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ORIGINAL ARTICLE

The relationship between sperm nuclear DNA fragmentation, mitochondrial DNA fragmentation, and copy number in normal and abnormal human ejaculates

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Abstract

Background: While it is common to clinically evaluate sperm nuclear DNA fragmentation, less attention has been given to sperm mitochondrial DNA. Recently, a digital PCR assay has allowed accurate estimation of the proportion of fragmented mtDNA molecules and relative copy number.

Objectives: To determine the correlation of classical sperm parameters, average mtDNA copies per spermatozoon and the level of mtDNA fragmentation (SDF-mtDNA) to that of nuclear DNA fragmentation (SDF-nDNA), measured as the proportion of global, single-strand DNA (SDF-SSBs) and double-strand DNA breaks (SDF-DSBs). To determine whether the level of nuclear and mitochondrial DNA fragmentation and/or copy number can differentiate normozoospermic from non-normozoospermic samples.

Materials and methods: Ejaculates from 29 normozoospermic and 43 non-normozoospermic were evaluated. SDF was determined using the sperm chromatin dispersion assay. mtDNA copy number and SDF-mtDNA were analyzed using digital PCR assays.

Results: Relative mtDNA copy increased as sperm concentration or motility decreased, or abnormal morphology increased. Unlike SDF-mtDNA, mtDNA copy number was not correlated with SDF-nDNA. SDF-mtDNA increased as the concentration or proportion of non-vital sperm increased; the higher the mtDNA copy number, the lower the level of fragmentation. Non-normozoospermic samples showed double the level of SDF-nDNA compared to normozoospermic (median 25.00 vs. 13.67). mtDNA copy number per spermatozoon was 3× higher in non-normozoospermic ejaculates (median 16.06 vs. 4.99). Although logistic regression revealed SDF-Global and mtDNA copy number as independent risk factors for non-normozoospermia, when SDF-Global and mtDNA copy number were combined, ROC curve analysis resulted in an even stronger discriminatory ability for predicting the probability of non-normozoospermia (AUC = 0.85, 95% CI 0.76–0.94, $p < 0.001$).

Conclusion: High-quality ejaculates show lower nuclear SDF and retain less mtDNA copies, with approximately half of them fragmented, so that the absolute number of non-fragmented mtDNA molecules per spermatozoon is extremely low.

KEYWORDS
mitochondrial DNA copy number, sperm chromatin dispersion test, sperm DNA fragmentation, sperm mtDNA damage

1 | INTRODUCTION

The human spermatozoan is a highly specialized cell, designed to contain, protect, and deliver the male DNA to the oocyte. Maintenance of the integrity of sperm DNA is an obligatory requirement for achieving a successful pregnancy and healthy offspring.[1–5] Even in a normal ejaculate, a certain proportion of spermatozoa show fragmented DNA. This proportion may be increased in males with low sperm quality, infertility, and those with pathologies such as varicocele, infections, cancer, or exposed to certain drugs or toxic agents.[1–6]

The mechanisms that result in sperm DNA fragmentation (SDF), have been shown to be a consequence of an apoptotic-like process,[7] impaired chromatin remodeling during spermiogenesis,[8] or due to attack by reactive oxygen species (ROS).[9] DNA breakages in the deoxyribose-phosphate backbone may be distributed throughout the molecule, affecting only one of the strands at a specific position (single-strand DNA breaks: SSBs) or both strands at a similar or close location (double-strand DNA breaks: DSBs).[10,11]

The current techniques for determining SDF, include the sperm chromatin structure assay (SCSA), the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay, the alkaline single-cell electrophoresis (alkaline comet) assay or the Sperm Chromatin Dispersion (SCD) test. A meta-analysis concluded that all these assays have a good sensitivity but possibly the SCSA and SCD could be less predictive of assisted reproduction outcome.[12] Nevertheless, recent reports indicate a good predictive value of the SCD test.[4,13] These assays do not distinguish if the DNA fragmentation of an individual sperm cell is only due to SSBs (SDF-SSBs) or DSBs (SDF-DSBs).[3,4,14] The presence and differentiation of DSBs, may however, be detected using the non-denaturing comet assay,[15] or a more recently reported variant of the SCD test (DSBs-SCD), where DNA fragments spread through passive diffusion.[16] The subtraction of SDF-DSBs value from that of global SDF obtained with the standard SCD test can thereby provide an estimate of the SSBs' sub-population.[16]

Sperm mitochondria form tight helices around the mid-piece of the flagellum to constitute the mitochondrial sheath and have particular features different than those of somatic cells, contributing to sperm motility, hyperactivation, capacitation, acrosome reaction, and fertilization.[17–22] While SDF is typically evaluated in nuclear DNA (nDNA), there also exists small 16,569 bp circular DNA copies present in human sperm mitochondria (mtDNA).[22] Some reports have estimated the average copy number of mtDNA per individual human spermatozoon, using real-time quantitative PCR normalized to a nuclear gene as control for relative quantitation.[23–26] The use of digital PCR (dPCR) of particular mtDNA sequences has allowed a direct and absolute estimation of the mtDNA copies.[27,28] In dPCR, the entire DNA sample is not processed in a single PCR, but it is distributed in thousands of individual and parallel end-point PCR reactions.[29]

Unlike the estimation of copy number, the evaluation of the breakage level of the human sperm mtDNA remains essentially unexplored due to the technical inability of current methods to distinguish between intact and fragmented mtDNA copies.[30] Recently, we have designed a dPCR assay that allows an accurate and reliable estimation of the proportion of fragmented mtDNA copies.[31] The assay amplifies two small target sequences, within ND1 and ND6, distantly located in the circular molecule, in independent microchambers running parallel end-point PCR reactions. Each target is detected by hybridization with hydrolysis-TaqMan probes labeled with different fluorochromes (HEX and FAM). A microchamber containing one mtDNA molecule without fragmentation will present both signals co-localized; that is, if HEX or FAM appears located alone in a different microchamber, then this corresponds to a fragmented mtDNA molecule, containing at least, two DSBs.[31]

In this study, the average number of mtDNA copies per sperm cell was determined and complemented with their corresponding fragmentation level obtained using our dPCR assay and may provide a more accurate estimation of potentially intact mtDNA molecules transmitted by the sperm to the oocyte. The relationship of mtDNA copies and fragmented mtDNA was also correlated with classical sperm quality parameters (concentration, motility, morphology, and vitality), as well as with the presence of SDF in nuclear DNA, assessed as either global SDF, or compared to the subpopulation with SSBs only or with DSBs.

2 | MATERIALS AND METHODS

2.1 | Semen samples

The study was performed using neat semen samples from 72 males attending the andrology laboratory from the Complexo Hospitalario Universitario A Coruña (CHUAC). All participants provided informed consent and the study was approved by the Institutional Review Board (reference number 2022/117). Individuals who required semen analysis and men from couples undergoing assisted reproduction techniques were included. Individuals with heavy alcohol and/or drug use in the previous 3 months, with a history of recent illness, fever, or exposure

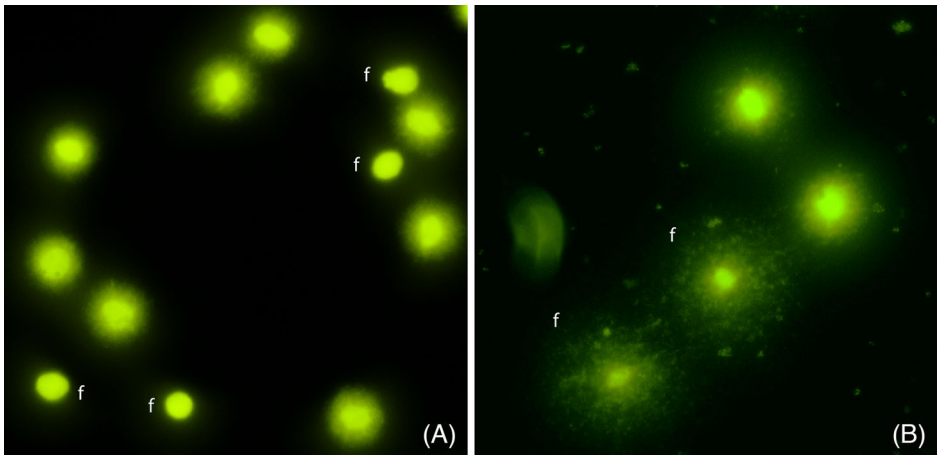


FIGURE 1 The sperm chromatin dispersion (SCD) assay for the detection of nuclear global-sperm DNA fragmentation (A) and DNA fragmentation containing DNA double-strand breaks (SDF-DSBs) (B). (a) Sperm with DNA fragmentation corresponded to those nucleoids with small halos. (B) Sperm with SDF-DSBs corresponded to those nucleoids with large dispersed halos. (f: fragmented).

to chemotherapy, radiotherapy, pesticides, heavy metals, or with positive serology for HIV, hepatitis B or C, syphilis, cytomegalovirus, *Chlamydia* and gonococcus, were excluded. The age of the patients was 37.4 ± 5.5 (mean ± sd) years. Concentration and motility were determined using CASA (Sperm Class Analyzer; Microptic SL, Barcelona, Spain). Morphology and vitality were evaluated under the microscope after Papanicolau and eosin–nigrosin staining, respectively.

Semen samples were categorized according to the WHO manual (5th edition, 2010)[32] as normozoospermic ($n = 29$;) or with abnormal semen parameters ($n = 43$; 12 asthenozoospermic, 2 teratozoospermic,14 asthenoteratozoospermic, 3 oligozoospermic, 4 oligoasthenozoospermic, 8 oligoasthenoteratozoospermic). The age of normozoospermic and non-normozoospermic individuals was 36.7 ± 4.8 and 37.8 ± 5.9 years, respectively. The fifth percentile of the fertile population was established as the cut-off point; concentration: 15 million/mL; total sperm number: 39 million; total motility: 40%; progressive motility: 32%; abnormal forms: 4%, and vitalilty: 58%.

2.2 | Sperm chromatin dispersion test

The SCD test was performed using the HalospermG2 kit (Halotech DNA SL, Madrid, Spain), accordingly to the manufacturer's recommendations.[14] In brief, an aliquot of each semen sample was diluted to 5−10 million spermatozoa mL^{-1}, mixed with molten agarose and pipetted onto a slide and covered with a coverslip. The slide was then placed onto a cold plate in the refrigerator (4°C) for 5 min to allow the agarose to form a microgel in which the sperm cells were embedded for further processing. The coverslip was subsequently removed, and the slide immediately covered with drops of HCl solution for 7 min at room temperature (22°C). The slide was then incubated with the lysing solution for 25 min. After washing with distilled water, the slide was dehydrated in increasing ethanol baths (70%–90%–100%), air-dried and stained with SYBR Gold and observed under the epifluorescence microscope. Sperm without DNA fragmentation showed large haloes of dispersed DNA loops, whereas those with fragmented DNA show no halo or a small halo (see Figure 1A).

2.3 | DSBs-SCD assay

For the DSBs-SCD assay, spermatozoa were processed in a similar manner to the standard SCD test but with modifications.[16] Spermatozoa in the microgel were only incubated with a specifically adapted lysing solution for 2 min (Halotech DNA SL, Madrid, Spain). Slides were washed, dehydrated, and stained as per the standard SCD assay. For the DSBs-SCD assay, spermatozoa without DSBs showed compact haloes of chromatin loops from the central core, whereas spermatozoa with fragmented DNA associated with DSBs exhibited large haloes of diffused "spotty" DNA fragments (Figure 1B). Rarely, extreme DNA fragmentation and mobilization away from the nucleoid made the corresponding halo very "faint" so that only a residual core of the nucleoid remained detectable.

2.4 | Isolation of sperm DNA

For isolation of sperm DNA, 150 μL of semen sample was mixed with 300 μL of PBS and centrifuged at 4000 rpm for 10 min. After the pellet was resuspended in 100 μL of PBS, 100 μL of extraction buffer (20 mM Tris-HCl pH 8; 20 mM Ethylenediaminetetraacetic acid (EDTA); 200 mM NaCl; 4% Sodium Dodecyl Sulfate (SDS) and 1 μL of DTT 1 M (final concentration 5 mM) were added. This mixture was incubated in the dark at room temperature for 60 min. Automated DNA extraction was performed with the QIAamp DNA Blood Mini Kit in QIAcube Connect (QIAgen) according to the manufacturer's instructions. Total DNA concentration and quality were estimated using Thermo Scientific NanoDrop One (Thermo Fisher Scientific, Madrid, Spain).

2.5 | mtDNA copy number per spermatozoon

The average copy number of mtDNA per individual spermatozoon was estimated using dPCR for concurrent amplification of a target within the mtDNA gene (ND6) and another target in a single-copy nuclear gene (RPP30) as a reference for relative quantitation, differentially labeled. Given the haploid nature of sperm, each RPP30 copy estimated by the dPCR assay must correspond to an individual spermatozoon present in the sample.

dPCR was performed using the microwell-on-chip system (Thermo Fisher Scientific). For this procedure, 1 ng of isolated DNA in 2 μL was mixed with 7.5 μL of master mix (Thermo Fisher Scientific), 0.75 μL of primer solution and TaqMan hydrolysis probe for target RPP30, HEX-labeled (ddPCR Gene Expression Assay RPP30, human, Bio-Rad), 0.75 μL of primer solution and TaqMan probe for target MT-ND6, FAM-labeled (ddPCR Gene Expression Assay MT-ND6, human, Bio-Rad) and 4 μL of water. This final mixture volume (15 μL) was then evenly distributed on a dPCR chip. dPCR was performed with a QuantStudio 3D Digital PCR System (Thermo Fisher Scientific). The PCR conditions were set at one cycle of 96°C, 10 min; 39 cycles of hybridization-extension at 55°C, 1 min and denaturation at 98°C, 30 s, and a final cycle of 60°C, 2 min.

Chip analysis was carried out by QuantStudio 3D AnalysisSuite software. This analysis allowed us to determine the number of copies of ND6 (mtDNA) and RPP30 (nuclear single-copy gene) present. The ratio between the two genes provided an estimate of the average number of mtDNA copies present in each spermatozoon from the sample.

2.6 | mtDNA fragmentation level

In parallel to the determination of mtDNA copy number per spermatozoon, mtDNA fragmentation was estimated by a different dPCR performed under the same technical conditions. The only difference was that the primer for the RPP30 nuclear gene was replaced by a primer to amplify a target within another mitochondrial gene, MT-ND1.[31] In brief, in our design, Target Sequence 1 was located within the ND1 mitochondrial gene, comprising bases 3,629 to 3,775 (146 pb); as indicated. Target Sequence 2 was found within the ND6 mitochondrial gene, comprising bases 14,250 to 14,382 (132 pb) (ddPCR Gene Expression Assay MT-ND1 and MT-ND6, human, Bio-Rad). The two targets were connected by two segments: the longer of 10,475 bp, and the shorter of 5,816 bp. Since the two DNA segments between the two targets were very long, if DSBs were present, they will be located almost exclusively in these regions.

Amplification of Target 1 was detected by hybridization with a TaqMan probe labeled with HEX, while amplification of Target 2 was detected with another probe labeled with FAM. Since mtDNA is circular, the joint amplification of both targets, that is, the colocalization of HEX + FAM in a microchamber, will correspond to a non-fragmented molecule. If the molecule has at least two DSBs, one in segment X and another in segment Y, each target will be located in a different fragment, so they can be separated and distributed in different microchambers ("break-apart"). When the PCR reaction takes place, there will be a chamber with a single HEX signal and another chamber with the FAM signal (Figure 2). As with all quantitative studies with dPCR,

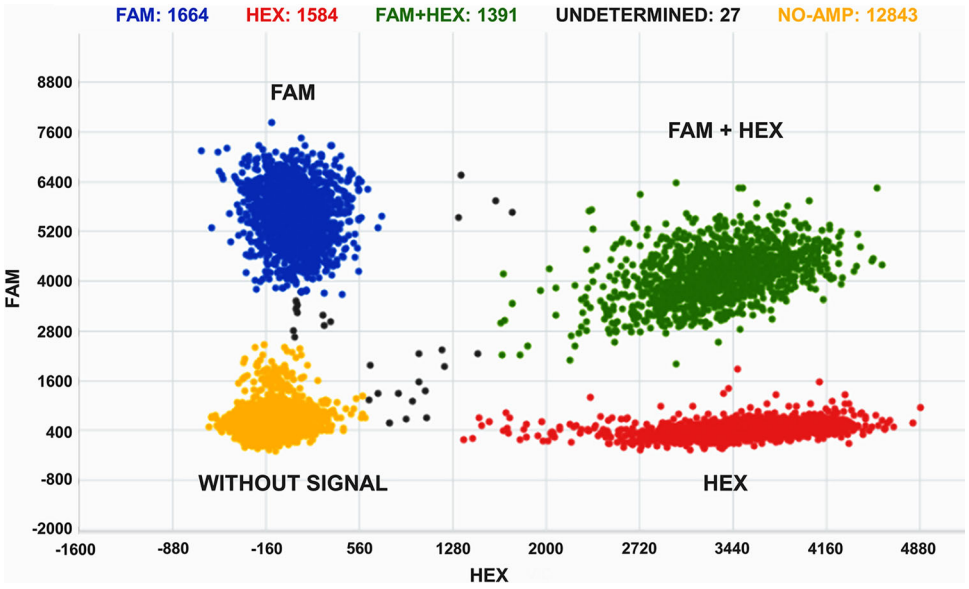


FIGURE 2 Graphic representation of the distribution of the number of microwells of dPCR chips, without and with amplification signals of the mtDNA targets in a sperm sample. The *X*-axis corresponds to the HEX signal intensity (signal from amplification of the target within the ND1 gen) and the *Y*-axis to the FAM signal intensity (signal from amplification of the target within the ND6 gen). Upper right: microwells with colocalized FAM + HEX signals (green dots), corresponded to non-fragmented mtDNA copies. Upper left: microwells with only a FAM signal (blue dots). Bottom right: microwells with only a HEX signal (red dots). The software indicated that these correspond to 60.68% fragmented mtDNA molecules. Bottom left: microwells without signal (yellow dots).

TABLE 1 Descriptive statistics of the parameters of semen samples.

	N	Mean	SD	Median	Minimun	Maximum
Concentration (million/mL)	72	62.52	60.92	36.25	1.30	303.00
Total sperm n° (million)	72	191.70	185.72	115.10	6.50	706.80
Total motility (%)	72	38.86	19.74	36.50	6.00	89.00
Progressive motility (%)	72	31.99	19.80	31.00	0.00	86.00
Abnormal forms (%)	72	93.46	4.10	94.00	80.00	99.00
Non-vital cells (%)	49	34.45	14.71	34.00	10.00	65.00
SDF-global nDNA (%)	72	26.02	16.39	22.84	4.33	78.33
SDF-DSBs nDNA (%)	72	10.86	11.09	7.67	0.67	66.67
SDF -SSBs nDNA (%)	72	15.16	10.19	12.67	1.67	37.33
Contribution DSBs (%)	72	41.00	20.70	37.14	4.11	85.11
Contribution SSBs (%)	72	59.00	20.70	62.86	14.90	95.89
mtDNA copy n°	72	16.33	18.71	9.24	1.17	107.91
SDF-mtDNA (%)	72	53.29	13.33	52.43	31.61	100

Abbreviations: DSBs, double-strand DNA breaks; SD, standard deviation; SSBs, single-strand DNA breaks.

this assay must be performed ensuring that an adequate proportion of microchambers are without signal, to avoid as far as possible the colocalization of both targets that were separated due to DSBs, but that may coincide in the same microchamber by random. Under these conditions, the Poisson distribution allows its estimation for subsequent correction.

2.7 | Statistical analysis

Three hundred spermatozoa were scored per SDF technique. All data were analyzed and graphs generated using the SPSS 26 software package for Windows (SPSS Inc., Chicago). Data were not normally distributed according to the Kolmogorov–Smirnov test so that Spearman's rho was employed to evaluate the correlation of distributions of SDF-Global, SDF-SSBs, SDF-DSBs, SDF-mtDNA, mtDNA copy number per spermatozoon, and seminal parameters. The distribution of SDF-SSBs and SDF-DSBs in the Global-SDF was evaluated with Chi-squared (χ^2) test. Regression analysis was used to analyze mathematical models of relationship between different parameters. Comparisons between samples with normal and abnormal semen parameters were performed using the Mann–Whitney *U*-test. Logistic regression and receiver operating characteristic curves (ROC) curves were constructed and used to investigate a model that allowed the prediction of the non-normozoospermia. Statistical significance was defined as $p < 0.05$.

3 | RESULTS

Descriptive statistics (mean ± SD) of the different parameters evaluated for the whole sample population are summarized in Table 1. Values of classical sperm parameters in normo- and non-normozoospermic samples are presented in Table S1.

3.1 | Sperm nuclear DNA

Table 2 reports the Spearman's rho correlation between SDF of nuclear DNA (nDNA), mtDNA copy number, and the DNA fragmentation of mtDNA (SDF-mtDNA). These resulted showed that the higher the level of the global SDF, the higher the spermatozoa with SSBs, and spermatozoa with DSBs in the sample (rho = 0.85 and 0.67, respectively; $p < 0.001$). Following logarithmic transformation of the data to obtain a normal distribution, linear regression was used to examine the relationship between SDF-SSBs and that of SDF-DSBs on global-SDF; global-SDF showed a coefficient of determination (r^2) value of 0.68 ($p < 0.001$) with a linear coefficient-slope of 0.68 when plotted against SDF-SSBs and an r^2 value of 0.52 ($p < 0.001$) with a linear coefficient of 0.54 for SDF-DSBs (Figure 3). These results suggest that sperm with SSBs contributed to a higher proportion of SDF-global sperm that those with DSBs. Significant correlations were also found between nuclear DNA damage and the classical seminal parameters, with the exception of sperm concentration (Table 3). Thus, the higher the level of SDF-Global, and/or SDF-SSBs and SDF-DSBs, the higher the frequency of non-motile sperm (total and progressive), sperm with abnormal morphology, and non-vital sperm.

3.2 | Sperm mitochondrial DNA

Table 2 shows that the higher the frequency of spermatozoa with fragmented global nuclear DNA, the higher the fraction of fragmented mtDNA molecules found in the sample (rho = 0.29; $p = 0.013$); a similar correlation was not found with SDF-DSBs but was evident with SDF-SSBs (rho = 0.35; $p = 0.003$; Table 2). Interestingly, while the copy number of mtDNA per sperm cell was not correlated with that of SDF of nuclear DNA, it was negatively correlated with the fragmentation level of mtDNA (rho = −0.51; $p < 0.001$) (Table 2); the data fitted signif-

TABLE 2 Correlations between SDF in nuclear DNA (nDNA), mtDNA copy number and SDF-mtDNA.

		SDF-global nDNA	SDF-DSBs nDNA	SDF-SSBs nDNA	mtDNA copy n°	SDF-mtDNA
SDF-global nDNA	*R*					
	p-Value					
SDF-DSBs nDNA	*R*	0.672**				
	p-Value	< 0.001				
SDF-SSBs nDNA	*R*	0.852**	0.284*			
	p-Value	< 0.001	0.016			
mtDNA copy n°	*R*	0.163	0.185	0.082		
	p-Value	0.172	0.121	0.496		
SDF-mtDNA	*R*	0.290*	0.034	0.348**	−0.512**	
	p-Value	0.013	0.777	0.003	< 0.001	

Abbreviations: DSBs, double-strand DNA breaks; SSBs, single-strand DNA breaks.
*$p < 0.05$.
**$p < 0.01$.

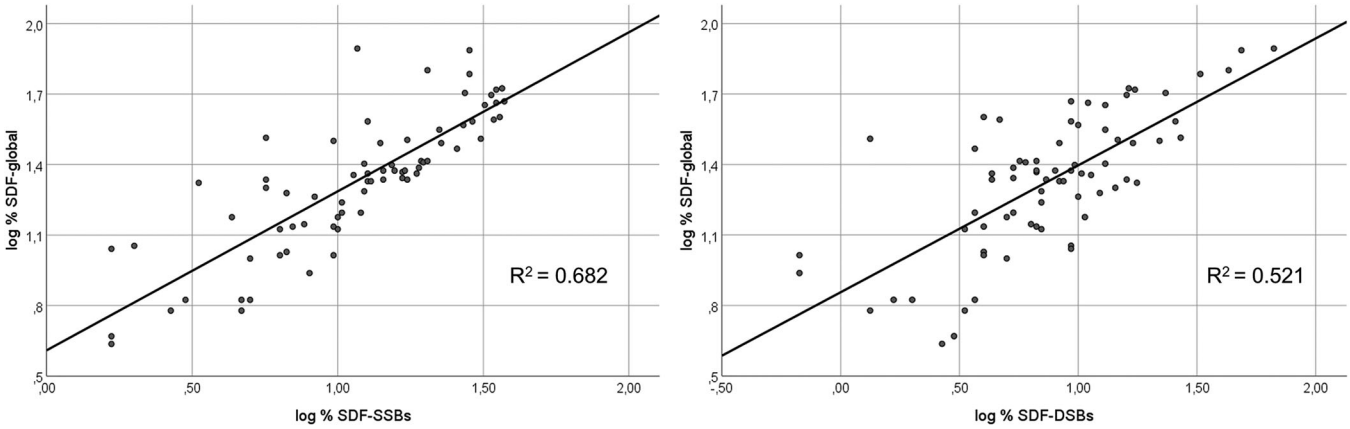


FIGURE 3 Linear regression to evaluate the influence of SDF-SSBs (left) and of SDF-DSBs (right) with respect to global-SDF.

TABLE 3 Correlations between classical sperm parameters, SDF in nuclear DNA (nDNA), mtDNA copy number, and mtDNA fragmentation.

		SDF-global nDNA	SDF-DSBs nDNA	SDF-SSBs nDNA	mtDNA copy n	SDF-mtDNA
Concentration	*r*	−0.196	−0.140	−0.134	**−0.805**	**0.440**
	p-Value	0.099	0.241	0.260	**< 0.001**	**< 0.001**
Total sperm n°	*r*	−0.205	−0.130	−0.141	**−0.815**	**0.488**
	p-Value	0.083	0.278	0.239	**< 0.001**	**< 0.001**
Total motility	*r*	**−0.541**	**−0.392**	**−0.454**	**−0.397**	−0.056
	p-Value	**< 0.001**	**0.001**	**< 0.001**	**0.001**	0.639
Progressive motility	*r*	**−0.546**	**−0.412**	**−0.443**	**−0.356**	−0.049
	p-Value	**< 0.001**	**< 0.001**	**< 0.001**	**0.002**	0.680
Abnormal forms	*r*	**0.481**	**0.279**	**0.477**	**0.328**	0.109
	p-Value	**< 0.001**	**0.018**	**< 0.001**	**0.005**	0.363
Non-vital cells	*r*	**0.653**	**0.557**	**0.406**	−0.182	**0.451**
	p-Value	**< 0.001**	**< 0.001**	**0.004**	0.212	**0.001**

Abbreviations: DSBs, double-strand DNA breaks; SSBs, single-strand DNA breaks.
Bold values correspond to significant correlations.

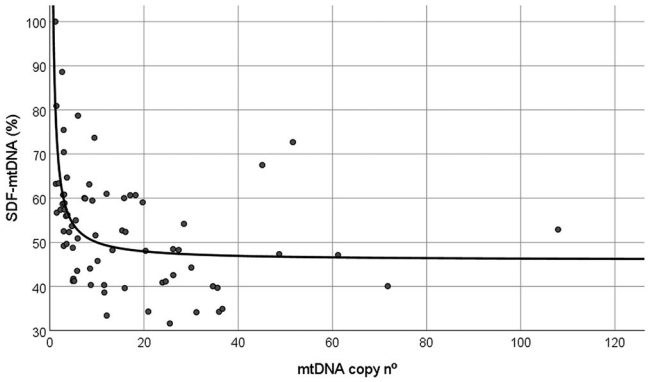


FIGURE 4 The regression curve of the influence of the number of mtDNA copies per sperm (*X*-axis) in the proportion of fragmented sperm mtDNA molecules (SDF-mtDNA; *Y*-axis). The data were consistent an inverse regression model; the higher the mtDNA copies, the fewer the proportion of fragmented ones.

icantly to an inverse regression model ($y = 45.91 + (40.75/x)$; $r^2 = 0.33$; $p < 0.001$), suggesting that the SDF-mtDNA fraction decreased very rapidly at first and then more slowly as the number of mtDNA copies increased (Figure 4).

Regarding the classical sperm parameters, SDF-mtDNA correlated positively only with concentration, total sperm number and with non-vital sperm (Table 3). The mtDNA copy number significantly correlated negatively with concentration, motility, either total or progressive, and positively with abnormal morphology (Table 3). An inverse regression model revealed a substantial influence of concentration on the variation of the number of mtDNA copies ($y = 8.24 + (151.96/x)$; $r^2 = 0.65$; $p < 0.001$).

3.3 | A comparison of non-normozoospermic and normozoospermic ejaculates

With respect to the fragmentation of nuclear DNA, non-normozoospermic samples ($n = 43$; Table 4) showed nearly double global SDF average values than normozoospermic samples ($n = 29$) (median: 25.0 vs. 13.7; Mann–Whitney *U*-test, $p < 0.001$). However, the differences were mostly dependent on SDF-SSBs (median: 16.7 vs. 9.7; $p < 0.001$). While SDF-DSBs were also significantly increased in non-normozoospermic, the difference was lower (median: 9.3 vs. 6.0; $p = 0.004$). The relative contribution of SDF-SSBs and SDF-DSBs to the SDF-Global sperm population was not found to be significant between non-normozoospermic and normozoospermic ($p = 0.487$), with the SDF-SSBs population representing 66.0% and 60.5% of the median, respectively.

However, when mtDNA was analyzed, non-normozoospermic samples showed three times more copies per sperm than normozoospermic (median: 16.1 vs. 5.0; $p = 0.001$). The dPCR assay for estimation of SDF-mtDNA also revealed that approximately half of these mtDNA copies were fragmented on average; this proportion was very similar in both groups (median: 52.4 vs. 53.7; $p = 0.570$).

When SDF-Global, SDF-DSBs, mtDNA copy number, and SDF-mtDNA were included in a multivariate analysis, using binary logistic regression, only SDF-Global and mtDNA copy number per spermatozoon remained as significant independent risk factors associated with non-normozoospermia (Figure 5; SDF-Global: OR 1.09, 95% CI 1.03–1.14, $p = 0.002$; mtDNA copy number: OR 1.10, 95% CI 1.03–1.18, $p = 0.003$). When SDF-Global and mtDNA copy number were combined, ROC curve analyses resulted in even stronger discriminatory ability for predicting the probability of a non-normozoospermic ejaculate (Area under the curve (AUC) = 0.85, 95% CI 0.76–0.94, $p < 0.001$), when compared to SDF-Global and mtDNA copy number, separately (Figure 5). The highest discriminant level of the combined factors, which corresponded to 72.10% sensitivity and 89.7% specificity, was obtained when the multivariant model results in a probability value ≥ 0.64.

Most of the non-normozoospermic samples showed mixed categories of oligozoospermia, and/or teratozoospermia and/or asthenozoospermia, so that there were not sufficient samples to make a confident comparison with normozoospermic. Nevertheless, a preliminary approach was attempted with those categories with a small but acceptable number (see Table S2). Asthenozoospermic samples ($n = 12$) showed near double levels of SDF-global (median: 25.5; $p = 0.015$), but mtDNA copy number and SDF-mtDNA were not found significantly different to those from normozoospermic samples. Asthenoteratozoospermic ($n = 14$) showed 2.3 times increase of the median of SDF-Global (median: 31.5; $p = 0.001$); in this category, mtDNA copies per sperm doubled that of normozoospermic (median: 11.8; $p = 0.01$), whereas their fragmentation level was not different (median: 55.7). Finally, oligoasthenoteratozoospermic ($n = 8$), that is, the most abnormal of the samples, also showed a median value 2.3 times higher for SDF-global levels (median: 31.3; $p = 0.004$) compared to normozoospermic samples. Moreover, this cohort also showed the highest level of mtDNA copies per sperm, 8 times higher (median: 40.9; $p < 0.001$) than normozoospermic samples. Again, no differences were found with respect to the fragmentation level of mtDNA (median: 47.8) between oligoasthenoteratozoospermic and normozoospermic samples.

4 | DISCUSSION

During spermatogenesis, there is a considerable depletion of mtDNA, so that a mature human spermatozoon typically only contains around ten copies, but ranging from 0 to 226, as estimated by qPCR.[23,33,34] This phenomenon is peculiar to sperm cells, since mtDNA depletion may be pathological if present in somatic cells. Moreover, other studies have pointed out that good quality-normozoospermic samples tend to have sperm with less mtDNA copy number than poorer quality samples, similar to what was found in the current study; a finding that also suggests that increased mtDNA copy number could serve as a potential biomarker for spermatogenic dysfunction.[24,26,35,36]

Estimation of mtDNA copy number in stallion sperm has been shown to vary depending on the mitochondrial target sequence

TABLE 4 Comparison between normozoospermic and non-normozoospermic samples, regarding SDF in nuclear DNA (nDNA), relative contribution of SDF-DSBs and of SDF-SSBs to SDF-global nDNA (%), mtDNA copy number and SDF-mtDNA.

		N	Mean	SD	Median	Minimum	Maximum
SDF-global nDNA	**Normo**	29	17.56	12.51	13.67	4.33	61.00
	Non-normo	43	31.73	16.35	25.00*	10.00	78.33
SDF-DSBs nDNA	**Normo**	29	7.72	7.52	6.00	0.67	32.67
	Non-normo	43	12.98	12.60	9.33*	1.33	66.67
SDF-SSBs nDNA	**Normo**	29	9.84	6.64	9.67	1.67	28.33
	Non-normo	43	18.75	10.64	16.67*	2.00	37.33
Contribution DSBs	**Normo**	29	42.09	19.49	39.42	6.49	84.82
	Non-normo	43	40.26	21.67	33.80	4.11	85.11
Contribution SSBs	**Normo**	29	57.92	19.48	60.54	15.18	93.61
	Non-normo	43	59.74	21.67	66.20	14.90	95.89
mtDNA copy n°	**Normo**	29	7.60	7.06	4.99	1.17	28.47
	Non-normo	43	22.21	21.69	16.06*	1.27	107.91
SDF-mtDNA	**Normo**	29	54.42	13.78	53.68	31.61	100
	Non-normo	43	52.53	13.13	52.36	34.15	88.61

Abbreviations: Normo, normozoospermia; Non-normo, non-normozoospermia; DSBs, double-strand DNA breaks; SD, standard deviation; SSBs, single-strand DNA breaks.

*$p < 0.05$ indication for significant differences between Normo and Non-normo.

amplificated by qPCR, so it has been suggested that this phenomenon could be a result of fragmentation of the mitochondrial genome during spermatogenesis.[35] Nevertheless, unlike nuclear DNA, sperm mtDNA fragmentation has typically not been routinely evaluated given the technical difficulty to distinguish fragmented and intact copies.[30] The majority of the reports concerning sperm mtDNA integrity have been related to current known deletions or point mutations, estimated by PCR and/or sequencing. These alterations have been found more frequent in poor quality sperm samples.[33,36–38]

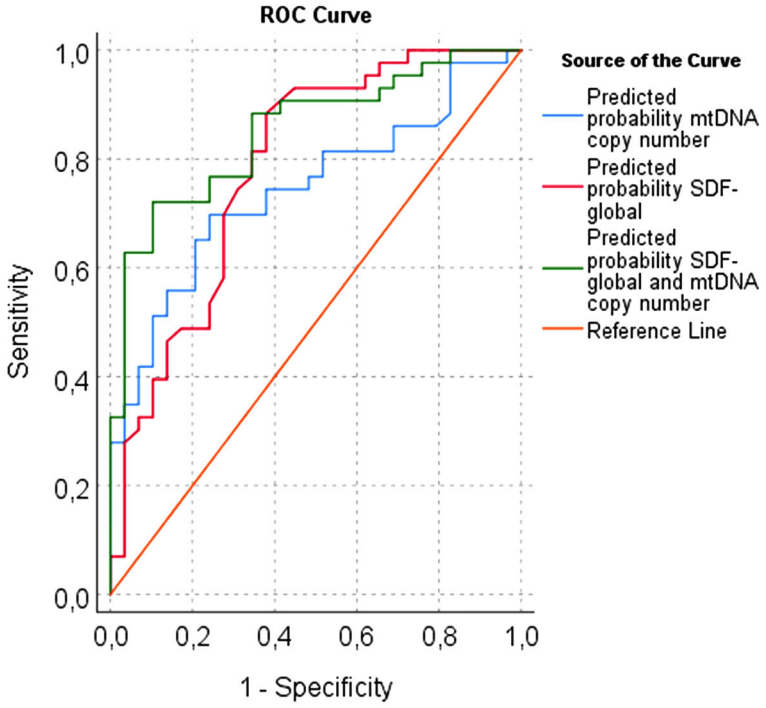
Song and Lewis[33] evaluated sperm mtDNA fragmentation and deletions using a long PCR assay for half of the mtDNA genome (8.7 kb). This procedure was based on the capacity of many DNA lesions, and obviously DSBs, to block or impede the progression of the DNA polymerase. The technique revealed that abnormal sperm samples had lower amplification levels than normozoospermic samples, showing a decrease in mtDNA integrity. Although the long PCR target may increase the possibility of finding a blocking DNA lesion, it also resulted in a significant processivity problem of the DNA polymerase, with low efficiency and low sensitivity and reproducibility.[39] Moreover, quantification using this procedure was semiquantitative.

An opposite result was reported in a recent study performed by next generation sequencing.[40] First, dPCR, confirmed a lower mtDNA copy number in good quality spermatozoa. Second, high-throughput sequencing revealed that mtDNA from good quality sperm showed a much higher frequency of large deletions and duplications, these being heterogeneous and random, so they could be potentially related to DSBs, presumably generated by mtDNA degradation.[40] Nevertheless, the described next generation sequencing procedure required an initial step, where the mitochondrial genome was amplified in two large segments of 8397 and 8289 bp. As previously indicated, if DSBs were present in these segments, they would not allow the amplification by DNA polymerase, so the resulting native fragments would not be detected.

Our dPCR methodology, which is based on an adapted "break-apart" approach, has been specifically designed to quantify accurately the proportion of fragmented mtDNA copies. Our dPCR mtDNA fragmentation assay revealed that sperm from normozoospermic samples, not only contained very few mtDNA copies, but also that an average of half of them appeared fragmented. For comparison, the same specific dPCR procedure revealed that mtDNA from human peripheral blood cells ($n = 10$ samples) showed a fragmented fraction of only 7.97 % ± 0.39% (mean ± sd).[31]

Although the normozoospermic samples in the current study showed three times lower mtDNA copy number than the non-normozoospermic samples, SDF-mtDNA was not found significantly different between these groups. However, when all the samples irrespective of categorization were examined, mtDNA fragmentation clearly increased as mtDNA copy number decreased. This finding may mean that good-quality sperm samples, which have less mtDNA copy number, express most of these copies as degraded, so that the absolute number of remaining complete circular mtDNA copies in sperm must be quite rare. mtDNA fragmentation also increased significantly with high sperm concentration, whereas correspondingly, mtDNA copies decreased as sperm concentration increased.

A complete and perfect spermatogenesis process should theoretically at least result in a high-quality mature sperm with a lower amount of mtDNA molecules and with most of these remaining copies being fragmented. This is likely to be a dynamically-progressive process, so



Area Under the ROC Curve

Test Result Variable(s)	Area	CI 95%		p-value
		Lower Bound	Upper Bound	
Predicted probability SDF-global	0.78	0.67	0.90	<0.001
Predicted probability mtDNA copy	0.74	0.63	0.86	<0.001
Predicted probability SDF-global and mtDNA copy	0.85	0.76	0.94	<0.001

FIGURE 5 Receiver operating characteristic (ROC) curves showing the discriminatory power of nuclear global sperm DNA fragmentation (global-SDF) and average mtDNA copy number per spermatozoon, either isolated or combined, to distinguish non-normozoospermic from normozoospermic samples. CI: confidence interval.

that a high frequency of mtDNA fragmentation would be evidence of effective mtDNA degradation and elimination. Conversely, a high relative amount of mtDNA, with a low frequency of fragmentation would be indicative of poor sperm quality, and a consequence of inefficient or defective spermatogenesis; in fact, the high copy number could be due to an impaired or slower degradation mtDNA process and reflect a spermatozoon of less maturity.

While the evolutionary reason of elimination of paternal mtDNA from the sperm cell is still equivocal, it is well established that human mtDNA shows a strict matrilineal inheritance,[41,42] although some exceptions have been reported.[43] It has generally been implied that mtDNA molecules from sperm cell must be eliminated in the oocyte after fertilization,[44,45] but perhaps this removal would be greatly facilitated if the male mtDNA copies are reduced in number prior to fertilization, or at least made susceptible to degradation through extensive fragmentation.[46]

We might suppose that good sperm quality means less nuclear SDF and less mtDNA copy number with the remaining mtDNA being more fragmented. However, the results of the current study revealed a positive correlation between mtDNA fragmentation and global-nuclear DNA fragmentation. Global-SDF determined by the alkaline-denaturant comet assay was also found correlated with the number and size of mtDNA deletions within a 8.7 kb segment amplified by long PCR.[47] Given the preceding discussion, it would seem counter-intuitive that more nuclear SDF be associated with more mtDNA fragmentation. In the current study, non-vital dead sperm and mtDNA fragmentation levels showed a positive correlation, so this phenomenon could be associated with the number of dead sperm cells, with these dead or dying cells contributing to a co-incident increase in both nuclear and mitochondrial DNA fragmentation.

If we focus on the DNA breakage type, SDF-mtDNA, in spite of being a consequence of DSBs, correlated more strongly with nuclear

SSBs. This association is somewhat difficult to explain and should be interpreted with caution, since nuclear and mtDNA fragmentation are estimated using different techniques. Nevertheless, it must be recognized that mtDNA nucleoids are less protected by associated proteins than nuclear DNA, so that SSBs produced by nucleases or ROS could be relatively more frequent in mtDNA than in nuclear DNA. In addition, the accumulation of SSBs could easily and rapidly result in the coincidence of SSBs in adjacent but opposite sites in both strands leading to DSBs.[16]

Unlike SDF-mtDNA, there was no significant correlation between nuclear-SDF and mtDNA copy number, further confirming the results of Faja et al.[24] In that report, SDF had been analyzed by the TUNEL assay. Our logistic regression identified SDF-Global and mtDNA copy number per spermatozoon as independent risk factors associated with non-normozoospermia. A similar conclusion was achieved by Shi et al., where SDF was evaluated using the SCSA.[48] A high predictive accuracy for consecutive diagnoses of clinical infertility has previously been described for mtDNA copy number (AUC: 0.91).[38] Our ROC curve analysis established strong potential discriminatory power of the two combined factors to distinguish non-normozoospermic samples.

The results from the analysis of the whole sperm samples studied here suggest that besides nuclear SDF, mtDNA copy number, and possibly mtDNA fragmentation are related to sperm quality and may serve as potential biomarkers for spermatogenic dysfunction. Expanding the study to larger cohorts would provide further detail about the relevance of mtDNA fragmentation in sperm quality and male fertility.

AUTHOR CONTRIBUTIONS

Experimental design: JLF, JG. *Experimental performing*: AT, FO. *Data analysis*: SJ, AT, FO, JG. *Manuscript writing*: JLF, JG, SJ.

CONFLICT OF INTEREST STATEMENT

JG and JLF are consultants of Halotech DNA SL.

DATA AVAILABILITY STATEMENT

Data are available upon request.

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