

DNA fragmentation in brighter sperm predicts male fertility independently from age and semen parameters

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Objective: To evaluate whether sperm DNA fragmentation (sDF), measured in brighter, dimmer, and total populations, predicts natural conception, and to evaluate the intra-individual variability of sDF.

Design: Prospective study.

Setting: Outpatient clinic and diagnostic laboratory.

Patient(s): A total of 348 unselected patients and 86 proven fertile men.

Intervention(s): None.

Main Outcome Measure(s): sDF was revealed with the use of terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL)/propidium iodide (PI). Receiver operating characteristic (ROC) curves were built before and after matching fertile men to patients for age (76:152) or semen parameters (68:136) or both (49:98). Intra-individual variability of sDF was assessed over 2 years.

Result(s): Brighter (area under ROC curve [AUC] 0.718 ± 0.54), dimmer (AUC 0.655 ± 0.63), and total (AUC 0.757 ± 0.54) sDF predict male fertility in unmatched and age- or semen parameters-matched subjects. After matching for both age and semen parameters, only brighter (AUC 0.711 ± 0.83) and total (AUC 0.675 ± 0.92) sDF predict male fertility. At high values of total sDF, brighter predicts natural conception better than total sDF. Intra-individual coefficients of variation of sDF were $9.2 \pm 8.6\%$ ($n = 25$), $12.9 \pm 12.7\%$ ($n = 53$), and $14.0 \pm 12.6\%$ ($n = 70$) over, respectively, 100-day and 1- and 2-year periods, appearing to be the most stable of the evaluated semen parameters.

Conclusion(s): The predictive power of total sDF partially depends on age and semen parameters, whereas brighter sDF independently predicts natural conception. Therefore, brighter sDF is a fraction of sDF that adds new information to the routine semen analysis. At high levels of sDF, distinguishing the two sperm populations improves the predictive power of sDF.

Overall, our results support the idea that TUNEL/PI can be of clinical usefulness in the male fertility workup. (Fertil Steril® 2015;104:582–90. ©2015 by American Society for Reproductive Medicine.)

Key Words: Sperm DNA fragmentation, natural conception, male fertility, TUNEL/PI

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One in seven couples of reproductive age will encounter problems with fertility and, in about one-half of them, the male factor is the sole or a contributory cause (1, 2). Semen analysis is routinely used

to evaluate the male factor of infertile couples and is considered to be the cornerstone evaluation in male fertility workups. However, whether or not semen analysis predicts natural conception is still controversial (3–5). The predictive power of standard semen parameters is further complicated by the high technical (intra- and interassay [6, 7]) and biologic (intra-individual [8, 9]) variability of the measurements of semen parameters. In such a situation, identifying other diagnostic tests to use in conjunction with, or in alternative to, semen analysis in the

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evaluation of infertile men has become an urgent issue. Among the several tests that might improve the prediction of natural conception by means of routine semen analysis, evaluation of sperm DNA fragmentation (sDF) appears to be promising. Indeed, sDF is higher in subinfertile patients (10) and only partially related to semen quality (11), and high sDF levels are associated with poor assisted reproductive technology (ART) outcomes (10). Several studies (12–16) investigated the impact of sDF on natural pregnancy with the use of different techniques to reveal DNA breaks, reporting variable thresholds of sDF discriminating between infertile and fertile subjects. Among these studies, that by Giwercman et al. (17) not only reported a odds ratio (OR) of 5.1 for infertility in men with an sDF >20%, but also showed that this prediction power greatly increases when considering men with at least one semen abnormality, suggesting that semen parameters might affect the prediction of natural conception with the use of sDF. Male age represents another variable that might have an effect on the ability of sDF to discriminate between fertile and infertile patients, because it has been reported that the amounts of sDF increase as a function of aging (18–20), possibly explaining the putative impact of advancing paternal age on pregnancy and offspring health (21). In most studies, however, the ages of fertile and infertile men were different (16) or unknown (12, 15). Furthermore, the semen quality of infertile men was poorer than in fertile ones (13, 17) or not reported (12, 14). Thus, the influence of semen quality and age on the predictive power of sDF is currently unknown. In addition, the lack of standardization of most methods (22) used to detect sDF hampers the clinical use of the reported threshold values.

Our group has developed a new version of flow cytometric terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) combining nuclear staining with propidium iodide (PI) for the detection of sDF and yielding precise, standardized (23), and more accurate measurements compared with simple TUNEL (24). Indeed, the nuclear staining with PI allows for the exclusion of semen apoptotic bodies, which otherwise cause large unsystematic underestimation of the measurements of sDF in most semen samples (24). In addition, TUNEL/PI reveals the occurrence of two sperm populations with a different nuclear stainability: one is more (brighter) and the other is less (dimmer) colored by PI (24). The two populations have several differences, including the fact that the dimmer population is completely formed by dead (25) and DNA-fragmented sperm (24) and contains cells with a large loss of chromatin material (26), whereas in the brighter one variable percentages of live and dead sperm with or without DNA fragmentation are present (24, 25, 27). Considering all of this, we reasoned that dead fragmented dimmer sperm have no chance to participate in the fertilization process and hypothesized that sDF might have a different impact on reproduction depending on whether it is measured in the brighter, the dimmer, or the total sperm population (i.e., dimmer + brighter sDF). To verify this hypothesis, by using TUNEL/PI, we investigated the predictive power of natural conception of sDF in total, brighter, and dimmer populations by comparing male

partners of infertile couples and fertile men. In addition, we verified whether the ability of total sDF and of the two fractions to distinguish between fertile men and patients depends on semen parameters and patients age and determined the intra-individual variability of sDF in 70 patients who repeated TUNEL/PI assay over a 2-year period.

MATERIALS AND METHODS

Chemicals

Human tubal fluid (HTF) medium and human serum albumin (HSA) were purchased from Celbio. Diff-Quick kit was purchased from CGA, Diasint. Bovine Serum albumin (BSA) was purchased from ICN Biomedicals. The other chemicals, unless otherwise indicated, were from Sigma Chemical.

Semen Samples: Collection and Preparation

Semen samples from subfertile and fertile men were collected during the period 2010–2014. Fertile men ($n = 86$) were subjects who had recently fathered a child (≤ 1 year from conception). Pregnancy obtained by means of ART was an exclusion criterion. Patients ($n = 348$) were male partners of infertile couples undergoing routine semen analysis in the andrology laboratory of the University of Florence. These men were unselected and represented a random cross-section of the male population attending the laboratory. Female factors of infertility in these couples were unknown. Semen samples were collected according to World Health Organization (WHO) criteria (7). The study was approved by the local Hospital Committee for Investigations in Humans (protocol no. 54/10) and all recruited men gave their informed consents. Sperm morphology and motility were assessed with the use of optical microscopy according to WHO criteria (7). Normal sperm morphology (subsequently called sperm morphology) was evaluated by determining the percentage of normal and abnormal forms after Diff-Quik staining, scoring ≥ 100 sperm/slide. Sperm motility was scored by determining the percentages of progressive motile, nonprogressive motile, and immotile spermatozoa, scoring ≥ 200 sperm/slide. The andrology laboratory participates in the external quality control programs United Kingdom National External Quality Assessment Service and Verifica Esterna di Qualità of Tuscany.

Matching Procedures

To assess whether the ability of sDF to predict male fertility status depended on age, semen parameters, or both, we matched fertile men to patients for: 1) age; 2) sperm count, progressive motility, and morphology; and 3) age, sperm count, progressive motility, and morphology.

To this aim, we calculated the tertile values of age (34–37 years), total sperm count (115.5–229.6 million/ejaculate), sperm progressive motility (47%–57%), and sperm morphology (6%–9%) in fertile men and established the following categories: A1 (age ≤ 34 y), A2 (age 34–37 y), A3 (age > 37 y), B1 (sperm count ≤ 115.5 million/ejaculate), B2 (sperm count 115.5–229.6 million/ejaculate), B3 (sperm count > 229.6 million/ejaculate), C1 (motility $\leq 47\%$), C2

(motility 47%–57%), C3 (motility >57%), D1 (morphology ≤6%), D2 (morphology 6%–9%), and D3 (morphology >9%), as well as all the possible combinations of the above categories (matching groups). Consequently, matching groups were three for only age matching, 27 for sperm number/motility/morphology matching, and 81 for sperm number/motility/morphology/age matching. Each fertile man and patient was assigned to the proper matching group. For example, the group A1B2C3D3 includes men with age ≤34 y, sperm count 115.5–229.6 million/ejaculate, motility >57%, and morphology >9%. Finally, within each matching group, fertile men were randomly matched with patients in a 1:2 ratio, resulting in 76 fertile men and 152 patients for age matching, 68 fertile and 136 infertile men for semen parameters matching, and 49 fertile men and 98 patients for age and semen parameters matching.

Interindividual Variability

To assess the intra-individual variability of sDF and semen parameters among patients undergoing sDF determination with the use of TUNEL/PI, we retrospectively selected those men ($n = 70$) who repeated the test twice over a 2-year period. Exclusion criteria were: pharmacologic therapies and high fever (28, 29) within the preceding 100 days; sexual abstinence >7 or <2 days (7); and incomplete collection of semen sample (30). sDF variability was expressed as coefficient of variation (CV) = (SD/mean of the two determinations) × 100. The intra-assay CV for sDF detected by TUNEL/PI is <5% (23).

TUNEL/PI Coupled with Flow Cytometry

Sperm DNA fragmentation was determined in neat semen samples after washing twice with HTF medium and fixing with paraformaldehyde (200 μ L, 4% in phosphate-buffered saline solution [PBS], pH 7.4) for 30 minutes at room temperature. Fixed samples were immediately processed for DNA break labeling because the storage of fixed sperm samples affects the measurement of sDF by means of TUNEL (23). To label DNA breaks, we used the In Situ Cell Death Detection Kit (Roche Molecular Biochemicals) as described elsewhere (23). Briefly, fixed spermatozoa were centrifuged at 500g for 10 minutes and washed twice with 200 μ L PBS with 1% BSA. Then spermatozoa were permeabilized with the use of 0.1% Triton X-100 in 100 μ L 0.1% sodium citrate for 4 minutes in ice. After washing 2 times, the labeling reaction was performed by incubating sperm in 50 μ L of labeling solution (supplied by the kit) containing the terminal deoxynucleotidyl transferase (TdT) enzyme for 1 hour at 37°C in the dark. Finally, samples were washed twice, resuspended in 500 μ L PBS, stained with 10 μ L PI (30 μ g/mL in PBS) and incubated in the dark for 10 minutes at room temperature. Sample measurements were acquired with the use of a FACScan flow cytometer (Becton Dickinson) equipped with a 15-mW argon-ion laser for excitation. For each test sample, three sperm suspensions were prepared for instrumental setting and data analysis: 1) by omitting both PI staining and TdT; 2) by omitting only TdT (negative control); and 3) by omitting only PI staining (for fluorescence compensation). Green fluorescence of nucleotides was

revealed with the use of an FL-1 (515–555 nm wavelength band, voltage set 590) detector; red fluorescence of PI was detected with the use of an FL-2 (563–607 nm wavelength band, voltage set 477) detector. For each sample, 10,000 events were recorded within the flame-shaped region (FR) characteristic of spermatozoa (23) in the forward-light scatter/side-light scatter dot plot. sDF was determined by gating the nucleated events (i.e., the events labeled with PI) within the FR (23). This strategy guarantees that fluorescence is analyzed in a population formed only by spermatozoa (24, 26), excluding debris, large cells, and semen apoptotic bodies (31, 32). For flow cytometric data analysis, in each of the two sperm populations (brighter and dimmer; Supplemental Fig. 1, available online at www.fertstert.org), a vertical marker was established in the TUNEL axis of the dot plot of negative control (TdT omitted), including 99% of total events. That marker was translated in the corresponding test sample, and all events beyond it were considered to be positive for TUNEL. Discrimination between dimmer and brighter sperm populations was established with the use of a horizontal marker in the PI axis (Supplemental Fig. 1). To assess whether such a discrimination was reproducible, in ten samples (five from fertile subjects and five from patients) we calculated the CVs of two measures of the amount of brighter population independently determined by two operators and found an average CV value of 1.1% (range 0%–4.0%). Dimmer sDF corresponds to the percentage of all dimmer sperm [(dimmer DNA sperm/total sperm population) × 100] because they are all DNA fragmented (24); brighter sDF was calculated as (brighter DNA fragmented sperm/total sperm population) × 100. Total sDF is dimmer sDF + brighter sDF.

Statistical Analysis

Unless otherwise indicated, data were analyzed with the use of Statistical Package for the Social Sciences (SPSS 20) for Windows. All variables were assessed for normal distribution with the use of the Kolmogorov-Smirnov test, and results expressed as mean ± SD or median (interquartile range [IQR]). Comparison of sDF and standard semen parameters between fertile men and patients was assessed with the use of the Mann-Whitney *U* test. To assess the ability of sDF to identify fertile men and patients, ROC curves were built as a binary classifier system to identify the sensitivity and the specificity of total, brighter, and dimmer sDF in predicting male fertility status. The ROC curves were built by iteratively using total, brighter, or dimmer sDF as “test variables,” and “fertile versus patient” (binary variable, with fertile = 0 and patient = 1) as “state variable” and setting the value of the “state variable” as 1. The optimal cutoff value was determined with the use of the Youden index to maximize the sum sensitivity + specificity (Analyse-it for Microsoft Excel, Method Validation Edition). For multivariate analysis, we used a binary logistic regression model, having as “dependent variable” the fertility status, defined as a binary variable (with fertile = 0 and patient = 1) and introducing as covariates, besides brighter and dimmer sDF, standard semen parameters (sperm count, sperm progressive motility, and sperm morphology) and age. Variables were introduced as centiles to normalize their variation,

and their specific contributions were compared in terms of OR for the fertility status.

In age/semen parameters-matched fertile men (n = 49) and patients (n = 98), the probability (power) of rejecting the null hypothesis (H₀) that dimmer sDF is equal in the two groups, being the mean difference in dimmer sDF of 0.84% (SD 1.94%), was calculated to be 0.7. The type I error probability associated with this test of this H₀ is 0.05.

For statistical tests, differences with a P value of <0.05 were considered to be significant.

RESULTS

SDF as a Predictor of Male Fertility Status: Unmatched Subjects

Patients recruited in this study were older and showed poorer semen parameters, except for motility, than fertile subjects (Table 1, unmatched data). Comparing the values of total, brighter, and dimmer sDF, we found that all were statistically higher in patients than in fertile men (Table 1, unmatched data). Interestingly, the contributions of brighter and dimmer fractions to total sDF were different in fertile men and patients (Fig. 1A). At high values (>70th percentile) of total sDF, similar percentages of brighter and dimmer fractions contribute to total sDF in patients (Fig. 1A, top), whereas the dimmer sDF is the prevailing fraction in fertile men (Fig. 1A, bottom).

The ability of sDF to discriminate patients from fertile men was studied by means of ROC curve analysis and calculating the corresponding AUCs (Fig. 2A; Supplemental Table 1 [unmatched data], available online at www.fertstert.org) and the Youden index; the latter was used to determine the optimized threshold value for each type of sDF. As shown, the total as well as the two fractions of sDF differentiated the two groups of men (i.e., AUC statistically >0.5, the AUC of the reference line; Fig. 2A). With the use of the Youden index, we found that 34.0% total and 22.4% brighter sDF maximize the sum of sensitivity and specificity. By 34.0% total sDF, 73% of the patients (true positive proportion [TPP]) were over the threshold versus 35% of the fertile men (false positive proportion [FPP]); similarly, by 22.4% brighter sDF, 58% of the patients were over the threshold versus 26% of the fertile men.

SDF as a Predictor of Male Fertility Status: Matched Subjects for Age, Semen Parameters, and Both

To verify whether the predictive ability of sDF is dependent on semen parameters and/or age, we matched fertile men to patients for age, semen parameters, and both. After matching the two groups for age (76 fertile men to 152 patients), semen parameters remained worse in patients compared with fertile men, and total, brighter, and dimmer sDF were still greater in the former (Table 1, age-matched data). In addition, the ROC curve analysis indicated that all three types of sDF still discriminated the two groups (Supplemental Table 1, age-matched data). After semen parameters matching (68 fertile men to 136 patients), age remained different between

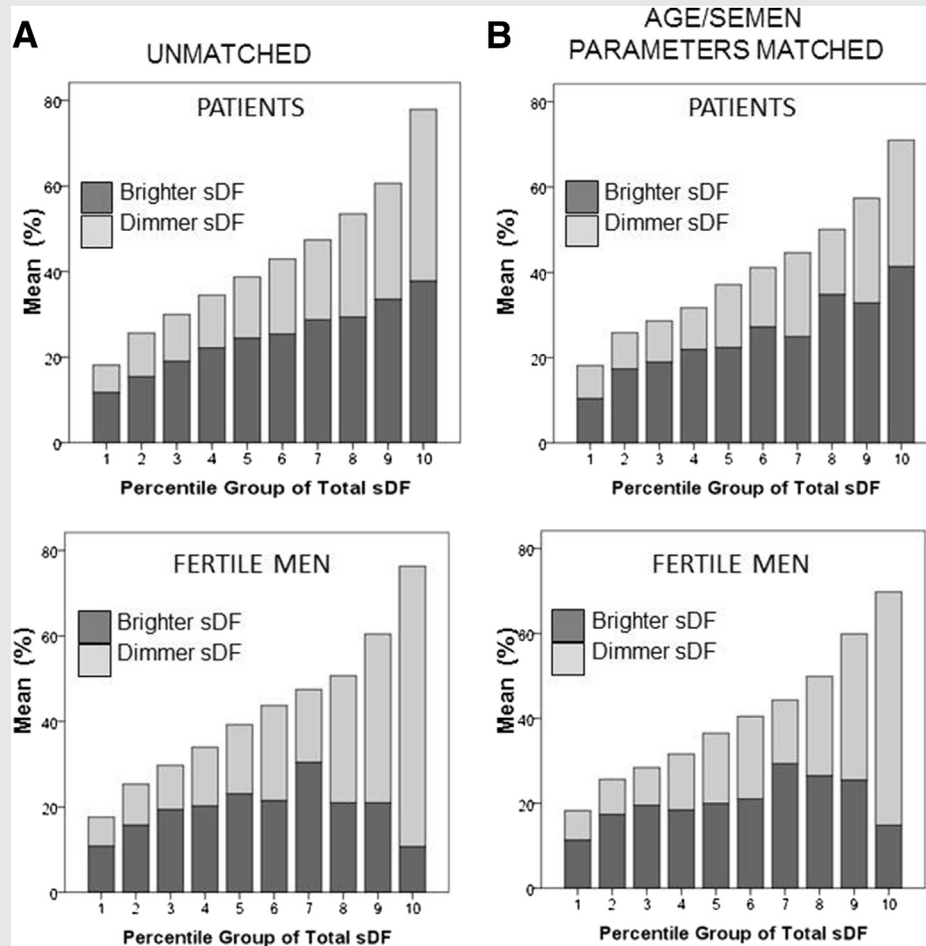
TABLE 1

Total, brighter, and dimmer sperm DNA fragmentation (sDF) and semen parameters in unmatched and matched fertile men and patients.

Parameter	Unmatched		Age-matched		Semen parameters-matched		Age- and semen parameters-matched	
	Fertile men	Patients	Fertile men	Patients	Fertile men	Patients	Fertile men	Patients
No. of subjects	86	348	76	152	68	136	49	98
Age (y)	36.0 (33.0–38.3)	39.0 ^a (37.0–43.0)	36.5 (35.0–39.8)	37.0 (35.0–41.0)	36.0 (33.3–39.0)	39.0 (36.0–42.0)	38.0 (36.0–41.5)	38.0 (36.0–41.3)
Sperm count (x 10 ⁹ /ejaculate)	174.2 (98.7–260.4)	101.3 ^b (36.4–249.0)	177.2 (91.1–269.1)	106.3 ^b (30.1–248.9)	162.2 (65.7–269.1)	151.3 (44.2–297.4)	156.8 (62.4–249.5)	144.6 (49.9–283.3)
Concentration (10 ⁶ /mL)	51.8 (32.3–80.6)	33.8 ^b (13.5–74.0)	52.5 (32.1–83.1)	34.0 ^a (10.0–73.5)	50.1 (26.0–83.1)	49.0 (15.6–86.3)	50.0 (28.0–67.3)	45.0 (17.0–83.6)
Total motility (%)	61.0 (53.0–71.3)	60.0 (47.3–71.0)	61.0 (53.0–70.8)	60.0 (43.3–72.8)	61.0 (52.0–69.5)	59.0 (49.3–69.8)	61.0 (49.5–71.0)	60.0 (47.0–71.0)
Progressive motility (%)	50.5 (43.0–62.5)	48.0 (31.0–62.0)	49.5 (43.0–62.0)	50.0 (27.3–63.8)	49.0 (42.3–62.0)	50.0 (38.0–61.8)	49.0 (37.0–62.0)	50.0 (35.8–61.3)
Morphology (%)	8.0 (5.0–10.5)	4.0 ^a (2.0–7.0)	7.0 (5.0–7.0)	4.0 ^a (2.0–8.0)	7.0 (5.0–10.0)	7.0 (2.0–10.0)	6.0 (4.0–9.0)	5.0 (2.0–9.0)
Total sDF (%)	28.9 (23.1–39.6)	43.9 ^a (33.0–55.7)	30.5 (23.8–39.7)	43.3 ^a (32.5–55.5)	30.3 (23.0–39.7)	42.5 ^a (33.8–56.5)	31.0 (25.8–40.5)	42.5 ^b (29.4–54.0)
Brighter sDF (%)	17.0 (12.3–23.3)	24.4 ^a (17.7–32.4)	17.5 (13.3–23.7)	24.3 ^a (17.7–31.0)	17.5 (12.3–23.1)	25.5 ^a (18.2–31.8)	18.1 (14.9–23.4)	25.2 ^b (18.7–33.3)
Dimmer sDF (%)	10.8 (7.1–17.0)	15.4 ^a (10.0–25.4)	10.9 (7.1–16.7)	16.3 ^a (9.8–27.0)	10.8 (7.0–17.8)	15.4 ^b (10.3–22.9)	13.4 (7.7–19.8)	14.0 (8.5–22.1)

Note: Data are expressed as median (interquartile range).
^aP<.001; ^bP<.01; patients versus corresponding fertile men.
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FIGURE 1



Contribution of brighter and dimmer fractions to total values of sperm DNA fragmentation (sDF) in fertile men and patients. Percentage of total sDF levels in (A) unmatched and (B) age- and semen parameters-matched (top) fertile men and (bottom) patients are expressed as deciles.

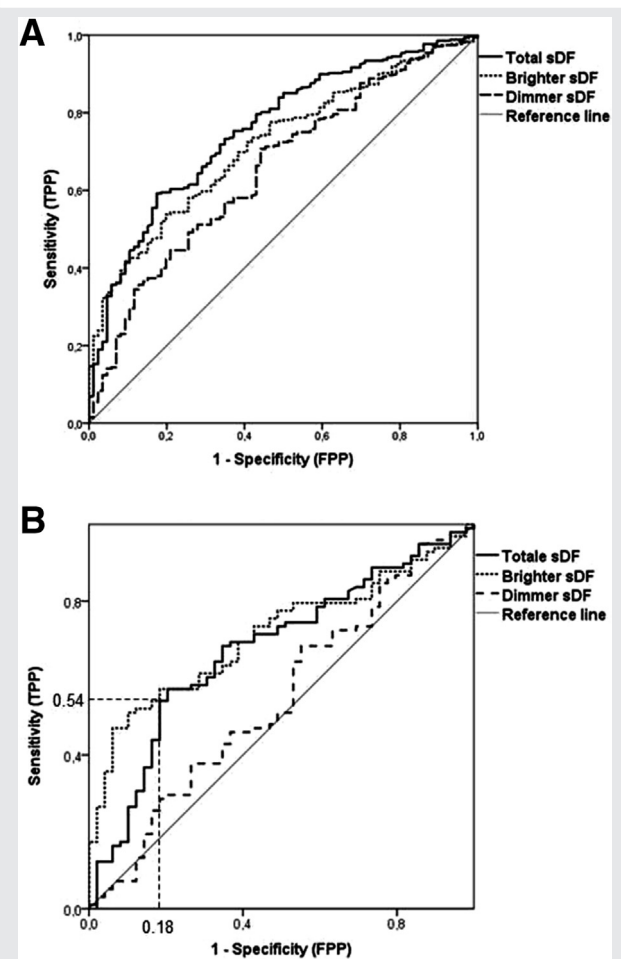
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the two groups and total as well as the two fractions of sDF were greater in patients than in fertile men (Table 1, semen parameters-matched data). Accordingly, all three types of sDF still successfully discriminated the two groups (Supplemental Table 1, semen parameters-matched data).

Finally, we matched men for both semen parameters and age (Table 1, age- and semen parameters-matched data). In this case, total and brighter sDF were higher in patients ($n = 98$) than in fertile men ($n = 49$), whereas no difference occurred in dimmer sDF (Table 1, age- and semen parameters-matched data). Accordingly, Figure 2B shows that only total and brighter sDF successfully discriminated patients from fertile men, whereas the AUC for the dimmer fraction was not different ($P > .05$) from the reference line (Fig. 2B). With the use of the Youden index to determine the value of sDF maximizing the sum of sensitivity and specificity, we found similar thresholds to those obtained with unmatched data: 36.0% of total and 22.4% of brighter sDF, yielding TPP of, respectively, 68% and 61% and FPP, respectively, of 35% and 28%.

In Figure 2B, it can be noted that the two ROC curves for total and brighter sDF, albeit including similar AUCs, are not identical (33): In the high specificity range (FPP $< .18$), the portion of AUC of brighter sDF is greater than that of total sDF. This finding is consistent with the different contribution of dimmer and brighter fractions to the high values of total sDF in fertile men compared with patients, which occurs both for unmatched (Fig. 1A; see above) and for age/semen parameters-matched data (Fig. 1B). In particular, at high values ($>41.7\%$, which corresponds to the operating point TPP, FPP 0.54, 0.18; Fig. 2B), total sDF is mainly composed of the dimmer fraction in fertile men, whereas in patients the two fractions contribute similarly to total sDF (Fig. 1B). To further investigate the relevance and independence of brighter sDF in predicting male fertility, we performed a binary logistic regression model, with fertility status as dependent variable and introducing as covariates—besides brighter sDF—dimmer sDF, sperm count, progressive motility, morphology, and age. We found that brighter, but not

FIGURE 2



Receiver operating characteristic curves analysis for total, brighter and dimmer sperm DNA fragmentation (sDF) in (A) unmatched and (B) age- and semen parameters-matched subjects. FPP = false positive proportion; TPP = true positive proportion.

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dimmer, sDF predicts male fertility status, independently from other semen parameters (Supplemental Table 2, available online at www.fertstert.org). In particular, for each centile increase in brighter sDF there is a 3.4% increase in the risk of being infertile. Standard semen parameters were also significantly associated with fertility, although with lower ORs (Supplemental Table 2).

Intra-individual Variation of sDF

To evaluate intra-individual variation of sDF as determined by TUNEL/PI, we selected men who executed the test at least twice over a 2-year period and calculated CV values for total, brighter, and dimmer sDF, as well as for standard semen parameters. As presented in Supplemental Table 3 (available online at www.fertstert.org), the total and two fractions of sDF show lower intra-individual variability regarding all standard semen parameters, over both a 1-year and a 2-year period.

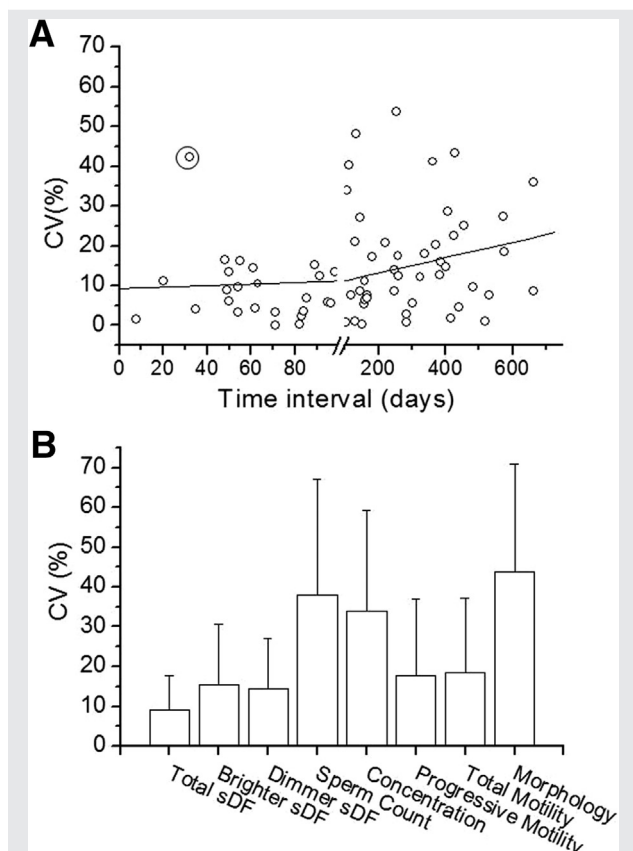
To verify whether there is a maximum time over which sDF is relatively stable, we plotted CV values for total sDF against the time between the first and the second tests (Fig. 3A). We found that the longer the time between the two tests, the greater the intra-individual variation of sDF (Fig. 3A; $r = 0.3 \pm 11.9$; $n = 69$; $P < .05$; note that the outlier, identified by a circle in the figure, was not considered in the linear regression analysis). Over a period of ~ 100 days, all the CVs ($n = 25$) were $< 20\%$ (except for the outlier; Fig. 3A), whereas for longer times, the sDF CVs ($n = 45$) were $\geq 20\%$ in 16 out of 45 patients and lower in 29 subjects (Fig. 3). In Figure 3B, the average CV ($9.2 \pm 8.6\%$) for total sDF, as assessed over a 100-day period, is compared with the average CVs for standard semen parameters as determined in the same semen samples.

DISCUSSION

In the present study we demonstrate that sDF evaluated with the use of TUNEL/PI is able to discriminate between male partners of infertile couples and fertile men, and that such an ability is partially dependent on the difference in age and semen quality between the two groups. Most importantly, we demonstrate that the two sperm populations detected with the use of our technique have a different predictive power of male fertility. Brighter sDF predicts fertility independently from age and semen parameters, whereas dimmer sDF is dependent on these parameters, indicating that the fraction of sDF that actually adds new information to routine semen analysis is the brighter one. In addition, we show that, at variance with patients, when high sDF levels are found in fertile men, these are mainly due to the dimmer fraction, which has no chance to participate in the fertilization process. This latter result appears clinically relevant because, in case of high sDF level, only the distinction between the two sperm populations can discern the fertility of the patient.

Results of the present study show that the levels of total, brighter, and dimmer sDF were all lower in men with proven fertility and successfully predicted fertility status. However, patients were older and their semen parameters poorer compared with fertile men, indicating that, at least in part, the ability of sDF to discriminate between the two groups may depend on such differences. Similar results were obtained by abolishing, alternately, the difference in age or semen parameters between the two groups of subjects. Only after matching for both age and semen parameters, or by a multivariate analysis after adjusting for age and introducing the main semen parameters, a difference between brighter and dimmer sDF becomes evident. Indeed, at variance with brighter sDF, the levels of dimmer sDF were similar in fertile men and patients, and dimmer sDF completely lost the ability to predict fertility status. Overall, these findings confirm that the ability of sDF to predict male fertility status in unmatched groups partially depends on semen parameters and age and that such a dependence is due to the dimmer sDF. Conversely, the independent diagnostic power of total sDF is completely due to the brighter fraction which predicted male fertility similarly in unmatched and age/semen parameters-matched subjects. The finding that the ability of sDF to predict fertility

FIGURE 3



(A) Intra-individual variability of sperm DNA fragmentation (sDF). Coefficient of variation (CV) of total sDF values are plotted against the time between the first and the second determinations. (B) Average CV values ($n = 25$) for total, brighter, and dimmer sDF and for all standard semen parameters as assessed over a 100-day period.

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status is not completely independent from semen parameters has already been underscored in a previous study (17). We now report that age as well influences such prediction, and we have identified the fraction of sDF whose predictive power is affected by age and standard semen parameters (i.e., the dimmer sDF).

The amount of dimmer sperm population is a sign of impaired testis function (24; unpublished results). However, because it is composed of 100% dead cells, the dimmer sDF likely has no impact on natural reproduction, at variance with the brighter sDF. Based on this, one would expect that the difference between fertile men and patients relies not only in the lower amounts of sDF in the former but also in the fact that in fertile men, sDF is formed mainly by the fraction of sperm DNA damage considered to have less impact on reproduction (i.e., dimmer sDF). The latter difference was evident only at the high values of sDF ($> \sim 40\%$) possibly justifying why certain men are fertile despite such high sDF levels. As a consequence, at these values of sDF, the brighter fraction was a better predictor of male fertility status than total sDF, because it still discriminated between fertile men and

patients with similar age and semen parameters, even if they exhibited equal amounts of total sDF. The difference in the brighter and dimmer compositions of sDF between fertile men and patients is not apparent in low values of total sDF. At present, we do not have an explanation for this finding, although we suppose that it is due to the fact that a certain percentage of fertile men are likely included in the patient population (see below), masking such a difference at low levels of total sDF.

Fertile men with high values of sDF (mainly composed of the dimmer fraction) resemble the subgroup of fertile men recently identified by Ribas-Maynou et al. (34) showing high percentages of sperm with double-strand sDF as detected by neutral comet assay. According to the same authors, DNA damage in those spermatozoa derives mainly from nuclease activity (35). Of interest, we recently demonstrated that the percentage of dimmer spermatozoa correlates with those of activated caspases and semen apoptotic bodies, also indicating that their damage is due to apoptotic nucleases (26).

With the use of ROC analysis, we found that the sensitivity and specificity obtained with a threshold of 34.0% total sDF are consistent with those found by Aitken et al. (15) but lower than those reported by other studies (13, 14) showing values for AUCs > 0.9 . The reason for the lower diagnostic performance observed by us likely relies on the fact that, in our series, female factors of couple infertility were not excluded. Indeed, the patient population of our study consists of men seeking fertility treatment (similar to the patient population presenting to the clinician in the basic infertility workup), and up to 40% of this group may be fertile subjects (4). Future studies should be directed to build cutoff values with the use of infertile couples excluding female factors. Presently the cutoff values of our study can be used by the clinicians to identify, with a certain probability and independently from semen parameters, an additional possible cause of male infertility. Moreover, the discrimination between brighter and dimmer sperm allows the clinician to identify fertile subjects even in the presence of high total sDF. Currently, very few diagnostic tests are available to infertile men, and evaluation of sDF with the use of TUNEL/PI could help in elucidating the reason for infertility. The requirement of both costly instruments and skilled operators, however, makes TUNEL/PI more suitable as a reference than a routine laboratory test.

The intra-individual variability of sDF greatly affects its predictive power regarding male fertility and, therefore, its clinical usefulness. For example, Erenpreiss et al. (36) reported that $\sim 40\%$ of men with amounts of sDF below the threshold for male infertility (30% as established by sperm chromatin structure assay [SCSA]), were above that threshold in the next measurement of sDF. In the present study, we found that the mean CV ($9.2 \pm 8.6\%$) for sDF is quite low over a 100-day period (a few percentage points over the mean intra-assay CV [23]), indicating that, within the mentioned period, the evaluation of a single semen sample provides a baseline data sample. Over a longer time the variability increases, but in a good percentage of subjects the values remain similar. In those subjects showing high variability, the occurrence of some unknown factor able to affect sDF

or of neglecting some requested information in the questionnaire by the patients (see below) could be hypothesized.

We also found that the variability of sDF was lower than that of any standard semen parameter, and lower than what had been previously reported (~30%) for similar periods with the use of both SCSA (36, 37) and TUNEL (38). Such a difference could be due to the recruitment criteria adopted in our study that, at variance with the above studies (36–38), excluded any conditions among those that so far are known to affect sDF (including recent pharmacologic therapies and fever episodes [28, 29]). Indeed, when some of these conditions are excluded, the variability of sDF results decreased (~20% [39]). It is also possible that the lower variations are due to use of the TUNEL/PI technique, which eliminates interference due to semen apoptotic bodies. The latter, indeed, are highly related to poor semen quality (31, 32, 40), so their inclusion in the analysis likely increases the dependence on semen parameters of sDF values, increasing its variability as well.

In conclusion, sDF successfully predicts fertility status and this ability partially depends on age and semen parameters when the latter are, respectively, greater and poorer in patients compared with fertile men. However, if the brighter fraction of sDF is considered, the predictive power becomes independent from both age and semen quality, also suggesting that it is the brighter fraction of sDF that actually adds new information in routine semen analysis. These findings, along with the low intra-individual variability of sDF, support the idea that the determination of sDF with the use of TUNEL/PI can be of clinical usefulness in the male fertility workup.

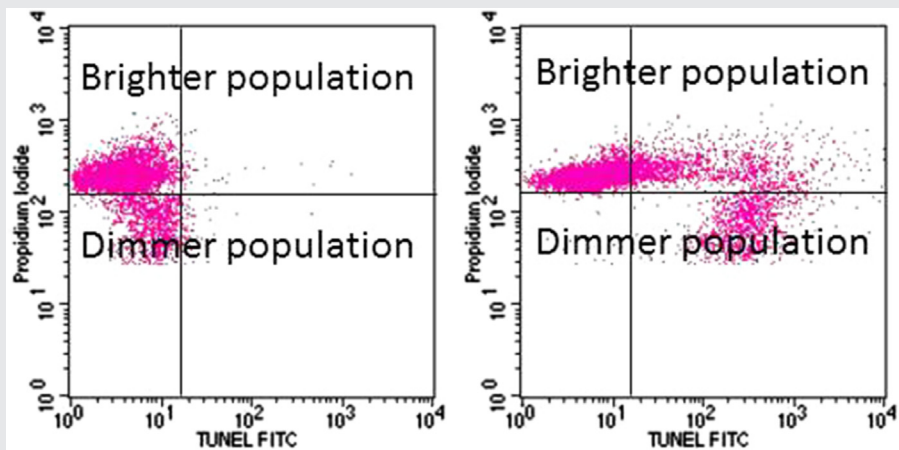
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SUPPLEMENTAL FIGURE 1



Typical dot plots of TUNEL/PI: (left) negative control and (right) test sample. Horizontal marker distinguishes the two sperm populations brighter and dimmer. The vertical marker is established in the negative control sample to include >99% of the events and is then translated to the test sample. Note that dimmer sperm are 100% DNA fragmented (24).

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SUPPLEMENTAL TABLE 1

Area under the receiver operating characteristic curve (AUC) values for total, brighter, and dimmer sperm DNA fragmentation (sDF) in unmatched and matched fertile men and patients.

Variable	Test	AUC (95% CI)	SE	P value
Unmatched	Total sDF	0.757 (0.703–0.812)	0.03	< .0001
	Brighter sDF	0.718 (0.664–0.772)	0.03	< .0001
	Dimmer sDF	0.655 (0.592–0.718)	0.03	< .0001
Age-matched	Total sDF	0.727 (0.659–0.795)	0.03	< .0001
	Brighter sDF	0.683 (0.614–0.753)	0.03	< .0001
	Dimmer sDF	0.645 (0.571–0.719)	0.04	< .0001
Semen parameters-matched	Total sDF	0.737 (0.664–0.809)	0.04	< .0001
	Brighter sDF	0.723 (0.653–0.793)	0.04	< .0001
	Dimmer sDF	0.638 (0.555–0.720)	0.04	.001
Age- and semen parameters-matched	Total sDF	0.675 (0.584–0.767)	0.05	< .0001
	Brighter sDF	0.711 (0.629–0.794)	0.04	< .0001
	Dimmer sDF	0.546 (0.445–0.647)	0.05	.1873

Note: CI = confidence interval; SE = standard error.

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SUPPLEMENTAL TABLE 2

Binary logistic regression model with age, brighter sDF, dimmer sDF, morphology, sperm count and progressive motility as introduced variables and fertile subjects versus patients as binary variable.

Variable	P value	OR	95% CI	
			Lower	Upper
Age	.000	1.023	1.012	1.034
Brighter sDF	.000	1.034	1.022	1.046
Dimmer sDF	.264	1.007	.995	1.018
Morphology	.000	0.975	.963	0.986
Sperm count	.003	.980	.966	.993
Progressive motility	.018	1.016	1.003	1.029

Note: CI = confidence interval; OR = odds ratio; sDF = sperm DNA fragmentation

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SUPPLEMENTAL TABLE 3

Intra-individual variability of total, brighter, and dimmer sperm DNA fragmentation (sDF) and of standard semen parameters over 1 y and 2 y.

Parameter	CV	
	1 y	2 y
No. of subjects	53	70
Total sDF (%)	12.9 ± 12.7	14.0 ± 12.6
Brighter sDF (%)	21.6 ± 20.2	23.9 ± 21.0
Dimmer sDF (%)	17.2 ± 15.4	20.1 ± 17.8
Sperm count ($\times 10^6$ /ejaculate)	39.6 ± 28.9	41.7 ± 30.0
Concentration (10^6 /mL)	34.9 ± 25.7	37.9 ± 26.5
Total motility (%)	24.5 ± 26.2	24.4 ± 24.5
Progressive motility (%)	28.9 ± 32.3	31.7 ± 34.7
Morphology (%)	43.0 ± 33.3	42.2 ± 32.9

Note: Data are expressed as mean ± SD.

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