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SHORT COMMUNICATION

Evaluation of sperm DNA quality in men presenting with testicular cancer and lymphoma using alkaline and neutral Comet assays

Correspondence:
Csilla Krausz, Sexual Medicine and Andrology Unit, Department of Clinical and Experimental Biomedical Sciences "Mario Serio", University of Florence, Italy, Viale Pieraccini 6, 50139 Florence, Italy.
E-mail: c.krausz@dfc.unifi.it
and
Kishlay Kumar, Centre for Public Health, Queen's University Belfast, Grosvenor Road, Belfast BT126BJ, Northern Ireland, UK.
E-mail: kkumar01@qub.ac.uk
and
Sheena Lewis, Centre for Public Health, Queen's University Belfast, Grosvenor Road, Belfast BT126BJ, Northern Ireland, UK.
E-mail: S.E.Lewis@qub.ac.uk

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[1]K. Kumar, [1]S. Lewis, [2]S. Vinci, [2]A. Riera-Escamilla, [2]M.-G. Fino, [2]L. Tamburrino, [2]M. Muratori, [3]P. Larsen and [2]C. Krausz

[1]Centre for Public Health, Queen's University Belfast, Belfast, Northern Ireland, UK, [2]Sexual Medicine and Andrology Unit, Department of Clinical and Experimental Biomedical Sciences "Mario Serio", University of Florence, Florence, Italy, and [3]Cryos International, Aarhus, Denmark

SUMMARY

Despite more cancers in young men over the past two decades, improvements in therapies give a greater chance to live full lives following treatment. Sperm genomic quality is variable following cancer diagnosis, so its assessment is important if sperm cryopreservation is being considered. Here, we evaluated DNA damage using two DNA damage assays: an alkaline and for the first time, a neutral Comet assays in men presenting with testicular cancer (n = 19 for alkaline and 13 for neutral group) and lymphoma (n = 13 for alkaline and 09 for neutral group) compared with fertile donors (n = 20 for alkaline and 14 for neutral group). No significant differences were observed in any semen analysis parameters. In contrast, sperm DNA damage was higher in men with testicular cancer than in donors as assessed by both the alkaline (12.4% vs. 37.4%, $p < 0.001$) and neutral (7.5% vs. 13.4%; $p < 0.05$) Comet assays. Similar trends were observed in men with lymphoma. Here, sperm DNA damage was higher using both the alkaline (35.0% vs. 12.4%) and neutral (10.7% against 7.5% ($p < 0.05$) Comet assays. Moreover, the DNA strand breaks (particularly double-strand breaks) were significantly more prominent in men with cancer having abnormal seminal parameters than normozoospermic ones. This study showed that sperm DNA testing using alkaline and neutral Comet assays is more sensitive than semen analysis in detecting impaired sperm quality in men presenting with cancer. It may provide a useful adjunct when considering storage prior to cancer investigations and assisted reproductive techniques (ART)-based treatment.

INTRODUCTION

The question about adverse effects of cancer on spermatogenesis is still debated and has stimulated further research on both quantitative and qualitative sperm parameters in oncological patients (O'Flaherty *et al.*, 2008; Smit *et al.*, 2010; McDowell *et al.*, 2013; Paoli *et al.*, 2015). Cryopreservation is the only available preventive measure prior to cytotoxic therapies, and it allows the use of frozen/thawed spermatozoa for assisted reproductive techniques (ART). As the most important sperm characteristic for fathering a healthy child is good sperm DNA quality, it is important to determine the DNA quality of spermatozoa from men with cancer at the time of sperm cryopreservation. Damaged sperm DNA is negatively associated with early fertility checkpoints such as fertilization rate, embryo quality, implantation and positively with miscarriage [reviewed by (Robinson *et al.*, 2012)]. The majority of studies included in

this meta-analysis of miscarriage data were by SCSA and TUNEL were recruited. The quality of the paternal genome is also associated with the later checkpoints in offspring health (Lewis & Kumar, 2015). To date, however, there are conflicting reports on the effect of cancer on sperm DNA (O'Donovan, 2005; O'Flaherty *et al.*, 2008; Stahl *et al.*, 2009; Smit *et al.*, 2010; McDowell *et al.*, 2013; Paoli *et al.*, 2015). Here, a novel sperm DNA test specifically for double-stranded breaks (the neutral single cell gel electrophoresis Comet) is compared with the alkaline Comet detecting a combination of single- and double-strand breaks. The study was designed to evaluate whether both types of damage were induced by disease in men presenting with testicular cancer and lymphoma in comparison with fertile donors.

Each test has its benefits and limitations and measures a unique aspect of DNA damage. It is hypothesized that double-stranded damage may have more adverse consequences than single-strand breaks for later stages of fertility because the oocyte has less capability to repair it following fertilization but before the first cleavage (Alvarez, 2005). In most of the previous studies, single assay was used to analyse the sperm DNA damage level in men diagnosed with different types of cancer. In this study, we evaluated the level of both double and double plus single-stranded sperm DNA damage in men with reproductive and non-reproductive cancers.

MATERIALS AND METHODS

Study samples

Men with testicular cancer or lymphoma attending the Andrology Unit, Department of Clinical and Experimental Biomedical Sciences "Mario Serio", University of Florence were invited to participate in this study. The project was approved by local research ethics and clinical governance committees and written informed consent for participation was obtained from each subject. Men presenting with testicular cancer (n = 19) or lymphoma (n = 13) with normal or abnormal semen parameters prior to cytotoxic therapy and 20 fertile donors (Cryos International, Denmark) were included in this study. Men with azoospermia were excluded from the study. All fertile controls had normal semen by WHO (2010) criteria, and none had any history of infertility. All semen samples were obtained after 2–7 days of sexual abstinence.

Semen and sperm DNA fragmentation (damage) analysis

All semen samples were examined for liquefaction time, pH, semen volume, sperm concentration, sperm morphology, sperm motility, according to World Health Organisation guidelines (WHO, 2010). After collection of semen immediately, it was incubated at 37 °C for 30–60 min for complete liquefaction. All the seminal parameters were evaluated once the samples were completely liquefied. All semen samples were cryopreserved for the later analysis of DNA damage. Rapid cryopreservation procedures were used to freeze the spermatozoa from both fertile donors and men presenting with cancer. Briefly, the sperm freezing media were slowly mixed (dropwise) with the semen and left for incubation for 10 min at room temperature. Then the mixture was transferred to straws or cryovials and equilibrated horizontally above liquid nitrogen vapour for 30 min. Finally, the cryovials/straws were plunged into liquid nitrogen and transferred to storage tank until further use. Unless otherwise stated, all the reagents were purchased from Sigma-Aldrich, England, UK.

Alkaline Comet assay

Sperm DNA fragmentation was assessed using an alkaline single cell gel electrophoresis assay as reported previously (Hughes *et al.*, 1997; Donnelly *et al.*, 1999). Briefly, the semen sample concentration was adjusted to 2×10^6/mL in PBS. Fully frosted slides (Surgipath Europe, UK) were layered with 150 μL of 1% normal melting agarose (NMA) and immediately covered with a coverslip. Once the NMA had solidified, the coverslip was removed and immediately layered with a mixture of 10 μL of diluted sample (2×10^6/mL in PBS) and 75 μL of 0.5% low melting agarose (LMA). The slides were quickly covered with a coverslip and allowed to solidify at room temperature. Once LMA solidified, the coverslip was removed and slides were immersed in a coplin jar containing lysis solution (2.5M NaCl, 100 mM Na_2EDTA and 10 mM Tris-HCl, pH 10) with 1% Triton X-100, for 1 h at 4 °C. Slides were further incubated for 30 min at 4 °C with dithiothreitol (10 mM) followed by 90 min incubation at room temperature with lithium diiodosalicyclate (4 mM) to decondense the DNA. Slides were then incubated with cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA) for 20 min to unwind the DNA. The slides were further subjected to electrophoresis using cold alkaline electrophoresis buffer for 10 min at 25 V, with the current adjusted to 300 mA. Then slides were removed from electrophoresis tank and were neutralized in neutralization solution (0.4 M Tris-HCl, pH 7.5). Finally, slides were stained with 30 μL of 20 mg/mL ethidium bromide and analysed immediately. At least 50 Comet images were analysed using image analysis software (Komet 6, Andor Technology, Belfast, UK), and the results were expressed in percentage tail DNA.

Neutral Comet assay

Double-stranded sperm DNA fragmentation was assessed using single cell gel electrophoresis (Neutral Comet) assay. To establish the efficient neutral Comet assays experimental conditions, the procedures described by Ribas-Maynou *et al.* (2014) were applied with modifications in the initial lysis steps and electrophoresis optimized in the first author's laboratory. The initial preparation of slides with agarose and sample was similar to the alkaline assay as stated above. Once the slide completely solidified with agarose, slides were then treated with 1% Triton X-100 solution for 30 min at room temperature (RT). Next, slides were washed in 0.9% NaCl solution three times for 5 min and then washed two times for 5 min in PBS. Control slides were treated with 15UI Alu I restriction enzyme for 30 min at 37 °C. All slides were then incubated for 30 min at RT in lysis buffer I (TRIS-HCl 0.4 M, DTT 0.8 M, SDS 1%, pH 7.5), followed by 30 min at RT in lysis buffer II (TRIS-HCl 0.4 M, NaCl 2 M, DTT 0.4 M, Na_2EDTA 50 mM, pH 7.5), followed by 30 min at RT in lysis buffer III (TRIS-HCl 0.4 M, SDS 1%, DTT 0.8 mM, pH 7.5) in the fume hood. Following this, slides were rinsed in cold TBE electrophoresis buffer (TRIS-HCl 0.445 M, Boric acid 0.445 M, 10 mM EDTA, pH 7.5) for 10 min. Electrophoresis was carried out in a cold room with cold TBE electrophoresis buffer. Slides were submerged, and electrophoresis was run at 20 V (1 V/cm) for 8 min. Following electrophoresis, slides were rinsed in 0.9% NaCl and stained with 30 μL of 20 μL/mL of ethidium bromide. Fifty Comets per slide were scored with KOMET 6 software (Komet 6, Andor Technology).

Table 1 Semen parameters in men at the time of cancer diagnosis compared to WHO reference limits for fertile men

Semen parameters	[a]WHO 2010 lower reference limits	Men with lymphoma (n = 13)	Men with testicular cancer (n = 19)	Mann–Whitney U-test
Sperm concentration (10^6/mL)	15 (12–16)	44.7 (9.0–80.0, 51.0)	24.0 (3.20–82.0, 25.0)	p = 0.099
Total sperm number	39 (33–46)	119.7 (20.37–336.0, 158.3)	38.0 (5.76–246.0, 100.2)	p = 0.120
Motility (PR, %)	32 (31–34)	63.0 (6.0–73.0, 22.5)	59.0 (3.0–83.0, 48.0)	p = 0.631
Morphology (%)	4 (3.0–4.0)	4.0 (0–8.0, 3.5)	4.0 (1.0–10.0, 3.0)	p = 0.684

[a]Lower reference limits (5th centiles and their 95% confidence intervals) for WHO semen parameters values; All values in patient groups are median (minimum–maximum, interquartile range.)

Statistical analysis was performed using the Statistics Package for the Social Sciences software, version 21 (SPSS Inc., Chicago, IL, USA). Comparisons of seminal parameters between (testicular cancer and lymphoma) men with cancer were assessed using the non-parametric Mann–Whitney U-test as the data were not normally distributed. Similarly, Mann–Whitney U-test was also used for the comparisons of DNA damage between men with testicular cancer and lymphoma and healthy donors. Data presented correspond to the median (minimum–maximum) and interquartile range. For all statistical analysis, $p < 0.05$ was considered significant.

RESULTS

Semen parameters in men with testicular cancer and lymphoma

All median semen parameters of both groups were above the WHO (2010) cut-off values with no significant differences were found in any conventional semen parameters between the two cancer groups (Table 1). However, abnormal semen parameters were present in 63% and 46% of men with testicular cancer and lymphoma respectively. All fertile donors had high semen quality with all sperm parameters above WHO (2010) lower reference limits.

Comparison of sperm DNA fragmentation between men presenting with testicular cancer, lymphoma and fertile donors

In contrast to seminal parameters, sperm DNA fragmentation was significantly higher in men with testicular cancer than in the control group as assessed by both the neutral (n = 13) (7.5% vs. 13.4%; $p < 0.05$) and alkaline (n = 13) (12.4% vs. 37.4%, $p < 0.001$, Table 2) Comet assays. Similar results were obtained for men presenting with lymphoma. Sperm DNA fragmentation (a combination of both double and single strands) of 35.0% against 12.4% in donor group ($p < 0.001$ using the alkaline Comet), whereas DNA fragmentation of 10.7% against 7.5 ($p < 0.05$; using the neutral Comet) (Table 2). Marked increases of 78% and 42% in double-strand breaks were observed in spermatozoa from men with testicular cancer and lymphoma, respectively.

Moreover, a comparison of double plus single- and double-stranded DNA damage only from men with each type of cancer subdivided into those with normal and abnormal semen parameters was also performed. DNA fragmentation level detected by Comet assays tended to be higher in those with abnormal semen parameters group compared to cancer men with normal semen.

In men presenting with testicular cancer, both those with normal and abnormal semen had high levels of single- and double-stranded DNA damage. In contrast, only those men with abnormal semen had high levels of double-stranded damage, in fact, it was 1.5 times higher than in those with normal semen and double than donor group (14.12% vs. 9.61&7.5%). Those with normal semen had levels of double-stranded breaks similar to the fertile donors (Table 3).

Also, in men presenting with lymphoma, the trends were similar. There was little difference with sperm DNA fragmentation levels of 34.81% and 36.05% being observed in normal and abnormal semen parameters groups, respectively, as measured by alkaline Comet assay. In contrast, the double-stranded damage was double in the men with abnormal semen compared to those with normal semen and also to donors (19.11% vs. 9.83 and 7.5%) (Table 3).

DISCUSSION

Data in the literature are conflicting as to the quality of semen, as assessed by a conventional semen analysis, in men presenting with cancer (Rives *et al.*, 2012; McDowell *et al.*, 2013; Caponecchia *et al.*, 2016). In this study, we found no significant reductions in sperm concentrations, motility or morphology in men

Table 2 Comparison of sperm DNA damage using alkaline and neutral Comet assays in men with testicular cancer and lymphoma

DNA damage assays	Men with lymphoma vs. donors sperm DNA damage (%)				Men with testicular cancer vs. donors sperm DNA damage (%)			
	Donors	DNA damage (%)	Percentage difference (%)	Mann–Whitney U-test	Donors	DNA damage (%)	Percentage difference (%)	Mann–Whitney U-test
Alkaline Comet assay	12.4 (5.3–18.8, 7.2) (n = 20)	35.0 (17.93–53.34,8.65) (n = 13)	+182	$p < 0.001$	12.4 (5.3–18.8, 7.2) (n = 20)	37.4 (17.42–49.42, 12.8) (n = 19)	+201	$p < 0.001$
Neutral Comet assay	7.5 (5.9–15.1, 3.7) (n = 14)	10.7 (7.9–37.1,9.3) (n = 09)	+42	p = 0.012	7.5 (5.9–15.1, 3.7) (n = 14)	13.4 (4.3–19.6, 5.7) (n = 13)	+78	p = 0.010

All values are median = /-minimum–maximum and interquartile range. Mann–Whitney U (M–W test)-test was used to test significance at $p < 0.05$.

Table 3 Comparison of sperm DNA fragmentation in cancer men with normal and abnormal semen parameters

Assays	Fertile donors	Men with lymphoma		M–W test (Control vs. NS)	M–W test (Control vs. AS)
	Donors	Normal semen (NS)	Abnormal semen (AS)		
Alkaline Comet	12.4 (5.3–18.8, 7.2)[a] (n = 20)	34.81 (17.93–45.86, 9.70)[b] (n = 07)	36.05 (33.91–53.34, 10.98)[c] (n = 06)	$^{avsb}p < 0.001$	$^{avsc}p < 0.001$
Neutral Comet	7.5 (5.9–15.1, 3.7)[d] (n = 14)	9.83 (8.14–16.14, 6.43)[e] (n = 04)	19.11 (10.61–37.12, 14.47)[f] (n = 05)	$^{dvse}p = 0.137$	$^{dvsf}p = 0.003$
Assays	Fertile donors	Men with testicular cancer		M–W Test (Control vs. NS)	M–W Test (Control vs. AS)
	Donors	Normal semen(NS)	Abnormal semen (AS)		
Alkaline Comet	12.4 (5.3–18.8, 7.2)[g] (n = 20)	39.06 (19.45–49.42, 17.46)[h] (n = 07)	37.28 (17.42–49.31, 12.50)[i] (n = 12)	$^{gvsh}p < 0.001$	$^{gvsi}p < 0.001$
Neutral Comet	7.5 (5.9–15.1, 3.7)[j] (n = 14)	9.61 (4.30–16.20, 6.73)[k] (n = 05)	14.16 (9.97–19.6, 3.50)[l] (n = 08)	$^{kvsm}p = 0.405$	$^{kvsn}p = 0.003$

All values are median = /-minimum–maximum and interquartile range. M–W Test (Mann–Whitney U-test) was used to test significance at $p < 0.05$.
[a]Median alkaline assay sperm DNA fragmentation level in Fertile donors.
[b]Median alkaline sperm DNA fragmentation level in men with lympohoma having normal semen parameters.
[c]Median alkaline sperm DNA fragmentation level in men with lympohoma having abnormal semen parameters.
[d]Median neutral assay sperm DNA fragmentation level in Fertile donors.
[e]Median neutral sperm DNA fragmentation level in men with lympohoma having normal semen parameters.
[f]Median neutral sperm DNA fragmentation level in men with lympohoma having abnormal semen parameters.
[g]Median alkaline assay sperm DNA fragmentation level in Fertile donors.
[h]Median alkaline sperm DNA fragmentation level in men with testicular cancer having normal semen parameters.
[i]Median alkaline sperm DNA fragmentation level in men with testicular cancer having abnormal semen parameters.
[j]Median neutral assay sperm DNA fragmentation level in Fertile donors.
[k]Median neutral sperm DNA fragmentation level in men with testicular cancer having normal semen parameters.
[l]Median neutral sperm DNA fragmentation level in men with testicular cancer having abnormal semen parameters.

with either form of cancer compared to WHO lower reference limit for fertile men (WHO, 2010). However, in agreement with other larger studies, we observed a trend towards poorer seminal values in men with testicular cancer compared to men presenting with lymphoma (Williams *et al.*, 2009; Caponecchia *et al.*, 2016). Furthermore, men with abnormal seminal parameters in testicular cancer and lymphoma were oligozoospermic (OZ), oligoasthenozoospermic (OAZ), oligoteratozoospermic (OTZ), oligoasthenoteratozoospermic (OATZ), asthenoteratozoospermic (ATZ) and teratozoospermic (TZ) wherein OZ, OAZ, OTZ, OATZ and ATZ were common in both types of cancer as observed by previous studies (Chung *et al.*, 2004; Djaladat *et al.*, 2014). In this study, semen parameters fell within the normozoospermic category in 37% and 54% men with testicular cancer and lymphoma, respectively.

The literature is also contradictory concerning sperm DNA fragmentation, reporting both significantly higher (Gandini *et al.*, 2000; O'Flaherty *et al.*, 2008, 2012; Stahl *et al.*, 2009) and similar (Ribeiro *et al.*, 2008; Smit *et al.*, 2010; McDowell *et al.*, 2013) DNA quality in men presenting with cancer to healthy men. In our study, by performing alkaline and neutral Comet assays, we observed high levels of both single and double DNA strand breaks in men presenting with cancer indicating a potential adverse genomic effects of this disease. The Comet assay has a higher sensitivity than other assays, and it can provide more information about the extent and heterogeneity of DNA (Godard *et al.*, 1999; Kindzelskii & Petty, 2002; Simon *et al.*, 2014). The ability of the Comet to detect degrees of DNA damage in individual spermatozoa rather than an overall percentage of damaged spermatozoa in a semen sample makes it more suitable than other assay as a diagnostic test in this context. The Comet assay under alkaline conditions (pH ≥13) detects both single- and double-strand breaks while under neutral conditions it detects only double-strand breaks (DSBs) (Olive *et al.*, 1991; Collins, 2002). A further advantage is that unlike some other tests that detect primarily breaks in histone-associated chromatin, the Comet assay has a greater capacity to detect DNA damage because the Comet procedure removes all nucleoproteins revealing breaks in DNA associated with both protamine- and histone-bound chromatin. In addition, Comet assay detects real than potential damage and our research group and others have reported that sperm DNA damage in native and density centrifuged spermatozoa measured by the alkaline Comet assay has been significantly associated with ART outcomes (Morris *et al.*, 2002; Simon *et al.*, 2013). In men with testicular cancer, O'Donovan (2005)reported a significant difference of Comet head DNA integrity between cancer patients and controls (49.87 vs. 86.91%). Similarly, O'Flaherty *et al.* (2008) reported higher levels of sperm DNA damage (comet tail extent moment) in men presenting with testicular cancer (≈12.0 vs. 30.0) and Hodgkin's lymphoma (≈12.0 vs. 25). Likewise, using the alkaline Comet assay in this study, we report three times more DNA damage in men with testicular cancer and lymphoma compared to donor groups.

Also, an increased sensitivity of the neutral Comet assay with respect to other assays have been demonstrated in somatic cells (Yasuhara *et al.*, 2003). The neutral version of the Comet is the latest DNA test to be added to the range of DNA tests currently being used in ART. Its major advantage is that, unlike any of the other tests, it detects double-stranded breaks exclusively. Although the number of studies on neutral Comet assays is limited, few studies showed that DNA damage detected by neutral Comet assay could prove to be good prognosis in cases of male infertility and male partners of unexplained recurrent abortion couples with no detectable female factors (Ribas-Maynou *et al.*, 2012a,b; Zhong *et al.*, 2015). Indeed, sperm double-strand DNA breaks fragmentation examined by the neutral Comet assay significantly correlated with sperm nuclear vacuoles, an important parameter for sperm selection for ART treatment (Pastuszek *et al.*, 2017). Moreover, sperm DNA fragmentation assessed by this assay associated with chromosome rearrangements causing aneuploidy in the fertilized embryos (Ramos *et al.*, 2015). Under neutral

conditions, here we report a significant difference in DNA double-strand breaks compared to fertile donors in men presenting with testicular cancer and lymphoma. Although some of the differences observed between donors and disease samples from men presenting with cancer may be due to inherent variability, of human of sperm quality, the marked differences suggest a specific contribution from the disease *per se*. Here, significantly higher double-strand breaks were observed in both groups of men with cancer compared with healthy donors, but double-strand breaks were more prominent in men with testicular cancer than in those affected by lymphoma suggesting a potentially stronger association of the reproductive testicular cancer with sperm chromatin quality than non-reproductive cancer. Interestingly, in the current study, as *double-strand breaks* were more prominent in men with *abnormal semen* in both types of *cancer*, a potential association that needs further investigation.

Moreover, fatherhood in cancer survivals is conflicting in the literature reporting both high (Brydoy *et al.*, 2005) and low paternity rate (Saxman, 2005). Knowing the detrimental effect of the cancer itself (especially in the case of testicular cancer) and the effect of cytotoxic treatments, men with cancer should be guided to preserve their reproductive potential through sperm cryopreservation. To date, this often results in men with oncological disease becoming fathers through ART. Double-strand DNA damage could be a predictor for successful ART outcomes in cancer survivors as proposed recently for infertility treatment (Garolla *et al.*, 2015). It is important clinically as the oocyte may have more difficulty locating and repairing double-strand breaks post-fertilization possibly leading to genomic instability in the developing embryo. Hence, clinicians undergoing subspeciality training in andrology should be taught about preventive procedures and monitoring of sperm DNA integrity in men presenting with cancer before and after cytotoxic treatments (Krausz *et al.*, 2015).

In summary, the characterization of single- and double-strand DNA breaks in spermatozoa has provided additional important data to indicate that sperm DNA is already damaged at diagnosis, irrespective of cancer treatment. Double- and single-strand breaks are common in both types of cancer and detected at higher levels in men who also have abnormal semen. Thus, the Comet assays provide the basis of potential tests for screening out samples that may be unsuitable for future clinical use. Sperm DNA damage is a useful bio-marker when considering storage prior to treatment and thus its future use for ART.

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AUTHORS' CONTRIBUTIONS

KK, SL and CK designed the research study. KK performed experimental work, acquisition of data, data analysis and wrote the manuscript. SV and ARE contributed to support of the experimental work and interpretation. MGF, LT and MM were involved in the recruitment of study subjects and data acquisition of the recruited subjects. CK and PL provided study material of the patients and controls, respectively. All authors critically reviewed the manuscript and approved the final version.

DISCLOSURES

SL is a director in SpermComet Ltd; a university spin out company. The other authors declare no conflict of interest.

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