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ORIGINAL ARTICLE

Role of intra-individual variation in the detection of thresholds for DFI and for misclassification rates: A retrospective analysis of 14,775 SCSA® tests

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Abstract

Background: Sperm DNA damage is associated with reduced male fertility after natural conception and intrauterine insemination. However, the impact on in vitro fertilization (IVF) and especially intracytoplasmic sperm injection (ICSI) treatments is still unclear. Few studies have focused on the intra-individual variation in DFI even though it may have an important role to play in terms of detection of thresholds and for misclassification rates.

Methods: Results for Sperm Chromatin Structure Assay (SCSA®) tests performed for 70 European fertility clinics between January 1st, 2008 and December 31st, 2022 were examined. A small retrospective study included 406 couples receiving their first treatment with IVF or ICSI. These results were then used for a mathematical simulation to investigate the role of intra-individual variation. The large retrospective study included a total of 14,138 diagnostic tests and 637 tests from an IUI study. The distribution of DFI was assessed for the IUI cohort and cohorts of patients attending Sims IVF and Fertility Center Hamburg (FCH). Descriptive analysis of the data was performed regarding time of year, male age, and year.

Results: When DFI was above the thresholds of 15 and 25, a significant reduction in ongoing pregnancies after 12 weeks of gestation was observed for IVF and ICSI treatments, respectively. For IVF treatments, the pregnancy rate was reduced from 45.1% to 24.6%, odds ratio = 2.58 ($p = 0.004$). For ICSI treatments, the pregnancy rate was reduced from 48.6% to 29.6%, odds ratio = 2.00 ($p = 0.032$). Intra-individual variation was significantly related to the misclassification rate and the sample size required to identify a threshold. The percentage of patients with a DFI below 15 was 64.8% for the IUI cohort and 51.7% and 41.6% for cohorts of patients attending Sims IVF and FCH, respectively. The median DFI for these cohorts differed significantly and was 11.6, 15.0 and 17.2, respectively. DFI shows a seasonal variation, and increases with male age. During the past 15 years, the median DFI has increased by 0.05% per year ($p = 0.02$).

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Discussion and conclusions: Ongoing pregnancy rates are reduced significantly for both IVF and ICSI treatments when DFI is above the thresholds of 15 and 25, respectively. The misclassification rate and the required sample size increase with increasing intra-individual variation. Couples with a DFI above 15 are more likely to experience failed assisted reproductive technology (ART) cycles. DFI appears to have increased during the past 15 years.

KEYWORDS
embryo mortality, ICSI, intra-individual variation, IVF, pregnancy rate, SCSA® sperm DNA integrity

1 | INTRODUCTION

It is now 25 years since Evenson et al. were the first to show a significant relationship between sperm DNA fragmentation (DFI) and reduced male fertility.[1] The authors established two thresholds for the clinical use of the results of the Sperm Chromatin Structure Assay (SCSA®). A DFI below 15 was indicative of "high fertility" with 84% of the couples in this group achieving pregnancy naturally within 3 months. In contrast, no couples achieved pregnancy if the DFI was above 30. A DFI between 15 and 30 was associated with "reduced fertility" and more time was required to achieve pregnancy. In addition, an increased risk of miscarriage was observed for this group of couples.

On the basis of these very promising results, it would seem reasonable to assume that this technology would have been well received by the reproductive societies since. However, in fact, sperm DNA fragmentation has had a long and bumpy road and today it is still a controversial topic.[2–4]

In our opinion, there are several reasons for the controversy and misunderstandings. Firstly, there are four different methods for assessing sperm DNA damage: COMET (single cell gel electrophoresis), SCD (sperm chromatin dispersion test), TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling), and SCSA® (Sperm Chromatin Structure Assay). The principles behind these methods differ, which means that the resulting measurements cannot be compared directly. For the sake of simplicity, we only focus on SCSA® in this paper. However, the same considerations and principles apply regardless of the method used for testing sperm DNA fragmentation.

The first source of disagreement arose in connection with the interpretation of the results of the study in 1999.[1] A commonly accepted perception back then was that sperm DNA fragmentation was a stable parameter almost like the man's fingerprint. Therefore, it was assumed that the reduction in reproductive outcome observed after natural intercourse would be the same for an intrauterine insemination (IUI), in vitro (IVF), and intracytoplasmic sperm injection (ICSI) treatments.[5] Yet this assumption was incorrect, and it was soon discovered that the outcome of IVF and especially ICSI treatments was affected to a lesser degree than the outcome of IUI treatments.[6–8] Another important and surprising observation was that prolonged abstinence time could increase DFI whereas a shorter abstinence time would lead to a reduction in DFI.[9–11]

The different impact on IUI, IVF, and ICSI treatments as well as the influence of the duration of abstinence made it clear that there was a need to seriously reconsider the way in which sperm DNA becomes damaged. To this end, in 2010, Aitken and De Iuliis developed a new hypothesis known as the Two-Step Hypothesis.[12] According to this, damage to sperm DNA occurs in two steps. The first step is a disturbance of spermiogenesis, which leads to the production of mature sperm with fragile DNA. The second step is oxidative damage to the sperm DNA, which mainly occurs after the sperm has left the testicle and increases its production of energy required for motility.

During spermiogenesis, the haploid spermatids undergo major morphological changes to form mature spermatozoa. In the last 21 days of spermiogenesis, histones are replaced by basic transition proteins and then protamines, which ensures the tight compaction of chromatin, so the DNA becomes transcriptionally inactive and inaccessible to DNA repair proteins.[13,14] Any disturbance during spermiogenesis (i.e., poor protamination or nicks in the DNA) reduces the stability of the sperm DNA and makes it vulnerable to reactive oxygen species (ROS). Although ROS may originate from external sources (i.e., activated leukocytes), the most common source of oxidative attack is in the form of H_2O_2, which is released from the spermatozoa's own mitochondria.[12] Therefore, for a spermatozoa with vulnerable DNA, the journey to the oocyte and the penetration of the zona pellucida is a race against the time. The vulnerable DNA will soon have several places with single stranded DNA breaks, but such nicks will quickly be transformed into double stranded breaks, thereby causing the DNA to break into fragments (fragmentation). IUI treatment is much more energy demanding for the sperm cell than IVF treatment, and the DNA will, therefore, have acquired more damage before fertilization has been completed. For a spermatozoon with vulnerable DNA, the least demanding treatment is ICSI as the spermatozoa does not need to produce energy to reach the oocyte or become hyperactivated and penetrate the zona pellucida. Clearly, a prolonged stay of the motile spermatozoa in the epididymis will also increase sperm DNA damage.

We commonly talk about sperm DNA fragmentation (abbreviated to SDF), but the Two-Step Hypothesis provides a more nuanced picture of the problem. The spermatozoa with fragmented DNA is only the tip

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of the iceberg. The underlying problem is the reduced stability of the sperm DNA, which makes it vulnerable to damage.

Assessing semen quality has traditionally been limited to methods that suffer from a high degree of random variation and a significant amount of intra-individual variation.[15,16] The study on the SCSA® by Evenson et al. was among the first to use flow cytometry to analyze spermatozoa. Flow cytometric analysis of sperm quality has the potential for an extremely high degree of precision.[17] Indeed, the theoretical binomial measurement error when analyzing 5,000 spermatozoa is <1%. Evenson et al. demonstrated that the intra-individual variation for DFI determined by the SCSA® was small in comparison to the classical parameters reported by Schrader et al.[15,18] In addition, DFI has also been shown to be highly repeatable between laboratories using the SCSA®.[18] Since, several studies have used flow cytometry as a technological platform to assess sperm concentration, sperm viability, mitochondrial function, and acrosomal status in samples from different species of animals as well as humans.[19–27] Several studies concerning the SCSA® have been conducted with spermatozoa from various animals and humans.[8,27–33]

Evenson et al. inspired other researchers, who published several studies during the following couple of decades.[1] However, a large proportion of the studies which followed were based on small sample sizes or had other shortcomings.[5,34,35] As a result, various thresholds for DFI were reported, and some authors were even unable to confirm a threshold.[30,31,36,37] One of the largest studies concerning the SCSA® and the outcome of IUI, IVF, and ICSI was performed by Bungum et al.[8] This study confirmed a threshold of 30 for IUI, but a threshold of 15 was not confirmed. Furthermore, a threshold was not confirmed for IVF or ICSI, but when the DFI was above 30, the treatment outcome for IVF was reduced in comparison to ICSI.

For a test to be useful, it must be reproducible, so similar results are obtained each time the same man is tested.[16,38] In the longitudinal study from 1991, Evenson et al. demonstrated that DFI is largely independent of classical semen parameters and that it is a very stable parameter.[18] The within-male coefficient of variation for DFI (CV_W) was 23%. Since then, only 3 studies have focused on intra-individual variation for DFI using the SCSA®. Erenpreiss et al. and Oleszczuk et al. estimated the intra-individual variation CV_W to 29.0% and 30.1%, respectively.[39,40] In contrast, Blomberg Jensen et al. conducted a randomized and controlled trial and estimated the intra-individual variation in DFI to a CV_w of 16.5%.[41] The discrepancy in the reported intra-individual variation in DFI may be important as a small degree of variation is essential for correct diagnosis and treatment. This led us to formulate the following research question:

Does intra-individual variation in DFI play a significant role in the ability to detect thresholds for DFI and for misclassification rates?

The secondary objectives of this work were to demonstrate the impact of DFI on assisted reproductive technology (ART) treatments and to test if DFI had stayed at the same level during the study period.

In the following, we perform a retrospective analysis of 14,775 SCSA® tests to answer these questions. The impact of DFI on the outcome of IVF and ICSI is demonstrated using a small cohort of 406 couples receiving their first treatment. Subsequently, we use these results in a mathematical simulation with different levels of intra-individual variation and calculate the required sample size to demonstrate a threshold for DFI as well as a misclassification rate. Finally, the impact of DFI on ART treatments is demonstrated in a retrospective analysis of all 14,775 SCSA® tests. Descriptive analysis of the data shows the effect of time of year, male age, well as development in DFI from 2008 to 2022.

2 | MATERIALS AND METHODS

2.1 | Preparation of semen samples

Semen samples were prepared by 70 European fertility clinics between January 1st, 2008 and December 31st, 2022 from a total of 14,138 clinical cases. Couples receiving fertility treatment signed a consent form in which they accepted that their data could be used anonymously for research. In addition, 637 tests from an IUI study were also included resulting in a total of 14,775 SCSA® tests.

Fertility Center Hamburg (FCH), Germany, started to use the SCSA® in 2007 and contributed 2591 of the clinical cases (average male age was 38.4 years, CI 38.2–38.7) from January 1st, 2008. Sims IVF (Sims IVF), Dublin, Ireland, used SPZ Lab's service from 2008 to 2012 and contributed 1971 of the clinical cases (average male age was 38.9 years, CI 38.7–39.2).

A standard abstinence time of 3 days was recommended. After liquefaction for a minimum of 20 min, 0.5 mL neat semen was diluted with a sperm wash medium or with a TNE buffer (0.01 M TrisCl, 0.15 M NaCl, 1 mM Disodium-EDTA, pH 7.4). Diluted semen samples were placed in Sarstedt CryoPure vials 1.8 mL and were frozen in a dry shipper or directly in liquid nitrogen. The clinics sent the dry shippers (MVE SC 4/3) to SPZ Lab by courier.

The concentration of spermatozoa was determined by the fertility clinics and reported on the order forms along with the man's name and date of birth. At Sims IVF and FCH, sperm concentration was determined using a Makler chamber. The same device was used for motility assessments, which were assessed in four categories: A (rapidly progressive motile), B (progressively motile), C (non-progressively motile) and D (non-motile). All assessments were performed twice and were repeated if the results did not agree. A+B motility was reported as the percentage of motility.

2.2 | Fluorescent Staining

The dilution and staining of samples was performed according to the procedure described by Evenson.[42] A vial was thawed in a water bath at 37°C for 4 min and then incubated on ice for 5 min.[32] Approximately 11% of the thawed semen samples were observed to be viscous when they were gently aspirated with a pipette. These samples were diluted and mixed carefully before processing. Sperm concentration was determined using a Nucleocounter SP-100 according to the manufacturer's instructions (Chemometec A/S). An aliquot of the sample

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was then diluted to a concentration of 2×10^6 spermatozoa/mL with a TNE buffer to a total volume of 200 µL in a 5 mL Falcon tube (Corning Science). An acid detergent solution (400 µL; 0.08 M HCl, 0.15 M NaCl, 0.1% v/v Triton X-100, pH 1.2) was added, and a stopwatch was started. After precisely 30 s, 1.20 mL of AO staining solution was added (6 µg/mL acridine orange (AO, Polysciences Inc.) 0.037 M citric acid, 0.126 M Na_2HPO_4, 1.1 mM Disodium-EDTA, 0.15 M NaCl, pH 6.0). The stained sample was then placed in a flow cytometer. Data acquisition of 5000 events was performed 3 min after initiation of the acid detergent treatment. For each semen sample, a minimum of two aliquots were stained and analyzed.

2.3 | Flow Cytometry

The samples were analyzed using a FACSCalibur or a FACSCan flow cytometer (BD Biosciences). Both instruments were operated with a 15 mW, 488 nm laser. Emission signals were separated by a 560 nm dichroic mirror. The green fluorescence (FL1) was collected through a 515–545 nm band-pass filter. The red signal was collected through a 650 nm long-pass filter. The flow rate was set to "high" (60 µL/minute) and resulted in an event rate below 150 per second.

The DNA fragmentation index (DFI) was determined by the CellQuest Pro Software (BD Biosciences) as the percentage of cells outside the main population exhibiting a red signal (single stranded DNA). The main population consisted of spermatozoa exhibiting a green signal (double stranded DNA).

To ensure stringent quality control, all flow cytometric analyses were inspected visually. DFI values for the two replicates had to agree with each other (SD below 2.5%). If this was not the case, two new replicates were analyzed. Approximately 7% of the samples were reanalyzed.

2.4 | Recommended use of the SCSA® test

In 2008, SPZ Lab's clinical recommendation was that IUI or IVF treatment should be performed when the DFI was below 25. ICSI treatment was recommended when the DFI was above 25, but it was also conducted according to the guidelines used by the clinics. This recommendation was according to the implementation of SCSA® in the Southern Sweden Hospital Region in 2007 following the study by Bungum et al.[8]

2.5 | Design of the small retrospective study

This study was based on cases from FCH and Sims IVF. Clinical IVF and ICSI records were assessed consecutively in the fall of 2012. Couples were only selected if they had received their first treatment within 3 months of the SCSA® test. Only the outcome of the first treatment cycle was included in the data. IVF (n = 210) and ICSI (n = 196) records were identified. The 25 couples did not have embryo transfer performed on the first treatment cycle and were, therefore, not included in the study. The women had an average age of 35 years with a range of 24–46 years. The age of the men ranged from 22 to 60 years with an average of 37 years. For each record, the treatment date, date of birth of the woman and man, and results for sperm concentration and motility were recorded. Pregnancy was confirmed by ultrasound after 12 weeks of gestation. Thresholds for DFI were 15 and 25 for IVF and ICSI, respectively. The effect on the outcome below and above the threshold was analyzed.

The effect on embryo morphology was assessed for FCH (IVF n = 75, ICSI n = 56). The mean grade for the best embryo and the average grade of the embryos was estimated with a 95% confidence interval for IVF and ICSI treatments below and above the respective thresholds. Similarly, the likelihood of pregnancy was estimated with a 95% confidence interval.

2.6 | Role of intra-individual variation

A mathematical simulation was performed to see how differences in intra-individual variation in the assessment of sperm DNA damage affect the observed pregnancy rates below and above a threshold. The work was based on the result of the small retrospective study in combination with the magnitude of the intra-individual variation as estimated in a randomized controlled trial.[41]

2.7 | Design of the large retrospective study

The large retrospective analysis was carried out in January 2023 and utilized data from the 14,138 diagnostic tests of sperm DNA damage, which were performed for 70 European fertility clinics between January 1st, 2008 and December 31st, 2022.

The median DFI was considered in relation to year, time of year, male age, and fertility clinics, and the comparison of two groups was quantified using a median ratio. To assess possible differences between clinics, the median DFI was calculated for Sims IVF and FCH. The median DFI was also calculated for a previously unpublished IUI study with 637 tests in connection with IUI treatments performed by one of the clinics. The median DFI for the IUI cohort was compared against the median DFI of all clinics. The median DFI for the Sims IVF and FCH cohorts was compared with the median of the other clinics.

2.8 | Statistical analyses

The analyses were performed using statistical software R version 4.4.1. In the small retrospective study, pregnancy rates between the DFI groups were compared using odds ratio in logistic regression adjusting for female age, sperm concentration and motility. The percent of the total variation of DFI described by sperm motility and concentration

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was assessed as the reduction in residual variation in a linear regression model of log-DFI with and without the inclusion of sperm motility and concentration as independent variables.

To perform the mathematical simulation, the pregnancy rates were estimated using the binomial model and compared between DFI < 15 and DFI 15–25 groups for IVF treatment and between DFI < 25 and DFI≥25 groups for ICSI treatment using the chi-squared test. The comparison of pregnancy rates between the DFI groups may have been confounded due to measurement error of the true male DFI level. To study the impact of measurement error, a fixed pregnancy rate was assumed within DFI < 15 and DFI 15–25 groups for IVF and within DFI < 25 and DFI ≥ 25 groups for ICSI. The true pregnancy rate without misclassification was then estimated in groups based on the true DFI level, and the assumption that DFI follows, approximately, a normal distribution on the logarithmic scale. Simulated sample size calculations were performed to ensure 80% power using 10,000 replications. The simulations are an illustration of non-differential misclassification yields risk difference toward the null value.[43,44] The necessary sample size was computed with the observed within subject CV_w of 16.5%,[41] including scenarios in which the CV_w was increased by 50% and 100%. For each scenario, the misclassification rate was calculated as the percent of the sample for which the true DFI was above the threshold (15 for IVF treatments and 25 for ICSI treatments), but the measured DFI was below the threshold, or the true DFI was below the threshold, but the measured DFI was above the threshold.

In the large retrospective study, the analysis of DFI was performed on the logarithmic scale using multiple linear regression with robust variance estimation to account for males with several DFI measurements. The association of DFI with year, time of year, and male age was assessed using restricted cubic spline.

3 | RESULTS

3.1 | The small retrospective study

The results from FCH for grading of embryo morphology are presented in Table 1. At the time of the treatments, German law only permitted a maximum of three oocytes to be fertilized and cultured. Regardless of the embryo morphology, all the embryos had to be transferred if alive. Transfers were performed on days 2 to 5.

Pregnancies were, in general, only achieved with embryo gradings of 3 or 4 (1 = poor, 4 = optimal morphology). Embryo grading did not differ below and above the threshold (DFI = 15) for IVF treatments 3.04 vs. 3.11 for the best embryo (p = 0.76), and 2.87 vs. 2.85 for the average grade ($p = 0.94$).

The grade for the best embryo morphology was 3.25 below vs. 3.34 above the threshold (DFI = 25) and not statistically different ($p = 0.69$). The average grade was 3.04 vs. 2.98 ($p = 0.81$).

Figure 1A shows the percentage of ongoing pregnancies after the first cycle of IVF treatment for 210 couples. Pregnancies were confirmed by ultrasound at 12 weeks of gestation. The pregnancy rate was 45.1% (95% CI: 36.5–54.0%) when the DFI was below 15. When the DFI was between 15 and 25, the pregnancy rate was 24.6% (15.6–35.8%). Odds ratio adjusted for female age, sperm concentration and motility was 2.58 (1.36–5.04, p = 0.004).

TABLE 1 Embryo morphology was graded on a scale from 1 to 4 (worst to best morphology). The numbers show the morphology grade and 95% CI for the best embryo and the average morphology grade (one to three embryos). Results are shown according to the type of treatment (in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI)) and the threshold for DNA fragmentation index (DFI, 15 for IVF; 25 for ICSI).

IVF treatments	Grade for the best embryo	Average grade
DFI < 15 (n = 48)	3.04 (2.78–3.31)	2.87 (2.59–3.14)
DFI 15 to 25 (n = 27)	3.11 (2.76–3.47)	2.85 (2.48–3.22)
p-value	0.76	0.94
ICSI treatments	**Grade for the best embryo**	**Average grade**
DFI < 25 (n = 24)	3.25 (2.90–3.60)	3.04 (2.68–3.40)
DFI ≥ 25 (n = 32)	3.34 (3.04–3.65)	2.98 (2.67–3.29)
p-value	0.69	0.81

For ICSI treatments, the ongoing pregnancy rate was 48.6% (39.3–58.2%) when the DFI was below 25 (Figure 1B). When DFI was above this threshold, the pregnancy rate was 29.6% (20.0–40.8%). Odds ratio adjusted for female age, sperm concentration and motility was 2.00 (1.07–3.80, p = 0.032).

For the 406 couples, the average female age was 35 years with a range of 24–46 years. The men had an average age of 37 years (range of 22–60 years). Sperm motility and sperm concentration had a low correlation to log (DFI) with a Pearson correlation of −0.34 and −0.15, respectively. Together, sperm motility and concentration explained 12% of the variation in DFI.

Figure 2 shows how results, illustrated as bar charts, are affected by intra-individual variation in the assessment of sperm DNA damage. The bar charts to the left show the pregnancy rates adjusting for misclassification of the true DFI level. The bar charts second from the left show the observed pregnancy rates when $CV_W = 16.5\%$.[41] Pregnancy rates with a 50% and 100% increase in the observed intra-individual variation are presented in the third and fourth position, respectively.

The difference in the calculated pregnancy rates below and above the threshold for DFI diminishes with increasing intra-individual variation. As a result, the required sample size increases when the intra-individual variation increases.

For IVF using the observed intra-individual variation, the required sample size in a prospective study would be 169 couples (significance level 0.05, statistical power 80%). With 50% and 100% increases in the intra-individual variation, the sample sizes increase to 207 and 260, respectively. An increase in the rate of misclassification is also seen with increasing intra-individual variation. With the observed intra-individual variation, 9.5% of the patients will be misclassified. This rate

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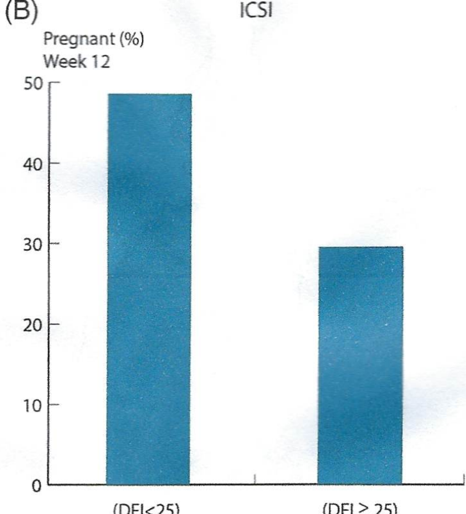

FIGURE 1 (A) Percentage of ongoing pregnancies after the first cycle of in vitro fertilization (IVF) treatments for 210 couples. Pregnancy was confirmed by ultrasound at 12 weeks of gestation. When the DNA fragmentation index (DFI) was below 15, the pregnancy rate was 45.1%. The pregnancy rate declined to 24.6% when the DFI was between 15 and 25. The odds ratio adjusted for female age, sperm concentration and motility was 2.58 ($p = 0.004$, 95% CI 1.36–5.04). (B) Results of 196 first cycle intracytoplasmic sperm injection (ICSI) treatments. When the DFI value was below 25, the pregnancy rate was 48.6%. When the DFI was above 25, the pregnancy rate was only 29.6%. The odds ratio adjusted for female age, sperm concentration and motility was 2.00 ($p = 0.032$, 95% CI 1.07–3.80).

TABLE 2 Distribution of DFI (DNA fragmentation index) in percent and the median DFI are shown for different fertility centers and treatments below.

	Median DFI	*p*-value	DFI < 15	15–25	25–40	40≤
IUI-treatments[a] (n = 637)	11.6 (11.0–12.2)	<0.001	64.8%	23.1%	10.2%	1.9%
Sims IVF[b] (n = 1971)	15.0 (14.6–15.4)	<0.001	51.7%	27.6%	15.3%	5.4%
FCH[c] (n = 2591)	17.2 (16.8–17.6)	<0.001	41.6%	30.3%	19.4%	8.7%
All tests[d] (n = 14,138)	15.6 (15.5–15.8)	-	47.5%	28.9%	16.9%	6.7%

[a]The median DFI for 637 tests of couples receiving intrauterine insemination (IUI) was significantly lower than the median for all tests ($p < 0.001$). Among the IUI-couples, 64.8% of the men had a DFI below 15.
[b]Sims IVF, Dublin, Ireland tested 1971 couples. The median DFI for this group was lower than the median for the other clinics ($p < 0.001$). Among the couples attending Sims IVF, 51.7% of the men had a DFI below 15.
[c]Fertility Center Hamburg, Germany (FCH) tested 2,591 couples. The median DFI for this group was 17.2, which was significantly higher than for the median for the other clinics ($p < 0.001$). Only 41.6% of couples in the FCH group had a DFI below 15.
[d]The median DFI was 15.6 for all tests (n = 14,138) performed between January 1st, 2008 and December 31st, 2022 for 70 European Fertility Centers.

increases to 13.7% and 17.6% when the intra-individual variation is increased by 50% and 100%, respectively.

For ICSI, the sample size is 336 with the observed intra-individual variation. With increases in the intra-individual variation of 50% and 100%, the sample sizes increase to 437 and 569, respectively. Corresponding misclassification rates for ICSI are 6.4%, 9.5% and 12.5%.

Figure 3 shows the distribution of the 14,138 diagnostic SDI®-tests. The median DFI is 15.6 (15.5–15.8) and the 95% central range is 4.6–53.2.

The observations are distributed with 47.3% classified as "low" DFI (< 15, Table 2). An "increased" DFI (between 15 and 25) accounted for 28.9% of the observations, whereas 16.9% of the DFI values were "high" (between 25 and 40). Additionally, 6.7% of the DFI values were "very high" (> 40).

Table 2 also presents the distribution and median DFI for three selected cohorts of patients.

The IUI cohort includes 637 tests of sperm DNA damage for couples receiving their first, second or third IUI treatment (unpublished data). The median DFI was 11.6, which was significantly lower than it was for

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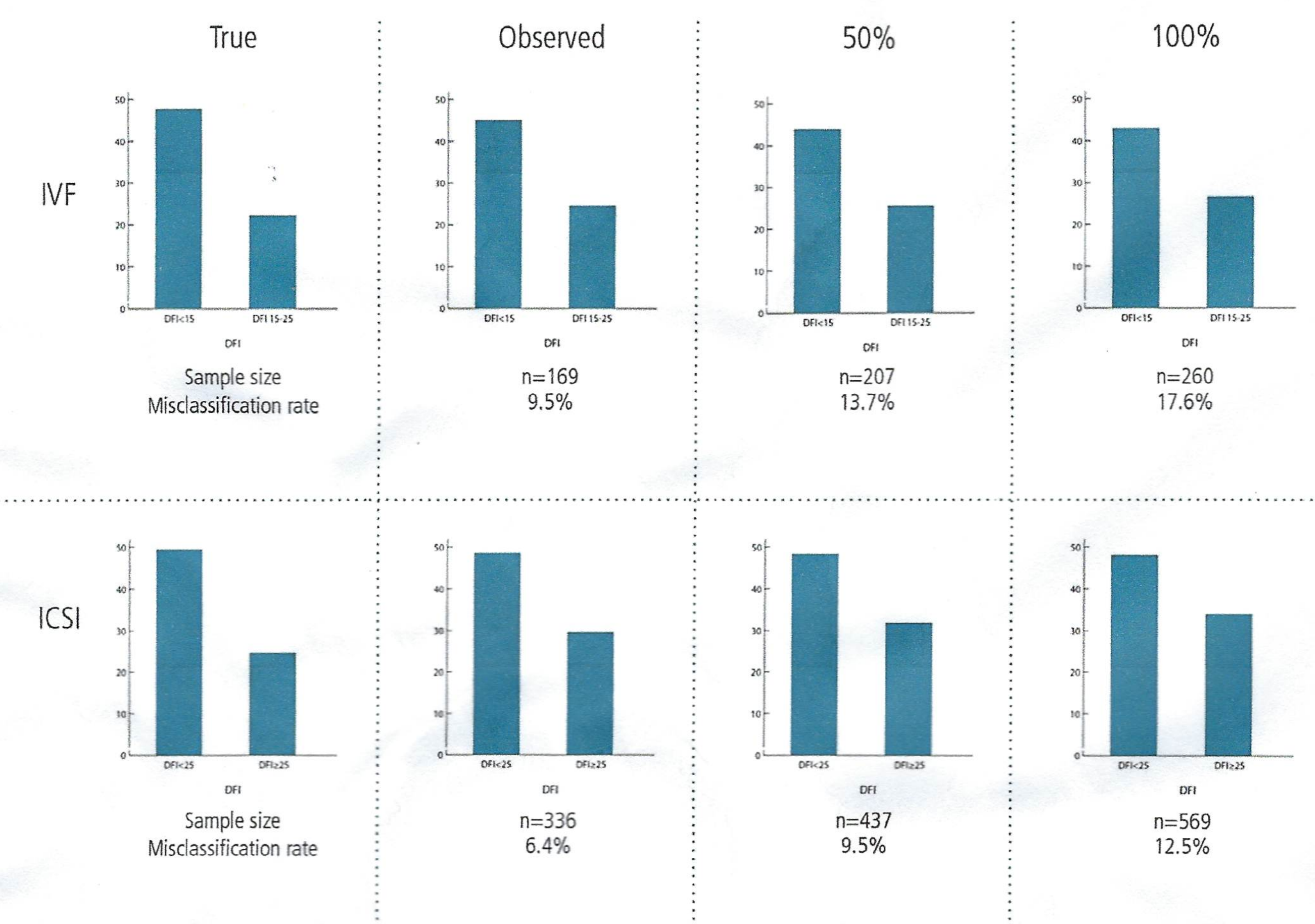

FIGURE 2 The bar charts illustrate how intra-individual variation in the assessment of sperm DNA integrity affects the observed pregnancy rates. Pregnancy rates at 12 weeks of gestation are shown for in vitro fertilization (IVF) treatments (upper row, DNA fragmentation index [DFI]-threshold = 15) and intracytoplasmic sperm injection (ICSI) treatments (lower row, DFI-threshold = 25). The bar charts second from the left show pregnancy rates with the **observed** intra-individual variation by SPZ Lab ($CV_w = 16.5\%$). Bar charts on the far left indicate estimated **true** pregnancy rates using a $CV_w = 0\%$. Pregnancy rates with a **50%** and **100%** increase in intra-individual variation are shown in the third and fourth position, respectively. The difference in pregnancy rates below and above thresholds for DFI diminish when moving from left to right in each row. A smaller difference in pregnancy rates implies that a larger sample size is required to demonstrate statistical significance. For IVF, sample sizes were calculated to n = 169 (**observed**) and increased to 207 (**50%** increase) and 260 (**100%**). For ICSI treatment, the corresponding sample sizes were calculated to n = 336, n = 437 and n = 569. The misclassification rates increase when moving from the left to the right in each row. The misclassification rate is the percentage of cases for which repeated SCSA® testing resulted in a switch in DFI category in relation to the threshold. For IVF (threshold = 15), the misclassification rates were calculated as 9.5%, 13.7% and 17.6%, respectively. For ICSI (threshold = 25), the misclassification rates were 6.4%, 9.5% and 12.5%, respectively.

all tests ($p < 0.001$). In the IUI cohort, 64.8% of the couples had a DFI below 15.

The cohort of patients attending Sims IVF comprises 1971 observations with a median DFI of 15.0. This median was statistically lower than the median DFI for the other clinics ($p < 0.001$). The percentage of couples with a DFI below 15 was 51.7%.

The cohort of patients attending FCH includes 2591 observations with a median DFI of 17.2 ($p < 0.001$). In this cohort, only 41.6% of the couples had a DFI below 15.

Comparison across the cohorts shows that the median DFI increases significantly: 11.6 (IUI cohort), 15.0 (Sims IVF cohort) and 17.2 (FCH cohort). For the cohorts, the corresponding proportion of observations with a DFI below 15 is 64.8%, 51.7% and 41.6%, respectively. In addition, the percentage of observations where DFI is above 25 are 12.1%, 20.7% and 28.1%, respectively.

Figure 4 illustrates how the median DFI changes during the time of the year. There appears to be a decline from February to May. From June to September, the median DFI increases, and the level remains high until the end of the year. This fluctuation is significant ($p = 0.006$) and accounts for 0.10% of the total variation in median DFI.

In Figure 5, the effect of male age on the median DFI can be seen. It appears that there is a significant increase in median DFI with increasing male age especially when the age is above 50 years. Male age had a statistically significant effect on DFI ($p < 0.001$) and accounted for 5.81% of the total variation.

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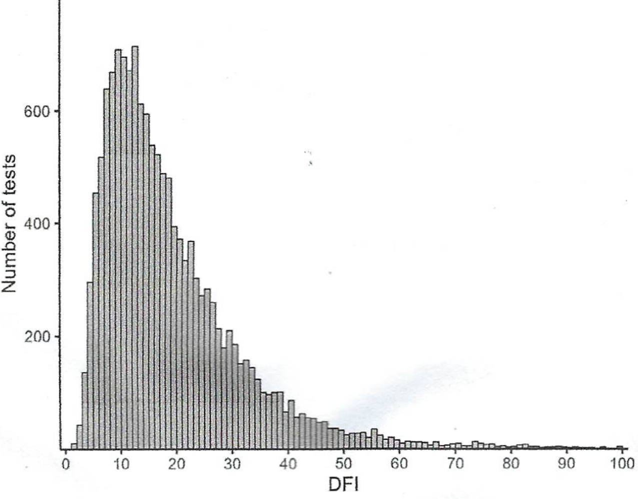

FIGURE 3 Distribution of DNA fragmentation index (DFI) for 14,138 diagnostic tests of sperm DNA damage performed for 70 European fertility centers from January 1st, 2008 to December 31st, 2022.

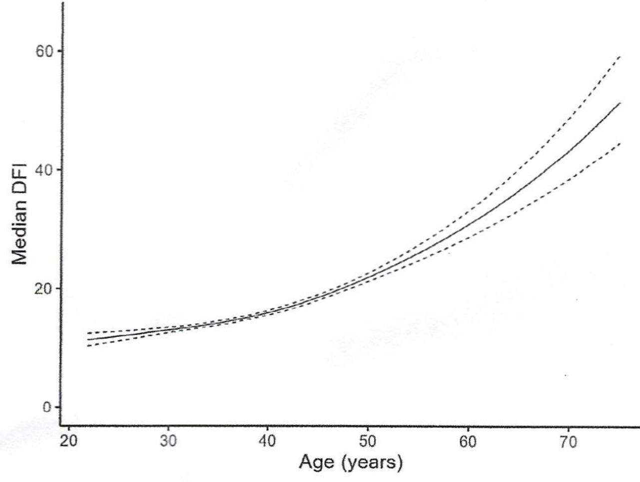

FIGURE 5 The median DFI (DNA fragmentation index) according to the age of the male for all diagnostic tests (n = 14,138) performed between January 1st, 2008 and December 31st, 2022. Dashed lines show the 95% confidence interval for the median DFI. Male age played a significant role for the DFI ($p < 0.001$) and accounted for 5.81% of the total variation in this parameter.

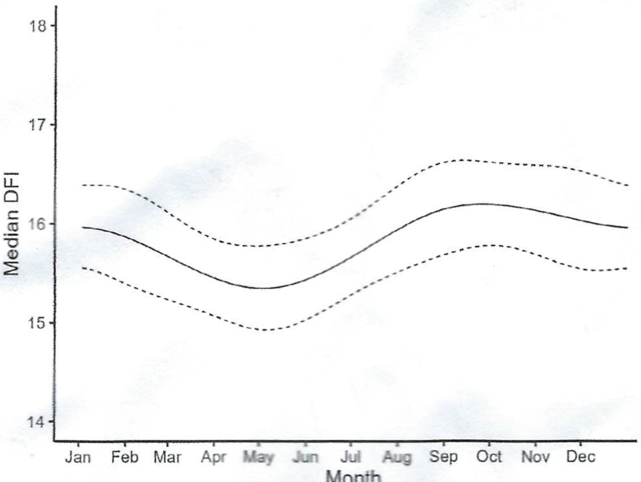

FIGURE 4 The relationship between the median DFI (DNA fragmentation index) and time of year for all diagnostic tests of sperm DNA damage (n = 14,138) performed between January 1st, 2008 and December 31st, 2022. Dashed lines show the 95% confidence interval for the median DFI. Time of year played a significant role for the DFI ($p = 0.006$) and accounted for 0.10% of the total variation in this parameter.

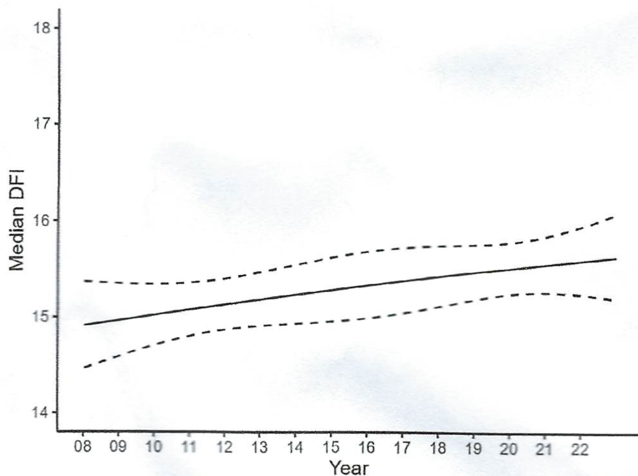

FIGURE 6 The relationship between the median DFI (DNA fragmentation index) and year for diagnostics tests performed between January 1st, 2008 and December 31st, 2022 (n = 14,138). Data are corrected for the effect of male age. Dashed lines show the 95% confidence interval for the median DFI. The year appeared to play a significant role for the DFI ($p = 0.02$) and accounted for 0.07% of the total variation in DFI.

Figure 6 shows the change in median DFI from January 1st, 2008 to December 31st, 2022 after correcting for the effect of male age. There appears to be a significant increase in median DFI of 0.05% per year ($p = 0.02$). This increase accounts for 0.07% of the total variation. For FCH, the increase in DFI per year was 0.13% ($p = 0.003$) and accounted for 0.61% of the total variation for the clinic.

Year, time of year, and male age together explain 5.91% of the total variation in median DFI.

4 | DISCUSSION

The results of the small retrospective study clearly demonstrate that DFI has a considerable impact on male fertility. When DFI was between 15 and 25, the outcome of IVF treatments was reduced significantly in comparison to treatments with a DFI below 15 (Figure 1A). This threshold has previously been demonstrated by Evenson et al.[1] For ICSI treatments, a significant reduction in pregnancy rates was observed when DFI was above 25 (Figure 1B). To our knowledge, this study is

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the first to demonstrate a threshold of DFI = 15 for IVF treatments, and a threshold of DFI = 25 for ICSI treatments using the SCSA®. The different thresholds for IVF and ICSI treatments are in line with the Two-Step Hypothesis, which predicts that the outcome of ICSI treatments would be affected to a lesser extent than the outcome of IVF treatments.[12]

An initial evaluation was conducted to determine whether embryological development and grading appeared to be significantly different below and above the selected thresholds for DFI. As shown in Table 1, this did not appear to be the case. We, therefore, chose to study whether implantation was affected or whether a miscarriage had occurred. Accordingly, pregnancy at 12 weeks of gestation was used as the clinical endpoint. Classical assessments of sperm concentration and motility accounted for only 12% of the variation in DFI. This confirms that DFI is an independent semen parameter as previously reported by Evenson et al.[18]

The couples in this study are typical of the population of couples attending European fertility clinics. In humans, it is difficult to obtain good fertility data for the male as couples may have reduced fertility due to female factors or issues for both partners. To minimize the influence of female and other factors, couples were only included if they had received their first treatment within 3 months of the SCSA® test.[27,28,38] A limitation of this study is that the selection procedure only allowed us to include 210 couples with IVF treatments and 196 with ICSI treatments.

To better understand the importance of the magnitude of the intra-individual variation, we performed a mathematical simulation study using the results of the small retrospective study. The observed intra-individual variation was compared with scenarios in which the variation was 50% and 100% higher (Figure 2).[41] The results show that the magnitude of the intra-individual variation plays a very significant role. With increasing intra-individual variation, the proportion of couples who are misclassified increases considerably. This is particularly clear for IVF treatments as the threshold (DFI = 15) is located in the part of the DFI distribution with the most observations (Figure 3). With a CW_w = 16.5%, 9.5% of the diagnoses will be incorrect for couples in the IVF group. This percentage increases to 13.7% and 17.6% with a 50% and 100% increase in intra-individual variation, respectively.

As the misclassification rate increases, the difference between the two bars becomes smaller (Figure 2) and, consequently, the required sample size to demonstrate a statistically significant difference increases dramatically. This mathematical simulation study shows that low intra-individual variation is essential when we want to detect a threshold of DFI = 15 for IVF treatments and DFI = 25 for ICSI treatments. It is likely that different levels of intra-individual variation are partly responsible for the various thresholds reported previously. The results show that the required sample size for IVF treatments is 169 couples with the observed intra-individual variation. The 210 couples included in the IVF group are thus a sufficient sample size. For ICSI treatments, the required sample size is 336 couples, which is higher than the 196 couples included. We have, therefore, initiated a larger prospective study to confirm the threshold for ICSI.

The small retrospective study was based on the first treatment cycle, and it does not help us in terms of understanding how DFI may affect the course of fertility treatments for a couple. To achieve this, the large retrospective study was conducted.

The distribution of DFI for the 14,138 diagnostic SCSA® tests is shown in Figure 3 and Table 2. Table 2 also contains an IUI-cohort (637 analyses) as well as two cohorts of patients attending Sims IVF and FCH. The couples in the IUI cohort were enrolled in a study (unpublished data) and had not received any prior treatment. In contrast, most couples had already received IUI treatments before attending Sims IVF. When Sims IVF started to use the test of sperm DNA damage in 2008, it was mandatory for couples to take the test prior to their first treatment with IVF or ICSI. From 2009, Sims IVF decided to just recommend that couples take the test prior to their first treatment. At FCH, the situation was slightly different. FCH is situated in the center of Hamburg and approximately half of the couples attending this clinic are referred from other clinics after failed cycles. When we view the distribution of DFI in Table 2, the proportion of patients with a DFI below 15 is 64.8% for the IUI cohort. This proportion is lower for patients attending Sims IVF (51.7%) and FCH (41.6%). In the three groups where DFI is above 15, the percentage of couples increases as we move from the IUI cohort to the Sims cohort and finally to the FCH cohort. The median DFI also increases significantly when we compare the three cohorts. Overall, these results demonstrate that all types of fertility treatment are more effective when the DFI is below 15. With a DFI above 15, couples are more likely to experience failed treatment cycles. This is particularly clear when DFI is above 25. This proportion of patients increases from 12.1% (IUI cohort) to 20.7% (Sims IVF cohort) and 28.1% (FCH cohort). Increase in male age could potentially be a bias, but male age was higher for the Sims IVF cohort (38.9 years) than it was for the FCH cohort (38.4 years). A limitation of this study is that male age was not available for the IUI cohort, which may also have had slightly better semen quality.

Data from the large retrospective study was also used for a descriptive analysis. An analysis of the effect of the time of year shows that DFI declines from January to May and then increases until September (Figure 4). From September to the end of the year, DFI remains on a stable plateau. Such fluctuations have been reported previously and may be caused by seasonal changes in melatonin and/or higher temperatures during the summer.[45] It is also possible that the decrease in DFI during the spring may be due to a shorter abstinence time as it has been shown that the frequency of ejaculation is significantly higher during the spring compared to the winter months.[46]

DFI increases with male age and accelerates after 50 years of age (Figure 5). This observation has been reported previously.[45,47,48] Wyrobek et al. included 97 healthy men aged between 20 and 80 and found that 40% of the variation in DFI could be explained by male age.[47] In the present study, only 5.81% of the variation in DFI was due to male age. Lifestyle and other factors may thus explain a large part of the variation in DFI, but these factors were not registered.

An important question is whether there has been any change in the DFI among males, who were the subject of infertility investigations from 2008 to 2022. As shown in Figure 6, the median DFI has increased

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significantly by approximately 0.05% per year ($p = 0.02$). This trend was also found for FCH with a 0.13% increase in the median DFI per year ($p = 0.003$). To our knowledge, this is the first study to demonstrate that DFI has increased among males who are the subject of infertility investigations. This result could be biased by couples taking the test later in their course of treatment. However, we do not consider this likely, as the test has been used more widely in recent years.

The increase in DFI is a worrying development and represents a serious challenge for human reproduction. Under natural conditions, the developmental failure rate of human embryos after fertilization is approximately 10–40% before implantation, and the total loss from fertilization to birth has been estimated to 40–60%.[49] For ART treatments, embryo mortality is significantly higher and is observed as poor embryo development, reduced implantation rate or increased miscarriage rate. De novo genetic and chromosomal defects in the embryo can originate from the female or male. It has been known for the past couple of decades that autosomal aneuploidy has a preferential maternal origin.[50] In contrast, de novo point mutations and structural chromosomal rearrangements have a preferential paternal origin.[51,52] Single stranded DNA damage may be repaired correctly by the oocyte or may result in de novo mutations.[52] More severe damage where the sperm DNA has suffered double-stranded breaks (fragmentation) is unlikely to be repaired correctly and may result in chromosomal aberrations.[14,52,53]

The increase in sperm DNA fragmentation is more than just a threat to our fertility as it will probably also affect the health of future generations. It is now more than a decade since we learned that de novo mutations in the paternal DNA can result in mental disorders such as autism or schizophrenia in the offspring, and that the risk of mental disorders is increased after fertility treatment.[54,55] In addition, children conceived with ART appear to have a higher risk of preterm birth, birth defects and cancer.[56–58]

During the last decade, it has become increasingly clear that men with reduced fertility already may have or will later develop comorbidities.[59–61] The connection between these observations may be damage to the DNA in the spermatozoa as well as the somatic cells. Indeed, Baumgartner et al. have demonstrated that sperm DNA fragmentation is often a biomarker for underlying instability of the DNA in the somatic cells.[62] DNA damage to the somatic cells is a central part of the pathogenesis in a wide range of diseases including cancers and cardiovascular disease.[63] Male comorbidity at the time of conception or sperm DNA fragmentation has been directly linked to miscarriage, stillbirth, preterm birth, and low birth weight.[58,60,64]

In the light of these considerations, clinicians and governments will have to adopt policies and strategies, which consider the health of both parents and the offspring. Shorter abstinence time, and procedures to remove spermatozoa with fragmented DNA have been suggested to increase male fertility.[10,11,65,66] According to the Two-Step Hypothesis, such methods may have a marginal impact on live birth rates and are unlikely to improve the health of the offspring. Clearly, such procedures will not improve paternal health.

The way forward appears to be to pay closer attention to the causative factors involved in sperm DNA integrity.[52] In order to identify the underlying factor in the individual man and retest once this has been corrected, it is paramount that the test used has a very high degree of precision. A more detailed fertility evaluation of the man can be conducted as an opportunity to improve his long-term health, as well as help him to produce spermatozoa with more robust DNA. This would result in higher fertility and the improved health of the offspring.

The recent WHO laboratory manual recommends that each laboratory determines and validates their thresholds for DFI.[67] We suggest that the first step of the validation process in a laboratory is to determine the intra-individual variation. Flow cytometric analysis of spermatozoa requires rigorous quality control.[32] Semen samples may be viscous and may stick to the flow system causing turbulence and incorrect results. To detect outliers and perform quality control, it is essential that at least two replicates per sample are analyzed. In addition, the flow cytometric plots should always be inspected carefully to detect any displacements or differences between the two replicates. The sample must be reanalyzed if there is any disagreement between the results and/or plots of the two replicates. Good quality control is time consuming but is essential to ensure highly reproducible results.[68,69]

In conclusion, sperm DNA damage has a negative impact on the outcome of IVF- and ICSI-treatments when the DFI is above 15 and 25, respectively. Couples with increased levels of sperm DNA damage are more likely to experience failed treatment cycles and complications for the offspring. DNA fragmentation appears to have increased during the past 15 years and is a significant challenge for human reproduction. Laboratories performing tests of sperm DNA damage must perform thorough quality control and ensure good preanalytical samples to achieve highly reproducible results.

AUTHOR CONTRIBUTIONS

Preben Christensen: The main contributor to the study; design; testing of sperm DNA integrity; data collection; and drafting. **Robert Fischer**: Clinician and project supervisor; review of final manuscript. **Wolfgang Schulze**: Clinician; project advisor; review of final manuscript. **Vera Baukloh**: Embryologist; data collection; drafting and review of final manuscript. **Kimberly Kienast**: Embryologist; review of final manuscript. **Graham Coull**: Embryologist; data collection; review of final manuscript. **Erik T. Parner**: Design; statistical analyses and mathematical simulations; drafting and review of final manuscript.

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CONFLICT OF INTEREST STATEMENT

Preben Christensen is a co-founder of SPZ Lab, which performs tests of sperm DNA integrity.

DATA AVAILABILITY STATEMENT

Due to EU GDPR legislation, data that support the findings of the retrospective studies and the mathematical simulation cannot be made

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available. Questions relating to data and statistical analyses should be addressed to ETP. Email: parner@ph.au.dk.

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REFERENCES

1. Evenson DP, Jost LK, Marshall D, et al. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum Reprod*. 1999;14:1039-1049. doi:10.1093/humrep/14.4.1039
2. ASRM Practice Committee. The clinical utility of sperm DNA integrity testing. *Fertil Steril*. 2006;86(Suppl 4):S35-37. doi:10.1016/j.fertnstert.2006.07.1482
3. ASRM Practice Committee. The clinical utility of sperm DNA integrity testing: a guideline. *Fertil Steril*. 2013;99:673-677. doi:10.1016/j.fertnstert.2012.12.049
4. ESHRE Add-ons working group, Lundin K, Bentzen JG, et al. Good practice recommendations on add-ons in reproductive medicine. *Hum Reprod*. 2023;38:2062-2104. doi:10.1093/humrep/dead184
5. Larson KL, De Jonge CJ, Barnes AM, Jost LK, Evenson DP. Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. *Hum Reprod*. 2000;15:1717-1722. doi:10.1093/humrep/15.8.1717
6. Virro MR, Larson-Cook KL, Evenson DP. Sperm chromatin structure assay (SCSA®) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. *Fertil Steril*. 2004;81:1289-1295. doi:10.1016/j.fertnstert.2003.09.063
7. Gandini L, Lombardo F, Paoli D, et al. Full-term pregnancies achieved with ICSI despite high levels of sperm chromatin damage. *Hum Reprod*. 2004;19:1409-1417. doi:10.1093/humrep/deh233
8. Bungum M, Humaidan P, Axmon A, et al. Sperm DNA integrity assessment in prediction of assissted reproduction technology outcome. *Hum Reprod*. 2007;22:174-179. doi:10.1093/humrep/del326
9. De Jonge C, LaFromboise M, Bosmans E, Ombelet W, Cox A, Nijs M. Influence of the abstinence period on human sperm quality. *Fertil Steril*. 2004;82:57-65. doi:10.1016/j.fertnstert.2004.03.014
10. Gonsalvez J, Gonzalez-Martinez M, Lopez-Fernandez C, Fernandez JL, Sanchez-Martin P. Shorter abstinence decreases sperm deoxyribonucleic acid fragmentation in ejaculate. *Fertil Steril*. 2011;96:1083-1086. doi:10.1016/j.fertnstert.2011.08.027
11. Sanchez-Martin P, Sanchez-Martin F, Gonzalez-Martinez M, Gonsalvez J. Increased pregnancy after reduced male abstinence. *Syst Biol Reprod Med*. 2013;59:256-260. doi:10.3109/19396368.2013.790919
12. Aitken RJ, De Iuliis GN. On the possible origins of DNA damage in human spermatozoa. *Mol Hum Reprod*. 2010 Jan;16(1):3-13. doi:10.1093/molehr/gap059
13. Holstein A, Schulze W, Davidoff M. Understanding spermatogenesis is a prerequisite for treatment. *Reprod Biol Endocrinol*. 2003;1:107. doi:10.1186/1477-7827-1-107
14. Marchetti F, Wyrobek AJ. DNA repair decline during mouse spermiogenesis results in the accumulation of heritable DNA damage. *DNA Repair (Amst)*. 2008;7:572-581. doi:10.1016/j.dnarep.2007.12.011
15. Schrader SM, Turner TW, Breitenstein MJ, Simon SD. Longitudinal study of semen quality of unexposed workers. *Reprod Toxicol*. 1988;2:183-190. doi:10.1016/0890-6238(88)90020-2
16. Matson PL. Clinical value of tests for assessing male infertility. *Baillieres Clin Obstet Gynaecol*. 1997;11:641-654. doi:10.1016/s0950-3552(97)80004-1
17. Evenson DP, Darzynkiewicz Z, Melamed MR. Relationship of mammalian sperm chromatin heterogeneity to fertility. *Science*. 1980;210:1131-1133. doi:10.1126/science.7444440
18. Evenson DP, Jost LK, Baer RK, Turner TW, Schrader SM. Individuality of DNA denaturation patterns in human sperm as measured by the sperm chromatin structure assay. *Reprod Tox*. 1991;5:115-125. doi:10.1016/0890-6238(91)90039-i
19. Johnson LA, Clarke RN. Flow sorting of X and Y chromosome-bearing mammalian sperm: activation and pronuclear development of sorted bull, boar, and ram sperm microinjected into hamster oocytes. *Gamete Res*. 1988;21:335-343. doi:10.1002/mrd.1120210402
20. Garner DL, Thomas CA, Allen CH. Effect of semen dilution on bovine sperm viability as determined by dual-DNA staining and flow cytometry. *Androl*. 1997;18:324-331. doi:10.1002/j.1939-4640.1997.tb01926.x
21. Garner DL, Thomas CA. Organelle-specific probe JC-1 identifies membrane potential differences in the mitochondrial function of bovine sperm. *Mol Reprod Dev*. 1999;53:222-229. doi:10.1002/(SICI)1098-2795(199906)53:2<222::AID-MRD11>3.0.CO;2-L
22. Christensen P, Stenvang JP, Godfrey WL. A Flow cytometric method for rapid determination of sperm concentration and viability in mammalian and avian semen. *J Androl*. 2004;25:255-264. doi:10.1002/j.1939-4640.2004.tb02786.x
23. Christensen P, Knudsen DB, Wachmann H, Madsen MT. Quality control in boar semen production by use of the FACSCount AF system. *Theriogenology*. 2004;62:1218-1228. doi:10.1016/j.theriogenology.2004.01.015
24. Christensen P, Hansen C, Liboriussen T, Lehn-Jensen H. Implementation of flow cytometry for quality control in four Danish bull studs. *Anim Reprod Sci*. 2005;85:201-208. doi:10.1016/j.anireprosci.2004.04.038
25. Birck A, Labouriau R, Christensen P. Dynamics of the induced acrosome reaction in boar sperm evaluated by flow cytometry. *Animal Reprod Sci*. 2009;115:124-136. doi:10.1016/j.anireprosci.2008.10.017
26. Birck A, Christensen P, Labouriau R, Pedersen J, Borchersen S. In vitro induction of the acrosome reaction in bull sperm and the relationship to field fertility using low dose inseminations. *Theriogenolgy*. 2010;73:1180-1191. doi:10.1016/j.theriogenology.2009.10.010
27. Christensen P, Labouriau R, Birck A, Boe-Hansen GB, Pedersen J, Borchersen S. Relationship among seminal quality measures and field fertility of young dairy bulls using low-dose inseminations. *J Dairy Sci*. 2011;94(4):1744-1754. doi:10.3168/jds.2010-3087
28. Boe-Hansen GB, Christensen P, Vibjerg D, Nielsen MBF, Hedeboe AM. Sperm chromatin structure integrity in liquid stored boar semen and its relationships with field fertility. *Theriogenology*. 2008;69(6):728-736. doi:10.1016/j.theriogenology.2007.12.004
29. Love CC, Kenney RM. The relationship of increased susceptibility of sperm DNA to denaturation and fertility in the stallion. *Theriogenology*. 1998;50:955-972. doi:10.1016/s0093-691x(98)00199-x
30. Spano M, Bonde JP, Hjøllund HI, Kolstad HA, Cordelli E, Leter G. Sperm chromatin damage impairs human fertility. The Danish first pregnancy planer study. *Fertil Steril*. 2000;73:43-50. doi:10.1016/s0015-0282(99)00462-8
31. Lin M, Lee R, Li S, Lu C, Sun F, Hwu Y. Sperm chromatin structure assay parameters are not related to fertilization rates, embryo quality, and pregnancy rates in in vitro fertilization and intracytoplasmic sperm injection, but might be related to spontaneous abortion

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rates. *Fertil Steril*. 2008;90:352-359. doi:10.1016/j.fertnstert.2007.06.018
32. Boe-Hansen GB, Ersbøll AK, Christensen P. Variability and laboratory factors affecting the sperm chromatin structure assay in human semen. *J Androl*. 2005;26:360-368. doi:10.2164/jandrol.04056
33. Boe-Hansen GB, Fedder J, Ersbøll AK, Christensen P. The sperm chromatin structure assay as a diagnostic tool in the human fertility clinic. *Hum Reprod*. 2006;21:1576-1582. doi:10.1093/humrep/del019
34. Larson-Cook KL, Brannian JD, Hansen KA, Kasperson KM, Aamold ET, Evenson DP. Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. *Fertil Steril*. 2003;80:895-902. doi:10.1016/s0015-0282(03)01116-6
35. Zini A, Meriano J, Kader K, Jarvi K, Laskin CA, Cadesky K. Potential adverse effect of sperm DNA damage on embryo quality after ICSI. *Hum Reprod*. 2005;20:3476-3480. doi:10.1093/humrep/dei266
36. Speyer BE, Pizzey AR, Ranieri M, Joshi R, Delhanty JDA. Fall in implantation rates following ICSI with sperm with high DNA fragmentation. *Hum Reprod*. 2010:1609-1618. doi:10.1093/humrep/deq116
37. Yang H, Li G, Jin H, Guo Y, Sun Y. The effect of sperm DNA fragmentation index on assisted reproductive technology outcomes and its relationship with semen parameters and lifestyle. *Transl Androl Urol*. 2019;8:356-365. doi:10.21037/tau.2019.06.22
38. Amann RP. Can the fertility potential of a seminal sample be predicted accurately?. *J Androl*. 1989;10:89-98. doi:10.1002/j.1939-4640.1989.tb00066.x
39. Erenpreiss J, Bungum M, Spano M, Elzanaty S, Orbidans J, Giwercman A. Intra-individual variation in sperm chromatin structure assay parameters in men from infertile couples: clinical implications. *Hum Reprod*. 2006;21:2061-2064. doi:10.1093/humrep/del134
40. Oleszczuk K, Giwercman A, Bungum M. Intra-individual variation of the sperm chromatin structure assay DNA fragmentation index in men from infertile couples. *Human Reprod*. 2011;26:3244-3248. doi:10.1093/humrep/der328
41. Blomberg Jensen M, Lawaetz JG, Pedersen JH, Juul A, Joergensen N. Effects of vitamin D and calcium supplementation on semen quality, reproductive hormones and live birth rate: a randomized clinical trial. *J Clin Endocrinol Metab*. 2018;103:870-881. doi:10.1210/jc.2017-01656
42. Evenson DP. Sperm chromatin structure assay (SCSA®) for fertility assessment. *Curr Protoc*. 2022;2:e508. doi:10.1002/cpz1.508
43. Copeland KT, Checkoway M, McMichael AJ, Holbrook RH. Bias due to misclassification of the estimation of relative Risk. *Am J Epidemiol*. 1977;105:488-495. doi:10.1093/oxfordjournals.aje.a112408
44. Kleinbaum DG, Kupper LL, Morgenstern H. *Epidemiologic research: principles and quantitative methods*. Lifetime Learning Publications; 1982.
45. Ghadikolaei PY, Ghaleno LR, Versali S, et al. Epidemiology of sperm DNA fragmentation in a retrospective cohort of 1191 men. *Andrology*. 2023;11(8):1663-1672. doi:10.1111/andr.13472
46. Carlsen E, Petersen JH, Andersson A, Skakkebaek NE. Effects of ejaculatory frequency and season on variations in semen quality. *Fertil Steril*. 2004;82:358-366. doi:10.1016/j.fertnstert.2004.01.039
47. Wyrobek AJ, Eskenazi B, Young S, et al. Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. *PNAS*. 2006;103:9601-9606. doi:10.1073/pnas.0506468103
48. Evenson DP, Djira G, Kasperson BS, Christianson J. Relationships between the age of 25,445 men attending infertility clinics and sperm chromatin structure assay (SCSA®) defined sperm DNA and chromatin integrity. *Fertil Steril*. 2020;114:311-320. doi:10.1016/j.fertnstert.2020.03.028
49. Jarvis GE. Misjudging early embryo mortality in natural human reproduction. *F1000Res*. 2020;9:702. doi:10.12688/f1000research.22655.1
50. Hassold T, Hunt P. To err (meiotically) is human: the genesis of human aneuploidy. *Nat Rev Genet*. 2001;2:280-291. doi:10.1038/35066065
51. Crow JF. The origins, patterns and implications of human spontaneous mutations. *Nat Rev Genet*. 2001;1:40-47. doi:10.1038/35049558
52. Aitken JR. Role of sperm DNA damage in creating de-novo mutations in human offspring: the 'post-meiotic oocyte collusion' hypothesis. *Reprod Biomed Online*. 2022;45(1):109-124. doi:10.1016/j.rbmo.2022.03.012
53. Fu W, Cui Q, Yang Z, et al. High sperm DNA fragmentation increased embryo aneuploidy rate in patients undergoing preimplantation genetic testing. *Reprod Biomed Online*. 2023. PMID: 37812976.
54. Kong A, Frigge ML, Masson G, et al. Rate of de novo mutations and the importance of father's age to disease risk. *Nature*. 2012;488:471-475. doi:10.1038/nature11396
55. Svahn MF, Hargreave M, Nielsen TSS, et al. Mental disorders in childhood and young adulthood among children born to women with fertility problems. *Hum Reprod*. 2015;30:2129-2137. doi:10.1093/humrep/dev172
56. Daltveit DS, Klungsøur K, Engeland A, et al. Cancer risk in individuals with major birth defects: large Nordic population based case-control study among children, adolescents, and adults. *BMJ*. 2020;371:m4060. doi:10.1136/bmj.m4060
57. Lu YMM, Liu LMM, Zhang P, Sun Y, Ma C, Li Y. Risk of birth defects in children conceived with assisted reproductive technology. A meta analysis. *Medicine (Baltimore)*. 2022;101(52):e32405. PMID: 36596016.
58. Boeri L, Belladelli F, Pozzi E, et al. Low birth weight is associated with sperm DNA fragmentation and assisted reproductive technology outcomes in primary infertile men: results of a cross-sectional study. *World J Mens Health*. 2024;42:384-393. doi:10.5534/wjmh.220283
59. Jensen TK, Jacobsen R, Christensen K, Nielsen NC, Bostofte E. Good semen quality and life expectancy: a cohort study of 43,277 Men. *Am J Epidemiol*. 2009;170(5):559-565. doi:10.1093/aje/kwp168
60. Kasman AM, Zhang CA, Li S, Stevenson DK, Shaw GM, Eisenberg ML. Association of preconception paternal health on perinatal outcomes: analysis of U.S. claims data. *Fertil Steril*. 2020;113:947-954. doi:10.1016/j.fertnstert.2019.12.026
61. Balladelli F, Muncey W, Eisenberg ML. Reproduction as a window for health in men. *Fertil Steril*. 2023;120:429-437. doi:10.1016/j.fertnstert.2023.01.014
62. Baumgartner A, Kurzawa-Zegota M, Laubenthal J, Cemeli C, Anderson D. Comet-assay parameters as rapid biomarkers of exposure to dietary/environmental compounds—an in vitro feasibility study on spermatozoa and lymphocytes. *Mutat Res*. 2012;743:25-35. doi:10.1016/j.mrgentox.2011.12.027
63. Cooke MS, Evans MD, Dizdaroglu M, Lunec J. Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J*. 2003;17:1195-1214. doi:10.1096/fj.02-0752rev
64. Kasman AM, Zhang CA, Li S, et al. Association between preconception paternal health and pregnancy loss in the USA: an analysis of US claims data. *Hum Reprod*. 2021;36:785-793. doi:10.1093/humrep/deaa332
65. Kabukcu C, Cil N, Cabus Ü, Alatas E. Effect of ejaculatory abstinence period on sperm DNA fragmentation and pregnancy outcome of intrauterine insemination cycles: a prospective randomized study. *Arch Gynecol Obstet*. 2021;303:269-278. doi:10.1007/s00404-020-05783-0
66. Leisinger CA, Adaniya G, Freeman MR, et al. Effect of microfluidic sperm separation vs. standard sperm washing process on laboratory outcomes and clinical pregnancy rates in an unselected patient population. *Reprod Med*. 2021;2:125-130. doi:10.3390/repromed2030013
67. WHO. WHO laboratory manual for the examination and processing of human semen. Sixth Edition WHO 27. July 2021. https://www.who.int/publications/i/item/9789240030787

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68. Plant AL, Locascio LE, May WE, Gallagher PD. Improved reproducibility by assuring confidence in measurements in biomedical research. *Nat Methods*. 2014;11(9):895-898. doi:10.1038/nmeth.3076
69. Björndahl L, Barrett CLR, Mortimer D, Jouannet P. 'How to count sperm properly': checklist for acceptability of studies based on human semen analysis. *Hum Reprod*. 2022;33:227-232. doi:10.1093/humrep/dev305

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