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ORIGINAL ARTICLE

Male Fertility

DNA fragmentation in two cytometric sperm populations: relationship with clinical and ultrasound characteristics of the male genital tract

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We investigated whether DNA fragmentation in two cytometric sperm populations (PI^{dimmer} and $PI^{brighter}$) with different biological characteristics and clinical relevance is related to clinical and color-Doppler ultrasound (CDUS) parameters of the male genital tract. One hundred and sixty males of infertile couples without genetic abnormalities were evaluated for clinical, scrotal, and transrectal CDUS characteristics, presence of prostatitis-like symptoms (with the National Institutes of Health-Chronic Prostatitis Symptom Index) and sperm DNA fragmentation (sDF) in PI^{dimmer} and $PI^{brighter}$ populations (using TUNEL/PI method coupled with flow cytometry). Data were adjusted for age (Model 1) along with waistline, testosterone levels, smoking habit, and sexual abstinence (Model 2). According to the statistical Model 2, PI^{dimmer} sDF was associated with testicular abnormalities, including lower clinical and ultrasound volume ($r = -0.21$ and $r = -0.20$, respectively; $P < 0.05$), higher FSH levels ($r = 0.34$, $P < 0.0001$) and occurrence of testicular inhomogeneity ($P < 0.05$) and hypoechogenicity ($P < 0.05$). $PI^{brighter}$ sDF was associated with prostate-related symptoms and abnormal signs, including higher NIH-CPSI total and subdomain scores, a higher prevalence of prostatitis-like symptoms and of CDUS alterations such as macro-calcifications, severe echo-texture inhomogeneity, hyperemia (all $P < 0.05$), and higher arterial peak systolic velocity ($r = 0.25$, $P < 0.05$). Our results suggest that DNA fragmentation in PI^{dimmer} sperm, which is related to poor semen quality, mainly originates in the testicles, likely due to apoptosis. Conversely, DNA fragmentation in $PI^{brighter}$ sperm appears to mainly originate during or after transit through the prostate, increasing with the presence of an inflammatory status of the organ. These results could lead to new perspectives for the identification of therapeutic targets to reduce sDF.

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INTRODUCTION

Male infertility affects about 7% of all men. Despite many technical advances, its etiology is still unknown in half of the cases reported.[1,2] To bridge this gap, new unconventional semen parameters likely affecting male fertility have been increasingly investigated, with more and more evidence for sperm DNA fragmentation (sDF).[3,4] sDF levels are higher in infertile than fertile men[5] and only partially correlate with conventional sperm parameters[6] with an additional value in the diagnosis of male partners of infertile couples. However, the causes and clinical features underlying sDF and its site(s) of origin have not been entirely clarified.[7]

Recently, we set up a new method to evaluate sDF by the terminal deoxynucleotidyl transferase-mediated-dUTP nick end labeling (TUNEL) assay.[8] This method, which we named TUNEL/PI, uses staining with propidium iodide (PI) to eliminate anucleated semen apoptotic bodies[9,10] which interfere with cytometric analysis, allowing for more accurate measures.[8,11] Based on PI staining, we identified two cytometric sperm populations, called $PI^{brighter}$ and PI^{dimmer}, which differ in several biological characteristics. In particular, PI^{dimmer} population is entirely formed by DNA fragmented spermatozoa and shows negative correlations with semen quality.[8] Recently, we demonstrated that PI^{dimmer} sperm are unviable[12,13] and show signs of apoptosis.[14] Conversely, $PI^{brighter}$ population consists of a variable percentage of sperm with DNA fragmentation,[8] is formed by both viable and unviable sperm,[12] and shows signs of apoptosis and DNA oxidation.[13] $PI^{brighter}$ sDF is independent from semen quality,[8] therefore, a DNA-fragmented sperm in this population may be motile and morphologically normal, and thus could possibly participate in the fertilization process. Accordingly, we recently provided evidence that $PI^{brighter}$ sDF is the fraction that best discriminates fertile and infertile men independently from semen quality.[15] Hence, according to our studies, $PI^{brighter}$ and PI^{dimmer} populations seem to reflect different biological/clinical aspects, with the former showing a greater clinical impact and the latter mainly reflecting testicular function. In light of this, the investigation of the relationship between sDF in the two populations and the clinical features of the patients may lead to the identification of the possible

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sites of origin of the damage and, thus, novel therapeutic targets aimed at reducing it in both sperm populations.

Although many studies have focused on the impact of sDF on reproductive outcome,[3] only a few studies have analyzed sDF in relation to clinical signs or symptoms. Such studies traced possible associations between sDF and body mass index,[16] blood hormonal levels,[17] varicocele,[18] and cryptorchidism.[19] However, so far, no study has systematically evaluated the possible associations between sDF and male genital tract abnormalities or prostatitis-like symptoms. Useful information in the assessment of male genital tract abnormalities are provided by color-Doppler ultrasound (CDUS), which is increasingly used in the evaluation of the infertile men.[2] With such a tool, signs of testicular dysgenesis, epididymal alterations, vascular features, and abnormalities of the prostate-vesicular region, including signs suggestive of sub-obstruction and inflammation, can be detected.[2] Concerning assessment of prostatitis-like symptoms, at present, the National Institutes of Health-Chronic Prostatitis Symptom Index (NIH-CPSI) is considered the gold standard instrument,[20] and symptom severity is classified according to Nickel's criteria.[21]

The aim of this study is to investigate the relationship between the percentage of sDF in $PI^{brighter}$ and PI^{dimmer} populations and the male clinical characteristics, focusing on CDUS features of the male genital tract and prostatitis-like symptoms as assessed by NIH-CPSI.

MATERIALS AND METHODS

Patients

A consecutive series of 160 male partners of infertile couples, without genetic abnormalities (karyotype abnormalities, chromosome Y micro-deletions, CFTR mutations, absence of at least one *vas deferens* and/or one seminal vesicle), attending our outpatients clinic from January 2010 to March 2014 for couple infertility, were included in the study. Couple infertility was defined as the inability of a sexually active couple to achieve pregnancy despite unprotected intercourse for a period >12 months, according to the World Health Organization (WHO).[22] Since the characteristics of female partners of the couples were unknown in most cases, our study population may contain both fertile, infertile, and subfertile subjects.

All patients were evaluated before beginning any treatment. The data reported in this study were collected during routine clinical procedures according to a "Day Service" standard protocol for males of infertile couples, encoded by PACC L-99 (D/903/110 Azienda Ospedaliera-Universitaria Careggi [AOUC], Florence, Italy) and approved by the Regional Health Care Service (§ DGRT n. 1045; § DGRT n. 722; § DGRT n. 867), as previously described.[23] In line with the PACC L-99 protocol, all patients underwent, within the same day, the following routine procedures: (i) medical history assessment, including screening of prostatitis-like symptoms (see below); (ii) a complete andrological and physical examination, including measurement of blood pressure, height, weight and waist circumference; (iii) hormonal assessment; (iv) scrotal and transrectal CDUS evaluation performed before and after ejaculation; (v) semen analysis including evaluation of sDF. In addition, at the time of the first visit, all patients gave their written informed consent to have their clinical records included in a dedicated database and they were aware that their data, after having been made anonymous, would be used for clinical research purposes.

Color-Doppler ultrasonography (CDUS)

All patients underwent scrotal and transrectal CDUS,[2] performed before and after ejaculation, during the same CDUS session using the ultrasonographic console Hitachi H21 (Hitachi Medical System, Tokyo, Japan).

Prostate and seminal vesicles were studied by scanning the organs at 5 mm intervals in various longitudinal, transverse and oblique scans, according to previous studies,[23] using a transrectal biplanar probe (linear transducer U533L 7.5 MHz; convex transducer U533C 6.5 MHz), which is more sensitive in the detection of prostatic features, and an "end-fire" probe (V53W 6.5 MHz, field of view 50°–200°) to better investigate seminal vesicles.[24] Prostate volume was measured using the planimetric method, as previously reported.[23] Prostate and seminal vesicle CDUS features were defined as previously reported.[2] In particular, prostate echogenicity and hyperemia were defined according to previous studies.[2] Prostate vascularization and arterial prostatic peak systolic velocity were evaluated before ejaculation, in order to avoid postejaculatory changes in the vascular flow pattern, as previously reported.[2] Seminal vesicle volume was calculated using the "ellipsoid/prolate spheroid" formula.[24] Total volume of seminal vesicles was calculated by the sum of the volumes of the right and left seminal vesicles.[24] Seminal vesicle echo-texture features were defined according to previous studies.[2] Ejaculatory duct CDUS characteristics were evaluated after ejaculation, in order to better emphasize indirect CDUS signs of partial or complete obstruction.

Scrotal CDUS was performed systematically in various longitudinal, transverse and oblique scans using a 7.5 MHz high-frequency linear probe (L54M 6–13 MHz). Testicular, epididymal, deferential and venous plexus CDUS features were defined as previously reported.[23] In particular, the mean testicular volume, as well as the mean size of epididymal heads and tails, proximal vas deferences and deferential ampullas, refer to the mean value of the respective parameters evaluated in the right and left organs. Testicular and epididymal inhomogeneity or hypoechogenicity were defined according to previous studies, as reviewed in Lotti and Maggi.[2] Epididymal and vas deferens CDUS characteristics were evaluated after ejaculation, to better emphasize indirect CDUS signs of partial or complete obstruction.

Semen analysis and hormonal evaluation

During the same ultrasound session, all patients underwent semen analysis, performed according to the WHO criteria.[25] Semen samples were obtained by masturbation following a recommended period of 2–7 days of sexual abstinence.[25] Sperm morphology, motility and viability were assessed using optical microscopy by scoring at least 100 spermatozoa for each parameter. Blood samples were drawn in the morning, after an overnight fast, for determination of total testosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by electrochemiluminescent method (Modular Roche, Milan, Italy).

Evaluation of sperm DNA fragmentation (sDF)

sDF was evaluated by the TUNEL/PI assay.[11] To allow the evaluation of sDF, only semen samples showing at least 1×10^6 sperm/ejaculate were included. After liquefaction (30 min following collection), spermatozoa were washed twice with HTF medium, fixed by 500 µl of 4% paraformaldehyde in PBS, pH 7.4, for 30 min at room temperature (RT). Sperm cells were centrifuged at 500 ×*g* for 10 min and washed twice with 200 µl of PBS with 1% bovine serum albumin (BSA). Then, spermatozoa were permeabilized with 0.1% Triton X-100 in 100 ml of 0.1% sodium citrate for 4 min in ice. After washed twice, the labeling reaction was performed by incubating spermatozoa in 50 µl of labeling solution (supplied with the *In Situ* Cell Death Detection Kit, fluorescein, Roche Diagnostics, Milan, Italy), containing the TdT enzyme, for 1 h at 37°C in the dark. Finally, samples were washed twice, resuspended in 500 µl of PBS, stained with 10 µl of PI (30 µg ml^{-1} in PBS), and incubated in the dark for 15 min at RT. For each test sample, a negative control (omitting TdT) and a

sample for fluorescence compensation (labeled only with TUNEL) were prepared. Green fluorescence (of nucleotide conjugated with fluorescein) and red fluorescence (of PI) were revealed, respectively, by the FL-1 (515–555 nm wavelength band) and the FL-2 (563–607 nm wavelength band) detectors of a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). For each sample, 10 000 events were recorded within the characteristic flame shaped region in the FSC/SSC dot plot which excludes debris and large cells, including leukocytes and germ cells (**Supplementary Figure 1a**). Since such a region also contains anucleated elements (apoptotic bodies, **Supplementary Figure 1b**) was not stained by PI, the percentage of sDF was calculated considering only the PI-positive events of the region. As mentioned above, nuclear staining with PI also unveils the occurrence of two sperm populations, $PI^{brighter}$ and PI^{dimmer}, based on a different intensity of such staining (**Supplementary Figure 1c**). Hence, we determined sDF within $PI^{brighter}$, PI^{dimmer} and total sperm populations.

Screening of prostate-related symptoms

Patients were asked to complete the Italian translation of the National Institutes of Health-Chronic Prostatitis Symptom Index (NIH-CPSI),[20] a brief self-reported questionnaire for the screening of prostatitis-like symptoms, which provides scores for pain, voiding symptoms and quality of life. NIH-CPSI total score was calculated as the sum of the scores of these domains. Patients were classified as having "prostatitis-like symptoms" if they complained of perineal and/or ejaculatory pain or discomfort and their pain index score was ≥4, according to Nickel *et al*.[21] Symptoms were classified as "mild" for a pain index score of 4–7 and "moderate-severe" for a pain index score of ≥8, according to Nickel's criteria.[21] This symptom scoring system was not used as a diagnostic tool, but rather to estimate the symptom's severity.[23,26]

Data analysis

All statistical analyses were performed using IBM SPSS Statistics (Statistical Package for the Social Sciences, Chicago, USA) for Windows 20.0. Kolmogorov–Smirnov test was used to test the distribution of parameters. Data were expressed as mean ± standard deviation (s.d.) when normally distributed, as medians (quartiles) for parameters with nonnormal distribution, and as percentages when categorical. Correlations were assessed using Spearman's or Pearson's method whenever appropriate. Unpaired two-sided Student's *t*-test was used for comparisons of means of normally distributed parameters; when distribution could be normalized through logarithmic transformation, as in the case of $PI^{brighter}$ and PI^{dimmer} sDF, LH, FSH or total seminal vesicles volume, the same test was applied to the logarithmically transformed data. In all other cases, Mann–Whitney U-test was used for comparisons between groups. Relative risk and 95% confidence interval were calculated for the association of categorical parameters, and Chi-squared test was used for comparisons. Step-wise multiple linear regression, logistic binary regression, or analysis of covariates (ANCOVA) with Bonferroni correction were applied for multivariate analyses whenever appropriate. Differences in percentages of total, $PI^{brighter}$ or PI^{dimmer} sDF have been reported in unadjusted and adjusted comparisons among groups, and respectively expressed as "d" (difference) and "adj. d" (adjusted difference). For graphical purposes, sDF in PI^{dimmer} and $PI^{brighter}$ populations in the figures are reported as quartiles.

RESULTS

The main clinical and laboratory parameters and the CDUS characteristics of the patients are shown in **Tables 1** and **2**, respectively, reporting the number and the prevalence of subjects with the evaluated features and the average values of the different parameters. In particular, 3.1% of the subjects studied had a history of cryptorchidism, 25% had a history of genito-urinary diseases, 37.5% and 25% showed the presence of clinical varicocele or CDUS-detected severe varicocele, respectively (**Tables 1** and **2**). Overall and "moderate to severe" prostatitis-like symptoms were detected in 8.2% and 4.5% of the patients studied, respectively (**Table 1**).

The average percentage median values of total, PI^{dimmer} and $PI^{brighter}$ sDF in our patients were respectively: 36.3 (11.6–95.7), 13.8 (8.1–23.7) and 18.9 (13.1–27.7). Age was positively associated with sDF measured in the three different populations ($r = 0.22$, $P < 0.01$ for total, $r = 0.20$, $P < 0.02$ for PI^{dimmer} and $r = 0.18$, $P < 0.05$ for $PI^{brighter}$). Hence, all the subsequent associations with clinical and CDUS characteristics of the male genital tract were adjusted for age (**Tables 3** and **4**, **Supplementary Table 1**, Model 1). In addition, since waist circumference, testosterone levels, and smoking habit have been reported to affect semen quality and/or sDF,[16,17,27] data have also been adjusted for these possible confounders (**Tables 3** and **4**, **Supplementary Table 1**, Model 2). Finally, since the duration of sexual abstinence (range 2–7 days) was significantly associated with PI^{dimmer} sDF ($r = 0.18$, $P = 0.03$), the former parameter was included as a further covariate in Model 2.

As reported in **Table 3**, although at univariate analysis PI^{dimmer} sDF was associated with several clinical and CDUS features of both scrotal and prostate-vesicular regions, after adjustment for confounders (Models 1 and 2) significant correlations were confirmed only with scrotal characteristics. In particular, lower mean testicular volume and higher LH and FSH levels were associated with a higher PI^{dimmer} sDF (**Table 3**). In addition, subjects with a positive history of cryptorchidism, testicular inhomogeneity or hypoechogenicity, or epididymal tail inhomogeneity at CDUS showed higher PI^{dimmer} sDF when compared with the rest of the sample (**Table 3**). Most of the relevant associations reported in **Table 3** are graphically represented in **Figure 1** showing the correlations between PI^{dimmer} sDF and quartiles of mean testicular volume (**Figure 1a**), FSH (**Figure 1b**)

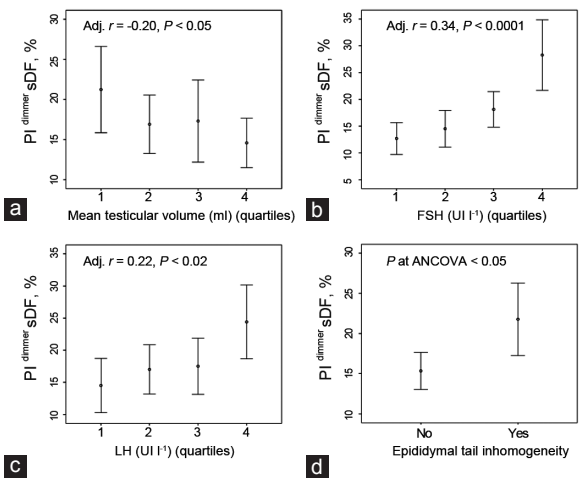

Figure 1: Main significant scrotal-related ultrasound and clinical parameters in relation to PI^{dimmer} sDF. (**a–c**) Stepwise relationships among PI^{dimmer} sDF and mean testis ultrasound volume, FSH and LH levels. The statistical analyses were performed using the mean testis volume, FSH or LH levels as continuous variables, although grouped here in quartiles for graphical purposes. Adjusted *r* (*Adj. r*) and *P* values derived from **Table 3**, Model 2 (linear regression analysis) are reported. (**d**) Difference in PI^{dimmer} sDF between subjects with or without epididymis tail inhomogeneity. *P* values derived from **Table 3**, Model 2 (ANCOVA) are reported.

Table 1: Clinical characteristics of the whole sample

	n (%)	*Mean±s.d. or median (quartiles)*	*Range (minimum, maximum)*
Clinical and laboratory parameters			
Age (years)		36.3±9.2	19.0–58.0
Waist circumference (cm)		100.8±20.1	75.6–162.0
Mean systolic blood pressure (mmHg)		123.8±10.9	100.0–160.0
Mean diastolic blood pressure (mmHg)		79.9±8.0	60.0–105.0
Current smoking	37 (23.1)		
Mean testicular volume (Prader, ml)		20.0±4.5	9.0–32.5
History of cryptorchidism	5 (3.1)		
History of genito-urinary diseases	40 (25.0)		
LH (UI l^{-1})		3.7 (2.6–5.1)	0.7–13.7
FSH (UI l^{-1})		4.3 (3.0–6.3)	1.0–17.6
Total testosterone (nmol l^{-1})		15.3±6.0	5.1–33.4
NIH-CPSI total score (0–43)		4.9±6.3	0.0–34.0
Pain domain (0–21)		2.1±3.5	0.0–16.0
Void domain (0–10)		1.1±1.7	0.0–10.0
Quality of life impact (0–12)		1.7±2.2	0.7–13.7
Prostatitis-like symptoms	13 (8.2)		
Prostatitis-like symptoms "moderate-severe"	8 (4.5)		
Semen parameters			
Sexual abstinence (days)		4.0±1.7	2.0–7.0
pH		7.6±0.2	7.2–8.6
Semen viscosity, positive subjects	27 (16.9)		
Semen volume (ml)		3.5±1.6	0.5–7.7
Sperm concentration, $\times 10^6$ ml^{-1}		24.5 (10.0–59.8)	0.3–272.0
Sperm progressive motility, %		40.9±19.5	0.0–82.0
Sperm morphology, percentage of normal forms		5.0 (2.0–8.0)	0.0–25.0
Leukocitospermia	14 (8.8)		
History of infertility			
Duration of infertility (years)		1.5 (1.0–3.0)	1.0–10.0
Primary infertility	125 (77.9)		
Secondary infertility	35 (22.1)		
Female partner age (years)		35.5±5.0	23.0–43.0

Data are expressed as mean±s.d. or as median (quartiles) when appropriate, or as percentages when categorical (in brackets), indicating the number (*n*) of subjects positive for each finding. Range of values for the 160 patients included in the study are also shown. FSH: follicle stimulating hormone; LH: luteinizing hormone; NIH-CPSI: National Institutes of Health-Chronic Prostatitis Symptom Index; s.d.: standard deviation

and LH (**Figure 1c**) levels, and occurrence of epididymal tail inhomogeneity (**Figure 1d**).

After adjustment for confounders (**Table 4**, Models 1 and 2), $PI^{brighter}$ sDF was significantly associated with prostate-related symptoms and signs. In particular, higher NIH-CPSI total or subdomains scores were associated with higher $PI^{brighter}$ sDF (**Table 4**). Subjects with overall (n = 13, 8.2%) "moderate to severe" (n = 8, 4.5%) prostatitis-like symptoms showed higher $PI^{brighter}$ sDF when compared to the rest of the sample (**Table 4**). In addition, subjects with prostate macro-calcifications (n = 37, representing 23.1% of the cases; **Table 2**), severe inhomogeneous texture (n = 7, 4.4%; **Table 2**) or hyperemia (n = 25, 15.6%; **Table 2**) at CDUS had higher $PI^{brighter}$ sDF when compared with those without these symptoms. Finally, detection of a higher mean arterial peak systolic velocity of the prostate was associated with higher $PI^{brighter}$ sDF levels (**Table 4**). Most of the relevant associations reported in **Table 4** are graphically represented in **Figure 2** showing $PI^{brighter}$ stepwise correlations with quartiles of NIH-CPSI total score (**Figure 2a**), prostatic arterial peak systolic velocity (**Figure 2b**), occurrence of prostate hyperemia (**Figure 2c**) or macro-calcifications (**Figure 2d**). For total sDF levels, correlations with clinical and CDUS features reflect those observed for both PI^{dimmer} and $PI^{brighter}$ sDF (**Supplementary Table 1**).

DISCUSSION

The association between DNA fragmentation in human spermatozoa and diminished reproductive outcomes highlights the clinical relevance of this semen parameter. Despite this fact, present knowledge about the endogenous origin of sDF and its relation to clinical features is rudimentary. Here, we report evidence that sDF may originate both in the testicles and during sperm transit in the genital tract.

In the present study, sDF was evaluated in two cytometric sperm populations, named $PI^{brigter}$ and PI^{dimmer},[8] which presented different biological characteristics[8,12,13] and, likely, different clinical relevance. The fact that sDF in $PI^{brighter}$ and PI^{dimmer} populations are associated with distinct characteristics of the patients in question suggests that their damage originates in different sites of the male genital tract (**Figure 3**). In particular, the association between PI^{dimmer} sDF and several clinical and ultrasound parameters suggestive of testicular damage, such as testicular inhomogeneity and hypoechogenicity, as well as higher FSH levels, indicates that this population, entirely formed by DNA fragmented and dead sperm,[8,12,13] results from an impairment of testicular function and/or alterations of spermatogenesis. This concept is reinforced by the previously reported positive correlation between PI^{dimmer} sDF and levels of apoptotic bodies,[14] round anucleated elements considered to be markers of excessive testicular apoptosis[10] and related

Table 2: Color-Doppler ultrasound characteristics of the whole sample

	n (%)	*Mean±s.d. or median (quartiles)*	*Range (minimum, maximum)*
Colour-Doppler ultrasound parameters			
Testis			
Mean testicular volume[Δ] (ml)		15.9±4.3	7.0–29.8
Testicular inhomogeneity[Δ]	41 (25.6)		
Testicular hypoechogenicity	18 (11.3)		
Testicular microcalcifications	11 (6.9)		
Severe varicocele[##]	40 (25.0)		
Epididymis			
Mean size of the heads (mm)		9.6±1.6	5.2–15.6
Mean size of the tails (mm)		4.8±1.2	2.3–8.0
Inhomogeneous tail[♦]	55 (34.4)		
Hypoechoic tail	19 (11.9)		
Hyperechoic tail	20 (12.5)		
Coarse tail calcifications	7 (4.4)		
Hyperemia	6 (3.8)		
Vas deferens			
Mean size of the proximal vas deferens (mm)		4.0±0.7	1.8–6.3
Mean size of the deferential ampullas (mm)		4.9±1.0	2.6–8.0
Prostate			
Prostate volume (ml)		23.1±8.0	11.5–52.2
Prostate macro-calcifications[ι]	37 (23.1)		
Major calcification diameter (mm)		8.2±5.4	0.0–24.0
Dilated ejaculatory duct	13 (8.1)		
Severe inhomogeneous texture[ǂ]	7 (4.4)		
Diffuse hypoechoic texture	14 (8.8)		
Prostatic hyperemia (before ejaculation)^	25 (15.6)		
Mean arterial peak systolic velocity^ (cm s^{-1})		9.1±3.3	5.0–19.0
Mean prostatic venous plexus (mm)		4.8±1.5	1.0–10.0
Seminal vesicles			
Total volume before ejaculation (ml)°		8.1 (4.9–16.8)	1.5–46.8
Total volume after ejaculation (ml)°		5.4 (3.2–8.7)	0.8–41.5
Inhomogeneity before ejaculation	53 (33.1)		
Inhomogeneity after ejaculation	48 (30)		
Areas of endocapsulation before ejaculation	34 (21.3)		
Areas of endocapsulation after ejaculation	21 (13.1)		
Wall thickening and septa	11 (6.9)		

[Δ]According to Lotti *et al.*[24]; [##]Severe echographic-defined varicocele, according to Lotti and Maggi[2]; [♦]According to Lotti and Maggi[2]; [ι]Calcifications with size >3 mm (according to Lotti *et al.*[23]); [ǂ]According to Lotti *et al.*[23]; [^]According to Lotti *et al.*[24]; °Calculated using the "ellipsoid/prolate spheroid" formula, according to Lotti *et al.*[24] Data are expressed as mean±s.d. or as median (quartiles) when appropriate, or as percentages when categorical (in brackets), indicating the number (*n*) of subjects positive for each finding. Range of values for the 160 patients included in the study are also shown. Ultrasound characteristics and abnormalities have been evaluated according to Lotti and Maggi[2] (see methods). The mean testicular volume, as well as the mean size of epididymal heads and tails, proximal vas deferens and deferential ampullas, refer to the mean value of the parameters evaluated in the right and left organs. Total volume of seminal vesicles refers to the sum of the volumes of the right and left seminal vesicles. CDUS: color-Doppler ultrasound; s.d.: standard deviation

to alterations of spermatogenesis.[28] The presence of apoptotic bodies of testicular origin in the semen supports the idea of the occurrence of abortive apoptosis in the testicles, as originally hypothesized by Sakkas *et al.*[29] Indeed, according to this theory, apoptosis, which initiates in the testicles, fails to complete, and sperm with apoptotic traits (including DNA fragmentation) are released from the testicles and found in the ejaculate. Overall, the association of PI^{dimmer} sDF with signs of testicular impairment (present study), the correlation with apoptotic testicular bodies,[14] and the demonstration that a high percentage of sperm in this population shows active caspase activity,[14] suggest a testicular origin of this sperm population as a result of abortive apoptosis. The occurrence of a positive association with inhomogeneity of the epididymal tail suggests that part of DNA fragmentation in the PI^{dimmer} sperm population may also originate at this level (**Figure 3**).

In contrast with PI^{dimmer}, sDF in the $PI^{brighter}$ population does not show any significant association with testicular features or FSH levels. Rather, it shows significant associations with prostatitis-like symptoms and several prostate CDUS abnormalities suggestive of inflammation.[2] This result, together with the absence of significant associations with signs of testicular or epididymal damage, suggests that DNA fragmentation in $PI^{brighter}$ spermatozoa largely originates downstream of the epididymis. Although our study does not allow us to establish exactly at which level, after sperm release from the epididymis, the damage occurs, the presence of a significant association between sDF in $PI^{brighter}$ population and signs or symptoms suggestive of prostate inflammation (CDUS prostate abnormalities, higher NIH-CPSI score and higher frequency of prostatitis-like symptoms) indicates that the transit of spermatozoa through the prostate and/or their contact with prostatic fluid during the ejaculation process may play a role. The fact that a certain level of $PI^{brighter}$ sDF is always present in semen samples implies that $PI^{brighter}$ sDF occurs even in the absence of clear symptoms or signs of prostatic inflammation. We here demonstrate that higher levels of $PI^{brighter}$ sDF are present in

Table 3: Significant associations between PI^{dimmer} sDF and main clinical and CDUS features of the male genital tract

	Unadjusted analyses	*Adjusted analyses*	
		*Model 1**	*Model 2***
Clinical and laboratory parameters			
Mean testicular volume (Prader, ml)	r=–0.19, P<0.02	r=–0.19, P<0.02	r=–0.21, P<0.02
History of cryptorchidism	d=17.2±6.0, P<0.02	d=15.5±5.9, P=0.02	d=16.7±6.1, P<0.02
Log_{10} [LH]	r=0.25, P<0.005	r=0.29, P<0.0001	r=0.22, P<0.02
Log_{10} [FSH]	r=0.41, P<0.0001	r=0.37, P<0.0001	r=0.34, P<0.0001
CDUS parameters			
Testis			
Mean testis volume (ml)	r=–0.19, P<0.02	r=–0.19, P<0.02	r=–0.20, P=0.02
Testicular inhomogeneity	d=6.4±2.5, P<0.05	d=5.1±2.5, P<0.05	d=6.3±2.9, P=0.02
Testicular hypoechogenicity	d=8.2±3.5, P<0.05	d=8.0±3.4, P<0.05	d=11.6±3.7, P<0.02
Epididymis			
Inhomogeneous tail	d=6.4±2.3, P<0.01	d=5.5±2.3, P<0.05	d=5.3±2.7, P<0.05
Prostate			
Prostate macro-calcifications	d=6.9±2.5, P<0.02	NS	-
Major calcification diameter, mm	r=0.216, P<0.01	NS	-
Prostatic hyperemia (before ejaculation)	d=7.8±3.0, P<0.05	NS	-
Seminal vesicles			
Total volume before ejaculation (ml)	r=0.18, P<0.05	r=0.23, P=0.005	NS
Total volume after ejaculation (ml)	r=0.16, P<0.05	r=0.21, P<0.01	NS

*Model 1: adjusted for age; **Model 2: adjusted for Model 1 + waist circumference, total testosterone, current smoking (no/yes) and sexual abstinence. All the clinical and CDUS characteristics reported in **Tables 1** and **2** were evaluated, whereas only parameters significantly associated with PI^{dimmer} sDF have been reported. CDUS features were defined as reported in Methods and **Table 2**. Unadjusted analyses were performed using: Spearman's or Pearson's method for linear independent variables, with data expressed as r and P value, or unpaired two-sided Student's t-test or Mann–Whitney U-test, whenever appropriate, for dummy independent variables, reporting the difference (d) between groups and related P value. The multivariate analyses were performed using linear regression analysis for linear independent variables, with adjusted data expressed as adjusted r and P value, or ANCOVA for dummy independent variables, reporting the adjusted difference between groups and related P value. NS: not significant; ANCOVA: analysis of covariates; CDUS: color-Doppler ultrasound; PI: propidium iodide; FSH: follicle stimulating hormone; LH: luteinizing hormone; sDF: sperm DNA fragmentation

Table 4: Significant associations between $PI^{brighter}$ DNA fragmentation and main clinical and CDUS features of the male genital tract

	Unadjusted analyses	*Adjusted analyses*	
		*Model 1**	*Model 2***
NIH-CPSI total score (0–43)	r=0.24, P<0.05	r=0.18, P<0.05	r=0.20, P<0.05
Pain domain (0–21)	r=0.26, P=0.02	r=0.24, P<0.005	r=0.18, P<0.05
Void domain (0–10)	r=0.18, P<0.05	NS	-
Quality of life impact (0–12)	r=0.19, P<0.02	r=0.163, P<0.05	r=0.23, P<0.05
Prostatitis-like symptoms#	d=14.3±4.0, P=0.002	d=14.1±4.0, P=0.002	d=13.2±4.7, P<0.05
Prostatitis-like symptoms "moderate-severe"#	d=19.9±5.1, P=0.002	d=20.0±5.1, P=0.001	d=18.6±6.2, P<0.02
Prostate			
Prostate macro-calcifications	d=3.1±2.4, P<0.05	d=3.0±2.9, P<0.05	d=5.7±3.3, P<0.05
Severe inhomogeneous texture	d=7.9±3.3, P=0.005	d=7.8±3.2, P<0.05	d=6.8±3.6, P<0.05
Prostatic hyperemia (before ejaculation)	d=7.3±3.3, P<0.005	d=7.2±3.2, P<0.02	d=7.6±3.5, P<0.05
Mean arterial peak systolic velocity (cm s^{-1})	r=0.24, P<0.05	r=0.20, P<0.05	r=0.25, P=0.02

#Prostatitis-like symptoms: "perineal and/or ejaculatory pain or discomfort and NIH-CPSI pain subdomain score ≥4," according to Nickel *et al*.[21] Symptoms were classified as "moderate-severe" for a pain index score of ≥8, according to Nickel's criteria[21]; *Model 1: adjusted for age; **Model 2: adjusted for Model 1 + waist circumference, total testosterone, current smoking (no/yes) and sexual abstinence. All the clinical and CDUS characteristics reported in **Tables 1** and **2** were evaluated, whereas only parameters significantly associated with $PI^{brighter}$ sDF have been reported. CDUS features were defined as reported in Methods and **Table 2**. Statistical analyses were performed as reported in **Table 3**

subjects with a higher frequency of signs and symptoms suggestive of prostatic inflammation; however, we cannot exclude that damage in $PI^{brighter}$ sperm may also originate in other parts of the genital tract.

Overall, our results indicate that DNA fragmentation of $PI^{brighter}$ sperm occurs much later in time respect to that of the PI^{dimmer} population. In addition, the relationship with inflammatory symptoms of the distal genital tract suggests that oxidative stress may be involved in inducing sDF in the $PI^{brighter}$ population, in agreement with recent findings of our group showing the concomitant presence of DNA fragmentation and oxidative DNA damage only in $PI^{brighter}$ spermatozoa.[13] The fact that a DNA fragmented $PI^{brighter}$ sperm is a result of a recent insult with respect to ejaculation and likely due to oxidative stress could explain why sDF in this population is unrelated to semen quality,[8] as this type of insult may affect any spermatozoon, regardless of its morphology and motility.

Previous attempts to correlate sDF levels and hormonal status of the patients gave rise to contrasting results. Indeed, most of these studies found a positive association with FSH,[17] while others did not.[30] A similar situation occurred for LH and testosterone levels.[17,30] A comparison with our study is possible only for sDF in the total sperm population, and, as such, our study agrees with those which found a positive association with FSH but not with LH or testosterone levels (**Supplementary Table 1**), suggesting that circulating androgens are not involved in generating or preventing sperm DNA damage.

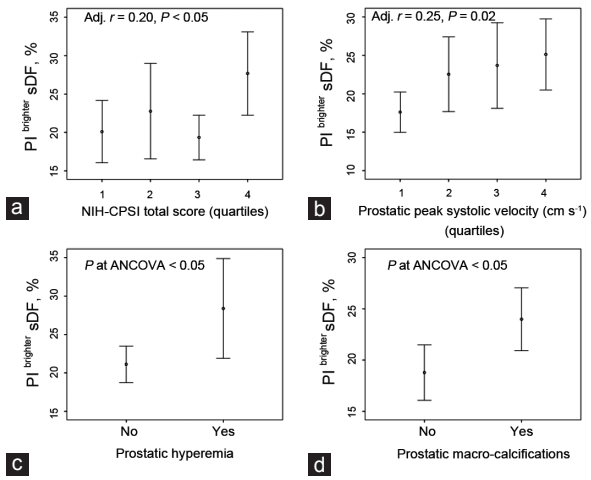

Figure 2: Main significant prostate-related ultrasound and clinical parameters in relation to $PI^{brighter}$ sDF. (**a** and **b**) Stepwise relationship among $PI^{brighter}$ sDF and prostate-related symptoms (NIH-CPSI total score) (**a**) or prostate arterial peak systolic velocity (**b**). The statistical analyses were performed using the NIH-CPSI total score or prostate arterial peak systolic velocity as continuous variables, although grouped here in quartiles for graphical purposes. Adjusted *r* (*Adj. r*) and *P* values derived from **Table 4**, Model 2 (linear regression analysis) are reported. (**c** and **d**) Difference in $PI^{brighter}$ sDF between subjects with or without hyperemia (**c**) or prostate macro-calcifications (**d**). *P* values derived from **Table 4**, Model 2 (ANCOVA) are reported.

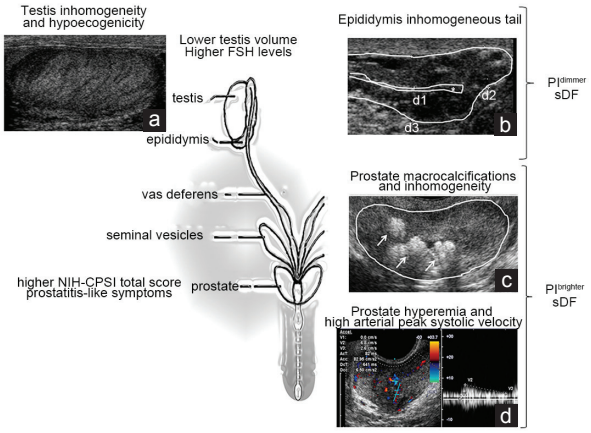

Figure 3: Schematic representation of the possible sites of origin of sDF in PI^{dimmer} and $PI^{brighter}$ populations. The parameters that correlate with PI^{dimmer} and $PI^{brighter}$ sDF are summarized in the figure. (**a**) Testis inhomogeneity and hypoechogenicity. (**b**) Epididymis inhomogeneous tail echo-texture. (**c**) Prostate inhomogeneity and macro-calcifications (arrows). (**d**) Prostate hyperaemia and high arterial peak systolic velocity.[23] Representative CDUS images are shown.

We found a strong correlation between sDF and patient age, in agreement with a recent meta-analysis.[31] Aging may cause degenerative alterations in the germinal epithelium affecting sperm quality.[32] The fact that correlation with age was present for both PI^{dimmer} and $PI^{brighter}$ sDF, suggests that age affects DNA sperm status independently from the site of origin and the mechanism generating the damage. Although several studies reported increased sDF in varicocele patients when compared to fertile subjects,[33] when men with idiopathic infertility with and without varicocele were compared, results were contradictory.[33] In our study, no relationship between sDF, either in total, $PI^{brighter}$ or PI^{dimmer} populations and detection of varicocele was observed, suggesting that the occurrence of varicocele does not worsen sperm DNA damage in subfertile men.

This study has some limitations. First, the present results are derived from patients consulting an Italian Andrology Clinic for couple infertility and could have different characteristics from the general male population or those males consulting general practitioners for reasons other than couple infertility. Furthermore, due to the cross-sectional nature of our study, neither a causality hypothesis nor mechanistic models can be drawn. In addition, the occurrence of CDUS abnormalities is suggestive but not necessarily indicative of pathology. Finally, another limitation is the low number of subjects with cryptorchidism, thus the higher PI^{dimmer} sDF in these subjects should be confirmed in further studies.

CONCLUSIONS

The results of our study suggest that sDF in the two cytometric sperm populations PI^{dimmer} and $PI^{brighter}$ may originate in different sites of the male genital tract. In particular, $PI^{brighter}$ damage, which is unrelated to semen quality, appears to occur mostly following contact with the prostatic fluid, increasing especially when inflammation is present. Future confirmation of these results may lead to new strategies for therapeutic interventions. Clarification of the relationship between sDF and clinical features might help clinicians to select cases where evaluation of the parameter may be of help in the diagnosis.

AUTHOR CONTRIBUTIONS

FL provided the conception of design of the study, drafted the article and interpreted the data, performed patients recruitment, arranged medical history and physical examination assessment, performed the color-Doppler ultrasound evaluation, data collection and analyses; LT took charge of evaluation of sperm DNA fragmentation, data collection and analyses; SM carried out flow cytometry analysis; EM and PV performed patient recruitment and data collection; Mo.Mu performed flow cytometric data interpretation and analysis; Ma.Ma and EB provided conception of the design of the study, drafted the article and interpreted the data and results. All the authors made substantial contributions in critically revising the article.

COMPETING INTERESTS

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Supplementary information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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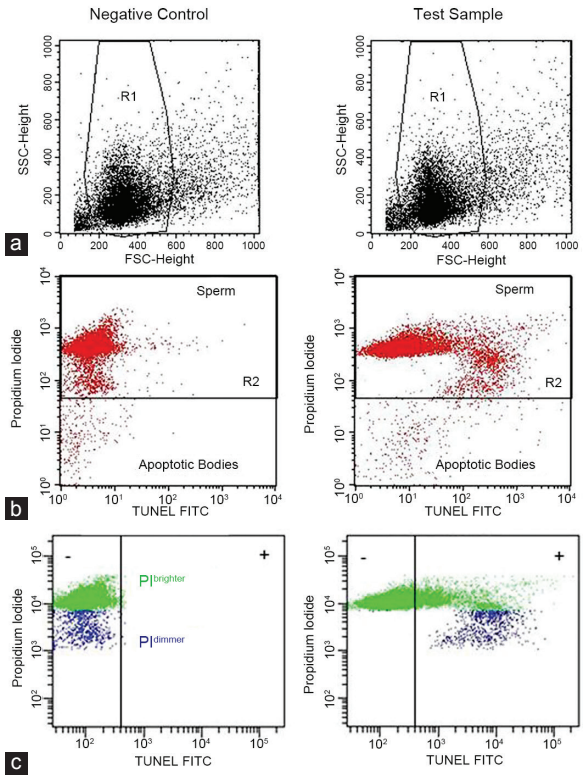

Supplementary Figure 1: Schematic representation of the cytofluorimetric analysis of sDF in $PI^{brighter}$ and PI^{dimmer} sperm by TUNEL/PI. (**a**) Typical flame shaped region (R1) in SSC/FSC scatter plot containing sperm and apoptotic bodies. (**b**) Within R1, the R2 region includes PI positive events (i.e., sperm) and excludes PI negative apoptotic bodies. (**c**) Within the R2 region the two sperm populations ($PI^{brighter}$ in green and PI^{dimmer} in blue) can be clearly distinguished. Left panels: negative control for TUNEL (omitting TdT); right panels: test samples.

Supplementary Table 1: Significant associations between total sperm DNA fragmentation and main clinical and CDUS features of the male genital tract

Clinical and laboratory parameters	*Unadjusted analyses*	*Adjusted analyses*	
		*Model 1**	*Model 2***
Mean testis volume (Prader, ml)	r=–0.173, P<0.05	r=–0.188, P=0.02	r=–0.185, P<0.05
History of cryptorchidism	d=18.9±8.3, P<0.05	d=16.8±8.2, P<0.05	d=17.5±8.4, P<0.05
Log_{10} [FSH]	r=0.256, P=0.002	r=0.207, P<0.02	r=0.188, P<0.05
CDUS parameters			
Testis			
Mean testis volume (ml)	r=–0.170, P<0.05	r=–0.188, P=0.02	r=–0.180, P<0.05
Testicular inhomogeneity	d=7.4±3.4, P<0.05	NS	-
Testicular hypoechogenicity	d=9.4±4.7, P<0.05	d=9.1±4.6, P<0.05	d=12.4±5.1, P=0.05
Prostate			
Prostate macro-calcifications	d=10.0±3.4, P<0.005	d=7.8±3.7, P<0.05	d=10.9±4.2, P<0.02
Major calcification diameter, mm	r=0.295, P<0.0001	r=0.168, P<0.05	r=0.257, P=0.02
Prostatic hyperemia (before ejaculation)	d=15.1±4.0, P<0.0001	d=13.3±4.1, P=0.002	d=13.7±4.5, P<0.005
Mean arterial peak systolic velocity (cm s^{-1})	r=0.220, P<0.01	r=0.201, P<0.02	r=0.314, P<0.005
Seminal vesicles			
Total volume before ejaculation (ml)	r=0.192, P<0.02	r=0.218, P<0.01	r=0.196, P<0.05
Total volume after ejaculation (ml)	r=0.210, P<0.02	r=0.239, P<0.005	r=0.206, P<0.05
Inhomogeneity before ejaculation	d=8.5±3.2, P<0.01	d=8.0±3.1, P<0.02	d=10.3±3.6, P<0.005
Inhomogeneity after ejaculation	d=9.1±3.7, P<0.01	d=8.9±3.2, P<0.01	d=10.7±3.6, P<0.005
Areas of endocapsulation before ejaculation	d=10.3±4.9, P<0.05	d=10.8±4.0, P<0.01	d=11.4±4.5, P<0.02

*Model 1: adjusted for age; **Model 2: adjusted for Model 1 + waistline, total testosterone, current smoking (no/yes) and sexual abstinence. All the clinical and CDUS characteristics reported in **Table 1** and **2** have been evaluated, whereas only parameters significantly associated with total sDF have been reported. CDUS features have been defined as reported in Methods and **Table 2**. Statistical analyses have been performed as reported in **Table 3**. CDUS: color-Doppler ultrasound; FSH: follicle stimulating hormone