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ARTICLE

Sperm mitochondrial DNA measures and semen parameters among men undergoing fertility treatment

BIOGRAPHY

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KEY MESSAGE

Sperm mitochondrial DNA copy number and DNA deletions are associated with diminished semen parameters and a markedly increased risk of male factor clinical infertility. These may serve as predictors of consecutive diagnoses of clinical infertility using consecutive semen samples, indicating their roles as stable measures of general long-term infertility status.

ABSTRACT

Research question: To examine associations between sperm mitochondrial DNA copy number (mtDNAcn), sperm mitochondrial DNA deletions (mtDNAdel), semen parameters and clinical infertility in an IVF setting.

Design: A total of 125 sperm samples were collected from men undergoing assisted reproductive procedures in an IVF clinic in Western Massachusetts, USA. Sperm mtDNAcn and mtDNAdel were measured by probe-based quantitative polymerase chain reaction. Semen parameters, clinical diagnoses of infertility, and infertility based on consecutive semen parameters, were fitted with mtDNAcn and mtDNAdel in linear models. The utility of sperm mtDNAcn and mtDNAdel to predict infertility was assessed by receiver operating characteristic curves.

Results: Adjusting for relevant covariates, both sperm mtDNAcn and mtDNAdel were associated with lower sperm concentration, count, motility and morphology ($P \leq 0.03$). Sperm mtDNAcn and mtDNAdel were also associated with increased risks of clinical infertility based on current and consecutive semen samples. Sperm mtDNAcn had high predictive accuracy for consecutive diagnoses of clinical infertility (C-statistic: 0.91), whereas sperm mtDNAdel had moderate predictive accuracy (C-statistic: 0.75).

Conclusions: Sperm mtDNAcn is a measure of consecutive abnormal semen parameters and has promise as a diagnostic test.

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KEYWORDS

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INTRODUCTION

Infertility affects 15% of all heterosexual couples (*Jungwirth et al., 2012*), and male factor infertility has been estimated to account for 30–50% of all infertile couples (*Winters and Walsh, 2014*). Poor reproductive health may be an indicator of overall health in men as well as subsequent progeny. Male infertility has been reported to be associated with an increased mortality rate (*Eisenberg et al., 2014*) and poorer overall health (*Ventimiglia et al., 2015*). A recent meta-analysis has shown that, after accounting for semen collection methods and other relevant factors, sperm counts of men from North America, Europe Australia and New Zealand declined by 59.3% between 1973 and 2011 (*Levine et al., 2017*), indicating a possible decline in male fertility. The biological determinants of semen parameters and male fertility need to be understood, as such investigations may help elucidate underlying contributors to male fertility and clarify the role of male infertility as a general health indicator.

Mitochondria are involved in a host of biological functions, most notably adenosine triphosphate production via oxidative phosphorylation of the electron transport chain. Mitochondria contain their own 16.6kb maternally inherited genome, which encodes 37 genes, including 13 proteins of the electron transfer chain, 22 tRNAs and 2 rRNAs (*Taanman, 1999*). The regulation and integrity of the mitochondrial genome is central for cellular bioenergetics. Mitochondrial DNA (mtDNA) is regulated by a combination of nuclear-encoded proteins, including DNA polymerase gamma (POLG) and mitochondrial transcript factor A (TFAM), the latter of which has been shown to coat mtDNA non-specifically (*Taanman, 1999; Malik and Czajka, 2013; Wang et al., 2013*). Compared with genomic DNA, mtDNA lacks protective histones, anti-oxidant rich cytoplasm and DNA repair mechanisms (*Kao et al., 1998*), rendering mtDNA more vulnerable to deletions and damage (*Lee et al., 2000*).

Mitochondrial DNA copy number (mtDNAcn), also known as mitochondrial DNA content, is defined as the number of copies of mtDNA per nuclear DNA copy. The turnover of mtDNA is highly regulated, variable by tissue type, and independent of the cell cycle (*Clay Montier et al., 2009*); therefore, mtDNAcn is suggested to be a measure of general mitochondrial dysfunction (*Malik and Czajka, 2013*). Changes in somatic tissue mtDNAcn have been linked to a range of adverse health outcomes, including various primary cancers (*Yu, 2011*), neurodegeneration (*Clay Montier et al., 2009*) and diabetes (*Clay Montier et al., 2009; Malik and Czajka, 2013*).

In spermatozoa, mitochondria form around the mid-piece of the flagella to form tight helices and contribute to sperm motility, hormone production, ion homeostasis and apoptosis (*Amaral et al., 2013*). It has been suggested that the over-proliferation of defective mitochondria (*Andreu et al., 2009*), abnormal spermatogenesis (*Song and Lewis, 2008*) and impaired autophagy of mitochondria in mature sperm (*Chan and Schon, 2012*) may all contribute to the propagation of sperm mtDNAcn. Two studies comparing normal and abnormal semen parameters reported that spermatozoa from individuals with abnormal semen parameters had significantly elevated mtDNAcn (*May-Panloup et al., 2003; Song and Lewis, 2008*). One cross-over study reported that sperm mtDNAcn is lower in normozoospermic donors compared with infertile men with clinical varicocele and with spermatozoa with poor motility, and that varicocelectomy improved sperm concentrations and chromatin structure parameters and lowered mtDNAcn in infertile patients with varicocele (*Gabriel et al., 2012*). More recently, a large cross-sectional study of young Chinese men reported that mtDNAcn was inversely associated with sperm concentration, count and motility (*Zhang et al., 2016*).

Deletions in mtDNA (mtDNAdel) are measures that reflect mtDNA integrity and damage. Studies comparing sperm fractions based on motility from gradient centrifugation reported that, within individuals, sperm fractions with poorer motility have higher frequencies of mtDNA deletions compared with sperm fractions with higher motility (*Kao et al., 1995; Kao et al., 1998; Ieremiadou and Rodakis, 2009; Gholinezhad Chari et al., 2015; Ambulkar et al., 2016a; 2016b*), with one exception (St *John et al., 2001*), which may have been driven by a limited sample size. Similarly, studies of men from different geographic regions, including the USA (*Song and Lewis, 2008*), England (St *John et al., 2001*), Greece (*Ieremiadou and Rodakis 2009*), Taiwan (*Kao et al., 1995; Kao et al., 1998*), Turkey (*Mughal et al., 2017*), Iran (*Bahrehmand Namaghi and Vaziri, 2017; Talebi et al., 2017*), and India (*Ambulkar et al., 2016A*) reported that sperm mtDNAdel were more frequent among men classified as infertile based on abnormal semen parameters compared with men with normal semen parameters. Investigators of an Australian study, however, did not observe higher mtDNAdel among men with oligospermia or azoospermia compared with men with normospermia (*Cummins et al., 1998*). With individual semen parameters, two cross-sectional studies reported that mtDNAdel was inversely associated with sperm concentration (*Zhang et al., 2016*), count (*Song and Lewis, 2008; Zhang et al., 2016*) and motility (*Zhang et al., 2016*).

Overall, evidence to suggest that both sperm mtDNAcn and mtDNAdel are related to sperm health and overall male fertility is compelling. Previous studies, however, did not evaluate the potential of sperm mtDNAcn and mtDNAdel as diagnostic tools for male infertility in clinical populations. In addition, semen parameters are known to vary considerably within individuals. Men who may be classified as clinically 'infertile' based on one semen sample may have normal subsequent semen parameters owing to natural variation or lifestyle and behaviour changes. This 'transient' infertility status is likely to differ from that of males who have abnormal semen parameters in consecutive semen samples, as they likely have different underlying causes; however, no study to date has examined whether sperm mtDNAcn or mtDNAdel are measures of the current clinical fertility status, general long-term clinical fertility status across consecutive semen samples, or both. Therefore, our study addressed these two research gaps by examining the associations of sperm mtDNAcn and mtDNAdel with both current clinical fertility status and long-term, persistent, fertility status using consecutive semen samples, as well as evaluating sperm mtDNAcn and mtDNAdel as potential diagnostic tools for clinical infertility.

MATERIALS AND METHODS

Study population and sample collection

This study comprised a convenience sample of the male partners of 125 couples recruited between 2014 and 2016 at Baystate Reproductive Medicine in Springfield, Massachusetts as part of the Sperm Environmental Epigenetics and Development Study (SEEDS). Couples were recruited if male partners were aged between18 and 55 years old without vasectomy, female partners were aged 40 years or younger with expected delivery at Baystate Medical Center, and fresh ejaculate sperm was used for IVF treatment. Written consent from eligible participants who were interested in participating was obtained by attending physicians. This study was approved by the Institutional Review Boards at Baystate Medical Center on 26 September 2017 and at the University of Massachusetts Amherst (reference BH-12-190).

Semen samples were collected as part of the IVF protocol in a sterile polypropylene specimen cup after a recommended 2–3 days of abstinence. Semen samples were processed using a two-step (80% and 40%) gradient fractionation, which separates motile sperm from abnormal and non-motile sperm and somatic cells (*Henkel and Schill, 2003*). 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 after gradient fractionation were observed to be WBC free. DNA from the motile fraction of sperm was isolated using our previously published protocol (*Wu et al., 2015*). Briefly, sperm are 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) before carrying out silica-column purification of total sperm DNA via Qiagen AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany).

Sperm mtDNAcn and mtDNAdel measurements

A triplex probe-based quantitative polymerase chain reaction (PCR) assay, based on a previously published method (*Phillips et al., 2014*), was used to quantify sperm mtDNAcn and mtDNAdel. The minor arc of the mtDNA was targeted for mtDNAcn assessment due to its stability within the genome, lack of interaction with other targets, and high amplification efficiency. In contrast, the 4977bp 'common deletion' region within the major arc is known to have high deletion rates and a region within this common deletion was used to quantify the rate of mtDNAdel. For each 10-µl PCR reaction, 10 µg of DNA was amplified with final primer concentrations of 250 µM for both minor and major arc and 1x concentration for RNAse P (ThermoFisher, cat# 4403326). Primer sequences can be found in Supplementary **TABLE 1**. 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.

All reactions were conducted in triplicate on the StepOnePlus Real-Time PCR. The inter-assay coefficients of variations were 4.7% for mtDNAcn and 8.3% for mtDNAdel, whereas intra-assay coefficients of variation were 3.5% for mtDNAcn and 7.3% for mtDNAdel. To normalize mtDNAcn data, RNAse P, the standard reference assay for copy number analysis (Applied Biosystems # A30064), was used to determine a nuclear DNA (nDNA) copy number reference, and sperm mtDNAcn was calculated via the ratio of mtDNAcn (minor arc) to nDNA (RNAseP). Similarly, % mtDNAdel (major arc) was normalized to mtDNAcn using the following formulas: $\text{mtDNAcn} = 2^{(\text{Ct:RNAseP} - \text{Ct:MinorArc})}$ and $\text{mtDNAdel}(\%) = 2^{(\text{Ct:MinorArc} - \text{Ct:MajorArc})} * 100$.

Outcome assessment

Five 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. Male clinical infertility status was characterized by two end-points: current clinical infertility and consecutive diagnoses of clinical infertility. Current clinical infertility status was derived from the sperm motility, sperm concentration and normal morphology assessments using the collected semen sample from which mtDNA data were generated. An individual was classified as infertile if at least one of these three measures were below World Health Organization (WHO) reference levels (*Cooper et al., 2010*). Because all individuals had at least one semen analysis before the study, individuals were classified as having consecutive diagnoses of clinical infertility if both the previous semen sample(s) and the current semen sample both had at least one of the three aforementioned measures below WHO reference levels. In this consecutive diagnoses definition, individuals who had abnormal semen parameters in the current sample but not in their prior sample(s) were not considered to be clinically infertile.

Covariate assessment

Data on relevant demographics (race, age, height, weight), lifestyle factors (current and past alcohol and cigarette use), and medical history (history of clinical infertility) were collected by clinic personnel during the IVF cycle.

Statistical analyses

Characteristics of the study participants (n = 125) were summarized using means, standard deviation, and percentages as appropriate, including proportions falling below WHO reference values for infertility based on semen parameters (*Cooper et al., 2010*). Relationships among the mtDNAcn, mtDNAdel and semen analysis parameters, e.g., % normal morphology, sperm concentration, volume, sperm count and motility, were evaluated by Spearman rank correlation analysis. Bivariate analyses comparing participant characteristics by clinical infertility status (both current and consecutive diagnoses) and by quartile of mtDNAcn and mtDNAdel were conducted using Wilcoxon rank sum tests for continuous variables or Fisher's exact test for categorical variables, the results of which were used to aid specification of multivariable models.

For multivariable analyses of the relationships of mtDNA measures with semen analysis parameters, continuous mtDNAcn and mtDNAdel were divided into quartiles with the first quartile as the reference group by multiple linear regression. Results of these models are interpreted as mean differences (MD) in semen parameters comparing each quartile to the lowest quartile. Semen volume (ml), sperm concentration (millions/ml), total sperm count (millions), sperm motility (%), and normal morphology (%) were evaluated in these models.

Generalized linear models were used to evaluate relationships between

mtDNA measures, semen parameters, and current and consecutive infertility as binary outcomes. These linear risk models specified a binomial distribution and an identity link function and were used to estimate risk differences comparing quartiles of mtDNAcn and mtDNAdel, with the lowest quartile serving as the reference group. For all quartile analyses, *P*-values were calculated by fitting mtDNAcn and mtDNAdel as continuous variables. To control for potential confounding, age, body mass index (BMI), race (white versus non-white), alcohol use (ever/never), cigarette smoking (ever/never, current/non-current) and measurement batch (categorical, 1–5) were considered as potential covariates. Covariates were included in the model if they were associated ($P < 0.10$) with both exposure and outcomes. Bivariate analyses of covariates with mitochondrial measures and the two clinical infertility measures showed no statistically significant associations (Supplementary TABLE 2 and Supplementary TABLE 3) except between ever smoking and current clinical infertility ($P = 0.04$). Therefore, the adjusted generalized linear models shown include only age and measurement batch as covariates whereas the potential influence of ever smoking was explored as a sensitivity analysis.

The predictive ability of sperm mtDNAcn and mtDNAdel for classifying current infertility and consecutive diagnoses of clinical infertility was evaluated using receiver operating characteristic (ROC) curve analysis. These analyses evaluated the extent to which continuous mtDNA measures are able to discriminate outcome status. The ROC curves were made for illustrative purposes, and predictive ability was quantified using C-statistics (area under the ROC curve).

All analyses were conducted using R (v3.3.0, R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Demographics and lifestyle data and semen parameters of the semen sample also used for mtDA analyses, are shown in TABLE 1. The mean age and BMI of

TABLE 1 SELECTED DEMOGRAPHICS AND SEMEN PARAMETERS OF THE SPERM ENVIRONMENTAL EPIGENETICS AND DEVELOPMENT STUDY GROUP (*N* = 125)

Demographics		
	Mean	**SD**
Age	36.2	5.5
Body mass index	29.2	5.9
	Number	**%**
Smoking (ever)		
Yes	37	29.6
No	72	57.6
Missing	16	12.8
Smoking (current)		
Yes	8	6.4
No	97	77.6
Missing	20	16.0
Race[a]		
Non-Hispanic White	96	76.8
Other	13	10.4
Missing	16	12.8
Semen parameters[b]		
	Mean (SD)	**% <WHO Reference**[a]
Volume (ml)	2.9 (1.4)	18
Count (million)	194.0 (192.0)	12
Motility (%)	56.1 (20.6)	18
Concentration (million/ml)	75.8 (77.2)	10
% Normal morph	6.0 (4.4)	36
Mitchondrial DNA parameters[b]		
	Mean (SD)	**Range**
mtDNAcn	3.3 (4.1)	0.2–34.7
mtDNAdel	20.1 (9.3)	3.2–39.0%

mtDNAcn, mitchondrial DNA copy number; mtDNAdel, mitchondrial DNA deletion; WHO, World Health Organization.

[a] According to *Cooper et al., (2010)*.

[b] The semen sample from which mtDNA measures were taken.

the population were 36.2 ± 5.50 years and 29.2 ± 5.9, respectively. Most of the participants self-identified as non-Hispanic white (76.8%) and non-smoking (77.6% non-current, 57.6% never). Using WHO reference values, the per cent of the study population who had semen parameters below the 5th centile were 18% for semen volume, 12% for sperm count, 18% for sperm motility, 10% for sperm concentration and 36% for normal morphology.

The correlations between semen parameters and sperm mitochondrial measures are presented in FIGURE 1. Sperm count, sperm motility, sperm concentration, and % normal morphology were all positively correlated with each other (Spearman's rho (r) ranging from 0.30 to 0.80; $P < 0.05$). Semen volume was not correlated with either sperm motility or per cent normal morphology. Sperm mtDNAcn and mtDNAdel were positively correlated (Spearman's $r = 0.35$, $P < 0.001$) and were inversely correlated with all semen parameters (Spearman's r ranging from −0.24 to −0.52; $P < 0.05$), with the exception of semen volume.

Next, to replicate findings from previous publications (*Kao et al., 1995*; 1998; St *John et al., 2001; May-Panloup et al., 2003; Song and Lewis, 2008; Ieremiadou and Rodakis, 2009; Gholinezhad Chari et al., 2015; Ambulkar et al., 2016A; Ambulkar et al., 2016B; Zhang et al., 2016; Gabriel et al., 2012; Mughal et al., 2017; Bahrehmand Namaghi and Vaziri, 2017; Talebi et al., 2017*), we examined the associations of mtDNAcn and mtDNAdel with semen parameters. The mean difference (MD) and 95% confidence intervals (CI) from the age and batch adjusted generalized linear models comparing semen parameter values by quartiles of sperm mtDNAcn and mtDNAdel are presented in TABLE 2. mtDNAcn was inversely associated with sperm concentration ($P = 0.03$), count ($P = 0.01$), motility ($P < 0.001$) and morphology ($P < 0.01$) in a dose-dependent manner (TABLE 2). Compared with the lowest quartile, the highest quartile of mtDNAcn was associated with lower sperm concentration (MD = −53.45, 95% CI −94.17 to −12.73), count (MD = −90.97, 95% CI −188.18 to −6.24), motility (MD = −22.86, 95% CI −32.60 to −13.13), and morphology (MD = −3.00, 95% CI −5.34 to −0.65). Also, mtDNAdel was significantly associated with lower sperm concentration ($P < 0.001$), count ($P < 0.001$), motility ($P = 0.01$), and morphology ($P < 0.01$), although, unlike mtDNAcn, a clear dose–response relationship was not observed. In all models, the addition of ever smoking status, BMI, or race as covariates did not appreciably alter any effect estimates (data not shown).

After demonstrating that mtDNAcn and mtDNAdel were associated with individual semen parameters, we next assessed their relationships with current and consecutive diagnoses of clinical infertility as determined by WHO reference levels for semen parameters. The distribution of clinical infertility diagnoses and the estimated risk differences by quartiles of mtDNAcn and mtDNAdel from generalized linear risk models of current and consecutive diagnoses of clinical infertility are presented in TABLE 3. Adjusted for age and measurement batch, there was a clear and statistically significant higher risk of current clinical infertility, as defined by a single semen sample, associated with mtDNAcn ($P < 0.001$) and mtDNAdel ($P = 0.04$). Additional analyses using the criterion of having two or more parameters below WHO cut-off to define clinical infertility showed similar results (results not shown). The results were even more striking for consecutive diagnoses of clinical infertility, defined by multiple semen samples. For example, none of the individuals classified as having consecutive diagnoses of clinical infertility were in the lowest quartile of mtDNAcn, whereas 16 out of 30 individuals in the highest quartile of mtDNAcn had consecutive diagnoses of clinical infertility. Compared with those in the lowest quartile, those in the highest quartiles of mtDNAcn and mtDNAdel had 47% (95% CI 26% to 69%) and 22% (95% CI 3% to 40%) increase in risk of consecutive diagnoses of clinical infertility, respectively. When individuals who had consecutive clinical infertility diagnoses were excluded, mtDNAcn and mtDNAdel were no longer associated with the remaining cases of current clinical infertility (Supplementary TABLE 4).

To establish the utility of mtDNAcn and mtDNAdel as predictors of clinical male infertility, we first compared the distribution of mtDNAcn and mtDNAdel by the two clinical infertility definitions. As shown in Supplementary TABLE 5, clear group differences exist in the distribution of mtDNAcn, with those who are diagnosed as infertile having generally higher values; a similar pattern was observed for mtDNAdel. Next, we calculated ROC curves and accompanying C-statistics (FIGURE 2). Both mtDNAcn and mtDNAdel demonstrate high predictive ability for consecutive diagnoses of clinical infertility, with C-statistic values of 0.91 and 0.75, respectively. Interestingly, the predictive value of mtDNAcn on consecutive diagnoses of clinical infertility is comparable or better than that of

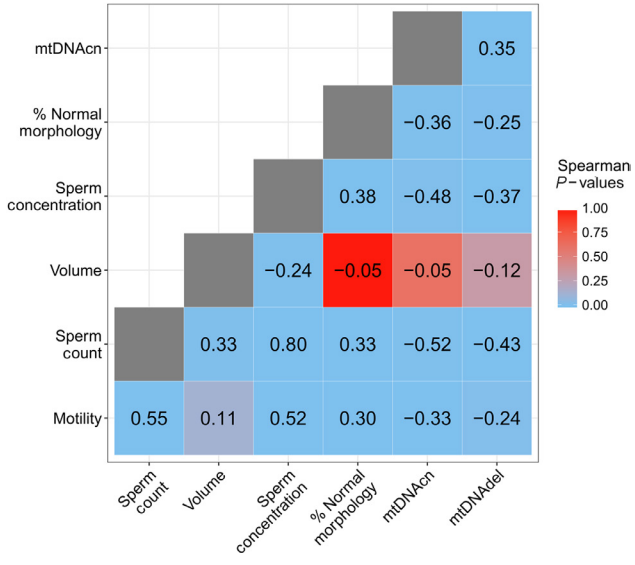

FIGURE 1 Correlation matrix of semen parameters, sperm mitochondrial DNA copy number (mtDNAcn), and sperm mitochondrial DNA deletions (mtDNAdel). The values in the figure show the coefficient estimates (rho) from Spearman correlation analyses and *P*-values for each estimate indicated by colour.

TABLE 2 GENERALIZED LINEAR MODEL RESULTS OF SEMEN QUALITY PARAMETERS FROM 119 PARTICIPANTS FROM THE SPERM ENVIRONMENTAL EPIGENETICS AND DEVELOPMENT STUDY BY QUARTILES OF SPERM MITOCHONDRIAL DNA COPY NUMBER AND MITOCHONDRIAL DNA DELETIONS

		Concentration (millions/ml)			Count (millions)			Motility (%)			Volume (ml)			Strict normal morphology (%)		
		MD	***95% CI***	***P*-value**	**MD**	**95% CI**	***P*-value**	**MD**	**95% CI**		**MD**	**95% CI**	***P*-value**	**MD**	**95% CI**	***P*-value**
mtDNAcn	Media, *n*															
Q1	0.95	Reference			Reference			Reference			Reference			Reference		
Q2	1.61	−4.90	−44.08 to 34.28	NS	111.71	18.17 to 205.24	0.02	−0.94	−10.30 to 8.42	NS	0.93	0.26 to 1.59	0.01	0.25	−2.01, 2.52	NS
Q3	2.47	−16.84	−56.60 to 22.91	NS	−33.49	−128.39 to 61.40	NS	3.35	−12.85 to 6.15	NS	-0.06	−0.73 to 0.62	NS	−1.28	−3.62, 1.07	NS
Q4	6.29	−53.45	−94.17 to −12.73	0.01	-90.97	−188.18 to 6.24	0.07	−22.86	−32.6 to −13.13	<0.001	0.28	−0.41 to 0.97	NS	−3.00	−5.34 to −0.65	0.01
P-value				0.03			0.01			<0.001			NS			<0.01
mtDNAdel																
Q1	7.44	Reference			Reference			Reference			Reference			Reference		
Q2	17.07	−80.63	−115.46 to −45.79	<0.001	−179.43	−266.84 to −92.03	<0.001	−11.27	−21.23 to −1.31	0.03	0.13	-0.55 to 0.81	NS	−0.94	−3.17 to 1.29	NS
Q3	23.80	−92.87	−128.08 to −57.67	<0.001	−224.06	−312.39 to −135.72	<0.001	−9.64	−19.71 to 0.42	0.06	0.09	-0.60 to 0.78	NS	−3.12	-5.36 to −0.88	0.01
Q4	31.78	−77.48	−113.12 to −41.83	<0.001	−209.7	−299.13 to −120.26	<0.001	−13.69	−23.88 to −3.50	0.01	-0.18	-0.87 to 0.52	NS	−2.82	−5.05 to −0.58	0.02
P-value				<0.001			<0.001			0.01			NS			<0.01

MD, mean difference; mtDNAcn, mitochondrial DNA copy number; mtDNAdel.

All models were adjusted for age and measurement batch. Race, smoking status and body mass index were also assessed, but these did not meaningfully change the effect estimates.

TABLE 3 RISK DIFFERENCE MODELS OF INFERTILITY BY QUARTILES OF SPERM MITOCHONDRIAL DNA COPY NUMBER AND SPERM MITOCHONDRIAL DNA DELETIONS

	Current infertility[a]			
	Fertile, *n*	**Infertile, *n***	**Risk difference[b] (95% CI)**	**Adjusted risk difference[c] (95% CI)**
mtDNAcn				
Q1	18	10	Reference	Reference
Q2	22	8	0.07 (–0.06 to 0.20)	–0.10 (–0.37 to 0.18)
Q3	16	12	0.21 (0.02 to 0.40)	0.10 (–0.22 to 0.41)
Q4	8	22	0.53 (0.31 to 0.75)	0.42 (0.14 to 0.71)
P-value				<0.001
mtDNAdel				
Q1	20	9	Reference	Reference
Q2	17	12	0.10 (–0.19 to 0.39)	0.11 (–0.19 to 0.42)
Q3	12	16	0.26 (–0.03 to 0.56)	0.26 (–0.04 to 0.56)
Q4	15	15	0.19 (–0.10 to 0.48)	0.23 (–0.08 to 0.53)
P-value				0.04
	Persistent infertility[d]			
	Fertile (n)	**Infertile (n)**	**Risk difference[b] (95% CI)**	**Adjusted risk difference[c] (95% CI)**
mtDNAcn				
Q1	30	0	Reference	Reference
Q2	28	2	–0.10 (–0.39 to 0.20)	0.03 (0 to 0.06)
Q3	26	3	0.18 (–0.13 to 0.49)	0.10 (0 to 0.21)
Q4	14	16	0.37 (0.10 to 0.64)	0.47 (0.26 to 0.69)
P-value				<0.001
mtDNAdel				
Q1	29	1	Reference	Reference
Q2	27	3	0.07 (–0.03 to 0.16)	0.06 (–0.02 to 0.13)
Q3	20	9	0.28 (0.08 to 0.47)	0.25 (0.06 to 0.45)
Q4	22	8	0.23 (0.05 to 0.41)	0.22 (0.03 to 0.4)
P-value				<0.001

mtDNAcn, mitochondrial DNA copy number; mtDNAdel, sperm mitochondrial DNA deletions.

[a] Infertility status based solely on the collected semen sample, which was also used for mitochondrial DNA measurements.

[b] Adjusted for age and measurement batch; also assessed body mass index, smoking status and race, but these did not meaningfully change the effect estimates.

[c] Infertility status based on several samples over time, including the collected semen sample as well as previous semen samples.

[d] Crude risk difference.

sperm concentration (C-statistic = 0.88), motility (C-statistic = 0.92), and morphology (C-statistic = 0.82) from the current semen sample. A composite model of both mtDNAcn and mtDNAdel (C-statistic = 0.91) did not demonstrate better predictive ability than mtDNAcn alone.

DISCUSSION

In this cross-sectional analysis of 125 men, we observed that sperm mtDNAcn and mtDNAdel were associated with markedly poorer semen parameters and higher risk of both current and consecutive diagnoses of clinical fertility. In addition, we found that both sperm mtDNAcn and mtDNAdel are accurate predictors of consecutive diagnoses of clinical infertility based on multiple semen samples. In ROC curve analysis, sperm mtDNAdel provided minimal to no incremental predictive ability to that of sperm mtDNAcn, suggesting that sperm mtDNAcn is the stronger predictor of the two, and comparable to semen parameters from which clinical infertility diagnoses are currently derived.

In the present study, although sperm mtDNAcn and mtDNAdel were associated with both current and consecutive diagnoses of clinical infertility, the associations with consecutive diagnoses of clinical infertility were stronger than that of current clinical infertility. For example, 16 out of the 21 cases of consecutive diagnoses of clinical infertility were in the highest quartile of sperm mtDNAcn, whereas 17 out of 21 were in top one-half of mtDNAdel. Once the consecutive diagnoses of clinical infertility cases were excluded, sperm mtDNAcn and mtDNAdel were no longer associated with increased risk of current clinical infertility, further demonstrating that sperm mtDNAcn and mtDNAdel

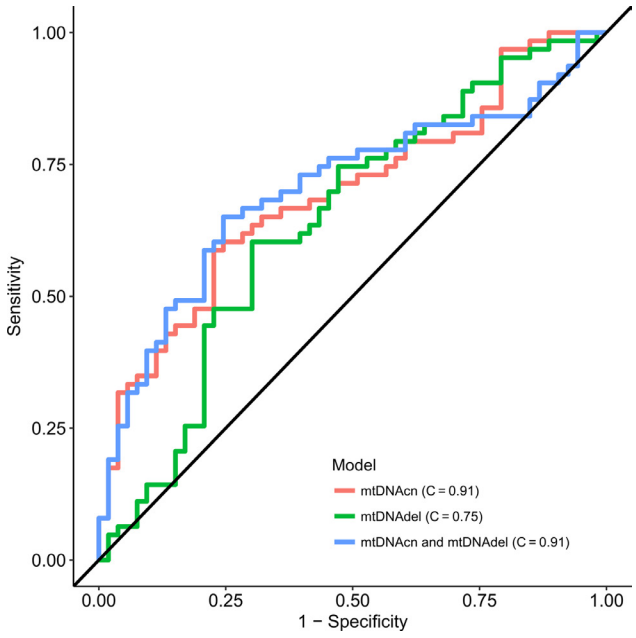

FIGURE 2 Receiver operating characteristic curves and accompanying C-statistics of sperm mitochondrial DNA copy number (mtDNAcn) and deletions (mtDNAdel) and consecutive diagnoses of clinical infertility. The curves and C-statistics were generated from logistic regression models with mtDNAcn, mtDNAdel, or both mtDNAcn and mtDNAdel as independent predictors and consecutive diagnoses of clinical infertility as outcome.

may be better suited as measures of consecutive diagnoses of clinical infertility rather than of current clinical infertility status. These results suggest that cases of current clinical infertility in the lower quartiles of sperm mtDNAcn and mtDNAdel did not have a history of poor semen samples. Those with current diagnoses of clinical infertility but without a history of poor semen parameters could have been cases of subfertility, natural variation, or lifestyle and behaviour changes that led to transiently abnormal semen parameters. In contrast to semen parameters, which have transient variation over time, the results of this study suggest that sperm mtDNAcn and mtDNAdel may be relatively stable measures of general long-term infertility status.

The underlying biological relationships among mtDNA measures, semen parameters and infertility are unclear. It is unknown whether sperm mtDNAcn and mtDNAdel are causal of poor semen parameters and increased risk of clinical infertility or if these associations are the result of some other factor that affects these measures. There are several possible biological explanations for the observed associations between poor semen parameters and elevated sperm mtDNAcn or mtDNAdel. First, higher sperm mtDNAcn can result from a compensatory feedback response that results in proliferation as a result of defective fragmented or mutated mtDNA (*Lee et al., 2000; Andreu et al., 2009*). Therefore, sperm mtDNAcn in our study may have been a proxy of sperm mtDNA integrity or some other general indicator of poor sperm mitochondrial quality. Second, because mtDNA depletion occurs during spermatogenesis (*Luo et al., 2013*), higher sperm mtDNAcn in infertile men may reflect abnormalities during spermatogenesis such as aberrant gene expression of *TFAM and POLG*, which are known regulators of mtDNAcn (*Amaral et al., 2007*). For example, DNA methylation of the CpG island of exon 2 of POLG has been shown to regulate mtDNAcn during cell differentiation in mice (*Kelly et al., 2012*) and in human stem and cancer cells (*Amaral et al., 2007; Lee et al., 2015*); however, a recent study reported no such relationship in human differentiated cells (*Steffann et al., 2017*). Thus, aberrant DNA methylation, gene expression of TFAM and POLG, or both, may result in higher sperm mtDNAcn observed in infertile men. Alternatively, oxidative stress has been linked to sperm mtDNAcn and mtDNAdel (*Abasalt et al., 2013; Bonanno et al., 2016*) as well as sperm parameters (*Agarwal et al., 2014; Aitken et al., 2014*). Therefore, sperm mtDNAcn and mtDNAdel may be indicative of an imbalance of redox signalling during spermatogenesis.

The results of our study are consistent with previous reports that sperm motility, concentration, count and morphology are associated with higher sperm mtDNAcn (*May-Panloup et al., 2003; Song and Lewis, 2008; Zhang et al., 2016*). Similarly, our findings on the associations of mtDNAdel with semen parameters and male clinical infertility diagnoses in a population of men seeking clinical fertility consultation in western Massachusetts are consistent with most previous reports comparing semen motility fractions (*Kao et al., 1995; Kao et al., 1998; Ieremiadou and Rodakis 2009; Gholinezhad Chari et al., 2015; Ambulkar et al., 2016a; 2016b*), individuals with varying semen parameters (*Song and Lewis, 2008; Zhang et al., 2016*), and samples from men of varying fertility diagnoses (*Kao et al., 1995; Kao et al., 1998; Song and Lewis, 2008; Ambulkar et al., 2016a; Bahrehmand Namaghi and Vaziri, 2017; Mughal et al., 2017; Talebi et al., 2018*).

To our knowledge, our study was the first to assess the predictive value of sperm mtDNAcn and mtDNAdel as diagnostic tests for clinical infertility. In addition, our study has several strengths. First, mtDNAdel was measured via probe-based quantitative PCR to target the *ND4* gene that resides within the 'common' 4977bp deletion. This approach overcomes the technical challenges of quantifying the 4977bp deletion via long PCR (St *John et al., 2001*) and allows for the normalization of the per cent of mtDNAdel by simultaneously assessing mtDNAcn in the same PCR reaction. Second, in our study, semen samples were processed via a two-step gradient fractionation protocol to enrich the motile fraction of sperm and to remove somatic cell contamination (*Henkel and Schill, 2003*). Spermatozoa possess a low number of mtDNA compared with somatic cells; therefore, any residual contamination of somatic cells in the final sperm population could affect the accurate quantification of sperm mtDNA measures. Third, previous studies conducted aong western populations (*May-Panloup et al., 2003; Song and Lewis, 2008; Gabriel et al., 2012*) were small clinical studies with limited scope

and sample size and none assessed the potential influences of demographic or lifestyle. Our study comprised a larger and more diverse population and was able to collect demographic and lifestyle data to assess their influence on the observed relationships. Additionally, the previous studies that examined sperm mtDNA measures and infertility often included only individuals with specific known diagnoses such as varicocele or oligoasthenozoospermia (Gabriel, 2012; *Ambulkar et al., 2016a; 2016b; Mughal et al., 2017; Bahrehmand Namaghi and Vaziri, 2017; Talebi et al., 2017*). In contrast, our study comprised a broader range of individuals, including all those who would be classified as infertile under WHO standards. Lastly, our study considered both current clinical fertility status as defined by a single semen sample as well as a more long-term general fertility status defined by multiple semen samples.

We also recognize some limitations of our study. First, our sample size only included 125 participants. Although we were able to provide evidence that sperm mtDNAcn could be a useful diagnostic measure, we have limited ability to derive a meaningful cut-off value as our population size was limited for a subsequent validation study. Second, our population, like most other studies examining sperm mtDNA measures and male fertility, was recruited from an IVF clinic and may not be generalizable to the broader general population. Our findings, however, are in line with those from a cross-sectional study of young Chinese men recruited from the general population (*Zhang et al., 2016*). Therefore, sperm mtDNAcn, either alone or in combination with sperm mtDNAdel may have a diagnostic potential in IVF settings as well as in the general population. Third, we have no data on mtDNA haplogroups, which are maternally inherited and have been associated with semen parameters in studies from Spain (*Ruiz-Pesini et al., 2000*) and China (*Feng et al., 2013*). Therefore, residual confounding is possible in this scenario where mtDNA haplogroups influence both semen parameters and mtDNA characteristics such as copy number and deletion. This would not, however, alter the predictive ability of mtDNA measures for persistent clinical male factor infertility. It is also worth noting that the relationships between mtDNA haplotype and sperm mtDNAcn and mtDNAdel are unknown. Lastly, sperm mtDNAcn and mtDNAdel were measured from sperm obtained after a two-step gradient (40% and 80%) centrifugation, which removes much of the abnormal and immotile sperm. Thus, it is unknown whether the magnitude of the results would be different when using sperm from combined 40% and 80% fractions. The use of the motile (80%) fraction of spermatozoa, however, demonstrates that changes in mtDNAcn and mtDNAdel are also detected in sperm with high fertilization capacity.

In conclusion, consistent with previous studies, our study of 125 men recruited from an IVF setting show that sperm mtDNAcn and mtDNAdel are clearly associated with diminished semen parameters and a markedly increased risk of male clinical infertility. In addition, our study also demonstrates that sperm mtDNAcn and mtDNAdel are useful predictors of consecutive diagnoses of clinical infertility. Finally, sperm mtDNAcn alone has comparable predictive performance of infertility diagnosis compared with semen parameters. Our study contributes to the existing literature that details the association between mtDNA measures and male clinical infertility. The influence of sperm mtDNA measures on IVF outcomes. However, is unknown. Therefore, future studies are needed determine if sperm mtDNA measures are related to fertilization rates, embryo quality and other pregnancy outcomes.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2018.10.004.

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