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Relationships between the age of 25,445 men attending infertility clinics and sperm chromatin structure assay (SCSA®) defined sperm DNA and chromatin integrity

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Objective: To determine relationships between age of men with potential male factor infertility and sperm chromatin structure assay (SCSA) measures of sperm DNA fragmentation (SDF) and high DNA stainable sperm (HDS), and to compare these data with those obtained from healthy donor men without reproductive issues.
Design: Retrospective study.
Setting: Infertility clinics and diagnostic laboratory.
Patients: A total of 25,445 men attending infertility clinics. Donors were 87 men working at Lawrence Livermore National Laboratory.
Intervention: None.
Main Outcome Measures: SCSA measures (% DNA fragmentation index (DFI), X DFI, SD DFI, and %HDS) of men aged 21–80 years.
Results: In the study population, advancing paternal age was associated with increased sperm DNA fragmentation (SDF) scored as increased percentage of sperm in semen ejaculates with measurable DNA strand breaks (%DFI). The slope of increase in % DFI prior to age 41.6 years was 0.39, which increased after age 41.6 to more than double at a slope of 0.86. These changes in DNA/chromatin in more than 25,000 aging men attending infertility clinics are similar to those seen over the same age span (20–80 years) in 87 nonpatient, healthy men without reproductive issues. For the age group 20–50 years, there was no major significant difference in %DFI between patients and donor men. According to a logistic regression model, the estimated probability is that, for example, a 40-year-old and a 50-year-old man have a 20% and 40% chance, respectively, to have a pathological DFI $\geq$25% by age factor alone. The condensation of sperm chromatin in patients increased with age in a linear fashion, from a mean of 12.2 %HDS at age 20–25 to a mean of 7.9 %HDS at age 60–65. Patients had a greater % HDS than donors across all ages.
Conclusions: The great heterogeneity of both DFI and HDS values at a specific age prevents the automatic translation of age into an index of DNA fragmentation. However, it reinforces the idea that both DFI and HDS evaluation can play a role in detecting potential male infertility in cases that are not resolved by routine testing and in cases of multiple miscarriages. DFI and HDS data can help clinicians to predict a man's fertility potential, to consider corrective therapeutic approaches, as well as to assess the risk to the offspring's health. (Fertil Steril® 2020;■:■–■. ©2020 by American Society for Reproductive Medicine.)
Key Words: Male infertility, healthy donors, age, SCSA test

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Delayed fatherhood has become socially acceptable in developed countries; however, the inherent consequences of this trend are poorly understood. Since 1980, U.S. birth rates have increased up to 40% for men 35–49 years of age and have decreased up to 20% for men less than 30 years of age (1). Although it is well known that maternal age is a significant contributor to human infertility (2), advanced paternal age has been associated with decreased semen parameters, chromosomal abnormalities, and reduced fertility (3), as well as with diseases such as schizophrenia, achondroplasia, and Apert syndrome (4). Sperm DNA is exquisitely sensitive to oxidative attack, resulting in impairment of embryo development, increased risk of gene mutations and miscarriage, congenital malformations. and a high frequency of diseases in the offspring (5, 6).

Our study here confirms results of many additional studies showing that older men produce more sperm with DNA damage (7, 8). The source of this damage includes oxidative stress, abortive Fas-mediated apoptosis, or deficiencies in natural processes such as recombination that induce DNA strand breaks (9). Indeed, oxidative stress is thought to be the pathologic molecular mechanism underpinning the majority of known clinical, environmental, and lifestyle causes of male infertility (10).

DNA strand breaks are significant for men attending reproductive clinics, as transmission of damaged DNA to the offspring may occur when levels exceed the DNA repair capacity of the oocyte (11, 12). These age-related factors may be attributed to older men producing more sperm with DNA fragmentation due to higher exposures of DNA-damaging oxidative stress in their reproductive tracts. It is reasonable to hypothesize that apoptotic functions of spermatogenesis are probably less effective in older men, resulting in the production of increased sperm DNA fragmentation.

Classical semen analysis of sperm density, motility, and morphology has been the gold standard for evaluating male factor infertility for many decades (13). Yet, these sperm parameters have generally failed to predict the outcome of male factor fertility. Numerous studies indicate that measures of sperm DNA fragmentation (SDF) can provide additional characterization of male factor infertility. The pioneering sperm DNA fragmentation study of Evenson et al. (14) strongly suggested that sperm DNA integrity was a significant indicator of pregnancy success in both bulls and humans. This 1980 study introduced the Sperm Chromatin Structure Assay (SCSA), which was followed by nearly two decades of extensive animal fertility and toxicology studies to validate the SCSA test (14–18). Current data suggest that the evaluation of sperm DNA integrity may serve as a valuable addition to the toolbox of infertility clinicians (19). The pioneering use of SCSA in the human clinic strongly validated that the SCSA test data were significantly related to human DNA pregnancy and miscarriage (20).

In our study with Wyrobek (3), age had the strongest effects on chromatin defects, explaining ~40% of the variance in DFI endpoints. Similar to Spano et al. (21), we found that average %DFI values more than doubled between 20 and 60 years of age, and in the Wyrobek et al. (3) study of healthy donors, a fivefold increase in %DFI between 20 and 80 years of age was observed. Older men may produce more sperm with DNA damage because of an age-associated increase in reproductive tract oxidative stress and/or altered testicular germ cell apoptosis (22).

Unique to SCSA data outputs in this study is the relationship between age of men and sperm chromatin condensation (3). Whereas the increase in %DFI with age is well established, the %HDS relationship is less understood. The abnormal sperm nuclear condensation process involves a complex sequence of events including topological rearrangements, transition of DNA-binding proteins, transcriptional alterations, nucleosomal structure loss, and abnormal condensation of chromatin, resulting in disturbances in the organization of genomic material in the sperm nuclei and decreasing sperm functional ability. Ultimately, this reduces normal fertilization, affects early embryonic development, and interferes with the primary mission of the sperm DNA, which is reliable transmission of paternal genetic information (23).

Interestingly, although it is known that an abnormally high HDS may lead to early embryo death and miscarriage (24–26), the rationale for this is controversial, with suggestions that it is related to increased aneuploidy (3, 24); however, it is more likely related to abnormality of tertiary chromatin structure, thereby causing an abnormal read-out of early embryo proteins needed for embryo growth and differentiation (26–29).

MATERIALS AND METHODS

Semen Samples

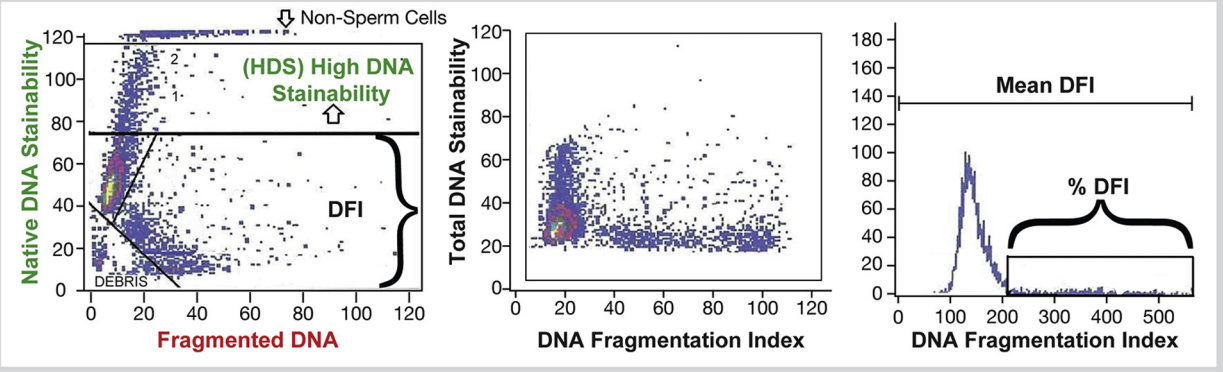
Clinical semen samples from men (n = 25,445) 21−80 years of age who provided signed consent forms were sent to SCSA Diagnostics (Brookings, SD) from approximately 200 North American and European (~15%) infertility clinics and home collections. These samples were collected after a recommended 2-to 5-day abstinence. Following liquefaction for ~1 hour at room temperature, ~0.2 mL of raw semen was transferred to one or two 2-mL cryovials and then flash-frozen in liquid nitrogen (LN_2). Some semen samples were collected at home using an SCSA semen collection kit followed by placing the raw semen−containing cryotubes into an LN_2 dry shipper.

The frozen semen samples were shipped to SCSA Diagnostics via Federal Express overnight either in LN_2 dry shippers or in boxes containing dry ice. Upon arrival at SCSA Diagnostics, the semen samples were transferred to a 45-L LN_2 tank and kept there until the time of SCSA measurements. All semen samples were received from assisted reproductive technology clinics that obtained permission from their patients.

SCSA Measurements

Individual samples were thawed in a 37°C water bath just until all ice had melted and then immediately diluted with TNE buffer (0.01 M Tris, 0.15 M NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 4°C) to a final concentration of 1-2 × 10^6 sperm/mL. A 200-μL sperm suspension was admixed with 400 μL acid solution (0.1%

FIGURE 1



File	% DFI	% HDS	Mean DFI	Std Dev DFI
1	17.66	8.40	222.4	182.2
2	18.00	8.80	224.7	186.9
mean	17.8	8.6	223.6	184.6
std dev	0.2	0.3	1.6	3.4

Sperm Chromatin Structure Assay (SCSA) test data (30). (**Left panel, top box**) Raw data from a flow cytometer showing each of 5,000 sperm as a single dot on a scattergram. Y axis = green fluorescence with 1,024 gradations (channels) of DNA stainability. X axis = red fluorescence with 1,024 gradations of red fluorescence (ss DNA). Axes shown are 1,024/10. Dotted line at Y = 75 marks the upper boundary of DNA staining of normal sperm chromatin; above that line are sperm (dots) with partially uncondensed chromatin allowing more DNA stainability. Bottom left corner shows gating out of seminal debris. (**Middle panel**) Raw data from left panel are converted by SCSAsoft software (or equivalent) to red/red + green fluorescence. This transforms the angled sperm display in the left panel to a vertical pattern that is often critical for accurately delineating the percentage of sperm with fragmented DNA. Y axis = total DNA stainability vs. X axis = red/red + green fluorescence (DFI). (**Right panel**) Frequency histogram of data from middle panel showing computer gating into %DFI and Mean DFI. (**Bottom box**) SCSAsoft software calculations of mean of two independent measures of mean and SD of DFI, SD DFI, and %DFI and %HDS. Inside the boxes are the means and standard deviations.

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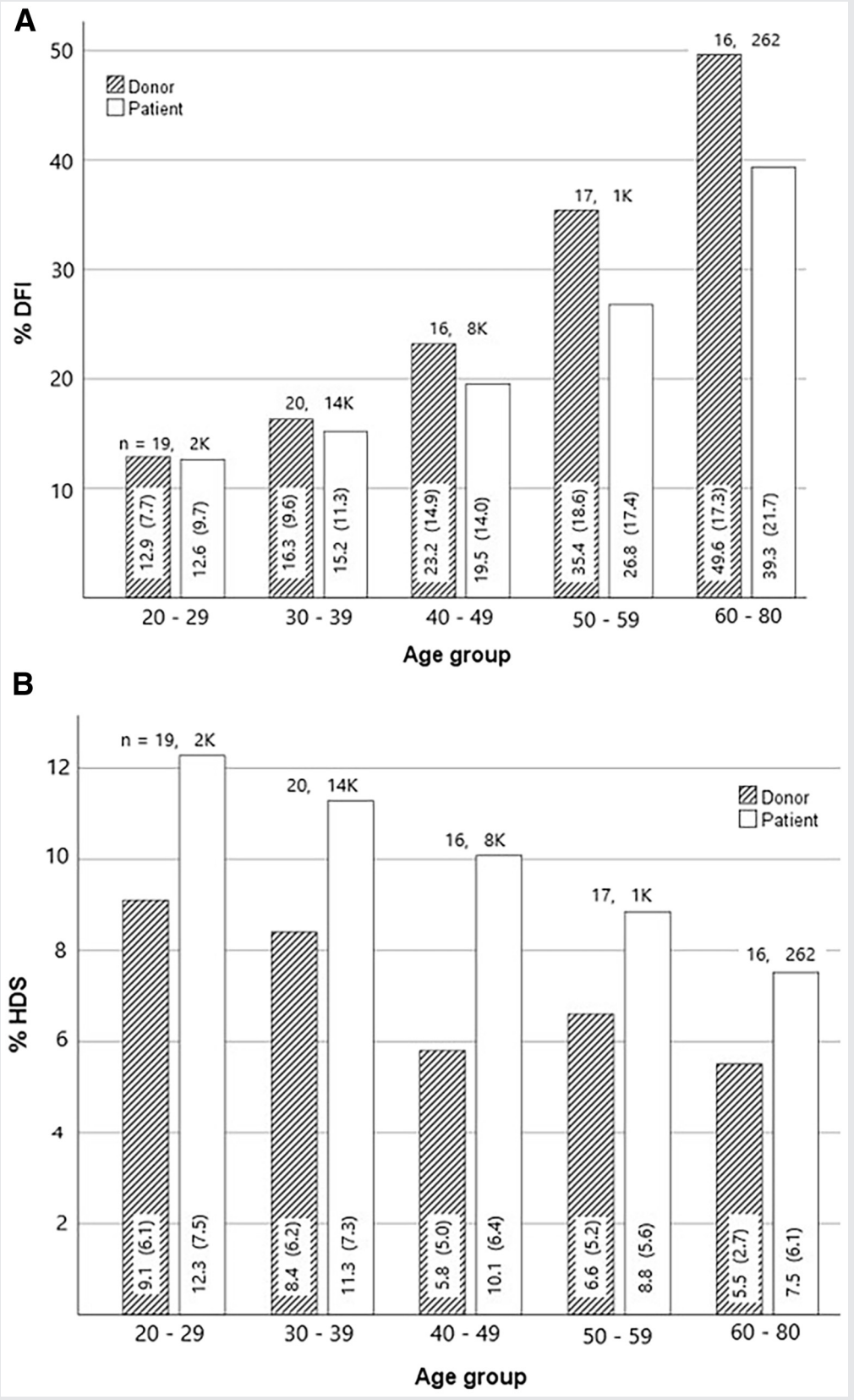
Triton X-100, 0.15 M NaCl, and 0.08 N HCl, pH 1.20, 4°C) for 30 seconds, followed by the addition of 1.20 mL of acridine orange staining solution (containing 6 μg chromatographically purified acridine orange (Polysciences Inc., Warrington, PA) per milliliter of acridine orange buffer (370 mL of stock 0.1 M citric acid, 630 ml of stock 0.2 M Na HPO, 1 mM disodium EDTA, 0.15 M NaCl, pH 6.0, 4°C) as previously described in detail (15, 30). Individual samples were placed into an Ortho Diagnostics L30 flow cytometer and after 2 minutes of hydrodynamic equilibration of sample and sheath flow, 5×10^3 sperm were measured at rates of $\leq$250 cells per second. All samples were measured independently twice. The mean values of the two independent measurements were calculated (n = 25,445; coefficient of variation 1–3%). These mean data were processed through SCSAsoft software (SCSA Diagnostics, Brookings, SD) to produce clinical reports, which were sent back to the clinics via a secured Web address.

Of great importance, all clinical semen samples obtained over 11 years, as well as the donor semen samples, were measured by nearly exactly the same strict protocol and with the same flow cytometer (15, 30). Positive (high %DFI) and a negative (low %DFI) semen samples were measured to verify the results as previously established. Then a reference semen sample from a set made up of hundreds of samples of small aliquots stored in LN_2 was used to set the red and green photomultipliers tubes to the same ($\pm$5/1,024 flow cytometer channels) X, Y coordinates, for example, 150 red/540 green. Notably, when one set of reference samples was nearly depleted, another set was made, even if from a different individual with different mean red (X) and green (Y) values. This was accomplished by first measuring the previous reference sample, then the measuring the new reference semen sample at the same red/green photomultiplier gains and noting the new mean red and green fluorescence values. In this fashion, samples measured years ago could be measured again with the new reference sample and obtain nearly exactly the same results. This is vitally important in order to make precise comparisons of all ~25,000 samples. Also, this is important for men who may have provided a semen sample years ago and want to see whether the new sample has increased or decreased in DNA integrity.

FIGURE 2



(**A**) Comparison of age-related %DFI from this study with that of our previous study of 87 men employed at or retired from the Lawrence Livermore National Laboratory (LLNL). (**B**) Comparison of age-related %HDS from this study of infertility patients with 87 donor men from the LLNL.

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Statistical Analysis

Graphical and numerical descriptive statistics, locally weighted scatterplot smoothing (LOWESS) techniques and binary logistic analysis were used to analyze the data. Boxplots were constructed at each age; LOWESS was used to study trends in %DFI and mean DFI; and binary logistic regression analysis was used to model patients reaching 25% DFI using age as a single predictor. SAS 9.3 (SAS Institute) and R 3.5.1 statistical software programs were used for the analyses. Statistical significance was defined as $P < .05$.

RESULTS

Data produced from SCSA measures are shown in Figure 1. A comparison of SCSA data between approximately 25,000 infertility patients and 87 healthy men without reproductive issues is summarized in Figure 2. Age-related %DFI from this study are compared in Figure 2A with data from our previous study of 87 men employed by or retired from the Lawrence Livermore National Laboratory (3, 31). Participants were currently employed or active retirees of LLNL, college and post-college educated, and in good to excellent health by self-report. These donors provided a convenience semen sample after an average of 5.1 days abstinence. In all, 79% had never smoked cigarettes regularly and 44% took a vitamin supplement regularly. Two-thirds (56/87) of the men had fathered a child (19/87 were in the age range of 20–29 years who may not have been married or attempted to father a child). Of course, having fathered a child once does not imply that men were fertile at the time of semen sampling. There is no significant difference for the first three age groups (20–50 years); however, there is a significant difference in %DFI in the last two age groups at the 20% level ($P = .03$ and .064). No explanation is known as to why these older donors had a higher %DFI than patients; however, because of the small number of donors in the 50- to 80-year age range, it was concluded that the donors and patients had the near same age-related changes. Figure 2B compares age-related %HDS from this study of infertility patients with that of 87 donor men from the LLNL (3, 31). Note that the patient mean %HDS and SD is higher than the donor mean %HDS and SD at all age groups.

Box plots in Figure 3 show %DFI and %HDS values of approximately 25,000 patients and compared them to to mean DFI. The box plots in Figure3A–C show %DFI, mean DFI and % HDS. All DFI plots show increase of values with age. %HDS values decreased linearly with age at an estimated slope of −0.137 (SE −0.0062). In all cases, there is great heterogeneity of values at all ages. Figure 3D compares the smoothed curves of %DFI and mean DFI. Note that the two curves are the same, which means that the sperm (dots on computer monitor) outside the main population as seen in Figure 1 represents the total amount of SCSA-defined DNA fragmentation. Thus, calculating the %DFI yields the same clinical interpretation as that obtained by the mean DFI as seen in Figure 1.

Supplemental Table 1 shows that in the age brackets of 20–25, 40–45, and 60–65 years, the %DFI is 12.1, 18.8, and 36.7 and the %HDS is 13.2, 10.2, and 7.6, respectively. Of note, the %DFI nearly doubled every two decades.

Supplemental Figure 1 shows a fit plot for age vs. average % DFI. The change point in slope of age vs. % DFI = age 41.6 (95% CI 40.4, 42.8). Of great interest, the slope < age 41.6 = 0.39, whereas the slope > age 41.6 = 0.86.

An important clinical question is as follows: what is the probability of a man reaching the detrimental 25% DFI clinical threshold by age factor alone? Supplemental Figure 2 shows the odds for a 40-year-old to be at 20% and a 50-year-old at 40% chance.

Supplemental Figure 2 shows predicted probabilities by age for reaching 25% DFI.

How do the %DFI values translate to predictive odds for live birth? Figure 4 is drawn from an estimated summary of published SCSA clinical data (20, 27, 32–37). A recent study (37) of patients receiving fertility treatment by intrauterine insemination (IUI) were inseminated by sperm with SCSA-derived %DFI of 0–9.9%, 10–19.9%, ≥20%, and ≥30% and had pregnancy rates of 28%, 26%, 10%, and 5% respectively. In this study, an increase from SCSA 20% DFI to 25% DFI caused a 50% drop in the IUI-derived pregnancy rate.

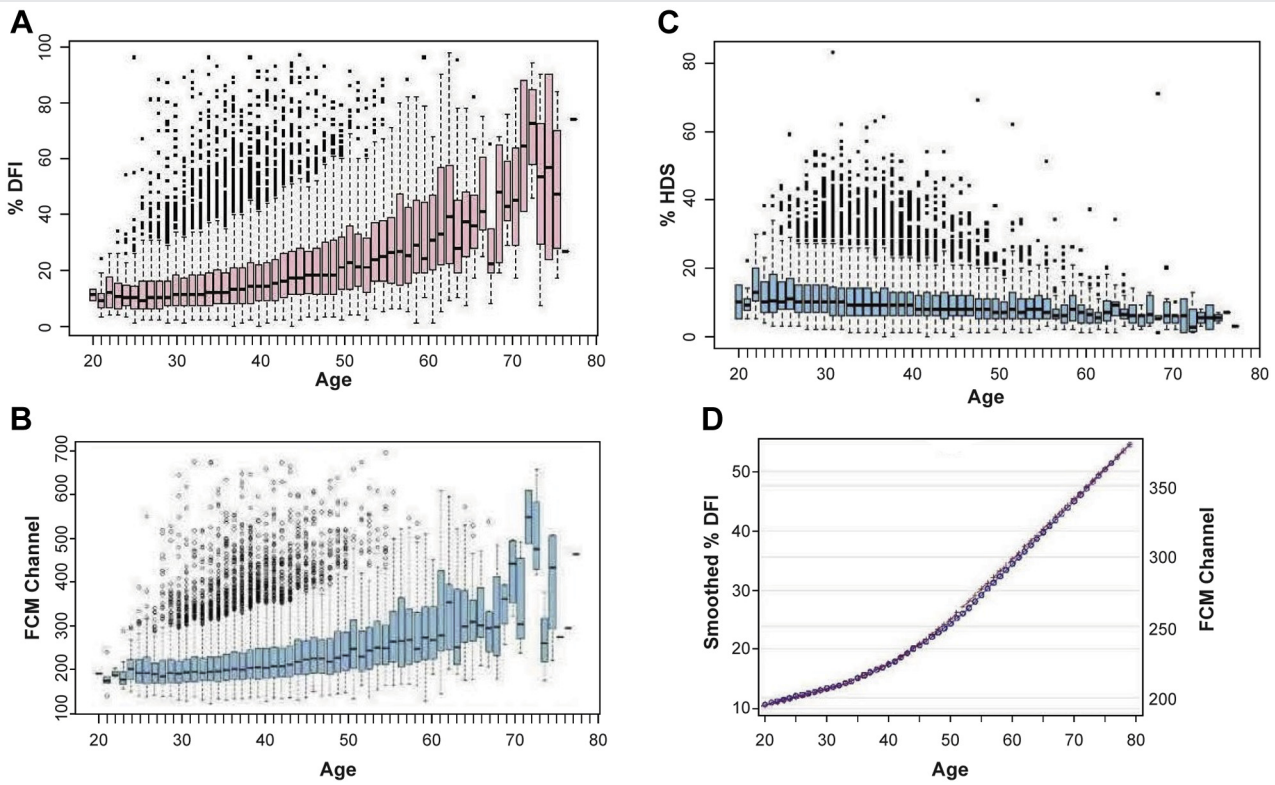
Figure 4 shows estimated decreasing odds for producing a live birth, moving from high odds at 0–15% DFI to decreasing odds with in vivo and IUI fertilization up to the clinical threshold of 25% DFI for couples without female infertility factors.

DISCUSSION

As men age, the integrity of paternal DNA is increasingly compromised (7, 8). It is important to note that the age-related increase of %DFI and decrease of %HDS observed for 87 healthy donor men, ranging in age from 20 to 80 years (median 44 years) and without any history of infertility or reproductive problems (3, 31), have nearly the same SCSA patterns as seen in this study of approximately 25,000 men attending infertility clinics. The shift to a significantly increased rate of %DFI for both is also at age ~41 years. Thus, the overall changes in %DFI and %HDS are driven primarily by age and are not specific to patients attending infertility clinics. As seen in Figure 2, the SD of %DFI, and the wide variations in the box plots in Figure 3, it is obvious that both infertility patients and donor men have a great heterogeneity of %DFI at all ages; thus, a young age does not ensure integrity of the male genetic package.

Mature sperm cannot repair their damaged DNA by themselves and rely on the oocyte repair machinery to correct paternal DNA alterations immediately after fertilization. Consequently, the sperm of aging men place an additional burden on the oocyte to eliminate alterations in the paternal DNA that can have dramatic consequences for the success of reproduction and the well-being of future individuals. Although we observe that there is quite significant heterogeneity for each age group with respect to the patient's DFI, which could logically be expected given that each patient has his own genetic and environmental history, the evaluation of the sperm DFI represents a valuable tool for the assessment of male infertility as well as the infertility of the couple (18, 19, 36, 39, 40). Nevertheless, as seen in Supplemental Table 1, the statistical evaluation of this large cohort of SCSA data show that patients 20–25 years of age have a

FIGURE 3



(**A**–**C**) Box plots of %DFI, mean DFI, and %HDS. (**D**) Comparison between %DFI and mean DFI in flow cytometer channels (0–1,024).

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DFI of ~12%. At ages 40–45, the average %DFI increases to ~19%, which has a decreasing of fertility potential, especially if one or more classical semen values are abnormal (36). At ages 60–65, the average %DFI nearly doubles to a pathological ~37%. In this study, 22.2% of the approximately 25,000 men reached ≥25% DFI, and 10.6% reached ≥20% HDS. Whether this pattern can be changed by various therapeutic actions and individualized medicine is now to be determined and is the goal to achieve. Vinnnakota et al. (41) described that repeat testing following lifestyle intervention in 29 couples in which the men had high initial DFI (35% ± 9.5%) showed that 71.4% had a decrease in DFI into the moderate to low range. It is noted, however, that the general approach to provide antioxidant treatments does not consistently provide increased fertility, and, when given at high doses, antioxidants might block essential oxidative processes such as chromatin compaction (42), resulting in increased %HDS, as has been reported by Ménézo et al. (43).

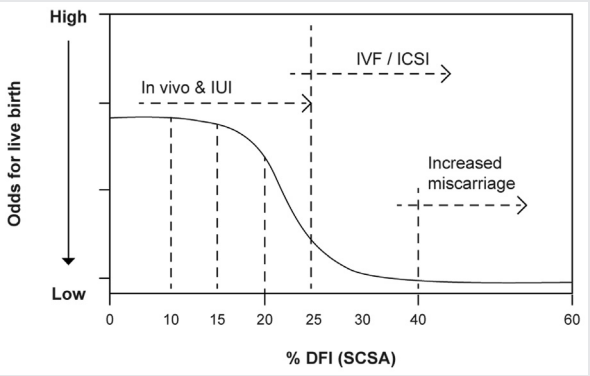
Comparison of %DFI versus Mean DFI

The %DFI is determined by computer gating of the sperm (dots on computer monitor) that have increased red fluorescence due to single (ss) and double (ds) DNA strand breaks. Mean DFI is the measure in flow cytometer channels (0–1,024) of the shift of the entire sperm sample population from normal DNA integrity to abnormal DNA strand breaks (Fig. 1). SCSA studies (20, 22, 26) and SCSA diagnostic clinical reports have shown both the %DFI and mean DFI as measures of sperm DNA damage. However, because it may be easier for the reproductive medicine community to understand "%DFI values" (% good vs. % bad) than mean DFI calculated in flow cytometer channel shifts, most authors reporting SCSA date have shown only %DFI. However, as shown in Figure 3D, the two methods produce the same results, which means that the commonly used %DFI is a measure of the entire sperm SCSA-defined DNA fragmentation.

HDS Sperm

Infertility is a condition associated with multiple etiologies including not only SDF but also abnormalities of sperm nuclear chromatin structure. During human spermiogenesis, all but ~15% of histones are replaced by protamines P1 and P2, resulting in sperm chromatin condensation followed by a halt to gene expression in haploid spermatids. The final structure of sperm chromatin is dependent upon post-translational methylation and acetylation that affects chromatin stability and the acquisition of epigenetic/imprinting marks with an impact on embryonic development (44). Faulty compaction makes an abnormal tertiary chromatin structure that likely prevents the embryo from accessing the correct sequences of the paternal genome for proper initiation of the embryonic developmental program (42). The observed

FIGURE 4



Estimated decreasing odds for producing a live birth, moving from high odds at 0–15% DFI to decreasing odds with >15% DFI.

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higher %HDS in patients across all ages relative to donors (Fig. 2) suggests that patients may experience a greater mean level of spermatogenesis dysfunction.

Given that acridine orange–stained histone-complexed DNA stains 2.3 times more than protamine-complexed DNA (45), this HDS sperm fraction is easy to detect with the SCSA test (Fig. 1). Flow cytometer–sorted HDS populations of human sperm showed that these sperm nuclei are more rounded, consistent with lack of full sperm chromatin maturity (46). These sorting experiments also showed that SCSA-defined moderate level DFI sperm had comet-defined DNA strand breaks but also a totally normal morphology (46, 47). Homa et al. (5) showed that HDS levels are not correlated with vitality but are negatively correlated with all other semen parameters; the strongest correlation is between HDS and morphology ($r = -0.488$, $P = .0001$).

High levels of sperm nuclear chromatin condensation abnormalities are associated with lower fertilization rates, impaired embryo quality, elevated arrested embryo rates, and decreased pregnancy rates (42). The most important is a block at the 2 PN stage or even an absence of decondensation of the sperm nucleus; this leads sometimes to a confusion with "absence of fertilization" in intracytoplasmic sperm injection (ICSI) (48). Sperm of men from repeated spontaneous abortion groups have been shown to have less chromatin condensation and poorer DNA integrity than sperm obtained from fertile men with no history of repeated spontaneous abortion (39)

In a clinical study of 1,417 infertility patients (26), the patients were divided according to three thresholds for % HDS (called SDI by Menezo et al.) as follows: <20% (77% of patients; no problem); 20–25% (10% patients; gray zone); and >25% (12% of patients; red zone).

DFI Sperm

It has been shown that %DFI measurements are predictive for unsuccessful pregnancy outcomes (14, 15, 18, 20, 21, 26, 36, 49–52). Patient data show that samples with SCSA %DFI values of <27–30% have higher probabilities of successful pregnancies by natural means (6.5- to 10-fold) (20), intrauterine insemination (7.0- to 8.7-fold) (33), routine in vitro fertilization (~2-fold), and intracytoplasmic sperm insemination (~1.4-fold) compared with samples with >30% DFI values (34). These thresholds are incorporated into Figure 4, showing three major clinical thresholds: 20% DFI for early loss of fertility potential, especially if one or more classical semen parameters are abnormal (36); 25% for natural and IUI fertilization (20, 21) with a switch to ICSI; and 40% for low probability for IVF/ICSI success and increased miscarriages (34). These threshold levels are supported by the clinical study of Menezo et al. (26). Three thresholds for %DFI were <20% (60% of patients; no problem); 20–25% (9.2% of patients; gray zone); and >25% (31% of patients; red zone infertility).

For the assisted reproductive technologies clinic, natural and IUI fertilization is likely successful with <25% DFI, whereas >25–30% DFI patients should be moved to ICSI. At the >40% DFI level, commonly seen in men more than 50 years of age, it becomes controversial whether to consider testicular sperm aspiration (TESA). The use of TESA in those patients failing with high %DFI ejaculate-ICSI have shown in some studies increased pregnancy rates (38). Others (39) had results suggesting that pregnancy outcomes and live birth rates with testicular-ICSI are not significantly superior to ejaculate-ICSI in patients with an elevated SCSA-defined sperm DNA fragmentation and prior ICSI failure(s).

Male partners of couples attending infertility clinics are very sensitive to the idea that it may be their sperm quality that is causing problems of pregnancy. If so, the couple may attribute the infertility to lifestyle factors in the male partner. This may be especially pertinent for men with secondary infertility and normal semen parameters; such samples may, by age alone, have high %DFI causing the infertility. Therefore, we consider that the information in Supplemental Figure 1 showing the average %DFI with age may be highly useful in showing that for the man in his 40s to 50s, age alone, through no fault of his lifestyle, is likely a significant factor in the couple's infertility situation.

Strengths and Limitations

This study has a number of strengths. For the first time, the changes in SCSA-defined DNA/chromatin with the age of men (20–80 years) attending infertility clinics are compared to changes seen in aging nonpatient healthy men without reproductive issues. Data are based on the world's largest number of men attending infertility clinics in any age study for more than 11 years, and semen samples were collected across all of North America and the United Kingdom for a broad sampling of environmental range. The data were collected by the laboratory of the inventor of the SCSA test, and all the approximately 25,000 patients and 87 donor samples underwent measurement by nearly exactly the same protocol. Rather than a simple measure of %DFI vs. age, this study calculated the shift point of age when the rate of %DFI doubles. Also calculated were %HDS, SD DFI, and mean DFI, as well as the age points of clinical thresholds.

Finally, the SCSA-derived curve plots of the originally used mean DFI are compared to the currently used %DFI.

Study limitations include men presenting with infertility, many of whom likely had a number of clinical conditions or adverse lifestyle factors (e.g., varicocele, tobacco or drug use, poor diet, and stress). Although pregnancy and miscarriage information was obtained on many patients for counseling, such data were not included in this article.

In conclusion, it is very clear that age compromises sperm DNA/chromatin integrity. As such, a man's biological clock is also "ticking," and by age 40 years, there is a 20% chance of reaching the pathological ~25% DFI by age alone, suggesting ICSI treatment. Notably, SDF testing not only suggests when ICSI should be used but it may also prevent unnecessary ICSI treatment if the %DFI is low, thus reducing the financial burden to infertility clinic patients.

REFERENCES

1. Martin JA, Hamilton BE, Sutton PD, Ventura SJ, Menacker F, Munson ML. Natl Vital Stat Rep 2005;54:1–116.
2. Sauer MV. Reproduction at an advanced maternal age and maternal health. Fertil Steril 2015;103:1136–43.
3. Wyrobek AJ, Eskenazi B, Young S, Arnheim N, Tiemann-Boege I, Jabs EW, et al. Advancing age has differential effects on DNA damage chromatin integrity, gene mutations, and aneuploidies in sperm. Proc Natl Acad Sci 2006;103:9601–6.
4. Kidd SA, Eskenazi B, Wyrobek AJ. Effects of male age on semen quality and fertility: a review of the literature. Fertil Steril 2001;75:237–48.
5. Homa ST, Vassiliou AM, Stone J, Killeen AP, Dawkins A, Xie J, et al. A comparison between two assays for measuring seminal oxidative stress and their relationship with sperm DNA fragmentation and semen parameters. Genes 2019;19:10–23.
6. Gavriliouk D, Aitken RJ. Damage to sperm DNA mediated by reactive oxygen species: its impact on human reproduction and the health trajectory of offspring. Adv Exp Med Biol 2015;868:23–47.
7. Kaarouch I, Bouamoud N, Madkour A, Louanjli N, Saadani B, Assou S, et al. Paternal age: negative impact on sperm genome decays and IVF outcomes after 40 years. Mol Reprod Dev 2018;85:271–80.
8. Alshahrani S, Agarwal A, Assidi M, Abuzenadah AM, Durairajanayagam D, Ayaz A, et al. Infertile men older than 40 years are at higher risk of sperm DNA damage. Reprod Biol Endocrinol 2014;12:103.
9. Wang M, Su P. The role of the Fas/FasL signaling pathway in environmental toxicant-induced testicular cell apoptosis: an update. Syst Biol Reprod Med 2018;64:93–102.
10. Drevet JR, Aitken RJ. Oxidative damage to sperm DNA: attack and defense. Adv Exp Med Biol 2019;1166:107–17.
11. Singh NP, Muller CH, Berger RE. Effects of age on DNA double-strand breaks and apoptosis in human sperm. Fertil Steril 2003;80:1420–30.
12. Rosiak-Gill A, Gill K, Jakubik J, Fraczek M, Patorski L, Gaczarzewicz D, et al. Age-related changes in human sperm DNA integrity. Aging 2019;11:5399–411.
13. World Health Organization. WHO laboratory manual for the examination and processing of human semen. Geneva: World Health Organization; 2010.
14. Evenson DP, Darzynkiewicz Z, Melamed MR. Relation of mammalian sperm chromatin heterogeneity to fertility. Science 1980;210:1131–3.
15. Evenson DP, Larson K, Jost LK. The Sperm Chromatin Structure Assay (SCSA): clinical use for detecting sperm DNA fragmentation related to male infertility and comparisons with other techniques. J Androl 2002;23:25–43.
16. Evenson DP, Jost L, Baer R, Turner T, Schrader S. Individuality of DNA denaturation patterns in human sperm as measured by the Sperm Chromatin Structure Assay. Reprod Toxicol 1991;5:115–25.
17. Evenson DP. SCSA test on its 40th anniversary. In: Agarwal A, Henkel R, Majzoub A, eds. Manual of sperm function testing in human assisted reproduction. Cambridge, UK: Cambridge University Press, in press.
18. Evenson DP. Sperm chromatin structure assay (SCSA®): Evolution from origin to clinical utility. In: Zini A, Agarwal A, editors. A Clinician's Guide to Sperm DNA and Chromatin. Springer International Publishing; 2018.
19. Drevet JR. Sperm DNA integrity testing: a valuable addition to the toolbox of infertility clinicians. Transl Androl Urol 2017;6:S590–1.
20. Evenson DP, Jost LK, Zinaman MJ, Clegg E, Purvis K, de Angelis P, et al. Utility of the Sperm Chromatin Structure Assay (SCSA) as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod 1999;14:1039–49.
21. Spano M, Bonde JP, Hjollund HI, Kolstad HA, Cordelli E, Leter G. Sperm chromatin damage impairs human fertility. The Danish First Pregnancy Planner Study Team. Fertil Steril 2000;73:43–50.
22. Brinkworth MH, Weinbauer GF, Bergmann M, Nieschlag E. Apoptosis as a mechanism of germ cell loss in elderly men. Int J Androl 1997;20:222–8.
23. D'Occhio MJ, Hengstberger KJ, Johnston SD. Biology of sperm chromatin structure and relationship to male fertility and embryonic survival. Anim Reprod Sci 2007;101:1–17.
24. Jerre E, Bungum M, Evenson D, Giwercman A. Sperm chromatin structure assay high DNA stainability sperm as a marker of early miscarriage after intracytoplasmic sperm injection. Fert Steril 2019;12:46–53.
25. Booze M, Brannian J, Von Wald T, Hansen K, Evenson D. High DNA stability in the SCSA® is associated with poor embryo development and live birth outcome. American Association of Bioanalysts Conference. May 16-18. New Orleans, LA. [abstract].
26. Menezo Y, Clement P, Amar E. Evaluation of sperm DNA structure, fragmentation and decondensation: an essential tool in the assessment of male infertility. Transl Androl Urol 2017;6:S553–6.
27. Dattilo M, Giuseppe D, Ettore C, Ménézo Y. Improvement of gamete quality by stimulating and feeding the endogenous antioxidant system: mechanisms, clinical results, insights on gene-environment interactions and the role of diet. J Assist Reprod Genet 2016;33:1633–48.
28. Talebi AR, Vahidi S, Aflatoonian A, Ghasemi N, Ghasemzadeh J, Firoozabadi RD, et al. Cytochemical evaluation of sperm chromatin and DNA integrity in couples with unexplained recurrent spontaneous abortions. Andrologia 2012;44:462–70.
29. Cornet D, Cohen M, Clement A, Amar E, Fournols L, Clement P, et al. Association between the MTHFR-C677T isoform and structure of sperm DNA. J Assist Reprod Genet 2017;34:1283–8.
30. Evenson DP. Sperm chromatin structure assay. In: Carrell D, Aston K, editors. Spermatogenesis: methods and protocols (Methods in molecular biology). New York: Humana Press; 2013.
31. Silver EW, Eskenazi B, Evenson DP, Block G, Young S, Wyrobek AJ. Effect of antioxidant intake on sperm chromatin stability in healthy nonsmoking men. J Androl 2005;26:550–6.
32. Virro MR, Larson-Cook KL, Evenson DP. Sperm chromatin structure assay (SCSA®) related to blastocyst rate, pregnancy rate and spontaneous abortion in IVF and ICSI cycles. Fertil Steril 2004;81:1289–95.
33. Bungum M, Humaidan P, Spano M, Jepson K, Bungum L, Giwercman A. The predictive value of Sperm Chromatin Structure Assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. Hum Reprod 2004; 19:1401–8.
34. Oleszczuk A, Giwercman A, Bungum M. Sperm chromatin structure assay in prediction of in vitro fertilization outcome. Andrology 2016;4:290–6.
35. Bungum M, Humaidan P, Spano M, Jepson K, Bungum L, Giwercman A. The predictive value of Sperm Chromatin Structure Assay (SCSA) parameters for the outcome of intrauterine insemination, IVF, and ICSI. Hum Reprod 2004; 19:1401–8.
36. Erenpreiss J, Elzanaty S, Giwercman A. Sperm DNA damage in men from infertile couples. Asian J Androl 2008;10:786–90.
37. Rex AS, Wu C, Aagaard J, Fedder J. Implementation of an in-house flow cytometric analysis of DNA fragmentation in spermatozoa. Asian J Androl 2020;22:246–51.
38. Esteves SC, Roque M, Bradley CK, Garrido N. Reproductive outcomes of testicular versus ejaculated sperm for intracytoplasmic sperm injection among men with high levels of DNA fragmentation in semen: systematic review and meta-analysis. Fertil Steril 2017;108:456–67.
39. Alharbi M, Hamouche F, Phillips S, Kadoch JI, Zini A, Alharbi M, et al. Use of testicular sperm in couples with SCSA-defined high sperm DNA

fragmentation and failed intracytoplasmic sperm injection using ejaculated sperm. Asian J Androl 2019 [Epub ahead of print].
40. Zini A, Albert O, Robaire B. Assessing sperm chromatin and DNA damage: clinical importance and development of standards. Andrology 2014;2:322–5.
41. Vinnakota C, Cree L, Peek J, Morbeck DE. Incidence of high sperm DNA fragmentation in a targeted population of subfertile men. Syst Biol Reprod Med 2019;24:1–7.
42. Dattilo M, Cornet D, Amar E, Cohen M, Menezo Y. The importance of the one carbon cycle nutritional support in human male fertility: a preliminary clinical report. Reprod Biol Endocrinol 2014;12:71.
43. Ménézo YJ, Hazout A, Panteix G, Robert F, Rollet J, Cohen-Bacrie P, et al. Antioxidants to reduce sperm DNA fragmentation: an unexpected adverse effect. Reprod Biomed Online 2007;14:418–21.
44. Puri D, Dhawan J, Mishra RK. The paternal hidden agenda: epigenetic inheritance through sperm chromatin. Epigenetics 2010;5:386–91.
45. Evenson DP, Darzynkiewicz Z, Jost L, Janca F, Ballachey B. Changes in accessibility of DNA to various fluorochromes during spermatogenesis. Cytometry 1986;7:45–53.
46. Evenson DP, Tritle D. Characterization of SCSA resolved sperm populations by Comet assay and image analysis. IFFS 8th World Congress on Fertility and Sterility; 2004 (abstract).
47. Evenson DP. The Sperm Chromatin Structure Assay (SCSA) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. Anim Reprod Sci 2016;169:56–75.
48. Junca A, Marti BG, Elisabetta Tosti E, Cohen M, De la fontaine D, Benkhalifa Moncef, et al. Sperm nucleus decondensation, hyaluronic acid (HA) binding and oocyte activation capacity: different markers of sperm immaturity. J Assist Reprod Genet 2012;29:353–5.
49. Agarwal A, Majzoub A, Esteves SC, Ko E, Ramasamy R, Zini A. Clinical utility of sperm DNA fragmentation testing: practice recommendations based on clinical scenarios. Transl Androl Urol 2016;5:935–50.
50. O'Neill CL, Palermo GD. A worldwide profile of the utilization of sperm DNA fragmentation testing in relation to reproductive outcome. Transl Androl Urol 2017;6:320–1.
51. Benchaib M, Lornage J, Mazoyer C, Lejeune H, Salle B, François Guerin J. Sperm deoxyribonucleic acid fragmentation as a prognostic indicator of assisted reproductive technology outcome. Fertil Steril 2007; 87:93–100.
52. Nicopoullos JD, Gilling-Smith C, Almeida PA, Homa S, Norman-Taylor JQ, Ramsay JW. Sperm DNA fragmentation in subfertile men: the effect on the outcome of intracytoplasmic sperm injection and correlation with sperm variables. BJU Int 2008;101:1553–60.