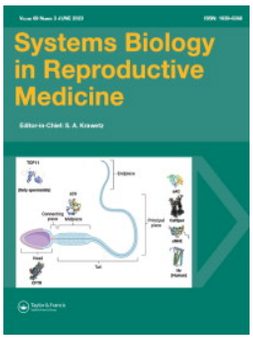

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# Intra-individual variation of sperm DNA fragmentation in the Human ejaculate

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**ABSTRACT**

This retrospective study assessed the biological intra-individual variability of the percentage of sperm with DNA damage (SDF) observed in subsequent ejaculates of the same individual. Variation in SDF was analyzed using the Mean Signed Difference (MSD) statistic based on 131 individuals, comprising 333 ejaculates. Either two, three or four ejaculates were collected from each individual. With this cohort of individuals two main questions were addressed; (1) does the number of ejaculates analyzed influence the variability in the level of SDF associated with each individual? and (2) is the variability observed in SDF similar when individuals are ranked according to their level of SDF? Results showed that the variation observed in mean SDF was not different when 2, 3 or 4 ejaculates were analyzed; consequently, we suggest that the assessment of SDF based on two ejaculates is likely to be representative of the mean SDF expected for the individual. In parallel, it was determined that the variation in SDF increased as SDF increased; in individuals presenting with an SDF value of lower than 30% (potentially fertile), only 5% possessed levels of MSD that could be considered as variable as that presented by individuals presenting with a recurrent high SDF. Finally, we showed that a single assessment of SDF in individuals with medium SDF (20-30%) was less likely to be predictive of the SDF value in the next ejaculate, and therefore, less informative of the patient's SDF status.

**Abbreviations:** CV: Coefficient of variation; ICSI: Intracytoplasmic sperm injection; IVF: In vitro fertilization; MSD: Mean signed difference; SDF: Sperm DNA fragmentation; L-SDF: Low SDF; M-SDF: Medium SDF; H-SDF: High SDF

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Sperm DNA fragmentation; male factor; ejaculate variability; human reproduction; clinical andrology

## Introduction

Human ejaculates with high SDF can compromise reproductive success as a result of failed fertilization and implantation, arrested development of the embryo and/or elevated rates of miscarriage (Robinson et al. 2012; Zhao et al. 2014; Borges et al. 2019; Esteves et al. 2021). For this reason, most innovative assisted reproductive laboratories have incorporated the assessment of SDF as an additional parameter to help better diagnose the etiology of male infertility. Although high SDF can be ascribed to cohorts of patients with specific pathologies, such as those with spinal cord injury (Gosálvez et al. 2022) or varicocele (Wang et al. 2012), there are other idiopathic causes of this phenomenon which still remain a mystery (Aktan et al. 2013). An interesting example of this phenomenon is the variation in SDF that presents when sequential ejaculates of the same individual are analyzed (Erenpreiss et al. 2006).

If intra-individual ejaculate variation of SDF does exist, then a single-point assessment of this parameter could significantly compromise a clinical decision and may have adverse consequences on reproductive outcomes. For example, if the clinician only relies on SDF data from the original ejaculate collected on the first presentation, without a follow-up evaluation closer to the time of the fertility treatment, this could lead to a misdiagnosis or inappropriate advice. Significant intra-ejaculate variation means that the ejaculate should be assessed as close as possible to the time of fertilization (either IVF or ICSI) so that suitable sperm selection protocols can be employed to reduce SDF.

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When assessing classical semen characteristics such as sperm concentration, morphology, and motility, it is well recognized that consecutive ejaculates of an individual can result in variable values (Alvarez et al. 2003; Oshio et al. 2004). While these sperm parameters have been traditionally used to classify men as infertile, sub-fertile or fertile, most are also considered relatively weak tools for infertility diagnosis (Guzick et al. 2001). In contrast, studies of variation with respect to the level of SDF in different ejaculates of the same individual are far less common, and where they do exist, there appears to be little agreement. For example, Smit et al. (2007) found that half the men attending their clinic showed an individual biological variation of SDF expressed as the coefficient of variation (CV) of two samples exceeding 10%; surprisingly, this variation was more profound in men with normal spermatogenesis than those with disturbed spermatogenesis. To explain their findings, they suggested chromatin structure may already be so impaired in men with alerted spermatogenesis that this phenomenon can overshadow any unidentified factors resulting in intra-ejaculate SDF variability. By contrast, when Erenpreiss et al. (2006) examined 282 infertile men, they reported a mean SDF coefficient of variation of 29%, with approximately one-third of their patients crossing the critical threshold for SDF between subsequent ejaculates. In a similar investigation, Zini et al. (2001) reported the coefficient of variation of SDF to be 9%, which was lower than that described for sperm concentration at 43.0%, progressive motility at 28.3% and sperm morphology at 28.3%; these results are equivalent to those previously reported by Evenson and coworkers in sperm donors (Evenson et al. 1991). Such disparity requires further investigation to validate the reliability of the SDF test as a diagnostic tool when the assessment of SDF is restricted to a single ejaculate, and whether or not this biological variability has a common etiological basis.

We propose that the phenomenon of intraindividual SDF variability is of crucial clinical importance since the tendency of many andrology laboratories is to assess SDF using one ejaculate, where it is assumed that this estimate is representative of the actual condition of the patient and that this value is both stable and strongly diagnostic. Nevertheless, the possibility exists that a single estimate may not be sufficient to provide an accurate assessment of SDF, and this may be further accentuated by the fact that those ejaculates that are used for fertilization (intrauterine insemination, IVF or ICSI) are typically not assessed simultaneously for SDF. Consequently, there is a serious risk, that an SDF value based on a previous analysis may not be representative of the semen quality of the patient at the time of fertilization. Establishing threshold values to predict the capacity of SDF for human fertility has attracted much attention (Esteves et al. 2021), but a better understanding of its intra-ejaculate repeatability is fundamental to assessing its capacity to do this.

The aim of this retrospective study was to explore two research questions. The first analysis examined whether a single assessment of SDF of a patient was sufficient to give a true representation of their ejaculate quality and whether variability of this estimate changed with repeated ejaculations. For this purpose, three experiment cohorts were defined from the dataset according to the number of repeat ejaculates observed; 2, 3, and 4 ejaculates per individual. The second analysis was to investigate whether variability observed from multiple ejaculates was dependent on the level of SDF of the patient.

## Results

### *MSD variability associated to number of ejaculates*

Results of SDF obtained in the different groups of individuals when categorized according to the number of ejaculates collected per individual are shown in (Figure 1A). (Figure 1B), reports the MSD in individuals presenting 2, 3 or 4 ejaculates, respectively.

The values of MSD within each ejaculate group failed to follow a normal distribution (2 ejaculates: Shapiro-Wilk - 0.838, $p = 0.0023$ ejaculates: Shapiro-Wilk = 0.904, $p = 0.0304$ ejaculates: Shapiro-Wilk - 0.885, $p = 0.013$), so that non-parametric statistics were used to compare the MSD values associated with each group. No statistical differences were observed in the MSD values when the different numbers of ejaculates were compared (Kruskal–Wallis test, $Chi^2 = 0.823$, $p = 0.663$).

To assess any interdependence in the SDF values with respect to sequential ejaculates within each individual, all possible correlation permutations between two different values of SDF of the same individual were tested. A strong correlation between SDF observed in one ejaculate and subsequent ejaculates of the same individual was observed (See Table 1).

### *The effect of the level of SDF on MSD variability*

Figure 2 shows a summary of the results obtained for SDF and MSD when the individuals were grouped

according to their respective SDF (L-SDF, M-SDF, H-SDF). In all cases, values associated with the variable MSD did not show a normal distribution (Group L-SDF: Shapiro-Wilk = 0.851; $p = 0.000$. Group M-SDF: Shapiro-Wilk = 0.895, $p = 0.008$. Group H-SDF: Kolmogorov-Smirnov 0.131; $p$ 0.005), so that non-parametric analyses were conducted.

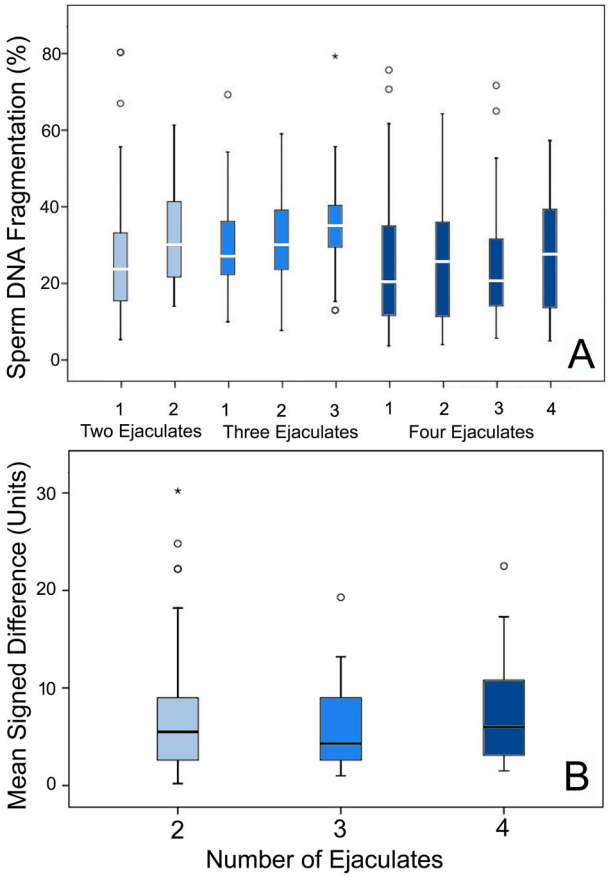


**Figure 1. Box and whisker plots representing the distribution of sperm DNA fragmentation (SDF) values in individuals after 2, 3 and 4 consecutive ejaculates and Mean Signed Differences (MSD) associated to each group according to the number of ejaculates.** A). Distribution of SDF values observed in the different ejaculates. SDF values were grouped considering two, three and four ejaculates. B) Mean Signed Differences (MSD) observed when individuals are grouped according to the number of repeated ejaculates assessed for SDF.

Table 2 reports the descriptive numerical values associated with MSD in the 3 pre-established groups according to the mean level of SDF observed. When values of MSD associated with L-SDF, M-SDF, and H-SDF were compared, significant differences were obtained (Kruskal-Wallis H - 22.2; $p = 0.000$). When the MSD values are compared pair-wise, as shown in Table 3, only the comparison between M-SDF and H-SDF failed to show a significant difference.

A correlation analysis was performed using the mean values of SDF and their respective MSD value (Figure 3). A Spearman Rho analysis revealed a significant positive correlation between both variables (Spearman Rho = 0.389: $p = 0.000$).

Based on these results, we further investigated if the MSD value had any power to discriminate between individuals presenting with low or high SDF. For this purpose, we assumed that individuals presenting with $\geq$ 30% of SDF represented a pathological condition (see Esteves et al. 2021). Two groups were constructed: individuals presenting less than a 30% of SDF (i.e., L-SDF + M-SDF) and individuals presenting $\geq$ 30% (H-SDF). Receiving Operator Characteristics (ROC) statistics were used to define the sensitivity and specificity associated with the MSD values. Results showed that an MSD value of 12.9 could discriminate both groups with a sensitivity of 80% and a specificity of 70% (Area Under the Curve: 0.796; CI 95%: High 0.699, Low 0.786; $p = 0.000$).

Figure 3 highlights the clinical cut-off SDF value of 30% (see red line in Figure 3) to distinguish potential fertile and infertile individuals and the cut-off value of 12.9 for MSD as derived from the ROC analysis (blue line in Figure 3); based on this presentation, it is possible to derive 4 quadrants that represent the whole distribution. These quadrants can be identified as the LL-quadrant (Individuals presenting Low SDF and Low MSD), LH-quadrant (Low SDF and High MSD), LH-quadrant (Low SDF and High MSD) and HH-quadrant (High SDF and High MSD).

If the proportion of individuals in each quadrant is examined, 45% of the population is represented in the LL-quadrant, 40.4% in the HL-quadrant, 2.3% in the LH-quadrant and 12.2% in the HH-quadrant.

**Table 1. Spearman Rho correlation (S-Rho) and associated P value after comparing paired SDF values in different ejaculates within the same individual. Individuals in which 2, 3 and 4 ejaculates were assessed have been included in the analysis. Spearman Rho $p = 0.05$.**

| Ejaculates Compared | 1–2 S-Rho (p) | 1–3 S-Rho (p) | 1–4 S-Rho (p) | 2–3 S-Rho (p) | 2–4 S-Rho (p) | 3–4 S-Rho (p) |
|---|---|---|---|---|---|---|
| 4 ejaculates | 0.609 (0.002) | 0.646 (0.001) | 0.648 (0.001) | 0.648 (0.001) | 0.589 (0.003) | 0.635 (0.001) |
| 3 ejaculates | 0.651 (0.000) | 0.541 (0.005) | | 0.724 (0.000) | | |
| 2 ejaculates | 0.437 (0.000) | | | | | |

S-Rho: Spearman Rho correlation.

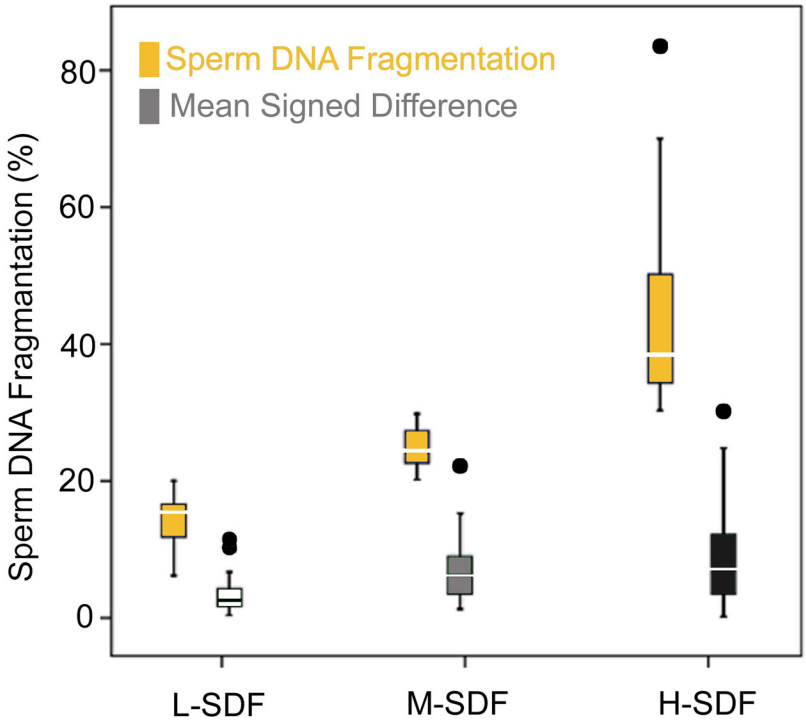


**Figure 2. Box and whisker plots representing the distribution of Sperm DNA Fragmentation (SDF) values and associated Mean Signed Difference (MSD).** The different patients were grouped considering the SDF threshold values fixed in this survey (SDF $\leq$ 20%: L-SDF), intermediate level of SDF (SDF ranging from 21%–29%: M-SDF) and a high level of SDF (SDF $\geq$30%; H-SDF). In yellow plots corresponding to SDF values and in grey plot corresponding to MSD values.

**Table 2. Descriptive statistical values associated to MSD in each cohort (Low L-SDF $\leq$ 20%, Medium M-SDF 20–29%, High H-SDF $\geq$ 30 %).**

| | N | MSD Mean | SD | Min value | Max value |
|---|---|---|---|---|---|
| L-SDF | 23 | 3.3 | 2.6 | 0.4 | 11.5 |
| M-SDF | 29 | 6.9 | 4.8 | 1.3 | 22.2 |
| H-SDF | 69 | 8.5 | 6.4 | 0.2 | 30.2 |

**Table 3. Pair-wise comparison of MSD values of L-SDF, M-SDF and H-SDF.**

| MSD comparison | N | Mean Rank | Wilcoxon W | *p* value (two-tailed) |
|---|---|---|---|---|
| L-SDF/M-SDF | 33/29 | 24.2/39.8 | 798.5 | 0.001 |
| L-SDF/H-SDF | 33/69 | 32.2/60.7 | 1064 | 0.000 |
| M-SDF/H-SDF | 29/69 | 45.4/60.7 | 1319 | 0.364 |

Interestingly, individuals observed presenting with low levels of variation (MSD lower than 12.9 units i.e., those in LL and HL quadrants) represented 83% of the total populations. In contrast, for those individuals presenting with an SDF lower than 30% (i.e., LL and LH quadrants or those typically considered as fertile individuals in terms of SDF), only 5% of the total showed MSD values greater than 12.9.

## Discussion

The results of this study indicate that while the estimates of SDF from two successive serial ejaculates of the same individual may show different quantitative values, the variability, expressed as the MSD of SDF, does not show significant differences, even if further ejaculates (up to 3) are collected for evaluation. This conclusion was reached after comparing all the MSD values observed in the groups presenting with a different numbers of ejaculates (Figure 1), but also after considering the correlation observed when paired SDF values in different ejaculates within the same individual are tested (Table 1). This result would appear at first glance to support the overall conclusion that based on the data from the whole population, the assessment of SDF using two ejaculates would be sufficiently accurate for documenting the range of dispersion of SDF that would typically be observed in an individual.

With regard to the effect of the level of SDF on the variation of SDF in subsequent ejaculates from the same individuals, the results showed an increase in MSD with increasing SDF (MSD L-SDF = 3.3 units; MSD M-SDF = 6.9 units; MSD H-SDF = 8.5 units). This observation is generally consistent with that of Zini et al. (2001) which used CV to express variability in an equivalent experimental design and reported the CV associated with SDF to be 9% in fertile men and around 20% in infertile men. However, these values are somewhat different from that of Erenpreiss et al.

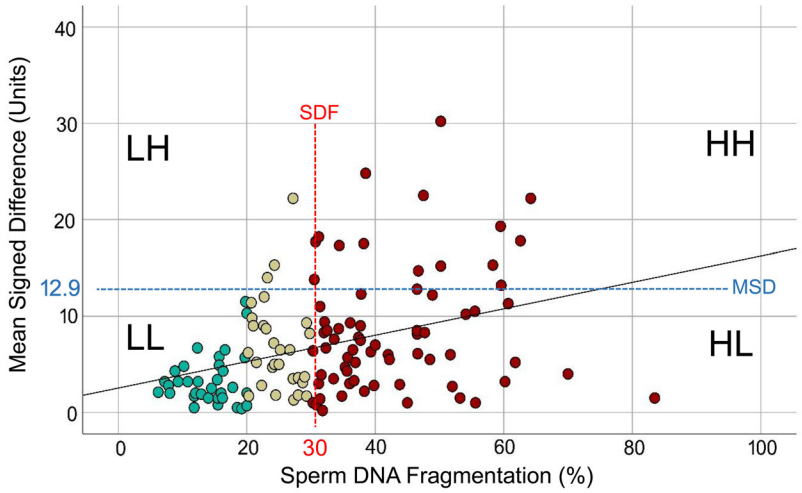


**Figure 3. Correlation of mean of sperm DNA fragmentation (SDF) of individuals grouped by L-SDF (green dots), M-SDF (yellow dots) and H-SDF (red dots) with that of the corresponding mean signed difference (MSD).** Using a threshold level of 30% for SDF and the MSD value derived from the receiving operator characteristics curve (MSD = 12.9) it was possible categorize 4 quadrants in the plot: (1) LL-quadrant individuals with a low level of SDF and a low MSD, (2) HL-quadrant individuals with a high SDF and low MSD, (3) LH-quadrant individuals with low SDF and high MSD and (4) HH-quadrant individuals with a high SDF and high MSD.

(2006) who reported a CV of 29% in patients with a high level of SDF.

Using MSD to investigate data dispersion, we observed that individuals presenting with MSD values lower than 12.9 units were those presenting with low and medium levels of SDF. This variation would be equivalent to a 9% CV as observed by Zini et al. (2001) in fertile individuals. Nevertheless, we must be cautious when making these comparisons for two reasons: (1) the established percentages of SDF to define low, medium, and high levels of SDF in both experiments were not identical and (2) as explained in the Material and methods section, the CV, defined as the ratio of the standard deviation (SD) to the mean, makes this statistic highly dependent on the observed mean of SDF.

When the level of variation of each group is compared using MSD, the variation between L-SDF and M-SDF and L-SDF and H-SDF was significant but the variation was not significant between M-SDF and H-SDF (Table 3). These results, together with the fact that the level of variation in the L-SDF group was the lowest observed, suggest that for ejaculates from individuals presenting with a low SDF ($\leq$ 20% or potentially good fertility), the expected level of inter-ejaculate variation is low, whereas when an increasing and an abnormal level of SDF is detected (more than 20%) there is a tendency to show more dispersed SDF values among different ejaculates. This observation is contrary to the results reported by Smit et al. (2007) who found that a large portion of the samples analyzed showed a more intense CV associated with the SDF in men with normal spermatogenesis than those with disturbed spermatogenesis.

Intuitively, but also supported by experimental evidence (Zini et al. 2001; Le et al. 2019; Campos et al. 2021), individuals presenting with low SDF, and coincidently a higher quality seminogram, also tended to exhibit a low level of variation in DNA damage in sequential ejaculates; conversely, large variations in SDF from sequential ejaculates might be expected when high percentages of SDF are observed since they are more likely to be associated with poorer quality seminograms. The results of our investigation show that for individuals presenting with SDF lower than 30%, only 5% present levels of MSD higher than 12.1 units (Figure 3; 3 individuals in region LH from a total of 62 [LL + LH]) presenting SDF lower than 30%). Taken together, these results point to the fact that the assessment of a single value of SDF would be only representative of the SDF status when low values of SDF are observed. Our results suggest that a single assessment of SDF, when the percentage of sperm with SDF surpasses 20–30%, is likely to be less predictive of the SDF value in the next and subsequent ejaculates, and is therefore, less informative of SDF status of the patient.

From a clinical perspective, our observations are important because in practice the level of DNA damage at first diagnosis and that used for fertilization can potentially differ. This may not be that critical in patients presenting with a low SDF, but it may compromise the results of pregnancy in patients categorized in M-SDF cohort, because of a biased

appreciation of the SDF based on a single ejaculate. Our observations are also congruent with the results recently reported by Esteves et al. (2022) in which paired ejaculates collected 3 months apart from 216 patients were tested for their degree of agreement using Cohen's kappa statistic based on the pre-classified SDF cohort as used in the current study (L-SDF, M-SDF, and H-SDF). Esteves et al. (2022) found that the agreement rate was highest (approximately 80%) in ejaculates initially classified as either normal or high but lowest (approximately 60%) among those with intermediate SDF levels. The frequency of intermediate SDF ejaculates downgraded to normal or upgraded to high SDF classes in the second test was similar (approximately 20%).

The data from Esteves et al. (2022) and the results presented in the current study appear to be pointing to the same conclusion, that individuals presenting with a low SDF, will show a low MSD, and subsequently, there is a low tendency to change from their pre-classified group at first diagnosis. Similarly, when individuals present with a high level of SDF, the MSD is high, but this disparity rarely changes the pre-classified group to where these patients were initially assigned. The issue of SDF variability, therefore, appears to become most problematic when the initial diagnostic SDF is in the 20-30% range; in this case, a second analysis would be advisable and, if possible, an SDF assessment of the actual sample used for fertilization would be also recommended.

Given the diverse range of parameters known to affect SDF over the reproductive life span of the individual, we must always consider that this type of retrospective analysis may be influenced by a series of uncontrollable confounding factors such as abstinence, conditions for semen collection and processing, time-lapse to assess sperm DNA fragmentation after collection, iatrogenic damage, patient age, time between ejaculations, or the type of patients to be included in the analysis. Other parameters such as spontaneous infections, pychological and physiological stress, and genetic background are more difficult to control. This fact not only applies when we are analyzing variations in SDF but for all parameters of the spermiogram. Nevertheless, these standard limitations, common to most retrospective studies do not, in our opinion, invalidate the results obtained in this study; in addition, what we have reported here is consistent with previously published information.

Additionally, the results of this study showed that the estimates of SDF from at least two successive serial ejaculates of the same individual, while showing different values, do not show significant differences in variability as measured by MSD, even if subsequent ejaculates (up to 3) are evaluated. However, when the individuals in the population are categorized further on the basis of SDF severity into LSDF, MSDF, and HSDF cohorts, those with a low SDF (lower than 20%), show less variability in the values of SDF among different ejaculates than that observed when SDF was 20–30% or higher than 30%. Therefore, we recommend at a minimum, that patients within the range of 20–30% sperm DNA fragmentation in their first ejaculate assessment, should be tested twice.

## Materials and methods

### Semen samples

This retrospective study was conducted to assess the natural biological intra-individual variability in SDF observed in different ejaculates of the same individual. A dataset incorporating SDF was obtained from a Spanish human reproduction clinic, considering the following inclusion criteria: (1) age range 22–35, (2) ejaculates obtained within a period of 4 months, (3) at least a 7-day minimum between ejaculates, (4) ejaculates collected after 3 days of declared abstinence, (5) ejaculates obtained from individuals that did not receive antioxidant supplementation, (6) all men as part of the assisted reproduction program of the clinic were considered as potential patients and (7) samples collected by masturbation and assessed for SDF 30 min after semen collection. One hundred and thirty-one individuals, comprising 333 ejaculates, were assessed for SDF using the Sperm Chromatin Dispersion test (Halospem; Halotech DNA, Madrid, Spain). The SCD process was performed by the same observer and followed the protocol as described in (Gosálvez et al. 2011).

### Retrospective cohort experimental design

This study retrospectively explored two research questions. The first analysis examined whether a single assessment of SDF of a patient was sufficient to give a true representation of their ejaculate quality and whether the variability of this estimate changed with repeated ejaculations. For this purpose, three experiment cohorts were defined from the dataset according to the number of repeat ejaculates observed; 2, 3, and 4 ejaculates per individual. The second analysis was to investigate whether variability observed from multiple ejaculates was dependent on the level of SDF of the patient. For this purpose, all individuals were ranked

according to their mean percentage of sperm with DNA Damage (SDF) obtained from different ejaculates in the neat ejaculate as defined by Esteves et al. (2021) into those with a low SDF (SDF $\leq$ 20%: L-SDF), intermediate SDF (SDF ranging from 21%–29%: M-SDF), and high SDF (SDF $\geq$ 30%; H-SDF). For this analysis of variability, data from sequential ejaculates (2, 3, and 4) were combined.

### *Statistical analysis*

Once the cohorts for analysis 1 and 2 were created, the mean signed difference (MSD) associated with the mean SDF of each individual was used for statistical comparison. To estimate possible variations in the SDF levels obtained within each individual among different ejaculates, the coefficient of variation (CV), was initially calculated as per the previous studies of Erenpreiss et al. (2006) and Smit et al. (2007). However, when our data were screened using this statistic, we observed a potential confounding issue, in that it can produce a bias in the perception of variability. CV is a measure of the dispersion of a probability or frequency distribution expressed as a percentage and is defined as the ratio of the standard deviation (SD) to the mean. Consequently, this statistic is highly dependent on the observed mean, so for a constant SD, the higher the mean, the lower the CV value. Given this limitation, we decided to employ a different statistic, known as the Mean Signed Difference (MSD) and which has been defined as

$$MSD\bar{x} = \frac{\sum_{i=1}^{n} |\mathrm{xi} - \bar{x}|}{\mathrm{N}}$$

where $\bar{x}$ is representative of the mean value of SDF associated to each individual, *xi* represents a single value of SDF of the series of ejaculates and N is the total number of ejaculates within one individual. This statistic reacts independently of the mean of the analyzed sample.

The data matrix for the mean of SDF and the MSD associated to each individual was exported from excel to SPSS (IBM SPSS Statistics Package, NY, USA). Normal distribution of the variable (MSD) was performed using Shapiro-Wilk or Kolmogorov-Smirnov test depending on the size of the sample. As the MSD data for both analyses did not conform to a normal distribution, non-parametric analyses were used for comparison; these included Mann-Whitney *U* and Kuskal–Wallis H. A correlation analysis was also performed using a Spearman Rho to test how the MSD varied with the mean of SDF observed in each individual. A receiver operating characteristic (ROC) curve was also used in analysis 2 to test the predictive value of MSD (Sensitivity and Specificity) in differentiating groups of individuals presenting with low or high SDF.

## Ethical approval

This study had ethical approval from the clinic and was approved by the University of Seville Ethics Committee (3375125c8b5f1d04c9511825aef98d3 09135328c – approved 04/08/2017). Informed consent was obtained.

## Acknowledgments

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## Disclosure statement

The authors report no conflict of interest.

## Authors' contributions

Patient recruitment and sperm sample recruitment, semiograment, SDF assessment: MG-M. Sperm DNA fragmentation assessment confirmation and graphic documentation: CL-F. Experimental design and clinical supervisor: PS-M. Experimental design, data processing, paper writing, and editing: SDJ, JG.

## Data availability statement

All data is available on request from Prof Jaime Gosalvez.

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