

SHORT COMMUNICATION

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

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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 μ L 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 μ L of diluted sample (2×10^6 /mL in PBS) and 75 μ L 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 Na₂EDTA 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 diiodosalicylate (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 μ L 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, Na₂EDTA 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 μ L of 20 μ L/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 (<i>n</i> = 13)	Men with testicular cancer (<i>n</i> = 19)	Mann–Whitney <i>U</i> -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)	<i>p</i> = 0.099
Total sperm number	39 (33–46)	119.7 (20.37–336.0, 158.3)	38.0 (5.76–246.0, 100.2)	<i>p</i> = 0.120
Motility (PR, %)	32 (31–34)	63.0 (6.0–73.0, 22.5)	59.0 (3.0–83.0, 48.0)	<i>p</i> = 0.631
Morphology (%)	4 (3.0–4.0)	4.0 (0–8.0, 3.5)	4.0 (1.0–10.0, 3.0)	<i>p</i> = 0.684

^aLower 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 <i>U</i> -test	Donors	DNA damage (%)	Percentage difference (%)	Mann–Whitney <i>U</i> -test
Alkaline Comet assay	12.4 (5.3–18.8, 7.2) (<i>n</i> = 20)	35.0 (17.93–53.34, 8.65) (<i>n</i> = 13)	+182	<i>p</i> < 0.001	12.4 (5.3–18.8, 7.2) (<i>n</i> = 20)	37.4 (17.42–49.42, 12.8) (<i>n</i> = 19)	+201	<i>p</i> < 0.001
Neutral Comet assay	7.5 (5.9–15.1, 3.7) (<i>n</i> = 14)	10.7 (7.9–37.1, 9.3) (<i>n</i> = 09)	+42	<i>p</i> = 0.012	7.5 (5.9–15.1, 3.7) (<i>n</i> = 14)	13.4 (4.3–19.6, 5.7) (<i>n</i> = 13)	+78	<i>p</i> = 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 Donors	Men with lymphoma		M–W test (Control vs. NS)	M–W test (Control vs. AS)
		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	^{dvst} p = 0.003
Assays	Fertile donors Donors	Men with testicular cancer		M–W Test (Control vs. NS)	M–W Test (Control vs. AS)
		Normal semen(NS)	Abnormal semen (AS)		
Alkaline Comet	12.4 (5.3–18.8, 7.2) ^a (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	^{gvst} p < 0.001
Neutral Comet	7.5 (5.9–15.1, 3.7) ^d (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	^{kvst} 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 $p < 0.05$.

^aMedian alkaline assay sperm DNA fragmentation level in Fertile donors.

^bMedian alkaline sperm DNA fragmentation level in men with lymphoma having normal semen parameters.

^cMedian alkaline sperm DNA fragmentation level in men with lymphoma having abnormal semen parameters.

^dMedian neutral assay sperm DNA fragmentation level in Fertile donors.

^eMedian neutral sperm DNA fragmentation level in men with lymphoma having normal semen parameters.

^fMedian neutral sperm DNA fragmentation level in men with lymphoma having abnormal semen parameters.

^aMedian alkaline assay sperm DNA fragmentation level in Fertile donors.

^hMedian alkaline sperm DNA fragmentation level in men with testicular cancer having normal semen parameters.

ⁱMedian alkaline sperm DNA fragmentation level in men with testicular cancer having abnormal semen parameters.

^dMedian neutral assay sperm DNA fragmentation level in Fertile donors.

^kMedian neutral sperm DNA fragmentation level in men with testicular cancer having normal semen parameters.

^lMedian 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 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 survivors 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 subspecialty 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.

REFERENCES

- Alvarez JG. (2005) The predictive value of sperm chromatin structure assay. *Hum Reprod* 20, 2365–2367.
- Brydoy M, Fossa SD, Klepp O, Bremnes RM, Wist EA, Wentzel-Larsen T & Dahl O. (2005) Paternity following treatment for testicular cancer. *J Natl Cancer Inst* 97, 1580–1588.
- Caponecchia L, Cimino G, Sacchetto R, Fiori C, Sebastianelli A, Salacone P, Marcucci I, Tomassini S & Rago R. (2016) Do malignant diseases affect semen quality? Sperm parameters of men with cancers. *Andrologia* 48, 333–340.
- Chung K, Irani J, Knee G, Efymow B, Blasco L & Patrizio P. (2004) Sperm cryopreservation for male patients with cancer: an epidemiological analysis at the University of Pennsylvania. *Eur J Obstet Gynecol Reprod Biol* 113(Suppl 1), S7–S11.
- Collins AR. (2002) The comet assay. Principles, applications, and limitations. *Methods Mol Biol* 203, 163–177.
- Djaladat H, Burner E, Parikh PM, Beroukhim Kay D & Hays K. (2014) The association between testis cancer and semen abnormalities before orchiectomy: a systematic review. *J Adolesc Young Adult Oncol* 3, 153–159.
- Donnelly ET, McClure N & Lewis SE. (1999) The effect of ascorbate and alpha-tocopherol supplementation in vitro on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. *Mutagenesis* 14, 505–512.
- Gandini L, Lombardo F, Paoli D, Caponecchia L, Familiari G, Verlengia C, Dondero F & Lenzi A. (2000) Study of apoptotic DNA fragmentation in human spermatozoa. *Hum Reprod* 15, 830–839.
- Garolla A, Cosci I, Bertoldo A, Sartini B, Boudjema E & Foresta C. (2015) DNA double strand breaks in human spermatozoa can be predictive for assisted reproductive outcome. *Reprod Biomed Online* 31, 100–107.
- Godard T, Deslandes E, Lebailly P, Vigreux C, Sichel F, Poul JM & Gauduchon P. (1999) Early detection of staurosporine-induced apoptosis by comet and annexin V assays. *Histochem Cell Biol* 112, 155–161.
- Hughes CM, Lewis SE, McKelvey-Martin VJ & Thompson W. (1997) Reproducibility of human sperm DNA measurements using the alkaline single cell gel electrophoresis assay. *Mutat Res* 374, 261–268.
- Kindzelskii AL & Petty HR. (2002) Ultrasensitive detection of DNA damage by the combination of the comet and TUNEL assays. *Methods Mol Biol* 203, 195–201.
- Krausz C, Brannigan RE & Sigman M. (2015) Subspecialty training in andrology. *Fertil Steril* 104, 12–15.
- Lewis SE & Kumar K. (2015) The paternal genome and the health of the assisted reproductive technology child. *Asian J Androl* 17, 616–622.
- McDowell S, Harrison K, Kroon B, Ford E & Yazdani A. (2013) Sperm DNA fragmentation in men with malignancy. *Fertil Steril* 99, 1862–1866.
- Morris ID, Ilott S, Dixon L & Brison DR. (2002) The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. *Hum Reprod* 17, 990–998.
- O'Donovan M. (2005) An evaluation of chromatin condensation and DNA integrity in the spermatozoa of men with cancer before and after therapy. *Andrologia* 37, 83–90.

- O'Flaherty C, Vaisheva F, Hales BF, Chan P & Robaire B. (2008) Characterization of sperm chromatin quality in testicular cancer and Hodgkin's lymphoma patients prior to chemotherapy. *Hum Reprod* 23, 1044–1052.
- O'Flaherty CM, Chan PT, Hales BF & Robaire B. (2012) Sperm chromatin structure components are differentially repaired in cancer survivors. *J Androl* 33, 629–636.
- Olive PL, Wlodek D & Banath JP. (1991) DNA double-strand breaks measured in individual cells subjected to gel electrophoresis. *Cancer Res* 51, 4671–4676.
- Paoli D, Gallo M, Rizzo F, Spano M, Leter G, Lombardo F, Lenzi A & Gandini L. (2015) Testicular cancer and sperm DNA damage: short- and long-term effects of antineoplastic treatment. *Andrology* 3, 122–128.
- Pastuszek E, Kiewisz J, Skowronska P, Liss J, Lukaszuk M, Bruszczyńska A, Jakiel G & Lukaszuk K. (2017) An investigation of the potential effect of sperm nuclear vacuoles in human spermatozoa on DNA fragmentation using a neutral and alkaline Comet assay. *Andrology* 5, 392–398.
- Ramos L, Daina G, Del Rey J, Ribas-Maynou J, Fernandez-Encinas A, Martinez-Passarell O, Boada M, Benet J & Navarro J. (2015) Comprehensive preimplantation genetic screening and sperm deoxyribonucleic acid fragmentation from three males carrying balanced chromosome rearrangements. *Fertil Steril* 104, 681–687. e2.
- Ribas-Maynou J, Garcia-Peiro A, Abad C, Amengual MJ, Navarro J & Benet J. (2012a) Alkaline and neutral Comet assay profiles of sperm DNA damage in clinical groups. *Hum Reprod* 27, 652–658.
- Ribas-Maynou J, Garcia-Peiro A, Fernandez-Encinas A, Amengual MJ, Prada E, Cortes P, Navarro J & Benet J. (2012b) Double stranded sperm DNA breaks, measured by Comet assay, are associated with unexplained recurrent miscarriage in couples without a female factor. *PLoS ONE* 7, e44679.
- Ribas-Maynou J, Gawecka JE, Benet J & Ward WS. (2014) Double-stranded DNA breaks hidden in the neutral Comet assay suggest a role of the sperm nuclear matrix in DNA integrity maintenance. *Mol Hum Reprod* 20, 330–340.
- Ribeiro TM, Bertolla RP, Spaine DM, Fraietta R, Ortiz V & Cedenho AP. (2008) Sperm nuclear apoptotic DNA fragmentation in men with testicular cancer. *Fertil Steril* 90, 1782–1786.
- Rives N, Perdrix A, Hennebicq S, Saias-Magnan J, Melin MC, Berthaut I, Barthelemy C, Daudin M, Szerman E, Bresson JL, Brugnon F & Bujan L. (2012) The semen quality of 1158 men with testicular cancer at the time of cryopreservation: results of the French National CECOS Network. *J Androl* 33, 1394–1401.
- Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S, Kirkman-Brown J & Coomarasamy A. (2012) The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. *Hum Reprod* 27, 2908–2917.
- Saxman S. (2005) Doctor... will I still be able to have children? *J Natl Cancer Inst* 97, 1557–1559.
- Simon L, Proutski I, Stevenson M, Jennings D, McManus J, Lutton D & Lewis SE. (2013) Sperm DNA damage has a negative association with live-birth rates after IVF. *Reprod Biomed Online* 26, 68–78.
- Simon L, Liu L, Murphy K, Ge S, Hotaling J, Aston KI, Emery B & Carrell DT. (2014) Comparative analysis of three sperm DNA damage assays and sperm nuclear protein content in couples undergoing assisted reproduction treatment. *Hum Reprod* 29, 904–917.
- Smit M, van Casteren NJ, Wildhagen MF, Romijn JC & Dohle GR. (2010) Sperm DNA integrity in cancer patients before and after cytotoxic treatment. *Hum Reprod* 25, 1877–1883.
- Stahl O, Eberhard J, Cavallin-Stahl E, Jepson K, Friberg B, Tingsmark C, Spano M & Giwercman A. (2009) Sperm DNA integrity in cancer patients: the effect of disease and treatment. *Int J Androl* 32, 695–703.
- WHO. (2010) *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction*, 5th edn. WHO, Geneva, Switzerland.
- Williams D, Karpman E, Sander JC, Spiess PE, Pisters LL & Lipshultz LI. (2009) Pretreatment semen parameters in men with cancer. *J Urol* 181, 736–740.
- Yasuhara S, Zhu Y, Matsui T, Tipirneni N, Yasuhara Y, Kaneki M, Rosenzweig A & Martyn JA. (2003) Comparison of comet assay, electron microscopy, and flow cytometry for detection of apoptosis. *J Histochem Cytochem* 51, 873–885.
- Zhong H-z, Lv F-t, Deng X-l, Hu Y, Xie D-n, Lin B, Mo Z-n & Lin F-q. (2015) Evaluating γ H2AX in spermatozoa from male infertility patients. *Fertil Steril* 104, 574–581.