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SYSTEMATIC REVIEW

Sperm mitochondrial DNA copy numbers in normal and abnormal semen analysis: A systematic review and meta-analysis

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Abstract

Background: Normal mature sperm have a considerably reduced number of mitochondria, which provide the energy required for progressive sperm motility. Literature suggests that disorders of sperm motility may be linked to abnormal sperm mitochondrial number and function.

Objectives: To summarise the evidence from literature regarding the association of mitochondrial DNA copy numbers and semen quality with a particular emphasis on the sperm motility.

Search strategy: Standard methodology recommended by Cochrane.

Selection criteria: All published primary research reporting on the association between mitochondrial DNA copy numbers and semen quality.

Data collection and analysis: Using standard methodology recommended by Cochrane we pooled results using a random effects model and the findings were reported as a standardised mean difference.

Main results: We included ten studies. The primary outcome was sperm mitochondrial DNA copy numbers. A meta-analysis including five studies showed significantly higher mitochondrial DNA copy numbers in abnormal semen analysis compared with normal semen analysis (standardised mean difference 1.08, 95% CI 0.74–1.43). Seven studies included in the meta-analysis showed a significant negative correlation between mitochondrial DNA copy numbers and semen parameters. The quality of evidence was assessed as good to very good in 60% of studies.

Conclusions: Our review demonstrates significantly higher mitochondrial DNA in human sperm cells of men with abnormal semen analysis in comparison to men with normal semen analysis.

KEYWORDS
abnormal semen parameters, mitochondrial DNA, sperm motility

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PROSPERO registration: CRD42019118841

Tweetable abstract: There is significantly higher mitochondrial DNA in sperm cells of men with abnormal semen analysis in comparison to men with normal semen analysis.

1 | INTRODUCTION

Mitochondria are one of the fundamental cell organelles providing the cell with energy in the form of adenosine triphosphate by the process of oxidative phosphorylation. The amount of mitochondria varies with cell type and function.[1] In sperm cells, the process of spermatogenesis results in a drastic decrease in the number of mitochondria.[2] This reduction is aimed to reduce/eliminate paternal mitochondrial DNA (mtDNA) transmission, resulting in uniparental inheritance. Mature sperm are thought to contain only 22–75 mitochondria. These provide the energy by oxidative phosphorylation for progressive sperm motility and other functions such as capacitation, hyperactivation, acrosome reaction and oocyte penetration.[3,4] Mitochondria contain their own DNA which codes for specific proteins involved in the respiratory chain.

It has been suggested that male infertility and disorders of sperm function may be linked to abnormalities of sperm mitochondria or mtDNA. Male infertility has been reported in men with mitochondrial disorders.[5] Also, associations between abnormalities of sperm mtDNA and abnormal sperm parameters have been reported.[6] Early reports available on mtDNA quantification in mammalian sperm present widely varying results.[2,7] In humans, few studies report the association of mtDNA copy number (mtDNAcn) with sperm motility and other semen characteristics.[8–10] A recently published narrative review highlights the important role of mitochondrial number, structure and function in male reproductive function.[11] However, no systematic review has reported on the effect of mtDNA copy numbers on sperm quality and function.

The aim of this review is to summarise the evidence from literature regarding the association of mtDNAcn and semen quality with a particular emphasis on sperm motility. This aims to guide clinical practice and provide direction for future research.

2 | MATERIALS AND METHODS

2.1 | Eligibility criteria

Our search aimed to identify all published literature reporting the association between mtDNAcn and semen quality. All types of studies published as primary research were included for the review. We included only those studies published in the English language, published as full manuscripts (not abstracts) and those involving humans only. We included studies where semen samples were analysed based on either the World Health Organization (WHO) 1999 or 2010 criteria. The methodology for undertaking the review was developed following the recommendations of the Centre for Reviews and Dissemination's guidance for undertaking reviews in health care.[12] Results were reported in accordance with PRISMA guidelines.[13] The review was prospectively registered with PROSPERO (CRD42019118841).

2.2 | Assessment of study quality and the risk of bias

Assessment of study quality was performed using the Newcastle–Ottawa Scale modified for cross-sectional studies. Further modification was used as only non-interventional observational studies were included. We conducted a comprehensive search for eligible studies to minimise the impact of reporting bias.

2.3 | Main outcome measures

The primary outcome measure was sperm mtDNA copy numbers.

2.4 | Data sources

DP and FD independently screened and identified studies that were relevant for the review. Standard Cochrane methodology was followed comprising electronic searches and hand searching. Embase Classic and Ovid MEDLINE were searched on 7 December 2020. The study period was from 1946 to 2020. We used the controlled vocabulary of Medical Subject Headings (MeSH) terms 'Male Infertility' and 17 additional keywords related to or describing the participants and/or outcome (e.g. asthenospermia, oligospermia, sperm quality). The detailed search strategy for MEDLINE and Embase can be found in Appendix S1. We updated our search by re-conducting the search 1 month before submission of the review for publication. The reference lists of relevant articles were screened to identify additional studies.

2.5 | Data collection

DP and FD independently screened the title, abstract and keywords (ti, ab, kw) of the retrieved articles. The full text

of potentially suitable articles was retrieved. From these, suitable articles were finalised for inclusion for the review. Agreement regarding potential relevance was reached by consensus. Inconsistencies were discussed among the reviewers and resolved by discussion with a third author. The only conference abstract retrieved was excluded from the review to avoid publication bias. The authors of the excluded conference abstract were contacted to get information concerning whether the abstract was finally published. Unfortunately, the authors did not respond.

DP and FD reviewed all selected articles and extracted relevant data regarding study characteristics independently. Data were collected on a bespoke data collection Excel sheet where data were collected for study design, methodology, participant characteristics and outcome variables. Multiple publications of a single study were pooled together under a single study ID. All identified references were exported to EndNote X 8.2 for Windows, where the list of publications was scanned for duplicates.

2.6 | Data analysis and synthesis

The pooled estimates for the outcome were presented as Standardised Mean Difference (SMD) with 95% CI using the random effects model and inverse variance method. Statistical significance was assumed when values of p were less than 0.05. If the information in the studies was not reported in the method appropriate for our data extraction, the authors were contacted. We conducted a subgroup analysis for studies using the WHO 1999 and WHO 2010 semen analysis criteria. Studies were excluded from meta-analyses if the data were presented using correlation analyses and without dividing the semen of patients into categories (normal/abnormal) or using different laboratory methodology. The results from these studies were presented as a narrative text.

3 | RESULTS

3.1 | General characteristics of studies

3.1.1 | Results of the search

The search of the two electronic databases retrieved 373 full-text articles after removal of duplicates. No further articles were retrieved by hand searching of the reference lists. After screening of the titles and abstracts, the full text of 19 studies were retrieved for further review. Ten of these studies were selected for the systematic review and nine were excluded. Of the ten selected studies, five were suitable for meta-analysis and were included for quantitative synthesis. The search and selection processes are documented with a PRISMA flow chart in Figure 1 and the list of included and excluded studies with reasons for exclusion is provided in Table S1.

3.1.2 | Included studies

The characteristics of the included studies are detailed in Table 1.

3.1.3 | Study design and setting

The ten studies included in this systematic review were all single-centre observational cross-sectional studies conducted across eight countries. Only five studies had a sample size of more than 100 participants, which we feel is satisfactory for providing good-quality evidence. The largest study was conducted by Diez-Sanchez et al.[14] from Spain and included 440 participants.

3.1.4 | Participants

Eight of the ten studies recruited participants from fertility clinics, denoting a convenience sampling strategy, with only one of these studies recruiting healthy volunteers as controls. Two studies recruited volunteer donors for their studies. Only five of the ten studies accounted for confounding factors such as age, body mass index and lifestyle factors in the design or analysis stage of their studies. Hence, the comparability of the participants in the included studies or within study groups cannot be estimated. The study group for five of the ten studies included in the meta-analyses comprised men with abnormal semen analysis. The criteria for abnormal semen analysis, however, showed significant heterogeneity. Some studies reported results based on the WHO 1999 criteria whereas others used the WHO 2010 criteria. Some studies included men with only reduced sperm motility and normal sperm counts as the abnormal semen analysis for the study group. Few studies divided the abnormal results into subgroups; however, these were dissimilar among the studies so it was not possible to conduct a subgroup analysis for a pooled estimate.

3.1.5 | Outcome

All studies reported the mtDNA/nuclear DNA ratio expressing the average mtDNAcn per sperm. The values for the ratio variables differed considerably between the studies, which might be explained by the methodological differences in interventions. The concept however remained constant across the studies. The ratios were either compared between patients with normal and abnormal WHO semen criteria or correlated to sperm parameters for the entire data set. Two studies compared mtDNA content between sperm cells from the same semen sample in addition to mtDNA content from the different patients.[8,14]

Two studies[14,15] have been excluded from the analysis because of different methodology to amplify the nuclear DNA and mtDNA, such as a hot-start concurrent polymerase

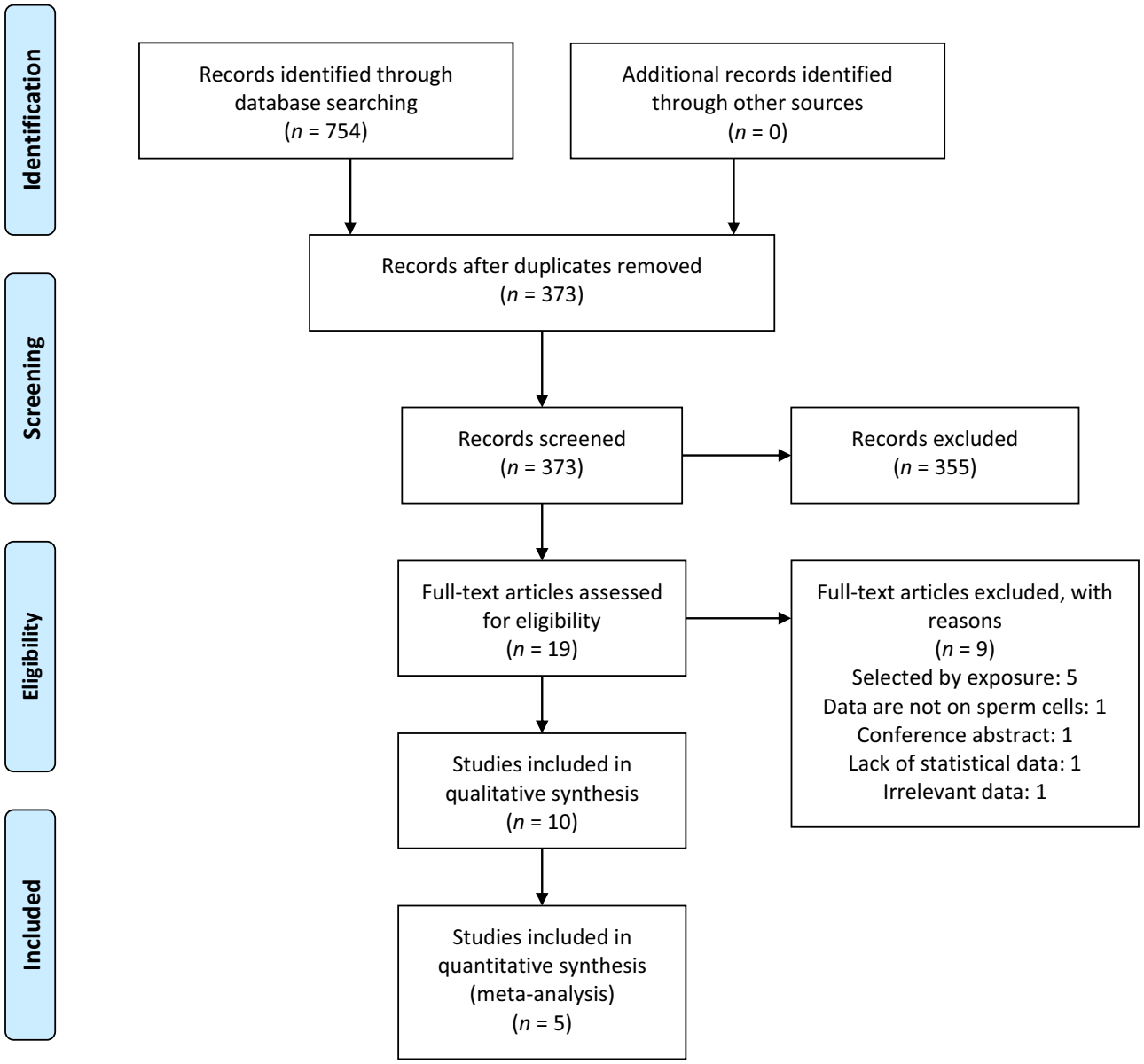

FIGURE 1 PRISMA flow chart. *From:* Moher et al.[13]

chain reaction (PCR) and slot-blot hybridisation, respectively. All studies using real-time PCR (quantitative PCR) to determine the amount of mtDNA relative to nuclear DNA have been included.

Seven studies compared the outcome between groups. Of these, five studies reported the primary outcome as a mean ± SD/SEM. Two studies reported the median + interquartile range /range. One study, which reported the mean without an SD, had to be excluded from the meta-analysis.[9] We contacted authors of four studies for additional and/or missing data. We were able to get this information for the study by Tian et al.,[10] and have updated our analysis accordingly. Seven studies reported the correlation between sperm mtDNA with sperm parameters across the entire data set rather than differences among defined groups with normal and abnormal semen parameters.[9,10,14,16–19] Two studies used a different methodology for estimation of DNA.[14,15] These studies were not included in the meta-analysis.

3.1.6 | Assessment of outcome

The method of mtDNAcn assessment is a multistep process and varied among studies. The time range between the first study and the last was 16 years, which may have an impact on the technical differences between the first and last experiments.

Before mtDNA extraction and quantification, semen samples were purified to remove non-sperm cell content using various techniques. Six of the ten studies used sperm washing techniques,[8,14,16,17,19,20] whereas two studies used osmotic shock.[15,18] One study used both.[15] The study by Tian et al.[10] applied experiments on cryopreserved sperm

TABLE 1 Characteristics of studies included in the (a) meta-analysis (b) review (excluded from the meta-analysis)

No.	Author/Year	Study design	Methods of mtDNAcn assessment	Participants			Comparison groups (number of participants)	Outcomes reported	Results (mean (SD) mtDNAcn per sperm)
				Number	Inclusion criteria	Exclusion criteria			
(a)									
1	May-Panloup/2003	Single centre, observational/cross-sectional study Country: France Dates of recruitment: Not available	qPCR	67	Couples who presented for semen analysis or ART and suffered from either male or female infertility, or infertility of unknown aetiology.	Not available	1: Semen analysis normal (WHO 1999) (32) 2: Semen analysis abnormal (35) 2a: Abnormal with 1 criterion (14) 2b: Abnormal with ≥2 criteria (21)	mtDNA/b-globin ratio, expressing the average copy number per haploid genome or per sperm the nucleotide positions of the primers on the light strand mtDNA: D41 (3254–3277) and D56 (3126–3147)	1: Semen analysis normal = 1.4 (0.98) 2: Semen analysis abnormal = 6.1 (6.88)
2	Amaral/2007	Single centre, observational/cross-sectional study Country: Portugal Dates of recruitment: Not available	qPCR	42	Participants undergoing routine semen analysis or fertility treatment involving both in vitro fertilisation and intracytoplasmatic sperm injection	Not available	1: Semen analysis normal (WHO 1999) (14) 2: Semen analysis abnormal (28) 2a: Abnormal with 1 or 2 criteria (14) 2b: Abnormal with >2 criteria (14)	mtDNA/b-globin ratio the nucleotide positions of the primers on the light strand mtDNA: D41 (3254–3277) and D56 (3126–3147)	1: Semen analysis normal = 6.8 (6.73) 2: Semen analysis abnormal = 29 (44.47)
3	Bonanno/2016	Single centre, observational/cross-sectional study Country: Italy Dates of recruitment: Not available	qPCR	59	Patients: Men who underwent semen analysis as a part of their fertility evaluation and whose sperm showed arbitrary progressive motility ($a + b \leq 20\%$) and non-progressive motility ($c > 50\%$). Those included in the study were diagnosed with idiopathic asthenozoospermia, clinical palpable varicocele, inflammation of the accessory sex glands or as overweight following physical examination and history taking. Controls: healthy men with normal sperm parameters (according to the *WHO 2010 guidelines*) whose fertility status was unknown and who volunteered to participate in the study	Patients: smokers, patients with known exposure to toxic chemicals, alcohol intake or drug abuse, systemic diseases, and recent hormonal treatment. Controls: cigarette smoking, history of cryptorchidism and varicocele, known exposure to toxic chemicals and presence of genital inflammation	1: Semen analysis with normal progressive motility (WHO 2010) (22) 2: Semen analysis with abnormal progressive motility (37) All participants had normal sperm concentrations	mtDNA 16S rRNA/GAPDH ratio	1: Semen analysis with normal progressive motility = 5.69 (2.33) 2: Semen analysis with abnormal progressive motility = 18.21 (14.12)

(Continues)

TABLE 1 (Continued)

No.	Author/Year	Study design	Methods of mtDNAcn assessment	Participants			Comparison groups (number of participants)	Outcomes reported	Results (mean (SD) mtDNAcn per sperm)
				Number	Inclusion criteria	Exclusion criteria			
4	Tian/2014	Single centre, observational/cross-sectional study Country: China Dates of recruitment: July 2009 to August 2010	qPCR	118	Male partners of couples who were undergoing fertility assessment because of an idiopathic inability to conceive	Not available	1: semen analysis with normal count (WHO 2010) (115) 2: semen analysis with reduced count (3) 3: semen analysis with normal motility (86) 2: semen analysis with reduced motility (32)	mtDNA MTF3212/R3319 gene/ACTB(ß-ACTIN) ratio	1: semen analysis with normal count/motility = 16.77 (3.74) 2: semen analysis with reduced count/motility = 103.11 (138.13)
5	Faja/2019	Single centre, observational/cohort study Country: Italy Dates of recruitment: Not available	qPCR	126	Men attending the andrological laboratory for preconception screening	Use of any medication (antibiotics, anabolic hormones), and/or the presence of medical conditions associated with impaired semen parameters (endocrine diseases, testicular trauma, urogenital tract infection, varicocele, cryptorchidism, testicular or other cancer, previous chemotherapy and/or radiotherapy, Klinefelter syndrome and other chromosome abnormalities or genetic syndromes). Leucocytospermia and/or increased viscosity	1: Group A with progressive motility <32% (asthenozoospermic samples) (63) 2: Group N with progressive motility ≥32% (normal sperm motility) (63)	Relative mtDNA content (COII – Cytochrome C oxidase) was obtained by calculating the cycle threshold $\Delta C_t = C_t COII - C_t$ calicin, and applying the exponential function $2-\Delta C_t$	1: semen analysis with normal sperm motility = 29.6 (23.3) 2: semen analysis with reduced sperm motility = 73 (57.6)
(b)									
6	Song/2008	Single centre, observational/cross-sectional study Country: USA Dates of recruitment: Not available	qPCR	57	Men whose wives were undergoing in vitro fertilisation. This included men with normal sperm count and motility and men with abnormal semen parameters (<20 million/ml or<50%motility)	Men with leucospermia (>1 million/ml)	1: Semen analysis showing normal count and motility (WHO 1999) (24) 2: Semen analysis abnormal (33) 2a: Abnormal motility (19) 2b: Oligozoospermia (3) 2c: OATS (11)	mtDNA 16S rRNA/GAPDH ratio	Average mtDNAcn was 15.7, 34.3, 56.7, and 73.7 in the patients with normal semen parameters, asthenozoospermia, oligozoospermia,and OATS, respectively. mtDNAcn was negatively correlated with sperm count ($r = -0.561$; $p < 0.01$).

TABLE 1 (Continued)

No.	Author/Year	Study design	Methods of mtDNAcn assessment	Participants			Comparison groups (number of participants)	Outcomes reported	Results (mean (SD) mtDNAcn per sperm)
				Number	Inclusion criteria	Exclusion criteria			
7	Kao/2004	Single centre, observational/cross-sectional study Country: Taiwan Dates of recruitment: Not available	hot-start concurrent PCR	86	Men who sought infertility therapy	Leucospermia and viscous semen	1: Normal semen analysis (WHO 1999) (29) 2: Sperm with poor motility (23)	Relative content of mtDNA _ (area of the ND1 band/ area of the _-actin band) × (315/450)	A decrease in sperm mtDNA content was detected in patients with poor sperm motility. The mean (SD) mtDNA was 1: Normal semen analysis = 74.1 (2.0) 2: Abnormal semen analysis = 7.2 (1.3)
8	Wu/2018	Single centre, observational/cross-sectional study Country: USA Dates of recruitment: 2014–2016	A triplex probe-based qPCR	125	Male partners 18–55 years. recruited as part of the Sperm Environmental Epigenetics and Development Study (SEEDS)	Men with vasectomy	Four quartiles of sperm mtDNAcn compared with semen analysis parameters (WHO 2010)	ratio of mtDNAcn (minor arc) to nDNA (RNAseP)	Sperm mtDNAcn was negatively correlated with sperm concentration, count, motility and morphology ($r = -0.24$ to -0.52, $p < 0.05$). Sperm mtDNA was also positively associated with a diagnosis of clinical infertilit9
9	Zhang/2016	Single centre, observational/ cohort study Country: China Dates of recruitment: 2013–2014	qPCR	386	Data collected from male participants in the MARHCS study, a prospective cohort study	<18 years old; <2 or >7 days of abstinence; a history of inflammation of the urogenital system, epididymitis or testicular injury; a history of incomplete orchiocatabasis; a history of varicocele treatment; absence of the pubis, prominentia laryngea or testis; abnormal breasts or penis; varicocele; or epididymal knob	Sperm mtDNAcn compared to semen analysis parameters (WHO 2010)	mtDNA 16S rRNA/ GAPDH ratio	mtDNAcn were significantly negatively correlated with sperm concentration, total sperm count, and progressive motility ($r = -0.214$, $p < 0.01$; $r = -0.232$, $p < 0.01$ and $r = -0.164$, $p = 0.01$, respectively)
10	Diez-Sanchez/ 2003	Single centre, observational/cross-sectional study Country: Spain Dates of recruitment: Not available	slot-blot hybridisation	440	Semen samples donated by healthy men	Not available	Sperm mtDNAcn compared to semen analysis parameters (WHO 1999)	mtDNA 16S rRNA/ nDNA 18S human rRNA ratio	The mean (SD) mtDNA was Normal sperm motility = 717 (394) Abnormal sperm motility = 1278 (477)

Abbreviations:: mtDNAcn, mitochondrial DNA copy numbers; qPCR, quantitative PCR.

cells followed by washing with sperm-wash buffer. Only the study by Song and Lewis did not report about semen purification.[9] The absence of round cells in sperm preparations was checked by light microscopy in all studies.

Various commercial DNA isolation kits were used to extract total DNA in eight of the ten studies. In studies by Kao et al.[15] and Diez-Sanchez et al.[14] the in-laboratory phenol–chloroform method was applied for DNA extraction. To quantify mtDNAcn, a quantitative PCR assay using specific primers to mitochondrial genes was used in eight of the ten included studies. The studies by Diez-Sanchez et al.[14] and Kao et al.[15] used a slot-blot hybridisation and a hot-start concurrent PCR to quantify mtDNAcn, respectively. To quantify the number of spermatozoa in the sample, nuclear DNA was determined. The relative mtDNA copy number was identified based on the mtDNA/nuclear DNA ratio.

The information about amplified genes to determine the amount of mtDNA relative to nDNA is presented in Table 1. The accuracy and specificity assessments of gene amplification have been performed accordingly to the method of mtDNA assessment.

3.1.7 | Quality of evidence and the risk of bias

The quality of evidence assessed by the Newcastle–Ottawa Scale was good to very good in six of the ten studies, and no study was considered unsatisfactory. Seventy percent of studies were downgraded because of the use of convenience sampling and 50% for small sample sizes included. The results are summarised in Appendix S2.

3.1.8 | Synthesis of the results

Meta-analysis

Of the ten studies reporting on differences in sperm mtDNA, five studies with 530 participants were included in the quantitative meta-analysis.[8,10,18–20] These compared mtDNAcn in men with normal and abnormal semen analysis. A significant difference in sperm mtDNAcn was seen between the two groups (SMD 1.08, 95% CI 0.74–1.43). All five included studies reported higher sperm mtDNAcn in abnormal semen samples compared with normal semen samples. Significant statistical heterogeneity was noted ($\tau^2 = 0.09$, $\chi^2 = 10.23$, df = 4, $p < 0.04$, $I^2 = 61\%$). The results are shown in Figure 2.

Qualitative description

Five studies were not included in the meta-analysis. The study by Song and Lewis[9] compared normal and abnormal semen analysis, but was not included because it did not present data in a suitable format (no SD/SEM provided). This study reported significantly higher mean mtDNAcn in men with abnormal semen parameters compared with men with normal semen parameters (15.7, 34.3, 56.7 and 73.7 in men with normal semen parameters, reduced motility, reduced sperm count, and both reduced count and motility, respectively). They also reported a significant negative correlation between mtDNAcn and sperm counts using the entire data set ($r = -0.561$, $p < 0.01$). The studies by Diez-Sanchez and Kao and their colleagues[14,15] were excluded from the meta-analysis because they used different methods to quantify the mtDNA, Diez-Sanchez et al.[14] reported a significant negative correlation between sperm mtDNAcn and sperm parameters in contrast to Kao et al.[15] who reported significantly lower mtDNAcn in men with abnormal semen analysis compared with men with normal semen analysis (7.2 ± 1.3 versus 74.1 ± 2). Two further studies, by Wu and Zhang and their colleagues,[16,17] were excluded from the meta-analysis because they presented results exclusively as a correlation analysis for all participants in the study. Both these studies reported a significant negative correlation between mtDNAcn and semen parameters. Three studies included in the meta-analysis also presented additional correlation analysis between sperm mtDNAcn and semen parameters in the entire data set.[10,18,19] They reported a significant negative correlation between mtDNA copy numbers and semen parameters.

Two studies reported mtDNA quantity specifically in men showing low sperm motility compared with healthy men. Bonanno et al.[20] reported increased quantity of mtDNAcn in 45.8% of patients, which correlated with reactive oxygen species production. Faja et al.[18] reported a significant correlation between mtDNAcn and total motile spermatozoa ($r = -0.51$, $p < 0.001$), Diez-Sanchez and May-Panloup and their colleagues[8,14] compared mtDNA content between sperm cells from different populations of the same sample without taking into account the initial sperm quality. They reported cells of worse quality to have higher mtDNA quantity than sperm cells of better quality.

The results for all the correlation analyses are given in Table 2

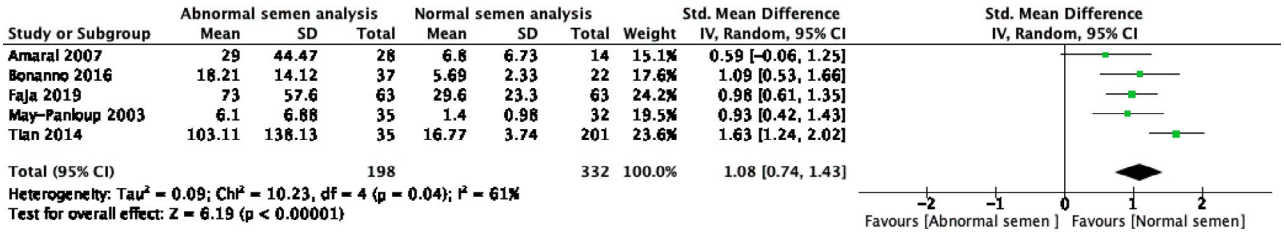

FIGURE 2 Forest plot of comparison of mtDNA between normal and abnormal semen analysis

TABLE 2 The correlations between semen parameters and mtDNAcn

No.	Study	Correlation test	Correlation coefficient (r) and p value			
			mtDNAcn/sperm progressive motility	mtDNAcn/sperm morphology	mtDNAcn/sperm concentration per ml	mtDNAcn/total sperm count
1	Diez-Sanchez/2003	Linear regression analysis	$r = -0.18$, $p = 0.0142$	–	$r = -0.2$, $p = 0.0007$	–
2	Amaral/2007	Spearman's test	$r = -0.29$, $p = 0.067$	$r = -0.45$, $p < 0.002$	$r = -0.56$, $p < 0.001$	–
3	Song/2008	Spearman's test	$r = -0.23$, $p > 0.05$	–	–	$r = -0.56$, $p < 0.01$
4	Tian/2014	Spearman's test	$r = -0.37$, $p < 0.001$	$r = -0.20$, $p < 0.05$	$r = -0.21$, $p = 0.02$	–
5	Zhang/2016	Spearman's test	$r = -0.16$, $p = 0.01$	–	$r = -0.21$, $p < 0.01$	$r = -0.23$, $p < 0.01$
6	Wu/2018	Spearman's test	$r = -0.33$, $p < 0.05$	$r = -0.36$, $p < 0.05$	$r = -0.48$, $p < 0.05$	$r = -0.52$, $p < 0.05$
7	Faja/2019	Spearman's test	$r = -0.51^{*}$, $p < 0.001$	–	$r = -0.50$, $p < 0.001$	$r = -0.44$, $p < 0.001$

* Correlation coefficient for total sperm motility.

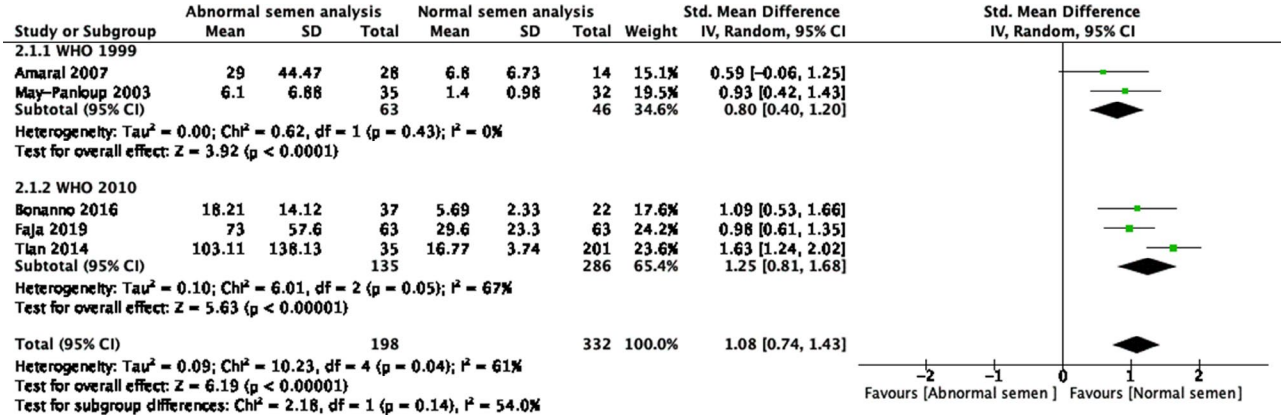

FIGURE 3 Subgroup analysis for the WHO semen analysis criteria (1999/2010) for comparison of mtDNA between normal and abnormal semen analysis

Sperm mtDNAcn in semen with multiple abnormalities

Amaral et al.[19] analysed the mtDNAcn between three male fertility groups: normal, with one or two sperm defects or with more than two defects. The group including three defects (low sperm number, decreased motility and abnormal morphology) showed significantly higher mtDNAcn compared with the normal group ($p < 0.01$) and compared with those with one or two defects ($p < 0.05$). These data are in agreement with the results of May-Panloup et al. and Song et al.,[8,9] where mtDNAcn of semen with only one abnormal criterion (count, motility or morphology) was not significantly different from the mtDNAcn in the normal group. However, highly significant differences were detected between patients with normal sperm and the group with multiple abnormalities

Subgroup analysis

We performed a subgroup analysis for the WHO 1999 and WHO 2010 criteria for semen analysis. This showed a similar direction and size of effect for both groups (WHO 1999: SMD 0.80, 95% CI 0.40–1.20, WHO 2010: SMD 1.25, 95% CI 0.81–1.68). The results are shown in Figure 3.

4 | DISCUSSION

4.1 | Main findings

Our systematic review and meta-analysis showed significantly higher sperm mtDNAcn in men with an abnormal semen analysis compared with those with a normal semen analysis. A significant negative correlation was seen between sperm mtDNAcn and all sperm parameters, including count, motility and morphology. Similar results were seen in comparisons between samples from different individuals and also within sperm populations from the same semen sample.

The WHO criteria classify abnormal semen analysis into three major groups: asthenospermia or reduced motility (A), oligospermia or reduced counts (O), and teratospermia or sperm with abnormal sperm morphology (T) and their different combinations such as OAT, AO, OT and AT.[21] Our review indicates that those who have more than two abnormal criteria have a significantly increased number of mtDNA copies compared with those with only one or two abnormalities (Figure 4).

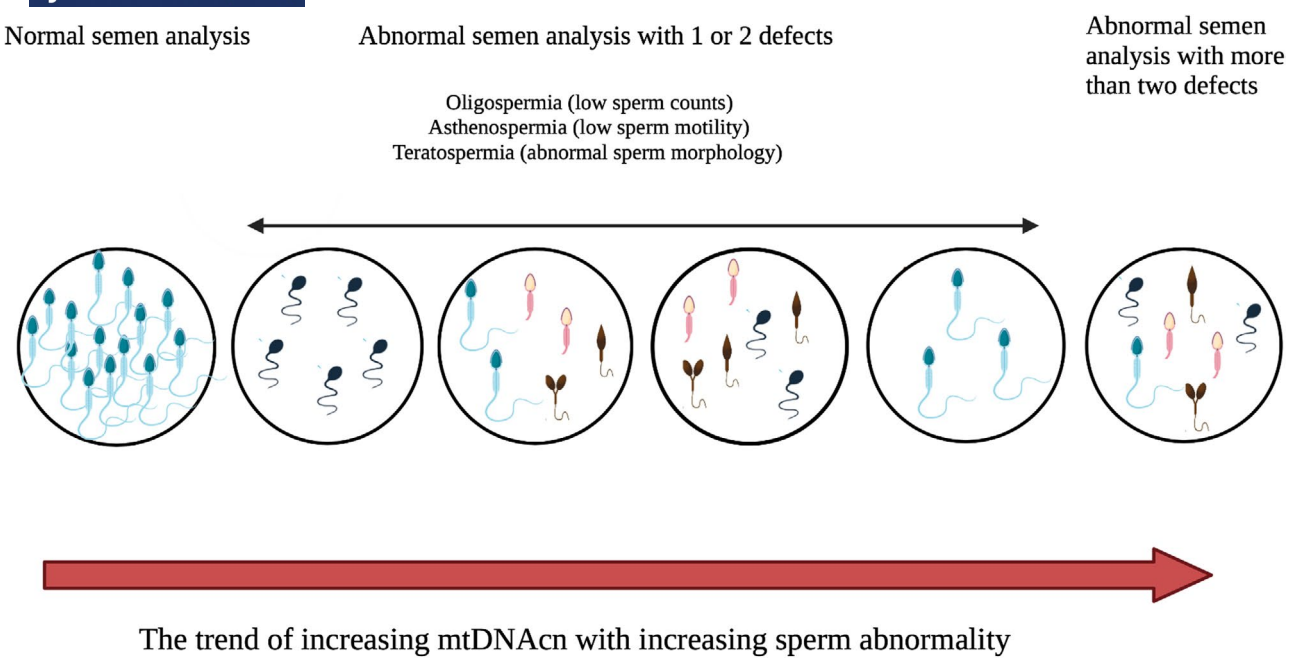

FIGURE 4 Trend of increasing mtDNA with increasing sperm abnormality

4.2 | Strengths and limitations

To our knowledge, the present systematic review is the first to assess the human sperm mtDNAcn. Although the narrative review by Boguenet et al.[11] provides a comprehensive narrative review of several aspects of sperm mitochondrial structure and function, our review is a systematic review, hence reducing the risk of bias. It also provides a pooled estimate of the direction and size of effect for sperm mtDNAcn between abnormal and normal sperm samples. We have carried out a comprehensive review of the literature, which aims to reduce the risk of publication bias. We have also assessed the risk of bias for each study, which gives a good indication of the quality of the included evidence and hence of the subsequent conclusions. Animal studies support the findings of this review, that increased mtDNAcn is associated with decreased total sperm motility.[22]

This review includes studies conducted over a span of more than a decade and hence not unexpectedly there are varying technologies used for sperm preparation, DNA extraction and mitochondrial quantification. The methodological variability in the process may affect sperm mtDNAcn quantification. However, despite this variability almost all included studies show a similar direction of effect. First, the variation in mtDNAcn might be explained by use of semen purification and variation in technique to perform this, as this affects the cell type and content of the analysed sample. Most studies used some semen purification method and hence they were all included in the review and analysis. Eight of the ten studies performed semen purification by sperm washing techniques. This separated the better-quality sperm in the sample from poor-quality sperm and non-sperm cells. As this enabled selection of the best sperm for that sample, these studies demonstrated lower total values of mtDNAcn in both normal and abnormal semen samples. Two studies (by Tian et al.[10] and Faja et al.[18]) used osmotic shock to separate sperm and non-sperm cell components. This did not separate sperm cells into subpopulations and analysed the whole sperm cell content. This resulted in increased total mtDNAcn both in normal and abnormal samples. To account for this discrepancy, we have used the SMD rather than the mean difference in the meta-analysis for the pooled estimate.

Second, the range of mtDNAcn may be affected by different techniques used for total DNA isolation and different primers sets.[23] Quantitative PCR is a more accurate method than older techniques such as the slot-blot method, which may overestimate mtDNA because of lower accuracy, lower sensitivity and subjective interpretation of the results.[24] We have therefore included only those studies that used quantitative PCR for DNA quantification. A single study reported a large effect size but an opposite direction of effect.[15] This could be attributed to an older method for estimation of DNA because the heat of the longer period during hot-start PCR may cause mtDNA degradation. Different sets of primers have been used between the studies to amplify mtDNA genes. It has been demonstrated that abnormal semen samples are prone to carry multiple deletions in the mitochondrial genome,[25] which may affect mtDNA quantification using single/ specific primers. The use of several primer sets for different mitochondrial genes can enhance the precision of DNA quantification. Another issue related to primers for mitochondrial genome amplification is nuclear insertions of mitochondrial origins. Hence, the specificity of mitochondrial primers has to be confirmed to avoid overestimation of mtDNA content. Although these methods for primer selection will increase the precision and reliability of the results,

we do not anticipate that these variations will affect the direction of effect for the meta-analysis as these changes will affect both normal and abnormal semen analysis.

The wide range of mtDNA quantities may also be due to the bias of dilution, and the low efficiency of total DNA extraction.[26] Considering the susceptible nature of mtDNA to degradation, there may be deletions in the analysed gene region due to oxidative stress. This may result from the presence of leucocytes, which are active producers of extracellular reactive oxygen species in semen.[27] Hence, it might be reasonable to determine mtDNAcn in sperm cells using several mitochondrial genes.

The study population may also affect the outcomes. It has been shown that the semen quality varies with the geographical region, as shown for the USA and Europe.[28,29] Moreover, seasonal variation of sperm concentration and total sperm count has also been reported.[29] All these factors may cause the mtDNA count variation in sperm cells.

4.3 | Interpretation

Sperm cells are highly specialised germ cells with very specific functions required to achieve fertilisation. Subsequently, the mitochondria in mature sperm are adapted to have a very distinctive structure and organisation linked to their function.[30] Although the sperm requires energy and functional mitochondria to fulfil its function, especially motility, normal spermatogenesis simultaneously needs to reduce the amount of mitochondria/mtDNA to achieve uniparental inheritance. It is reported that mature sperm cells continue to produce proteins required for the final stages of maturation despite very low numbers of mtDNA and low levels of transcription of mtDNA to RNA.[31] It has been suggested that this could be the result of an increased stability of the pre-existing transcripts, which continue the process of translation to produce proteins independent of reduced mtDNA and new transcription.[2]

The mechanisms behind the association of mitochondrial and/or mtDNA abnormalities and abnormal semen parameters are still unknown. Several explanations have been proposed. Rantanen and Larsson proposed the hypothesis that increased mtDNAcn may be due to an abnormality in the normal physiological downregulation of the Transcription factor A, mitochondrial protein, which is a regulator of mtDNA.[32–34] It is also proposed that increased mtDNA may be compensatory, secondary to inefficient energy production by the mitochondria, defective, mutated or deleted mtDNA.[35]

Based on the results mentioned above, the mtDNA copy number may potentially have a prognostic value for fertility and ART outcomes. A few studies presented the connection between mtDNAcn in sperm and clinical outcomes during ART procedures.[36–38] For example, Tiegs et al.[36] reveals no relationship between live birth rates, fertilisation, usable blastocyst development and blastocyst euploid rates with sperm mtDNAcn from infertile patients undergoing in vitro fertilisation with intracytoplasmic sperm injection. It is possible that the sperm cell selected for intracytoplasmic sperm injection had lower mtDNAcn than other cells from the same semen because of a heterogenic population of sperm cells. Simultaneously, the analysis by Tiegs et al. has confirmed the association of lower relative mtDNAcn with increased sperm motility.[36] Another study, by Rosati et al., revealed the association of mtDNAcn with lower pregnancy probability within 12 months and a longer time to pregnancy; the pregnancy probabilities decreased linearly with higher mtDNAcn.[38] The association of mtDNAcn of sperm cells and early ART outcomes was also analysed by Wu et al. in 2019.[37] The results suggest that sperm with higher mtDNAcn may result in lower odds of embryo development to Day 3 and Day 5.[37]

Regardless of the effect on ART's clinical outcomes, the levels of mtDNAcn may be used as a predictor of spermatogenic dysfunction in men. Gabriel et al.[39] suggested mtDNAcn as an indicator of spermatogenesis's efficiency based on the significant decrease of mtDNA quantity after varicocelectomy. Furthermore, the mtDNA content may play the role of a bioindicator of environmental pollutants such as air pollutants exposure;[40] polycystic aromatic hydrocarbons resulted in reproductive health problems[41] and synthetic organic chemicals, such as monocarboxy-isononyl phthalate, were positively associated with mtDNAcn.[42] Prolonged exposure to SO_2 is negatively associated with mitochondrial quantity.[40] Another study by Luo et al.[43] revealed the increase of mtDNAcn with hypoxic conditions at high altitudes (5300 m). Given the reversible effect on sperm quality and mtDNA content of environmental factors and some external factors such as sexual abstinence before the collection of the semen, heating, cigarette smoking and lifestyle, Wu et al.[16] suggest that mtDNAcn might be suited as an indicator of male reproductive status on the grounds of consecutive diagnoses rather than a single abnormal sample.

5 | CONCLUSION

In this review, we have demonstrated a significantly higher number of mtDNA in human sperm cells of men with abnormal semen analysis compared with men with normal semen analysis. It is important to note that the quantity of mtDNA rises with the increase in semen abnormal parameters. In addition, the heterogeneous sperm cell population in the semen creates sperm variation of mtDNAcn within the same sample. These findings would seem to suggest the predictive value of mtDNA quantification for male reproductive status assessment.

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CONFLICT OF INTERESTS

None declared. Completed disclosure of interests forms available to view online as supporting information.

AUTHOR CONTRIBUTION

DP and PB contributed equally to the conception, planning, execution, analysis, writing and final approval of the manuscript. FD contributed to literature search, study selection, data extraction and assessment of study quality. PBa revised the article critically for important intellectual content. GA contributed to the conception and planning, and revised the article critically for important intellectual content.

ETHICAL APPROVAL

Not needed.

DATA AVAILABILITY STATEMENT

Data available on request from the authors. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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