Received: 6 July 2022 | Revised: 12 January 2023 | Accepted: 13 January 2023

DOI: 10.1111/andr.13395

ANDROLOGY WILEY

ORIGINAL ARTICLE

# Sperm DNA fragmentation and idiopathic recurrent pregnancy loss: Results from a multicenter case–control study

Andrea Busnelli[1,2] | Andrea Garolla[3] | Elena Di Credico[1] | Silvia D'Ippolito[4] | Anna Maria Merola[4] | Domenico Milardi[4,5,6] | Alfredo Pontecorvi[4,5,6] | Giovanni Scambia[4,7] | Nicoletta Di Simone[1,2]

[1]Department of Biomedical Sciences, Humanitas University, Pieve Emanuele, Milan, Italy

[2]IRCCS Humanitas Research Hospital, Rozzano, Milan, Italy

[3]Unit of Andrology and Reproductive Medicine & Centre for Male Gamete Cryopreservation, Department of Medicine, University of Padova, Padua, Italy

[4]Dipartimento di Scienze della Salute della Donna, del Bambino e di Sanità Pubblica, Fondazione Policlinico Universitario A. Gemelli Istituto di Ricovero e Cura a Carattere Scientifico, Rome, Italy

[5]International Scientific Institute "Paul VI," Catholic University, Rome, Italy

[6]Unità Operativa Complessa (UOC) Endocrinology and Diabetology, Fondazione Policlinico Universitario A. Gemelli, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Rome, Italy

[7]Dipartimento di Scienze della Vita e Sanità Pubblica, Università Cattolica del Sacro Cuore, Rome, Italy

**Correspondence**
Andrea Busnelli, Department of Biomedical Sciences, Humanitas University, Via Rita Levi Montalcini, 4, 20090, Pieve Emanuele, Milan, Italy.
Email: andrea.busnelli@hunimed.eu

## Abstract

**Background:** Sperm DNA fragmentation was hypothesized to have a role in the pathogenesis of recurrent pregnancy loss. Unfortunately, the quality of already published evidence is low.

**Objectives:** To investigate the association between sperm DNA fragmentation and idiopathic recurrent pregnancy loss by limiting, as much as possible, the interference of confounding factors.

**Materials and methods:** This was a retrospective multicenter case–control study conducted in two Italian University Hospitals (i.e., Policlinico Gemelli, Rome and Humanitas S. Pio X, Milan) from July 2020 to March 2022. Cases were men belonging to couples affected by first trimester idiopathic recurrent pregnancy loss, defined as the previous loss of two or more pregnancies. Two control groups were selected: (i) men belonging to couples with proven fertility (i.e., at least two previous full-term pregnancies) (control group A); (ii) men belonging to couples with proven infertility (i.e., the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse) (control group B). The sperm DNA fragmentation index was measured by the terminal deoxynucleotidyl transferase dUTP nick end labeling assay.

**Results:** We included 74 cases, 37 men with proven fertility (control group A) and 100 men belonging to infertile couples (control group B). The median sperm DNA fragmentation index was significantly lower in control group A (17%, interquartile range: 14.3%–20.6%) compared to both case group (24.5%, interquartile range: 17%–32%; $p < 0.0001$) and control group B (24%, interquartile range: 18.9%–30%; $p = 0.001$). The rate of subjects with sperm DNA fragmentation index greater than 30% was significantly higher in both case groups (28%, 95% confidence interval [18%–40%]) and control group B (26%, 95% confidence interval [18%, 36%]) compared to control group A (0%, 95% confidence interval [0%–10%]) ($p < 0.001$). Multivariate regression models yielded a significant association between sperm DNA fragmentation index and

recurrent pregnancy loss (adjusted odds ratio 1.13, 95% confidence interval [1.04–1.23], $p = 0.006$), but failed to show an association between sperm DNA fragmentation index and infertility (adjusted odds ratio 1.13, 95% CI [1–1.29], $p = 0.05$).

**Conclusions:** Men within couples affected by recurrent pregnancy loss or infertility had a significantly higher rate of sperm DNA fragmentation compared to fertile controls. However, after adjusting for covariates, sperm DNA fragmentation index was associated only with recurrent pregnancy loss.

**KEYWORDS**
natural pregnancy, recurrent pregnancy loss, sperm DNA fragmentation, TUNEL assay

## 1 | INTRODUCTION

Recurrent pregnancy loss (RPL) is diagnosed after the spontaneous termination of two or more clinical pregnancies occurring before the legal definition of fetal viability, which ranges, depending on the country's laws, from 20 to 24 weeks of gestation. RPL is one of the most frustrating conditions in reproductive medicine for both patients and clinicians.[1–4] The extent of the problem is not negligible. In fact, large epidemiological studies conducted both in Europe and in the United States reported that the average prevalence of RPL was between 1% and 4% of all women who achieve pregnancy.[2,5]

The etiology of RPL remains poorly understood and, despite extensive testing, the underlying cause of RPL is identified in less than 50% of cases.[6] Idiopathic RPL is associated with substantial adverse clinical and psychological consequences for affected couples. Therefore, identifying the root causes of RPL is critical.[6,7] For many years, both clinical and scientific interests have focused on the possible female risk factors for RPL. Only more recently, different lines of research have also investigated the role of male factors (i.e., sperm quality, occupational exposure, pollution, and lifestyles) in RPL pathophysiology.[6–8] In this context, the integrity of sperm DNA, a measure of chromatin condensation damage, has gained particular attention. Spermatozoa with a high DNA fragmentation level can be alive, motile, and have normal morphology. However, if the oocyte, once fertilized, fails to restore DNA integrity, the embryo development can be impaired. The consequences in terms of reproductive outcomes can be manifold and range from infertility to pregnancy loss.[8] Interestingly, Robinson et al. showed an increased miscarriage incidence in patients with a high rate of fragmented sperm DNA compared with their negative counterparts (risk ratio [RR]: 2.16, 95% confidence interval [CI] [1.54–3.03]).[9] Their data laid the rational foundation to investigate the role of sperm DNA fragmentation (SDF) in RPL pathophysiology. Four recent systematic reviews and meta-analysis tested this hypothesis and showed a significantly higher SDF in couples who experienced idiopathic RPL[7,10–12] (Table 1). Unfortunately, the quality of the evidence is hampered by considerable weaknesses. First of all, the majority of selected studies included both subjects with infertility and subjects with an unknown fertility status. Such inaccuracy in population selection might have introduced a confounding factor, as SDF can be increased in infertile subjects.[7] Second, a few authors performed statistical analysis to test the association between SDF level and other spermatozoa characteristics evaluated according to the World Health Organization (WHO) guidelines. Third, the RPL evaluation is inconsistent among studies and often deviates considerably from the diagnostic workup recommended by both the American Society of Reproductive Medicine (ASRM) and the European Society of Human Reproduction and Embryology (ESHRE).[1,13] Finally, the vast majority of meta-analyzed studies are underpowered to draw meaningful conclusions.[7,14]

Considering the poor reliability of the available evidence, we deemed of relevance to further investigate the association between SDF and RPL by limiting as much as possible, the interference of confounding factors.

## 2 | MATERIALS AND METHODS

### 2.1 | Design

This research comprised a retrospective multicenter case–control study conducted across two Italian University Hospitals (i.e., Policlinico Gemelli Hospital, Catholic University of Scared Heart, Rome, Italy; and Humanitas S. Pio X Hospital, Humanitas University, Milan, Italy) between July 2020 and March 2022. The study protocol was approved by the Institutional Review Board (IRB) at both Institutions (Policlinico Gemelli Hospital, determination nr. 0003977/17; Istituto Clinico Humanitas, determination nr. 3133/22). All included subjects signed an informed consent form before enrollment.

### 2.2 | Participants

Men who referred to the obstetrics and gynecology departments of the two participating hospitals could be considered for study entry. Cases included men within couples affected by idiopathic RPL. This was defined as the previous loss of two or more pregnancies, according to the ESHRE guidelines and the ASRM committee opinion.[1,3] Couples who conceived their previous pregnancy within 1 year and experienced spontaneous pregnancy losses within the first trimester of pregnancy were eligible for participation. Biochemical, ectopic, and

**TABLE 1** Published meta-analyses investigating the association between HPV sperm infection and RPL

| Author | Design of included studies | Number of studies in quantitative analysis | RPL definition | RPL etiology | Total number of cases | Control group | Result of data pooling |
|---|---|---|---|---|---|---|---|
| McQueen et al., 2019[7] | Prospective studies | 13 | Two or more previous pregnancy losses | Idiopathic | 579 | Fertile couples | Significantly higher rate of sperm DNA fragmentation in cases when compared to fertile controls (MD 11.91, 95% CI: 4.97–18.86). |
| Tan et al., 2019[11] | Prospective and retrospective studies | 14 | Two or more previous pregnancy losses | Idiopathic | 530 | Fertile couples | Significantly higher rate of sperm DNA fragmentation in cases when compared to fertile controls (MD 11.98, 95% CI: 6.64–17.32) |
| Yfu et al., 2020[12] | Prospective and retrospective studies | 21 | Two or more previous pregnancy losses | Idiopathic | Not clearly reported | Fertile couples | Significantly higher rate of sperm DNA fragmentation in cases when compared to fertile controls. TUNEL test: 8 studies (MD12.12, 95% CI: 3.34–20.91); SCSA: 7 studies (MD 5.40, 95% CI: 1.76–9.03); SCD: 9 studies (MD 11.16, 95% CI: 6.70–15.62) |
| Dai et al., 2022[10] | Prospective and retrospective studies | 19 | Two or more previous pregnancy losses | Idiopathic | 1182 | Fertile couples | Significantly higher rate of sperm DNA fragmentation in cases when compared to fertile controls (WMD = 8.45, 95% CI: 1.48–15.42) |

Abbreviations: HPV, human papilloma virus; MD, mean difference; RPL, recurrent pregnancy loss; SCD, sperm chromatin dispersion; SCSA, sperm chromatin structure assay; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine (TdT) triphosphate (dUTP) nick end labeling assay; WMD, weighted mean difference.

molar pregnancies were not identified as RPL in this study. Women experiencing RPL underwent the following diagnostic workup: (i) genetic analysis of the pregnancy tissue; (ii) parental karyotyping; (iii) screening for antiphospholipid antibodies (lupus anticoagulant [LA], and anticardiolipin antibodies [ACA IgG and IgM]); (iv) screening for $\beta$2 glycoprotein I antibodies (a$\beta$2GPI); (v) screening for antinuclear antibodies (ANA); (vi) thyroid screening (thyroid-stimulating hormone [TSH] and thyroid peroxidase [TPO] antibodies); (vii) transvaginal 3D ultrasound; (viii) cervico-vaginal infections screening; and (ix) glucose metabolism assessment.[1,13] If the full diagnostic workup was negative, the couple was identified as experiencing "idiopathic RPL" and was eligible for the present study. Two control groups were selected: (a) men belonging to couples with proven fertility (i.e., at least two previous full-term pregnancies with live birth, the last achieved within the year preceding study enrollment, and no history of RPL) (control group A); (b) men belonging to couples with proven primary male infertility (i.e., the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse and no history of proven conception, neither natural nor through assisted reproductive technology [ART]) (control group B). All included subjects signed an informed consent form before enrollment.

## 2.3 | Hormonal serum concentration assessment

Blood samples were collected and then centrifuged at room temperature. Serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), 17-$\beta$ estradiol, prolactin (PRL), and testosterone concentration were assessed by using electrochemiluminescence dosage technique (ECLIA) (Roche Diagnostics GmbH, Mannheim, Germany). In addition to the daily quality checks performed according to the institution's protocol, the assays were calibrated both when a new reactive batch was used and when an outcome outside the normal range was observed.

## 2.4 | Varicocoele diagnosis and grading

All patients were examined to determine the presence of varicocoele by an experienced andrologist or urologist. The examinations were carried out with the patients in an upright position. Varicocoele was classified according to the modified Dubin and Amelar criteria as: (i) absent (not palpable), (ii) grade 1 (palpable with the aid of the Valsalva maneuver), (iii) grade 2 (palpable without Valsalva), and (iv) grade 3 (visible).[15,16] A scrotal and testicular color Doppler ultrasound was also

performed. All patients were examined in standing and supine positions by an experienced sonographer. Varicocoele was diagnosed if both the following ultrasound criteria were satisfied: (a) at least two venous channels with a diameter greater than 3 mm, and (b) flow reversal with or without Valsalva maneuver for 1 second or more.[15]

## 2.5 | Semen analysis and TUNEL assay

Semen samples were obtained via masturbation after 2–5 days of sexual abstinence and stored in sterile containers. Samples were allowed to liquefy for 30 min and were examined for seminal parameters according to World Health Organization (WHO) guidelines.[17] SDF was assessed by using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay in a commercially available kit (Cell Death Detection Kit, Roche Diagnostics, Milan, Italy). Next, cells were analyzed using a Becton Dickinson FACScan System for measuring and analyzing flow cytometry in Cellquest software (Becton Dickinson, Oxford, UK). This measure was expressed as a percentage and labeled the "sperm DNA fragmentation index" (SDFI) (i.e., the ratio of the number of spermatozoa with fragmented DNA to the total number of spermatozoa).[18] Seminal fluid analyses were carried out by trained technicians in the participating hospital laboratories a few days following recruitment and sample collection to allow compliance with the period of sexual abstinence. We ensured a high-quality testing by implementing regular external quality assessment programs.

## 2.6 | Statistical analysis

The target sample size was calculated based on results of a previous meta-analysis that reported a mean SDFI difference (MD) of 10.7% (95% CI [5.82–15.58]) between RPL patients and controls.[7] By setting alpha and beta values at 0.05 and 0.20, respectively, the estimated sample size required in this study was at least 70 cases and 35 controls. We also planned to recruit a number of men with infertility at least equal to that of men belonging to RPL couples.

The sample size was estimated using STATA 15.0 (StataCorp LP, College Station, TX, USA). Secondary analyses and subanalyses are considered exploratory, as this study lacked sufficient power to assess the impact of additional exposures.

Two multiple logistic regression analyses including, respectively, RPL and infertility as the dependent variables were performed to identify potential confounding factors. Two multivariate logistic regression models yielded adjusted measures of association between SDFI and (i) RPL and (ii) infertility, respectively. As continuous data were found not to be normally distributed, nonparametric tests were used (Mann–Whitney *U* test or Kruskal–Wallis *H* test, as appropriate). The chi-square test was carried out to compare categorical variables. To test the accuracy of sDFI in predicting both RPL and infertility, we performed a receiver operator curve (ROC) analysis. The Spearman's rank correlation was calculated to test the association between WHO sperm parameters and SDFI. Data analysis was performed using the Statistics Package for Social Sciences (SPSS 18.0, Chicago, IL, USA). Statistical significance was set to an alpha level of 0.05.

# 3 | RESULTS

We included 74 men belonging to couples affected by RPL (case group), 37 men with proven fertility (control group A) and 100 men belonging to infertile couples (control group B). Baseline characteristics did not differ significantly between the study groups (Table 2). The hormonal serum concentrations as well as the prevalence of varicocoele were also similar between cases and controls (Table 2). Sperm concentration and total sperm motility were significantly lower in control group B compared to both case group ($p < 0.001$) and control group A ($p < 0.001$). Progressive motility was significantly higher in control group A compared to both case group ($p = 0.03$) and control group B ($p = 0.02$) (Table 3). The percentage of abnormally shaped spermatozoa was significantly higher in control group B compared to both case group ($p = 0.01$) and control group A ($p = 0.04$). Sperm vitality was significantly higher in control group A compared to both control group B ($p = 0.003$) and case group ($p = 0.01$) (Table 3). A significant negative correlation between total sperm motility and the sDFI was observed both in the whole cohort (Spearman's correlation coefficient: −0.19, $p = 0.01$) and in the case group (Spearman's correlation coefficient: −0.26, $p = 0.03$) (Table S1).

The median sDFI was significantly lower in control group A compared to both case group ($p < 0.0001$) and control group B ($p = 0.001$) (Figure 1). Accordingly, the rate of subjects with sDFI greater than 30% was significantly higher in both case group and control group B compared to control group A ($p < 0.001$). The rate of subjects with SDF greater than 20% was significantly higher in both case group and control group B compared to control group A ($p = 0.008$ and <0.001, respectively) (Table 4). We observed a significant association between the SDFI and both RPL (OR 1.11; 95% CI [1.05–1.18], $p < 0.001$) and infertility (OR 1.14; 95% CI [1.07–1.22], $p < 0.001$). Multiple logistic regression analyses showed that (i) BMI, sperm progressive motility, and sperm vitality were associated with RPL (Table S2); and (ii) sperm concentration and sperm vitality were associated with infertility (Table S3). Multivariate regression models yielded a significant association between the SDFI and RPL (adjusted odds ratio [aOR] 1.13, 95% CI [1.04–1.23], $p = 0.006$), but failed to confirm an association between sDFI and infertility (aOR 1.13, 95% CI [1–1.29], $p = 0.05$) (Table S4).

The ROC analyses aimed at assessing the accuracy of sDFI in predicting RPL, and infertility showed an area under the curve (AUC) of 0.73 (95% CI [0.63–0.82]) and 0.76 (95% CI [0.68–0.84]), respectively (Figure 2).

Baseline characteristics as wells as hormonal serum concentration, varicocoele prevalence, and semen parameters of cases with high and normal sDFI (i.e., sDFI > 30% vs. sDFI ≤ 20%) were compared, but no significant differences emerged (Table S5). Cases were divided into subgroups based on the number of previous pregnancy losses. The median sDFI of each subgroup was significantly higher when compared to the median sDFI of fertile controls. We did not observe significant

**TABLE 2** Baseline characteristics of cases and controls

| Characteristics | Case group ($N = 74$) | Control group A ($N = 37$) | Control group B ($N = 100$) | *p*-Value |
|---|---|---|---|---|
| Age (years) | 39.5 [35–44] | 38 [35.5–41.5] | 39 [35.3–44] | 0.86 |
| BMI (kg/m$^2$) | 24.3 [22.1–26.3] | 23 [22.2–24.4] | 23.9 [22.2–25.5] | 0.14 |
| Partner's age (years) | 34 [31.8–37] | 35 [32–37] | 35 [33–37] | 0.65 |
| FSH (mUL/mL) | 6 [3.9–8.2] | 6 [5–7] | 6.5 [4.8–8] | 0.8 |
| LH (mUL/mL) | 4.8 [4–6] | 4 [3–5.4] | 4.9 [2.9–6.2] | 0.22 |
| Testosterone (nm/L) | 5.1 [4–6.7] | 6 [4.3–7] | 5.3 [4.3–6.5] | 0.60 |
| PRL (ng/mL) | 11 [6.6–18] | 15 [10.4–18] | 12 [7.1–18] | 0.83 |
| E2 (pg/mL) | 28 [24–30] | 25.5 [21–30] | 28 [23–30] | 0.46 |
| Smoke | | | | 0.93 |
| Nonsmokers | 62 (83.8%) | 32 (86.5%) | 85 (85%) | |
| Smokers | 12 (16.2%) | 5 (13.5%) | 15 (15%) | |
| Varicocoele | | | | 0.6 |
| Absent | 63 (85.1%) | 34 (91.9%) | 92 (92%) | |
| I grade | 5 (6.8%) | 0 (0.0%) | 3 (3%) | |
| II grade | 4 (5.4%) | 2 (5.4%) | 4 (4%) | |
| III grade | 2 (2.7%) | 1(2.7%) | 1 (1%) | |
| Lower urinary tract symptoms | | | | 0.13 |
| Absent | 66 (89.2%) | 37 (100%) | 92 (92%) | |
| Present | 8 (10.8%) | 0 (0.0%) | 8 (%) | |
| Prostatitis | | | | 0.22 |
| Absent | 68 (91.9%) | 37 (100%) | 94 (94%) | |
| Present | 6 (8.1%) | 0 (0.0%) | 6 (6%) | |

*Note*: Data are expressed as median and interquartile range and number (%). Case group: men belonging to couples affected by recurrent pregnancy loss. Control group A: men belonging to fertile couples. Control group B: men belonging to infertile couples.
Abbreviations: BMI, body mass index; E2, estradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PRL, prolactin.

**TABLE 3** Baseline semen parameters in the two study groups

| Semen parameters | Case group ($N = 74$) | Control group A ($N = 37$) | Control group B ($N = 100$) | *p*-Value |
|---|---|---|---|---|
| Volume (mL) | 3 [2–3.9] | 3 [2.5–3.7] | 2.7 [2–3.8] | 0.75 |
| pH | 7.8 [7.5–8] | 8 [7.5–8.3] | 7.8 [7.5–8] | 0.66 |
| Concentration (millions of spz/mL) | 45 [27.8–90.8] | 80 [66–96.5] | 16.5 [7.7–30.8] | <0.001[a] |
| Total motility (%) | 42 [29.5–55.3] | 39.5 [35–45] | 32 [25–40] | <0.001[a] |
| Progressive motility (%) | 25 [11–35] | 32 [23–37] | 22 [15–33.8] | 0.06[b] |
| Normal spz (%) | 3 [2–7] | 3 [2.3–8] | 3 [2–5.7] | 0.03[c] |
| Vitality (%) | 55 [48–61] | 58 [54–60] | 55 [45–60] | 0.01[d] |

*Note*: Data are expressed as median and interquartile range. Case group: men belonging to couples affected by recurrent pregnancy loss. Control group A: men belonging to fertile couples. Control group B: men belonging to infertile couples.
Abbreviation: spz, spermatozoa.
[a]Significantly lower in control group B compared to both case group ($p < 0.001$) and control group A ($p < 0.001$).
[b]Significantly higher in control group A compared to both case group ($p = 0.03$) and control group B ($p = 0.02$).
[c]Significantly lower in control group B compared to both case group ($p = 0.01$) and control group A ($p = 0.04$).
[d]Significantly higher in control group A compared to both case group ($p = 0.01$) and control group B ($p = 0.003$).

**TABLE 4** Sperm DNA fragmentation in the two study groups

| | Case group ($N = 74$) | Control group A ($N = 37$) | Control group B ($N = 100$) | *p*-Value |
|---|---|---|---|---|
| sDFI (%) | 24.5 [17–32] | 17 [14.3–20.6] | 24 [18.9–30] | <0.001[a] |
| Subjects with sDFI > 30%, *N* (%, 95% CI) | 21 (28%, 18%–40%) | 0 (0%, 0%–10%) | 26 (26%, 18%–36%) | 0.002[b] |
| Subjects with sDFI > 20%, *N* (%, 95% CI) | 47 (64%, 52%–74%) | 13 (35%, 20%–53%) | 72 (72%, 62%–81%) | <0.001[c] |
| Subjects with sDFI > 15%, *N* (%) | 62 (84%, 73%–91%) | 28 (76%, 59%–88%) | 89 (89%, 81%–94%) | 0.15 |

*Note*: Data are expressed as mean ± SD or as number (%). Case group: men belonging to couples affected by recurrent pregnancy loss. Control group A: men belonging to fertile couples. Control group B: men belonging to infertile couples.

Abbreviations: CI, confidence interval; *N*, number; sDFI, sperm DNA fragmentation index.

[a]sDFI was significantly lower in control group A compared to both case group ($p < 0.0001$) and control group B ($p = 0.001$).

[b]The rate of subjects with sDFI > 30% was significantly higher in both case group and control group B compared to control group A ($p < 0.001$).

[c]The rate of subjects with sDFI > 20% was significantly higher in both case group and control group B compared to control group A ($p = 0.008$ and <0.001, respectively).

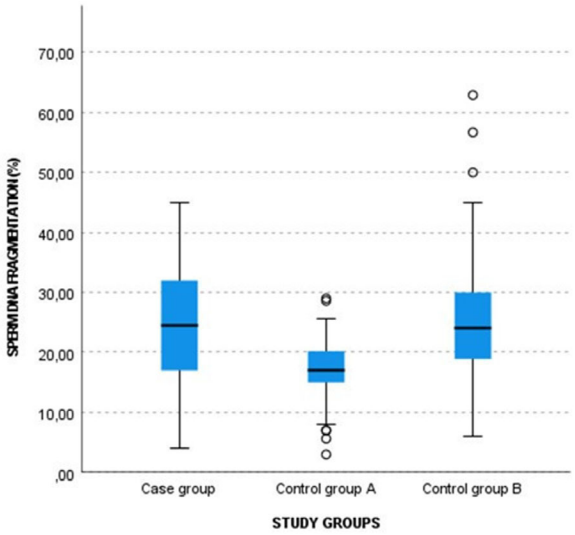


**FIGURE 1** Sperm DNA fragmentation index (sDFI) in study groups. Case group: men belonging to couples affected by recurrent pregnancy loss (RPL). Control group A: men belonging to fertile couples. Control group B: men belonging to infertile couples. Data are expressed as median and interquartile range (IQR). The sDFI was significantly lower in control group A compared to both case group ($p < 0.0001$) and control group B ($p = 0.001$).

differences in sDFI between cases with two, three, or four or more previous pregnancy losses (Figure S1). The median sDFI was compared between cases with primary ($N = 60$) (i.e., couples who never gave birth to a live infant) and secondary ($N = 14$) (i.e., couples who gave birth to, at least, one live infant) RPL without observing any difference ($p = 0.32$).

## 4 | DISCUSSION

In the present study, partners of women experiencing unexplained RPL had a significantly increased SDF, scored as sDFI, compared to fertile controls. Among cases, about one of four subjects had an sDFI greater than 30% and about three of five subjects had an sDFI greater than 20%. It is also relevant that the ROC analysis resulted in an AUC value of 0.73, suggesting a good performance of sDFI assessment in predicting RPL. Infertile controls, compared to cases, had a similar sDFI but lower sperm concentration, total sperm motility, and rate of normally shaped spermatozoa.

Several retrospective studies have suggested a possible association between SDF and fertility outcomes.[19–24] However, there are no well-conducted prospective studies investigating the impact of SDF on natural fecundity.[25,26] Accordingly, the most updated guidelines recommend that SDF analysis should not be considered part of the initial male infertility evaluation.[25,26] Our findings are consistent with this recommendation. In fact, the multivariate analysis did not confirm the association between sDFI and infertility that emerged from the univariate analysis. Overall, our results are in line with the most accepted theories claiming that a high SDF can negatively influence the ability to conceive only if associated with other favorable conditions, such as severe alterations of other seminal parameters or advanced maternal age.[27,28] Recent evidence showing that older oocytes, when injected with spermatozoa derived from samples with high SDF index, develop into embryos of poor quality, corroborates this theory.[27]

On the other hand, the results of both the multivariate analysis and Spearman's rank correlation suggest that covariates do not influence the association between sDFI and RPL. On this basis, one can speculate that the hypothetical role of sDFI in RPL pathophysiology might be independent of other semen parameters. Moreover, the concordance of available data showing an association between high sDFI and pregnancy loss after both natural and assisted conception suggests that SDF might exert its detrimental impact on embryo implantation and early pregnancy development.[29] In particular, it has been proposed that elevated SDF may trigger a non-apoptotic mechanism within the zygote, which slows paternal DNA replication and produces chromosomal rearrangements.[19] Exogenous (such as smoking, alcohol, air pollution, occupational exposure, and oncological therapies) and/or endogenous (i.e., increased BMI, varicocoele, genital tract infection, diabetes mellitus, cancer) factors have been demonstrated to exacerbate Reactive Oxygen Species (ROS) production fueling this process.[21] In the cohort of men with a positive history of RPL, we did not observe an association between the above-mentioned exposure factors and the

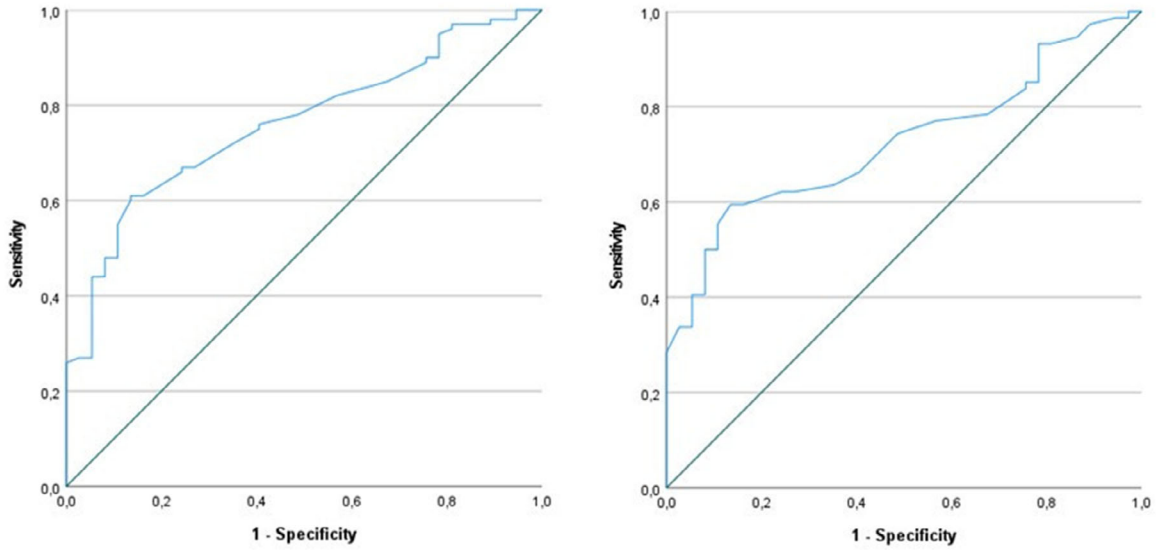


**FIGURE 2** Receiver operating characteristic (ROC) analysis aimed at assessing the accuracy of sperm DNA fragmentation index (SDFI) in predicting recurrent pregnancy loss (RPL) (left panel) and infertility (right panel). The areas under the curve (AUC) were 0.73 (95% confidence interval [CI] [0.63–0.82]) (left panel) and 0.76 (95% CI [0.68–0.84]), respectively.

level of SDF. However, it must be recognized that the sample size of the present study was insufficient to investigate such epidemiological issues. Implementing lifestyle changes (smoke cessation, decrease in bodyweight, decrease in alcohol consumption) that have a known beneficial effect on the general health and could potentially improve live birth rates, thus remains mandatory.[2]

## 4.1 | Strengths

The present study was conceived to overcome the weaknesses of already published evidence. The inclusion of only fertile couples who conceived naturally allows to overcome all the possible confounding factors associated with both infertility per sé and ART. On the basis of recent evidence, one can in fact hypothesize that ART and, in particular, intracytoplasmic sperm injection (ICSI) may have a beneficial effect on the reproductive outcomes of patients with increased SDF.[25] A further strength is the exclusion of couples with female partners aged more than 39 years. It is in fact known that female age constitutes the main risk factor for RPL and that the risk of pregnancy loss rapidly increases after the age of 40.[1] Furthermore, advanced maternal age could impair the oocyte ability to repair the SDF and make the association between high sDFI and both infertility and RPL seemingly more pronounced.[27]

Finally, also the other adopted selection criteria for both cases and controls were particularly strict and can accurately represent the intended populations. Couples with RPL could be included only after a comprehensive diagnostic workup. The ability to carry a fetus to viability in at least two distinct pregnancies ensured the selection of a fertile population. On the other hand, the inability to conceive guaranteed the selection of an infertile group without previous pregnancy losses.

## 4.2 | Limitations

Some limitations of the present study deserve to be mentioned. First of all, the retrospective design introduced uncontrolled biases. On the other hand, conducting a prospective study is particularly challenging for several reasons: (i) the need to recruit couples in the preconception period; (ii) difficulty in selecting men willing to undergo semen sample collection in the absence of fertility problems (which represents an obstacle in any study because of the social and cultural implications of this exam modality); (iii) the low incidence of idiopathic RPL); and (iv) the length of follow-up required.

Second, we do not have information on some variables that have been hypothesized to influence the characteristics of the seminal fluid. Among these, in particular, occupational exposure, nutrition and alcohol consumption. The degree of air pollution exposure is not known either. However, as these couples are all coming from two large Italian cites (i.e., Rome and Milan), it is plausible that this variable does not differ between the study groups. Third, SDF was assessed, in all semen samples, using the TUNEL assay, which analyzes the presence of DNA fragmentation by linking labeled nucleotides at the DNA 3-OH free-ending. Despite the TUNEL assay being technically able to analyze double-strand breaks (DSB), the fact that a few DSB may be present in the sperm cell makes this method suboptimal for this assessment.[30,31] This could limit the reliability of our findings as DSB compared to single-strand breaks (SSB) have a more significant impact on reproductive outcomes including both fecundity and miscarriage. Studies analyzing the same semen sample with different techniques for the assessment of SDF are thus welcomed not only to test the reliability of each method but also to further clarify the role of DSBs in the pathophysiology of RPL.[30–32]

## 5 | CONCLUSIONS

In conclusion, our data demonstrated an association between an increased sperm DNA fragmentation and idiopathic recurrent pregnancy loss. Although our results are in agreement with previous evidence, to date, inferences regarding the causal relationship between the exposure to a high sperm DNA fragmentation level and recurrent pregnancy loss cannot be made. Adequately powered prospective studies aimed at assessing the impact of sperm DNA fragmentation on the risk of recurrent pregnancy loss regardless of the covariates are urgently needed not only to clarify the pathophysiological issue but also to lay the foundation for the long-awaited intervention trials.

### AUTHOR CONTRIBUTIONS

Andrea Busnelli, Giovanni Scambia, and Nicoletta Di Simone conceived and designed the study. Elena Di Credico, Andrea Garolla, Silvia D'Ippolito, and Anna Maria Merola selected patients and collected data. Anna Maria Merola formatted the dataset. Domenico Milardi, Andrea Garolla, and Alfredo Pontecorvi analyzed the semen samples. Andrea Busnelli performed the statistical analysis and drafted the first version of the manuscript. Andrea Busnelli and Elena Di Credico conceived and drafted figures and tables. Nicoletta Di Simone critically revised the first version of the manuscript. All authors approved the final version of the manuscript to be published.

### CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available because of privacy or ethical restrictions.

### ORCID

*Andrea Busnelli* https://orcid.org/0000-0001-9870-5241
*Andrea Garolla* https://orcid.org/0000-0003-4736-9051

### REFERENCES

1. ESHRE Guideline Group on RPL, Bender Atik R, Christiansen OB, et al. ESHRE guideline: recurrent pregnancy loss. *Hum Reprod Open*. 2018;2018:hoy004.
2. Dimitriadis E, Menkhorst E, Saito S, et al. Recurrent pregnancy loss. *Nat Rev Dis Primers*. 2022;6:98.
3. Practice Committee of the American Society for Reproductive Medicine. Definitions of infertility and recurrent pregnancy loss: a committee opinion. *Fertil Steril*. 2020;113:533-535.
4. Zegers-Hochschild F, Adamson GD, Dyer S, et al. The international glossary on infertility and fertility care. *Fertil Steril*. 2017;108:393-406.
5. Quenby S, Gallos ID, Dhillon-Smith RK, et al. Miscarriage matters: the epidemiological, physical, psychological, and economic costs of early pregnancy loss. *Lancet*. 2021;397:1658-1667.
6. Coomarasamy A, Williams H, Truchanowicz E, et al. A randomized trial of progesterone in women with recurrent miscarriages. *N Engl J Med*. 2015;373:2141-2148.
7. McQueen DB, Zhang J, Robins JC. Sperm DNA fragmentation and recurrent pregnancy loss: a systematic review and meta-analysis. *Fertil Steril*. 2019;112:54-60.e3.
8. Kamkar N, Ramezanali F, Sabbaghian M, et al. The relationship between sperm DNA fragmentation, free radicals and antioxidant capacity with idiopathic repeated pregnancy loss. *Reprod Biol*. 2018;18:330-335.
9. Robinson L, Gallos ID, Conner SJ, et al. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. *Hum Reprod*. 2012;27:2908-2917.
10. Dai Y, Liu J, Yuan E, et al. Relationship among traditional semen parameters, sperm DNA fragmentation, and unexplained recurrent miscarriage: a systematic review and meta-analysis. *Front Endocrinol*. 2022;12:802632.
11. Tan J, Taskin O, Albert A, et al. Association between sperm DNA fragmentation and idiopathic recurrent pregnancy loss: a systematic review and meta-analysis. *Reprod Biomed Online*. 2018;38:951-960.
12. Yifu P, Lei Y, Shaoming L, et al. Sperm DNA fragmentation index with unexplained recurrent spontaneous abortion: a systematic review and meta-analysis. *J Gynecol Obstet Hum Reprod*. 2020;26:101740.
13. Practice Committee of the American Society for Reproductive Medicine. Evaluation and treatment of recurrent pregnancy loss: a committee opinion. *Fertil Steril*. 2012;98:1103-1111.
14. Lin L. Bias caused by sampling error in meta-analysis with small sample sizes. *PLoS One*. 2018;13:e0204056.
15. Cocuzza MS, Tiseo BC, Srougi V, et al. Diagnostic accuracy of physical examination compared with color Doppler ultrasound in the determination of varicocoele diagnosis and grading: impact of urologists' experience. *Andrology*. 2020;8:1160-1166.
16. Dubin L, Amelar RD. Varicocoele size and results of varicocoelectomy in selected subfertile men with varicocoele. *Fertil Steril*. 1970;21:606-609.
17. Cooper TG, Noonan E, von Eckardstein S, et al. World Health Organization reference values for human semen characteristics. *Hum Reprod Update*. 2010;16:231-245.
18. Sharma R, Iovine C, Agarwal A, et al. TUNEL assay - standardized method for testing sperm DNA fragmentation. *Andrologia*. 2021;53:e13738.
19. Esteves SC, Zini A, Coward RM, et al. Sperm DNA fragmentation testing: summary evidence and clinical practice recommendations. *Andrologia*. 2021;53:e13874.
20. Agarwal A, Majzoub A, Baskaran S, et al. Sperm DNA fragmentation: a new guideline for clinicians. *World J Mens Health*. 2020;38:412-471.
21. Panner Selvam MK, Ambar RF, Agarwal A, et al. Etiologies of sperm DNA damage and its impact on male infertility. *Andrologia*. 2021;53:e13706.
22. Li J, Luo L, Diao J, et al. Male sperm quality and risk of recurrent spontaneous abortion in Chinese couples: a systematic review and meta-analysis. *Medicine*. 2021;100:e24828.
23. Zhang X, Wang H, Feng T, et al. The relationship between semen factors and unexplained recurrent spontaneous abortion. *Clin Chim Acta*. 2020;510:605-612.
24. Zidi-Jrah I, Hajlaoui A, Mougou-Zerelli S, et al. Relationship between sperm aneuploidy, sperm DNA integrity, chromatin packaging, traditional semen parameters, and recurrent pregnancy loss. *Fertil Steril*. 2016;105:58-64.
25. Spandorfer SD. Sperm DNA fragmentation: a need to fully address the impact on fertility outcomes. *Fertil Steril*. 2021;116:69-70.
26. Schlegel PN, Sigman M, Collura B, et al. Diagnosis and treatment of infertility in men: AUA/ASRM guideline part I. *Fertil Steril*. 2021;115:54-61.
27. Setti AS, Braga DPAF, Provenza RR, et al. Oocyte ability to repair sperm DNA fragmentation: the impact of maternal age on intracytoplasmic sperm injection outcomes. *Fertil Steril*. 2021;116:123-129.

28. Xie P, Cheung S, Kocur OM, et al. Sperm DNA fragmentation: what have we learned so far? *Fertil Steril*. 2021;116:1491.
29. Malić Vončina S, Stenqvist A, Bungum M, et al. Sperm DNA fragmentation index and cumulative live birth rate in a cohort of 2,713 couples undergoing assisted reproduction treatment. *Fertil Steril*. 2021;116:1483-1490.
30. Ribas-Maynou J, Benet J. Single and double strand sperm DNA damage: different reproductive effects on male fertility. *Genes (Basel)*. 2019;10:105.
31. Agarwal A, Barbăroșie C, Ambar R, et al. The impact of single- and double-strand DNA breaks in human spermatozoa on assisted reproduction. *Int J Mol Sci*. 2020;21:3882.
32. Hervás I, Pacheco A, Rivera-Egea R, et al. IVF/ICSI cumulative live birth rates per consumed oocyte remain comparable regardless of sperm DNA fragmentation by TUNEL. *Reprod Biomed Online*. 2022;44:1079-1089.

## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Busnelli A, Garolla A, Di Credico E, et al. Sperm DNA fragmentation and idiopathic recurrent pregnancy loss: Results from a multicenter case–control study. *Andrology*. 2023;11:1673–1681.
https://doi.org/10.1111/andr.13395