STUDY DESIGN, SIZE, DURATION: A population-based prospective cohort study of couples discontinuing contraception to become pregnant recruited from 16 US counties from 2005 to 2009 followed for up to 16 months.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Sperm mtDNA_{cn} and mtDNA_{del} from 384 semen samples were assessed via triplex probe-based quantitative PCR. Probability of pregnancy within 1 year was compared by mitochondrial DNA, and discrete-time proportional hazards models were used to evaluate the relations with time-to-pregnancy (TTP) with adjustment for covariates.

MAIN RESULTS AND THE ROLE OF CHANCE: Higher sperm mtDNA_{cn} was associated with lower pregnancy probability within 12 months and longer TTP. In unadjusted comparisons by quartile (Q), those in Q4 had a pregnancy probability of 63.5% (95% CI: 53.1% to 73.1%) compared to 82.3% (95% CI: 73.2% to 89.9%) for Q1 ($P=0.002$). Similar results were observed in survival analyses adjusting for covariates to estimate fecundability odds ratios (FORs) comparing mtDNA_{cn} in quartiles. Relative to those in Q1 of mtDNA_{cn}, FORs (95% CI) were for Q2 of 0.78 (0.52 to 1.16), Q3 of 0.65 (0.44 to 0.96) and Q4 of 0.55 (0.37 to 0.81), and this trend of decreasing fecundability with increasing mtDNA_{cn} quartile was statistically significant (FOR per log mtDNA_{cn} = 0.37; $P < 0.001$). Sperm mtDNA_{del} was not associated with TTP.

LIMITATIONS, REASONS FOR CAUTION: This prospective cohort study consisted primarily of Caucasian men and women and thus large diverse cohorts are necessary to confirm the associations between sperm mtDNA_{cn} and couple pregnancy success in other races/ethnicities.

WIDER IMPLICATIONS OF THE FINDINGS: Our results demonstrate that sperm mtDNA_{cn} has utility as a biomarker of male reproductive health and probability of pregnancy success in the general population.

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Introduction

Infertility, clinically defined as the inability to achieve pregnancy within 12 months of regular unprotected sexual intercourse, is estimated to affect about 15% of couples globally (Jungwirth et al., 2012; Thoma et al., 2013). The prevalence of male infertility in the USA is reported at 12% (Louis et al., 2013) and accounts for approximately 50% of couple-based infertility (Thonneau et al., 1991). Clinically, male infertility is typically determined using measures of semen quality dictated by World Health Organization (WHO) cut points (Cooper et al., 2010). A recent meta-analysis suggested that sperm concentration and total sperm count among men in developed countries have declined over 50% in the past four decades, raising concerns about temporal trends in male fertility (Levine et al., 2017). However, importantly, semen parameter measures have been shown to poorly predict reproductive success and have minimal association with fecundability (Jungwirth et al., 2012; Buck Louis et al., 2014), raising questions about the significance and interpretation of conventional approaches for measuring male infertility. Thus, the identification of biomarkers that accurately predict male reproductive health and success is of major significance due to the potential to impact clinical care and to improve our understanding of the roots of male factor infertility.

Characteristics of mitochondrial DNA (mtDNA) and results of research to date provide motivation to evaluate the relations between mtDNA copy number (mtDNA_{cn}) and deletions (mtDNA_{del}) and couple fecundity. Mitochondria are involved in a variety of biological functions, most predominately ATP production via oxidative phosphorylation of the electron transport chain (ETC) but also hormone production, ion homeostasis and apoptosis. Importantly, mtDNA is highly susceptible to damage and dysfunction due to its proximity to the ETC in conjunction with its less robust repair mechanisms compared to those of nuclear DNA (Phillips et al., 2014). Propagation of the mtDNA genome can occur to compensate for defective mitochondria or damaged mtDNA. MtDNA_{cn} and mtDNA_{del} have been used as biomarkers of mitochondrial function in cancer, in neurodegenerative conditions, and in aging (Taylor and Turnbull, 2005; Greaves and Turnbull, 2009; Coskun et al., 2010).

Mitochondria form tight helices at the mid-piece of sperm during spermatogenesis and contribute to the motility of sperm (Amaral et al., 2013). Sperm mtDNA_{cn} is reduced during spermatogenesis by approximately 8- to 10 folds (Hecht et al., 1984; St John et al., 2010), ensuring sperm mtDNA_{cn} is low upon fertilization because mtDNA is maternally inherited. We (Wu et al., 2019a) and others (Song and Lewis, 2008; Zhang et al., 2016) have shown that retention of mtDNA during spermatogenesis is strongly related to semen quality, such that higher sperm mtDNA_{cn} and mtDNA_{del} were associated with decreases in sperm concentration, total sperm count and progressive motility. Moreover, we have recently shown that higher sperm mtDNA_{cn} and mtDNA_{del} were associated with lower odds of fertilization among men seeking fertility treatment and that these mtDNA biomarkers provided incremental predictive ability of fertilization independent of male age and semen parameters (Wu et al., 2019b). Elevated sperm mtDNA_{cn} and mtDNA_{del} may indicate lower quality spermatozoa resulting from damage due to oxidative stress (Chen et al., 2018), aborted apoptosis or abnormal spermatogenesis (Song and Lewis, 2008). In addition, given that various mechanisms are proposed to prevent the transmission of paternal mtDNA (Sutovsky

et al., 1999; St John et al., 2005; Al Rawi et al., 2011), elevated sperm mtDNA_{cn} is not desirable and could negatively impact fertilization and embryo development and these defenses may result in a direct impact of elevated mtDNA_{cn} on fertilization (Darr et al., 2017). Thus, mtDNA biomarkers in sperm may have utility as an indicator of reproductive health. Research to date, however, is largely limited to clinical populations of couples seeking fertility treatment.

To our knowledge, no studies have investigated the relation between sperm mtDNA biomarkers and couple fecundity as measured by time-to-pregnancy (TTP) among couples recruited from the general population. TTP is a functional measure of couples' individual and combined fecundity in keeping with the couple dependent nature of human reproduction. Using a prospective cohort, we examined the relations between sperm mtDNA_{cn} and mtDNA_{del} and TTP, and their role as biomarkers of couple fecundity.

Materials and methods

Study population

Whole semen samples were provided by male participants of the Longitudinal Investigation of Fertility and the Environment (LIFE) study, a prospective cohort for which details have been previously published (Buck Louis et al., 2011; Buck Louis et al., 2014). In brief, 501 couples were recruited from 16 counties in Michigan and Texas using a population-based sampling frame (Buck Louis et al., 2011). By design, eligibility criteria were minimal: (i) in a committed relationship and planning to discontinue contraception to become pregnant; (ii) no injectable contraceptive use in past year or off contraception for >2 months; (iii) females aged 18–40 and males aged 18 years or older; (iv) females' self-reported menstrual cycles between 21 and 42 days; and (v) an ability to communicate in English or Spanish (Buck Louis et al., 2014). Couples reporting prior infertility treatment were not eligible. Previous data has shown that environmental exposure levels among LIFE participants are comparable to those in the general population (Buck Louis et al., 2013). The current study includes 391 couples who had an aliquot of semen available for sperm purification and DNA isolation. Seven samples had limited sperm DNA and were excluded from analyses, which resulted in a final sample size of 384 couples. Study participants gave written informed consent before any data collection, and full institutional review board approval for human subjects was received from all collaborating institutions.

Data collection

Samples and data were collected in the homes of each participant as previously described (Buck Louis et al., 2014). Briefly, whole semen samples were collected in the home of each participant, including a baseline sample at entry into the study and a second sample 1 month later. Both samples were collected after a period of abstinence via masturbation with no lubricant use. Details regarding the sample collection procedures, shipping materials provided to participants and semen parameter quantification methods using computer-assisted semen analysis have been described in detail in prior analyses of the LIFE study (Buck Louis et al., 2014; Bloom et al., 2015). For the current analysis, only semen samples collected at baseline were evaluated.

TTP was considered as the number of completed menstrual cycles before an hCG confirmed pregnancy (Buck Louis *et al.*, 2011).

Sample preparation and DNA isolation

Semen samples were subjected to a one-step 40% gradient centrifugation to separate sperm from seminal plasma and somatic cells. Sperm DNA was isolated with our previously published method (Wu *et al.*, 2015), which first homogenizes sperm with 0.2 mm steel beads in RLT buffer (Qiagen, Hilden, Germany) containing 50 mM of tris(2-carboxyethyl)phosphine (Pierce, Rockford, IL, USA) before total sperm DNA is extracted via silica-column purification.

mtDNA quantification

A probe-based triplex quantitative PCR assay was used to simultaneously quantify mtDNA_C and mtDNA_D and a nuclear target based on a previously published method (Phillips *et al.*, 2014; Huffman *et al.*, 2018). The minor arc located in the D-Loop of mtDNA was targeted for mtDNA_C assessments because of its high stability and the rarity of deletions in this region. The major arc was targeted for mtDNA_D assessments because of the large prevalence of deletions common to this region. For genomic DNA reference, we used RNase P (ThermoFisher, cat# 4403326), the standard reference assay for copy number analysis. Sperm mtDNA_C was calculated via the cycle threshold (Ct) ratio of minor arc and nuclear DNA copy number determined by RNase P: $2^{-(Ct^{RNase P} - Ct^{MinorArc})}$ and the % mtDNA_D was calculated using the Ct ratio of minor arc and major arc: $mtDNA_{D\%} = 2^{-(Ct^{MinorArc} - Ct^{MajorArc})} * 100$. Reactions were performed in triplicate.

Statistical analysis

Participant characteristics were compared by quartile of mtDNA_C to describe participant characteristics and evaluate relations with mtDNA_C using ANOVA or χ^2 , as appropriate. Tests for trend were conducted using Spearman correlation analysis. MtDNA_C was right skewed and a log (base 10) transformation was used to meet assumptions for models of continuous mtDNA_C. MtDNA_D followed an approximately normal distribution and was not transformed.

We compared the probability of achieving pregnancy within 12 menstrual cycles between couples grouped by quartile of mtDNA_C, given that couples are classified as infertile after 12 months of unprotected sex. These probabilities were evaluated in unadjusted models using exact tests and the Cochran–Armitage test for trend for *P*-values.

Discrete-time proportional hazards models were used to evaluate the relation between mtDNA biomarkers and fecundability, as measured by TTP, allowing for covariate adjustment. Factors considered *a priori* as potential confounders included male and female age, male and female BMI, male race and ethnicity, male smoking status based on serum cotinine, study site of sample collection (i.e. Michigan or Texas), and qPCR batch. No variable was observed to represent a confounder based on bivariate analyses, but these factors were included as covariates in fully adjusted models. Active smoking was determined by a serum cotinine concentration of 10 ng/ml or greater (Bernert *et al.*, 1997). Estimates from discrete time survival models are interpreted as fecundability odds ratios (FORs); estimates <1.0

indicate a lower probability of pregnancy and longer TTP, while FOR >1.0 indicate a higher probability of pregnancy or a shorter TTP. Models were run with mtDNA biomarkers (i.e. log copy number and untransformed deletions) in their continuous form as well as in quartiles to allow for non-linear relations with outcomes. Additionally, survival curves from Kaplan–Meier analysis were generated to display and compare pregnancy probabilities by cycle throughout follow-up between mtDNA_C quartiles.

Results

Men were, on average \pm SD, 31.8 \pm 4.8 years of age with a BMI of 29.9 \pm 5.8. The majority of men were white (*n* = 311; 81.0%). Mean sperm mtDNA_C and mtDNA_D were 5.55 \pm 5.3 and 0.31 \pm 0.08, respectively. On average, female partners were 29.9 \pm 4.1 years of age with a BMI of 27.5 \pm 7.6. A total of 167 (43.5%) women reported never having been pregnant; 182 (47.4%) reported having 1+ live births. Comparisons of participant characteristics by quartiles of sperm mtDNA_C are shown in Table 1. Sperm mtDNA_C and mtDNA_D displayed a strong positive association (Table 1; *P* < 0.001). Sperm mtDNA_C was not associated with participant characteristics, otherwise.

In unadjusted models, a strong inverse association between mtDNA_C and cumulative probability of achieving pregnancy within 12 months of trying was observed, whereby pregnancy probabilities decreased linearly with higher mtDNA_C (Fig. 1). Probabilities (95% CI) were: Q1 = 82.3% (95% CI: 73.2%, 89.3%); Q2 = 75.0% (95% CI: 65.1%, 83.3%); Q3 = 67.7% (95% CI: 57.4%, 76.9%); and Q4 = 63.5% (95% CI: 53.1%, 73.1%). This represents an 18.8% lower probability of pregnancy for couples with male partners in the highest quartile of mtDNA_C than for those in the lowest quartile (*P* = 0.003). Tests for trend strongly suggest a dose–response, with decreasing pregnancy probability with increasing mtDNA_C (*P* = 0.002).

Sperm mtDNA_C was negatively associated with TTP (FOR = 0.37, 95% CI: 0.22, 0.60; per log unit increase), indicating significantly lower fecundability (and, correspondingly, longer TTP) with higher sperm mtDNA_C (Table 2). Similar results were observed when assessing mtDNA_C in quartiles. FOR estimates were: Q2 vs Q1, FOR = 0.78 (95% CI 0.52, 1.16); Q3 vs Q1, FOR = 0.65 (95% CI 0.44, 0.96); and Q4 vs Q1, FOR = 0.55 (95% CI 0.37, 0.81). Sperm mtDNA_D was not associated with fecundability. Adjustment for covariates was observed to have minimal to no impact on model estimates, providing indication of no substantial confounding.

Kaplan–Meier survival curves illustrating the adjusted cycle-specific probabilities of pregnancy by quartiles of mtDNA_C are shown in Fig. 2. Couples whose male partners were in Q1 of sperm mtDNA_C were observed to have a higher pregnancy probability as early as cycle two and throughout the study. A dose-dependent relation was observed for Q2, Q3 and Q4 of sperm mtDNA_C and pregnancy probabilities.

Discussion

Higher sperm mtDNA_C was strongly associated with diminished fecundability among couples trying to become pregnant. Couples with male partners in the fourth quartile of sperm mtDNA_C had an FOR

Table 1 Characteristics of the study participants (n = 384) by quartile of mtDNAcn.

Sperm mtDNAcn	Q1 (0.80–2.60)	Q2 (2.61–3.96)	Q3 (3.98–6.38)	Q4 (6.40–61.94)	P-value ¹	P-trend ²
	Mean (SD)					
Male age	31.8 (4.1)	31.6 (5.0)	32.1 (4.7)	31.4 (5.2)	0.73	0.53
Male BMI ^a	30.2 (5.6)	29.5 (4.9)	29.7 (6.0)	30.3 (6.5)	0.80	0.98
Sperm mtDNA _{del}	0.27 (0.09)	0.29 (0.07)	0.33 (0.06)	0.37 (0.06)	<0.001	<0.001
Female age	29.6 (3.6)	30.3 (4.5)	30.0 (3.9)	29.8 (4.3)	0.92	0.80
Female BMI ^b	28.2 (8.5)	26.4 (6.5)	26.9 (7.2)	28.4 (7.9)	0.77	0.56
	n (%)					
Male current smoking ^c	19 (19.8)	16 (16.7)	26 (27.1)	21 (21.9)	0.35	0.14
Male race ^d					0.69	0.70
White	80 (83.3)	76 (79.2)	75 (78.1)	80 (83.3)		
Non-white	15 (15.6)	19 (19.8)	21 (21.9)	16 (16.6)		
Female parity ^e					0.87	0.71
Never pregnant	45 (46.9)	37 (38.5)	41 (42.7)	44 (45.8)		
Pregnant, no birth	8 (8.3)	7 (7.3)	10 (10.4)	9 (9.4)		
Pregnant, live birth	43 (44.8)	51 (53.1)	45 (46.9)	43 (44.8)		

mtDNAcn, mitochondrial DNA copy number; mtDNA_{del}, mitochondrial DNA deletions.

¹P-value determined by ANOVA for continuous variables and χ^2 for categorical variables.

²P-trend determined by Spearman correlation analysis.

^aMissing n = 3.

^bMissing n = 1.

^cSmoking status determined by a serum cotinine >10 ng/ml; Missing n = 4.

^dMissing n = 2.

^eMissing n = 1.

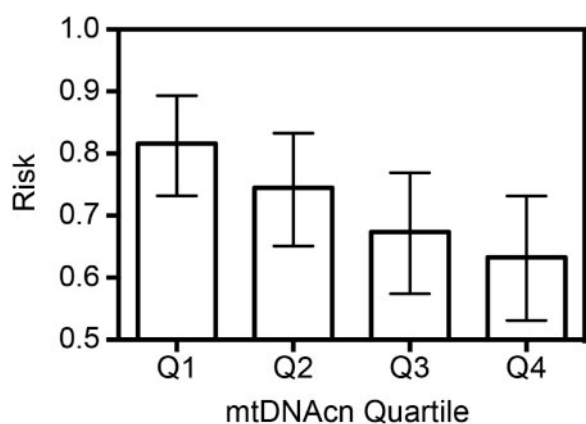


Figure 1. Observed proportions of participants having achieved pregnancy within 12 cycles of trying by quartile (Q) of mitochondrial DNA copy number (mtDNAcn) with 95% CIs. Pregnancy proportions shown above are: Q1 = 0.82 (95% CI: 0.73, 0.89); Q2 = 0.75 (95% CI: 0.65, 0.83); Q3 = 0.68 (95% CI: 0.57, 0.77); and Q4 = 0.63 (95% CI: 0.53, 0.73). Cochran–Armitage P-trend = 0.002.

of 0.55 and 18.8% lower probability of pregnancy within 12 cycles in comparison to couples with male partners in the first quartile of sperm mtDNAcn. This relationship persisted in multivariable models adjusting for potential confounding factors. These findings among pregnancy

planners in the general population who are not seeking clinical care are novel and highlight a potential promising biomarker for couple fecundity.

Clinical assessment of male infertility is largely based on WHO 2010 semen reference guidelines and is used to inform decision-making in the setting of ART. Nevertheless, research suggests semen analyses may be limited with regard to prognostic value of successful reproductive outcomes in couples (Esteves et al., 2012; Jungwirth et al., 2012). In a comparison of semen analysis results between fertile and infertile couples, no single semen parameter was observed to strongly predict fertility status (Guzick et al., 2001). Additionally, a 2019 meta-analysis reported a link between leukocytospermia and semen parameters, exhibiting significant decreases in progressive motility and sperm concentration; however, no significant differences in fertilization and pregnancy rates were observed (Castellini et al., 2020). In previous research from the LIFE study that evaluated 36 measures of semen quality parameters and TTP, model estimates were of low magnitude and only one parameter (percent coiled tail) was associated with TTP in multivariable models (Buck Louis et al., 2014). In the current analysis, we observed that sperm mtDNAcn was strongly associated with TTP and with probability of pregnancy within 12 cycles of trying and may serve as a single-measure parameter of sperm quality.

As the mitochondrial genome is maternally inherited in most species including humans, sperm mtDNAcn is depleted, an estimated 8- to 10 folds, during spermiogenesis (Hecht et al., 1984; St John et al., 2010). Thus, sperm mtDNAcn may represent a sensitive molecular marker of abnormal spermatogenesis and serve as an integrated measure of

Table II Fecundability odds ratios (FOR) estimated using Cox proportional hazards models relating sperm mtDNAcn and mtDNAdeI to time-to-pregnancy.

Biomarker	Unadjusted FOR (95% CI) ^b	Adjusted ^c FOR (95% CI)
mtDNAcn^a		
Continuous	0.36 (0.22, 0.58)***	0.37 (0.22, 0.60)***
Quartiles		
Q1	Reference	Reference
Q2	0.77 (0.53, 1.10)	0.78 (0.52, 1.16)
Q3	0.61 (0.42, 0.88)**	0.65 (0.44, 0.96)*
Q4	0.53 (0.37, 0.77)***	0.55 (0.37, 0.81)**
mtDNAdeI		
Continuous	0.25 (0.05, 1.21)	0.46 (0.06, 3.48)
Quartiles		
Q1	Reference	Reference
Q2	0.85 (0.59, 1.22)	0.87 (0.58, 1.31)
Q3	0.82 (0.56, 1.19)	0.87 (0.56, 1.35)
Q4	0.74 (0.51, 1.07)	0.89 (0.56, 1.40)

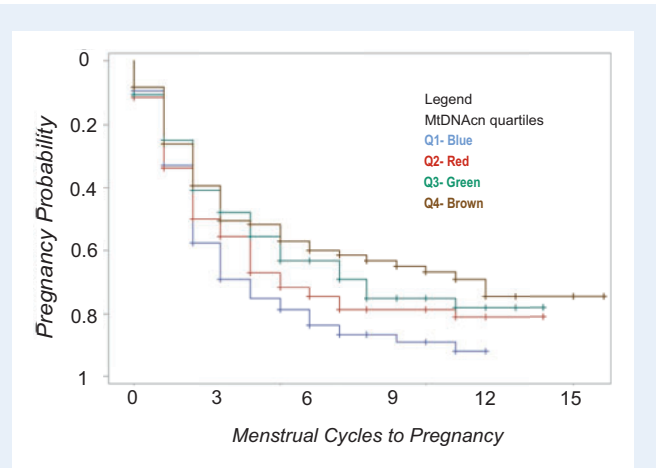
^aLog10 transformed.^bn = 384.^cAdjusted for male and female age, male and female BMI, male serum cotinine, male race/ethnicity, study site of sample collection and qualitative PCR batch; n = 374.

*P < 0.05;

**P < 0.01;

***P < 0.001.

conventional individual semen parameters. Work to date is consistent with this notion; we have previously described that sperm mtDNAcn is correlated with multiple sperm parameters and sperm quality (Zhang *et al.*, 2016; Wu *et al.*, 2019a). Additionally, in an ART cohort of 119 couples, we have observed higher sperm mtDNAcn and mtDNAdeI associated with lower rates of fertilization, even after adjustment for male age and semen parameter measures (Wu *et al.*, 2019b). The nature of causal relations among semen parameters is uncertain, raising questions about the causal interpretation of estimates from statistical models adjusting for multiple semen parameters. Nevertheless, the above-described results (Guzick *et al.*, 2001) demonstrate the incremental predictive ability of mtDNA biomarkers beyond semen characteristics alone. The use of a non-IVF cohort in this study suggests that our observations regarding sperm mtDNAcn in clinical infertility populations may have potential applicability to the general population. The biological basis for mtDNAcn as an integrated, global measure of semen quality is unclear. Previous research has speculated that the susceptibility of mtDNA to oxidative damage may provide a mechanistic link between sperm mtDNAcn and sperm quality, yet results have yet to be conclusive (Zhang *et al.*, 2016). Environmental chemicals, among other exposures, can lead to elevated oxidative stress; oxidative stress-related effects on mtDNA may represent a mechanism through which chemical exposures can affect reproductive health. However, this hypothesis remains relatively unexplored. Although the LIFE cohort recruited couples with presumed environmental chemical exposure, we note that persistent chemical levels among participants in the LIFE study have been observed to be comparable to those in the general population (Buck Louis *et al.*,

**Figure 2.** Survival curve indicating time (in menstrual cycles) to pregnancy by quartiles of sperm mtDNAcn.

Respective fecundability odds ratios using Q1 as the referent are: Q2 = 0.78 (0.52, 1.16); Q3 = 0.65 (0.44, 0.96)*; Q4 = 0.55 (0.37, 0.81)**. Models adjusted for male and female age, male and female BMI, male serum cotinine, male race/ethnicity, site of sample collection and quantitative PCR batch. *P < 0.05; **P < 0.01.

2013). Additional research is necessary to clarify the mechanism explaining the relation of sperm mtDNAcn with male fecundity.

Conclusion

There is a critical need for accurate measures of male fecundity for assessing overall reproductive health given the inherent limitations associated with conventional semen analysis. Research to date has largely been limited to clinical populations of patients seeking infertility treatment. Though somewhat modest in sample size, our study provides novel data regarding mtDNA biomarkers as predictors of reproductive potential beyond those seeking clinical treatment. Results of our study provide suggestion that sperm mtDNAcn may represent a more accurate, single-measure predictor of male fecundity in the general population. The identification of sperm mtDNAcn as a novel biomarker of male reproductive health and pregnancy success is of potential importance for both clinical care and general population reproductive health.

Authors' roles

A.J.R. isolated sperm DNA, performed statistical analyses, interpreted results and drafted the manuscript. B.W.W. oversaw study design, statistical analyses, and interpreted results. N.B. generated the sperm mtDNA biomarker data. G.M.B.L., S.L.M. and E.F.S. oversaw study design and interpreted the results. J.R.P. oversaw study design, sperm mtDNA biomarker data and interpreted results.

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Conflict of interest

The authors declare no competing interests.

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