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

Francesco Lotti*, Lara Tamburrino*, Sara Marchianii, Elisa Maseroli, Pasquale Vitale, Gianni Forti, Monica Muratori, Mario Maggi, Elisabetta Baldi

We investigated whether DNA fragmentation in two cytometric sperm populations (PI\textsuperscript{dimmer} and PI\textsuperscript{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\textsuperscript{dimmer} and PI\textsuperscript{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\textsuperscript{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\textsuperscript{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-calculations, 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\textsuperscript{dimmer} sperm, which is related to poor semen quality, mainly originates in the testicles, likely due to apoptosis. Conversely, DNA fragmentation in PI\textsuperscript{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.

Asian Journal of Andrology (2017) 19, 272–279; doi: 10.4103/1008-682X.174854; published online: 26 February 2016

Keywords: color-Doppler ultrasonography; DNA fragmentation; male infertility; male sex organs; spermatozoa

INTRODUCTION

Male infertility affects about 7% of all men. Despite many technical advances, its etiology is still unknown in half of the cases reported.\textsuperscript{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).\textsuperscript{3,4} sDF levels are higher in infertile than fertile men\textsuperscript{5} and only partially correlate with conventional sperm parameters\textsuperscript{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.\textsuperscript{7}

Recently, we set up a new method to evaluate sDF by the terminal deoxynucleotidyl transferase-mediated-dUTP nick end labeling (TUNEL) assay.\textsuperscript{4} This method, which we named TUNEL/PI, uses staining with propidium iodide (PI) to eliminate anucleated semen apoptotic bodies\textsuperscript{8,9} which interfere with cytometric analysis, allowing for more accurate measures.\textsuperscript{4,10} Based on PI staining, we identified two cytometric sperm populations, called PI\textsuperscript{brighter} and PI\textsuperscript{dimmer}, which differ in several biological characteristics. In particular, PI\textsuperscript{dimmer} population is entirely formed by DNA fragmented spermatozoa and shows negative correlations with semen quality.\textsuperscript{4} Recently, we demonstrated that PI\textsuperscript{dimmer} sperm are unviable\textsuperscript{12,13} and show signs of apoptosis.\textsuperscript{14} Conversely, PI\textsuperscript{brighter} population consists of a variable percentage of sperm with DNA fragmentation,\textsuperscript{4} is formed by both viable and unviable sperm,\textsuperscript{12} and shows signs of apoptosis and DNA oxidation.\textsuperscript{12} PI\textsuperscript{brighter} sDF is independent from semen quality,\textsuperscript{4} 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\textsuperscript{brighter} sDF is the fraction that best discriminates fertile and infertile men independently from semen quality.\textsuperscript{15} Hence, according to our studies, PI\textsuperscript{brighter} and PI\textsuperscript{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...
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, 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, blood hormonal levels, and cryptorchidism. 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. With such a tool, signs suggestive of sub-obstruction and inflammation, can be detected. 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, and symptom severity is classified according to Nickels criteria.

The aim of this study is to investigate the relationship between the percentage of sDF in P⁰ and P² 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).

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. 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, 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, 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 (V33W 6.5 MHz, field of view 50°–200°) to better investigate seminal vesicles. Prostate volume was measured using the planimetric method, as previously reported. Prostate and seminal vesicle CDUS features were defined as previously reported. In particular, prostate echogenicity and hyperemia were defined according to previous studies. Prostate vascularization and arterial and venous peak systolic velocity were evaluated before ejaculation, in order to avoid postejaculatory changes in the vascular flow pattern, as previously reported. Seminal vesicle volume was calculated using the “ellipsoid/prolate spheroid” formula. Total volume of seminal vesicles was calculated by the sum of the volumes of the right and left seminal vesicles. Seminal vesicle echo-texture features were defined according to previous studies.

Evaluation of sperm DNA fragmentation (sDF)
SDF was evaluated by the TUNEL/PI assay. To allow the evaluation of sDF, only semen samples showing at least 1 × 10⁶ 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 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, P favourable and P adverse, based on a different intensity of such staining (Supplementary Figure 1c). Hence, we determined sDF within P favourable, P adverse 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), 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. 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. This symptom scoring system was not used as a diagnostic tool, but rather to estimate the symptom's severity.

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 P favourable and P adverse 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, P favourable or P adverse 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 P adverse and P favourable 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 percent median values of total, P favourable and P adverse 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 P favourable and $r = 0.18, P < 0.05$ for P adverse). 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, 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 P adverse 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 P adverse 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 P adverse sDF (Table 3). In addition, subjects with a positive history of cryptorchidism, testicular inhomogeneity or hypoechogenicity, or epididymal tail inhomogeneity at CDUS showed higher P adverse 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 P adverse sDF and quartiles of mean testicular volume (Figure 1a), FSH (Figure 1b)
and LH (Figure 1c) levels, and occurrence of epididymal tail inhomogeneity (Figure 1d).

After adjustment for confounders (Table 4, Models 1 and 2), PI\textsuperscript{inflamer} sDF was significantly associated with prostate-related symptoms and signs. In particular, higher NIH-CPSI total or subdomains scores were associated with higher PI\textsuperscript{inflamer} sDF (Table 4). Subjects with overall (n = 13, 8.2%) “moderate to severe” (n = 8, 4.5%) prostatitis-like symptoms showed higher PI\textsuperscript{inflamer} 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\textsuperscript{inflamer} 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\textsuperscript{inflamer} sDF levels (Table 4). Most of the relevant associations reported in Table 4 are graphically represented in Figure 2 showing PI\textsuperscript{inflamer} stepwise correlations with quartiles of NIH-CPSI total score (Figure 2a), prostatic arterial peak systolic velocity (Figure 2b), occurrence of prostatic hyperemia (Figure 2c) or macro-calcifications (Figure 2d). For total sDF levels, correlations with clinical and CDUS features reflect those observed for both PI\textsuperscript{dimmer} and PI\textsuperscript{inflamer} 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\textsuperscript{inflamer} and PI\textsuperscript{dimmer}, which presented different biological characteristics and, likely, different clinical relevance. The fact that sDF in PI\textsuperscript{inflamer} and PI\textsuperscript{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\textsuperscript{dimmer} sDF and several clinical and ultrasound parameters suggestive of testicular damage, such as testicular inhomogeneity and hypoechochogenicity, as well as higher FSH levels, indicates that this population, entirely formed by DNA fragmented and dead sperm, results from an impairment of testicular function and/or alterations of spermatogenesis. This concept is reinforced by the previously reported positive correlation between PI\textsuperscript{dimmer} sDF and levels of apoptotic bodies, round anucleated elements considered to be markers of excessive testicular apoptosis and related...
Table 2: Color-Doppler ultrasound characteristics of the whole sample

| Colour-Doppler ultrasound parameters | n (%) | Mean±s.d. or median (quartiles) | Range (minimum, maximum) |
|-------------------------------------|-------|--------------------------------|--------------------------|
| Testis                              |       |                                |                          |
| Mean testicular volume (ml)          |       | 15.9±4.3                       | 7.0–29.8                 |
| Testicular inhomogeneity             | 41 (25.6) |                                |                          |
| Testicular hypoechochogenicity       | 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-califications         | 37 (23.1) |                                |                          |
| Major calcification diameter (mm)   | 8.2±5.4 | 0.0–24.0                       |                          |
| Dilated ejaculatory duct            | 13 (8.1)  |                                |                          |
| Severe inhomogeneous texture        | 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)   |                                |                          |

According to Lotti et al., 23 Severe echographic-defined varicocele, according to Lotti and Maggi; 23 According to Lotti and Maggi; 23 Calculations with size ≥3 mm (according to Lotti et al.); 23 According to Lotti et al. Calculated using the “ellipsoid/prolate spheroid” formula, according to Lotti et al. 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 (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.

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 PΠinner sDF with signs of testicular impairment (present study), the correlation with apoptotic testicular bodies, 30 and the demonstration that a high percentage of sperm in this population shows active caspase activity, 31 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 PΠinner sperm population may also originate at this level. 23 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 PΠproximal 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 PΠproximal 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 PΠproximal sDF is always present in semen samples implies that PΠproximal sDF occurs even in the absence of clear symptoms or signs of prostatic inflammation. We here demonstrate that higher levels of PΠproximal sDF are present in...
Table 3: Significant associations between PI<sup>dimmer</sup> sDF and main clinical and CDUS features of the male genital tract

| Clinical and laboratory parameters | Unadjusted analyses | Adjusted analyses |
|-----------------------------------|---------------------|------------------|
|                                   | Model 1*            | Model 2**         |
| 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<sub>10</sub> [LH]              | $r=0.25, P=0.005$  | $r=0.29, P<0.0001$| $r=0.22, P<0.02$ |
| Log<sub>10</sub> [FSH]             | $r=0.41, P<0.0001$ | $r=0.37, P<0.0001$| $r=0.34, P<0.0001$ |

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<sup>dimmer</sup> 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<sup>brighter</sup> 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**         |
| 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-calculations        | $d=3.1±2.4, P<0.05$ | $d=3.0±2.9, P<0.05$| $d=5.7±3.3, P<0.05$ |
| 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<sup>-1</sup>) | $r=0.24, P<0.05$    | $r=0.20, P<0.05$  | $r=0.25, P<0.02$ |

*Prostatitis-like symptoms: “perineal and/or ejaculatory pain or discomfort and NIH-CPSI pain subdomain score ≥4,” according to Nickel et al<sup>21</sup>. Symptoms were classified as “moderate-severe” for a pain index score of ≥8, according to Nickel’s criteria<sup>21</sup>. **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<sup>brighter</sup> 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 prostatitis inflammation; however, we cannot exclude that damage in PI<sup>dimmer</sup> sperm may also originate in other parts of the genital tract. Overall, our results indicate that DNA fragmentation of PI<sup>brighter</sup> sperm occurs much later in time relative to that of the PI<sup>dimmer</sup> 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<sup>brighter</sup> population, in agreement with recent findings of our group showing the concomitant presence of DNA fragmentation and oxidative DNA damage only in PI<sup>brighter</sup> spermatozoa.<sup>21</sup> The fact that a DNA fragmented PI<sup>brighter</sup> 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,<sup>6</sup> 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,<sup>17</sup> while others did not.<sup>10</sup> A similar situation occurred for LH and testosterone levels.<sup>17,20</sup> 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$^{\text{dimmer}}$ sDF. (a and b) Stepwise relationship among PI$^{\text{brighter}}$ 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$^2$ (Adj. r$^2$) and P values derived from Table 4, Model 2 (linear regression analysis) are reported. (c and d) Difference in PI$^{\text{brighter}}$ sDF between subjects with or without prostatic hyperemia (c) or prostate macro-calculations (d). P values derived from Table 4, Model 2 (ANCOVA) are reported.

We found a strong correlation between sDF and patient age, in agreement with a recent meta-analysis. Aging may cause degenerative alterations in the germinal epithelium affecting sperm quality. The fact that correlation with age was present for both PI$^{\text{dimmer}}$ and PI$^{\text{brighter}}$ 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, when men with idiopathic infertility with and without varicocele were compared, results were contradictory. In our study, no relationship between sDF, either in total, PI$^{\text{brighter}}$ or PI$^{\text{dimmer}}$ 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$^{\text{dimmer}}$ 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$^{\text{dimmer}}$ and PI$^{\text{brighter}}$ may originate in different sites of the male genital tract. In particular, PI$^{\text{brighter}}$ 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; MoMu performed flow cytometric data interpretation and analysis; MaMa 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.

ACKNOWLEDGMENTS

We thank Drs. Erminio Filimberti, Selene Degl’Innocenti and Maria Grazia Fino (AOUC Careggi) for semen analysis. The study was supported by grants from Ministry of University and Scientific Research (FIRB project to S. Marchiani, protocol number: RBFR10VJ56_001, SIR project to F. Lotti, protocol number: RBSI14LMQ). L. Tamburrino was recipient of a grant from Accademia dei Lincei (Rome, Italy).

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

REFERENCES

1. Krausz C. Male infertility: pathogenesis and clinical diagnosis. Best Pract Res Clin Endocrinol Metab 2011; 25: 271–85.
2. Lotti F, Maggi M. Ultrasound of the male genital tract in relation to male reproductive health. Hum Reprod Update 2015; 21: 56–83.
3. Tamburrino L, Marchiani S, Montoya M, Elia Marino F, Natali L, et al. Mechanisms and clinical correlates of sperm DNA damage. Asian J Androl 2012; 14: 24–31.
4. Zhao J, Zhang Q, Wang Y, Li Y. Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic...
sperm injection: a systematic review and meta-analysis. *Fertil Steril* 2014; 102: 998–1005.

5 Ribas-Maynou J, Garcia-Peiró A, Abad C, Amengual MJ, Navarro J, et al. Alkaline and neutral comet assay profiles of sperm DNA damage in clinical groups. *Hum Reprod* 2012; 27: 652–8.

6 Eugeni E, Charalabopoulos K, Asimakopoulos B. Human sperm DNA fragmentation and its correlation with conventional semen parameters. *J Reprod Infertil* 2014; 15: 2–14.

7 Muratori M, Marchiani S, Maggi M, Forti G, Baldi E. Origin and biological significance of DNA fragmentation in human spermatozoa. *Front Biosci* 2006; 11: 1491–9.

8 Muratori M, Marchiani S, Tamburro L, Tocci V, Failli P, et al. Nuclear staining identifies two populations of human sperm with different DNA fragmentation extent and relationship with semen parameters. *Hum Reprod* 2008; 23: 1035–43.

9 Muratori M, Porazzi I, Luceni M, Marchiani S, Forti G, et al. Annexin V binding and merocyanine staining fail to detect human sperm capacitation. *J Androl* 2004; 25: 797–810.

10 Marchiani S, Tamburro L, Maggi A, Vannelli GB, Forti G, et al. Characterization of M540 bodies in human semen: evidence that they are apoptotic bodies. *Mol Hum Reprod* 2007; 13: 621–31.

11 Muratori M, Tamburro L, Costantino A, Marchiani S, et al. Small variations in crucial steps of TUNEL assay coupled to flow cytometry greatly affect measures of sperm DNA fragmentation. *J Androl* 2010; 31: 336–45.

12 Marchiani S, Tamburro L, Giuliani L, Nosi D, Sarli V, et al. Sumo1ylation of human spermatozoa and its relationship with semen quality. *Int J Androl* 2011; 34: 581–93.

13 Muratori M, Tamburro L, Marchiani S, Cambi M, Olivotto B, et al. Investigation on the origin of sperm DNA fragmentation: role of apoptosis, immaturity and oxidative stress. *Mol Med* 2015; 21: 109–22.

14 Marchiani S, Tamburro L, Olivotto B, Betti L, Azzari C, et al. Characterization and sorting of flow cytometric populations in human semen. *Andrology* 2014; 2: 394–401.

15 Muratori M, Marchiani S, Tamburro L, Cambi M, Lotti F, et al. DNA fragmentation in brighter sperm predicts male fertility independently from age and semen parameters. *Fertil Steril* 2015; 104: 582–90.

16 Dupont C, Faure C, Sermondade N, Boubya M, Eustache F, et al. Obesity leads to higher risk of sperm DNA damage in fertile patients. *Asian J Androl* 2013; 15: 622–5.

17 Wdowiak A, Raczkiewicz D, Stasiak M, Bojar I. Levels of FSH, LH and testosterone, and sperm DNA fragmentation. *Neuro Endocrinol Lett* 2014; 35: 73–9.

18 Wang YJ, Zhang RQ, Lin YJ, Zhang RG, Zhang WL. Relationship between varicocele and sperm DNA damage and the effect of varicocele repair: a meta-analysis. *Reprod Biomed Online* 2012; 25: 307–14.

19 Smith R, Kaune H, Parodi D, Madariaga M, Morales I, et al. (Extent of sperm DNA damage in spermatozoa from men examined for infertility. Relationship with oxidative stress). *Rev Med Chil* 2007; 135: 279–86.

20 Litwin MS, McNaughton-Collins M, Fowler FJ Jr., Nickel JC, Calhoun EA, et al. The National Institutes of Health chronic prostatitis symptom index: development and validation of a new outcome measure. Chronic Prostatitis Collaborative Research Network. *J Urol* 1999; 162: 369–75.

21 Nickel JC, Downey J, Hunter D, Clark J. Prevalence of PLS in a population based study using the National Institutes of Health chronic prostatitis symptom index. *J Urol* 2001; 165: 842–5.

22 World Health Organization. WHO Manual for the Standardized Investigation and Diagnosis of the Infertile Couple. Cambridge: Cambridge University Press; 2000.

23 Lotti F, Corona G, Mondaini N, Masero E, Rossi M, et al. Seminal, clinical and colour-Doppler ultrasound correlations of prostatitis-like symptoms in males of infertile couples. *Andrology* 2014; 2: 30–41.

24 Lotti F, Corona G, Colpi GM, Filimberti E, Innocenti SD, et al. Seminal vesicles ultrasound features in a cohort of infertility patients. *Hum Reprod* 2012; 27: 974–82.

25 World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th ed. Geneva, Switzerland: WHO Press; 2010.

26 Bartoletti R, Cai T, Mondaini N, Dinelli N, Pinzi N, et al. Prevalence, incidence estimation, risk factors and characterization of chronic prostatitis/chronic pelvic pain syndrome in urological hospital outpatients in Italy: results of a multicenter case-control observational study. *J Urol* 2007; 178: 2411–5.

27 Taha EA, Ez‑Aldin AM, Sayed SK, Ghandour NM, Mostafa T. Effect of smoking on sperm vitality, DNA integrity, seminal oxidative stress, zinc in fertile men. *Urology* 2012; 80: 822–5.

28 Lotti F, Tamburro L, Marchiani S, Muratori M, Corona G, et al. Semen apoptotic M540 body levels correlate with testis abnormalities: a study in a cohort of infertile subjects. *Hum Reprod* 2012; 27: 3393–402.

29 Sakkas D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG. Origin of DNA damage in ejaculated human spermatozoa. *Rev Reprod* 1999; 4: 31–7.

30 Appasamy M, Jauniaux E, Serhal P, Al‑Qahtani A, Groome NP, et al. Evaluation of the relationship between follicular fluid oxidative stress, ovarian hormones, and response to gonadotropin stimulation. *Fertil Steril* 2008; 89: 912–21.

31 Johnson SL, Dunleavy J, Gemmell NJ, Nakagawa S. Consistent age-dependent declines in human semen quality: a systematic review and meta-analysis. *Aging Res Rev* 2015; 19C: 22–33.

32 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.

33 Zini A, Dolhe G. Are varicoceles associated with increased deoxyribonucleic acid fragmentation? *Fertil Steril* 2011; 96: 1283–7.
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 |
|-----------------------------------|---------------------|-------------------|
| Mean testis volume (Prader, ml)   | \( r = -0.173, \ P < 0.05 \) | \( r = -0.188, \ P = 0.02 \) |
| History of cryptorchidism         | \( d = 18.9 \pm 8.3, \ P < 0.05 \) | \( d = 16.8 \pm 8.2, \ P < 0.05 \) |
| \( \log_{10} \) [FSH]            | \( r = 0.256, \ P = 0.002 \) | \( r = 0.207, \ P < 0.02 \) |

CDUS parameters

| Testis                           |                     |
|----------------------------------|---------------------|
| Mean testis volume (ml)          | \( r = -0.170, \ P < 0.05 \) |
| Testicular inhomogeneity         | \( d = 7.4 \pm 3.4, \ P < 0.05 \) |
| Testicular hypoechochogenicity    | \( d = 9.4 \pm 4.7, \ P < 0.05 \) |

| Prostate                         |                     |
|----------------------------------|---------------------|
| Prostate macro-calcifications     | \( d = 10.0 \pm 3.4, \ P < 0.001 \) |
| Major calcification diameter, mm | \( r = 0.295, \ P < 0.001 \) |
| Prostatic hyperemia (before ejaculation) | \( d = 15.1 \pm 4.0, \ P < 0.001 \) |
| Mean arterial peak systolic velocity (cm s\(^{-1}\)) | \( r = 0.220, \ P < 0.01 \) |

| Seminal vesicles                 |                     |
|----------------------------------|---------------------|
| Total volume before ejaculation (ml) | \( r = 0.192, \ P < 0.02 \) |
| Total volume after ejaculation (ml) | \( r = 0.210, \ P < 0.02 \) |
| Inhomogeneity before ejaculation  | \( d = 8.5 \pm 3.2, \ P < 0.01 \) |
| Inhomogeneity after ejaculation   | \( d = 9.1 \pm 3.7, \ P < 0.01 \) |
| Areas of endocapsulation before ejaculation | \( d = 10.3 \pm 4.9, \ P < 0.05 \) |

*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.

Supplementary Figure 1: Schematic representation of the cytofluorimetric analysis of sDF in PI\(^\text{brighter}\) and PI\(^\text{dimmer}\) 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\(^\text{brighter}\) in green and PI\(^\text{dimmer}\) in blue) can be clearly distinguished. Left panels: negative control for TUNEL (omitting TdT); right panels: test samples.