Human Reproduction, Vol.29, No.10 pp. 2148–2155, 2014
Advanced Access publication on July 27, 2014 doi:10.1093/humrep/deu177

human reproduction

ORIGINAL ARTICLE *Andrology*

Human sperm sex chromosome disomy and sperm DNA damage assessed by the neutral comet assay

M.E. McAuliffe[1,2], P.L. Williams[3], S.A. Korrick[1,4], R. Dadd[1], F. Marchetti[5], S.E. Martenies[6], and M.J. Perry[6,*]

[1]Department of Environmental Health, Harvard School of Public Health, Boston, MA 02115, USA [2]Millennium: The Takeda Oncology Company, Cambridge, MA 02139, USA [3]Department of Biostatistics, Harvard School of Public Health, Boston, MA 02115, USA [4]Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA [5]Environmental Health Science Research Bureau, Health Canada, Ottawa, ON K1A 0K9, Canada [6]Department of Environmental and Occupational Health, Milken Institute School of Public Health, The George Washington University, Washington, DC 20052, USA

*Correspondence address. Department of Environmental and Occupational Health, Milken Institute School of Public Health, The George Washington University, 950 New Hampshire Ave NW Suite 400, Washington, DC 20052, USA. Tel: +1-202-994-1734; Fax: +1-202-994-0011; E-mail: mperry@gwu.edu

Submitted on June 13, 2013; resubmitted on May 20, 2014; accepted on June 24, 2014

STUDY QUESTION: Is there an association between human sperm sex chromosome disomy and sperm DNA damage?

SUMMARY ANSWER: An increase in human sperm XY disomy was associated with higher comet extent; however, there was no other consistent association of sex chromosome disomies with DNA damage.

WHAT IS KNOWN ALREADY: There is limited published research on the association between sex chromosome disomy and sperm DNA damage and the findings are not consistent across studies.

STUDY DESIGN, SIZE, AND DURATION: We conducted a cross-sectional study of 190 men (25% ever smoker, 75% never smoker) from subfertile couples presenting at the Massachusetts General Hospital Fertility Clinic from January 2000 to May 2003.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Multiprobe fluorescence *in situ* hybridization for chromosomes X, Y and 18 was used to determine XX, YY, XY and total sex chromosome disomy in sperm nuclei using an automated scoring method. The neutral comet assay was used to measure sperm DNA damage, as reflected by comet extent, percentage DNA in the comet tail, and tail distributed moment. Univariate and multiple linear regression models were constructed with sex chromosome disomy (separate models for each of the four disomic conditions) as the independent variable, and DNA damage parameters (separate models for each measure of DNA damage) as the dependent variable.

MAIN RESULTS AND THE ROLE OF CHANCE: Men with current or past smoking history had significantly greater comet extent (μm: regression coefficients with 95% CI) [XX18: 15.17 (1.98, 28.36); YY18: 14.68 (1.50, 27.86); XY18: 15.41 (2.37, 28.45); Total Sex Chromosome Disomy: 15.23 (2.09, 28.38)], and tail distributed moment [XX18: 3.01 (0.30, 5.72); YY18: 2.95 (0.24, 5.67); XY18: 3.04 (0.36, 5.72); Total Sex Chromosome Disomy: 3.10 (0.31, 5.71)] than men who had never smoked. In regression models adjusted for age and smoking, there was a positive association between XY disomy and comet extent. For an increase in XY disomy from 0.56 to 1.47% (representing the 25th to 75th percentile), there was a mean increase of 5.08 μm in comet extent. No other statistically significant findings were observed.

LIMITATIONS, REASONS FOR CAUTION: A potential limitation of this study is that it is cross-sectional. Cross-sectional analyses by nature do not lend themselves to inference about directionality for any observed associations; therefore we cannot determine which variable is the cause and which one is the effect. A small sample size may be a further limitation. Comparison of these findings to other studies is limited due to methodological differences.

WIDER IMPLICATIONS OF THE FINDINGS: Although consistent associations across sex chromosome disomies or DNA damage measures were not observed, this study highlights the need to explore etiologies of sperm DNA damage and sex chromosome disomy to better understand the potential mechanistic overlaps between the two.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by NIOSH Grant T42 OH008416, and NIH/NIEHS Grants ES 009718, ES 000002, and R01 ES017457. During the study M.E.M. was affiliated with the Department of Environmental Health at the Harvard School of Public Health.

TRIAL REGISTRATION NUMBER: N/A.

Key words: fluorescence *in situ* hybridization / aneuploidy / semen / DNA damage / neutral comet assay

Introduction

Spermatogenesis involves mitotic and meiotic divisions that differentiate germ cells, transforming them from spermatogonia to mature spermatozoa. Recent research has shown that apoptosis might be the mechanism responsible for tempering cell proliferation in the testes, as well as normal spermatogenesis (Liu *et al.*, 2004; Perrin *et al.*, 2009). Double-stranded DNA fragmentation occurs during apoptosis, and this DNA damage can be assessed by the comet assay. The presence of DNA fragmentation in ejaculated sperm has been well characterized, and is increased in infertile men (Gandini *et al.*, 2000; Shen *et al.*, 2002; Moustafa *et al.*, 2004). It is unclear, however, whether numerical chromosomal abnormalities, such as sex chromosome disomy, may also be associated with increased DNA damage in sperm.

Sperm aneuploidy originates from segregation errors during the two meiotic divisions of spermatogenesis. Exact causes of sperm aneuploidy are unknown; however associations with potential genetic and environmental risk factors have been explored, including exposures to pesticides, chemotherapeutic agents, smoking and alcohol, although findings are inconsistent (Hassold and Hunt, 2001). Immature spermatozoa have a 1.5- to 4-fold higher rate of chromosomal aneuploidy (X, Y and 17) than mature spermatozoa (Kovanci *et al.*, 2001), as well as higher levels of DNA fragmentation (Ollero *et al.*, 2001; Fernandez *et al.*, 2003). Several studies have shown associations between the percentages of aneuploid and apoptotic sperm (Carrell *et al.*, 2003; Schmid *et al.*, 2003; Liu *et al.*, 2004). Thus, it has been hypothesized that sperm aneuploidy could trigger sperm DNA damage through an apoptotic process (Muriel *et al.*, 2007). Further, it has been proposed that mis-segregated chromosomes may activate apoptosis (Dobles *et al.*, 2000), possibly through checkpoint pathways, as seen with other cell types (Castedo *et al.*, 2004), acting as a quality control mechanism (Evan and Littlewood, 1998).

After meiosis, male germ cells are most susceptible to induction of DNA lesions, including DNA strand breaks, DNA adducts and protamine adducts, as shown in animal studies (Sega *et al.*, 1989; Marchetti and Wyrobek, 2005). The potential mechanisms of DNA damage in spermatozoa include: (i) defective chromatin condensation during spermiogenesis due to unrepaired DNA breaks (McPherson and Longo, 1993; Manicardi *et al.*, 1995); (ii) initiation of apoptosis during spermatogenesis (Gorczyca, *et al.*, 1993; Sakkas, *et al.*, 1999; Aitken and Koppers, 2010); and (iii) oxidative stress by reactive oxygen species (Gagnon, 1997; Aitken *et al.*, 1998; Aitken and Krausz, 2001). It is unclear whether these mechanisms of action work independently or together to induce DNA damage. Sperm DNA damage has been shown to be induced by radiotherapy and chemotherapy (Sakkas and Alvarez, 2010), and environmental and occupational toxicants (Xu *et al.*, 2003). Increased sperm DNA damage is correlated with impaired fertility, defective embryo development, high rates of miscarriage and an increased risk of defects in offspring (Aitken and De Iuliis, 2010).

A limited number of studies have been published exploring the relation between sex chromosome aneuploidy and DNA damage in human sperm, and findings have been inconsistent. Among a group of men in couples experiencing recurrent pregnancy loss, sperm DNA fragmentation assessed using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was significantly correlated with the percentage of aneuploidy in chromosomes X, Y, 13, 18 and 21 (Carrell *et al.*, 2003). Using a modified sperm chromatin dispersion fluorescence *in situ* hybridization (SCD-FISH) assay, Muriel *et al.* (2007) found an increase in sex chromosome disomy in sperm with fragmented DNA, when compared with sperm without fragmented DNA. Based on these findings, Muriel *et al.* (2007) hypothesized that sperm aneuploidy may lead to an increase in sperm DNA damage as part of a genomic screening mechanism to reduce the ability of defective sperm to successfully fertilize an egg. Using the same combined SCD-FISH methodology, however, Balasuriya *et al.* (2011) reported no significant associations between DNA damage and aneuploidy; they also reported no significant associations between DNA fragmentation measured using the sperm chromatin structure assay and aneuploidy assessed by FISH. When evaluating the sperm samples of men belonging to couples experiencing recurrent pregnancy loss or implantation failure, Bronet *et al.* (2012) found no association between DNA fragmentation measured by the TUNEL assay and aneuploidy determined by FISH in either fresh semen samples or those processed for ICSI. In a recently published study, Enciso *et al.* (2013) reported a significant association between DNA fragmentation and total numerical abnormalities for chromosomes X, Y, 13, 16, 18, 21 and 22. Additionally, the Enciso *et al.* (2013) study replicated the modified SCD-FISH assay used by Muriel *et al.* (2007) and found a significantly higher frequency of disomy among sperm with higher levels of DNA fragmentation. Three other studies have explored the relationships between abnormal chromosomal karyotypes and sperm DNA damage. Perrin *et al.* (2009) found that men with structural chromosomal abnormalities (reciprocal translocations, Robertsonian translocations and pericentric inversions) had higher rates of DNA fragmentation than men without abnormal karyotypes. They concluded that DNA fragmentation rates depend solely on the presence of structural chromosomal abnormalities. Brugnon *et al.* (2006) found that DNA fragmentation rates were significantly higher in men that were translocation carriers compared with men who were not translocation carriers. Likewise, García-Peiró *et al.* (2012) observed statistically higher levels of DNA damaged sperm in men with chromosomal rearrangements compared with men with proven fertility.

The aim of this study was to investigate the relation between sex chromosome disomy, using FISH, and DNA damage, assessed by the neutral comet assay, to better understand whether men with increased rates of sperm sex chromosome disomy have increased sperm DNA damage. For human sperm, disomies in the sex chromosomes are most common and, upon fertilization, can result in viable offspring with mild to severe adverse health outcomes (Hassold and Hunt, 2001;

Martin, 2008). Unlike autosomal aneuploidies, which are largely maternal in origin, sex chromosome aneuploidies largely arise from segregation errors during spermatogenesis (Martin, 2008). For these reasons, sex chromosome disomy served as the aneuploidy outcome of interest in this study.

Materials and Methods

Subjects

Study recruitment procedures have been described elsewhere (Hauser *et al.*, 2003). Briefly, a subset of male partners from couples seeking fertility evaluation from the Massachusetts General Hospital (MGH) Fertility Center between January 2000 and May 2003 who were enrolled in an ongoing study assessing the impacts of environmental factors on fertility were participants in this study. Approximately 65% of eligible men agreed to participate in the parent study. Exclusion criteria for the parent study included presenting for post-vasectomy semen analysis, and/or receiving treatment for infertility (i.e. hormonal treatments). Men were eligible for this sub-study if they had sufficient semen sample for FISH analysis and data available on sperm DNA damage parameters. Of the 341 eligible men, sufficient semen sample was available for 192 (56%) and DNA damage analysis was available for 190 of the 192. The parent study was approved by the Harvard School of Public Health and MGH Human Subjects Committees. All subjects filled out a medical history and lifestyle questionnaire and signed an informed consent prior to participation.

Table I shows the demographic characteristics of the 190 study subjects. Overall, the subjects had a mean (SD) age of 35.4 years (5.1). A majority of the subjects were Caucasian (85%), with 3% African American, and 4% Hispanic. Most men (75%) had never smoked, with 15 (8%) current smokers, and 32 (17%) ex-smokers. Participants in this study did not differ with respect to their demographic characteristics compared with those in the full parent study cohort ($n = 341$) (data not shown).

Table I Demographic characteristics for a subset of men seeking fertility evaluation at the Massachusetts General Hospital Fertility Clinic ($n = 190$ patients).

Characteristic	*n* (%)	Mean (SD)
Age (years)	190	35.4 (5)
Race		
White	161 (85)	
African American	5 (3)	
Hispanic	8 (4)	
Other	16 (8)	
Abstinence time[a]		
≤2 days	41 (22)	
3-4 days	88 (46)	
≥5 days	60 (32)	
Smoking status[a]		
Never smoker	141 (75)	
Ever smoker	47 (25)	
Current smoker	15 (8)	
Ex-smoker	32 (17)	
Semen parameters*		
Concentration (10^6/ml)		
<15 mil/ml	18 (10)	103.4 (87)
Motility (% motile)		
<40% motile	42 (22)	52.5 (23)
Morphology (% normal sperm)		
<4% normal morphology	35 (18)	7.4 (4)

[a]Smoking status missing for two men, abstinence data missing for one.
*Reference values are defined as abnormal according to the World Health Organization (2010) guidelines.

Semen analysis

Details of the sample collection, storage and semen analysis are available in full elsewhere (Hauser *et al.*, 2003). Briefly, a single semen sample was collected from participants by masturbation after an abstinence period. Information on abstinence time was collected from each participant. Semen samples were liquefied for 20 min at 37°C prior to andrology laboratory analysis. Sperm concentration and motility were assessed using computer-aided sperm analysis (CASA; version 10HTM-IVOS, Hamilton-Thorn, Beverly, MA, USA). Percentage motile sperm was defined using World Health Organization (WHO, 2010) criteria for sperm velocity at 37°C. Morphology was assessed using the feathering method to create two thin smear slides per sample that were stained with a Diff-Quik staining kit (Dade Behring AG, Düdingen, Switzerland). Strict criteria from Kruger *et al.* (1988) were applied to cells viewed under a Nikon microscope with an oil immersion ×100 objective (Nikon Company, Tokyo Japan). Sperm samples were considered to be 'normal' for a parameter when the measured parameter met or exceeded the WHO reference values: concentration ≥15 million sperm per milliliter, motility scores A + B ≥ 32% and morphology ≥4% normal (WHO, 2010). After analysis of conventional semen parameters on fresh samples, remaining unprocessed volumes of the semen samples were transferred to 250 μl capillary straws (Cryo Bio System, IMV Technologies, San Diego, CA, USA) and frozen by immersion in liquid nitrogen (−196°C) (Duty *et al.*, 2003). Prior work has demonstrated that freezing samples without cryoprotectant in liquid nitrogen is a reliable method for storing semen samples prior to the comet assay and produces results similar to those with fresh semen samples (Duty *et al.*, 2002).

Neutral comet assay

The single cell gel electrophoresis, or comet assay, was used to measure the amount of DNA fragmentation in individual cells from the frozen samples. In this assay, a cell with fragmented DNA has the appearance of a comet with a brightly fluorescent head and a fluorescent tail. The intensity of the comet tail relative to the head reflects the number of DNA strand breaks present in the cell (Singh *et al.*, 1988; Hughes *et al.*, 1997; Singh and Stephens, 1998).

Methods used to conduct the neutral comet assay have been previously described in detail (Trisini *et al.*, 2004). Briefly, thawed semen samples were prepared with 0.7% agarose and embedded between two additional layers of agarose on specially designed glass slides (Erie Scientific, Portsmouth, NH, USA). The slides underwent an enzyme treatment to decondense sperm chromatin and allow migration of broken DNA out of the nucleus. Next, slides were electrophoresed, followed by precipitation of the DNA and its fixation in microgels. Fluorescent comet patterns were examined under ×400 magnification with a Leica fluorescence microscope model DMLB (Leica, Wetzlar, Postfach, Germany) equipped with a fluoroisothiocyanate filter combination (Leica, Wetzlar), and using excitation at 490 nm, dichromic 500 nm, emission 510 nm.

Image analysis

VisComet image analysis software was used to measure comet extent, percentage DNA in tail (%Tail) and tail distributed moment (TDM) on 100

sperm in each semen sample. Cells too long to measure with VisComet (>300 μm) were excluded from this analysis. Comet extent is a measure of total comet length from the beginning of the head to the last visible pixel in the tail. This measurement is similar to that obtained by manual analysis using an eyepiece micrometer. %Tail is a measure of the proportion of the total DNA that is present in the tail. The TDM is an integrated value that takes into account both the distance and intensity of comet fragments. The formula used to calculate the TDM is:

$$\text{TDM} = \frac{\sum(I \times X)}{\sum I},$$

where $\sum I$ = sum of all intensity values that belong to the head, body or tail; X = x-position of intensity value.

FISH

A single laboratory technician (M.E.M.) performed FISH, imaging and nuclei scoring analyses on all samples. The technician was blinded to DNA damage outcomes while preparing and analyzing all samples. FISH was carried out for chromosomes X, Y and 18 (autosomal control). Chromosome 18 was chosen as the autosomal control due to its low frequency of disomy in human sperm (Hassold and Hunt, 2001) to help distinguish between disomy, the outcome of interest in this study, and diploidy, which was not assessed as an outcome. Although of clinical importance, nullisomies were also not evaluated because of their rarity. Image processing and scoring were performed using custom software developed in MATLAB® (The Mathworks, Inc., Natick, MA, USA) using algorithms based on criteria described by Baumgartner *et al.* (1999). The overall hybridization efficiency was 97% and is consistent with the hybridization efficiency reported by other groups (Martin *et al.*, 1996; Johannisson *et al.*, 2002; Tiido *et al.*, 2005). These methods have been previously described in detail (Perry *et al.*, 2007, 2011).

Statistical analysis

We explored the relationship between sperm sex chromosome disomy (XX18, YY18, XY18, total sex chromosome disomy) and sperm DNA damage (mean comet extent, %Tail and TDM). First, univariate and multiple linear regression models were constructed with sex chromosome disomy (separate models for each of the four disomic conditions) as the independent variable and DNA damage parameters (separate models for each DNA damage measure) as the dependent outcome variable. Although %Tail was not normally distributed, analyses conducted using log-transformed %Tail yielded similar conclusions to those of untransformed %Tail; thus, only the model results for the untransformed outcome are presented for ease of interpretation.

The regression coefficients were expressed as a change in comet assay parameter per 0.1% change in disomy parameter. Potential confounders considered in models included age (modeled as a continuous and categorical variable); abstinence time (ordinal three-category variable: ≤2, 3–4, ≥5 days); and smoking status (never when compared with current or past smoker). Inclusion of confounding variables in final models was based on a change of at least 10% in the disomy measure. Although age (either continuous or categorical) was not a significant predictor and did not alter the estimated association with disomy measures, it was retained in the final models based on *a priori* assumptions about its likely associations with DNA damage and aneuploidy.

Additionally, we evaluated possible effect modification by semen quality to determine whether men with poor semen quality showed a different relationship between sex chromosome disomy and DNA damage than men with normal semen quality. Specifically, effect modification was examined in separate models for men with WHO-defined abnormalities in 1, 2 or all 3 semen parameters (concentration, motility and morphology) compared with men with normal values for all semen parameters, as well as for men with abnormal semen concentration (<15 million/ml), motility (<40% motile) and morphology (<4% normal) compared with men with all normal semen parameters.

Spearman correlation coefficients were used to quantify associations among DNA damage parameters and between DNA damage and disomy measures. Wilcoxon rank-sum tests were used to compare DNA damage and disomy conditions by categorical variables, such as smoking status (ever smoker versus never smoker) and abstinence time (≤2, 3–4, ≥5 days). Statistical Analysis Software (SAS) version 9.2 (SAS Institute, Inc., Cary, NC, USA) was used for all data analyses. Two-sided *P*-values <0.05 were considered significant.

Results

The distribution of the three comet assay parameters is shown in Table II. Figure 1 shows a sperm cell after undergoing the comet assay with the comet tail visible. Comet extent ranged from 47.6 to 222.7 μm, with a median of 123.8 μm; %Tail ranged from 9.9 to 64.4%, with a median of 25.6%; and TDM ranged from 13.8 to 59.5 μm, with a median of 28.5 μm.

Figure 2 shows two sperm cells after FISH for chromosomes 18, X and Y. A median (25th, 75th percentile) of 5895 (3248, 9960) sperm nuclei were scored per subject in this study by sperm FISH. The observed median percentages (25th, 75th percentile) of XX18, YY18, XY18 and total sex chromosome disomy were 0.27 (0.16, 0.50), 0.30 (0.19, 0.46), 0.89 (0.56, 1.46) and 1.56 (1.10, 2.47), respectively (Table III).

As expected, there were moderate to strong correlations among comet assay parameters. Comet extent was highly correlated with TDM ($r = 0.89$, $P < 0.001$), and moderately correlated with %Tail

Table II Distribution of comet assay parameters for a subset of men seeking fertility evaluation at the Massachusetts General Hospital Fertility Clinic (n = 190 patients).

		Percentile						
Comet assay parameter	Minimum	10th	25th	50th	75th	90th	Maximum	Geometric mean
Comet extent (μm)	47.6	78.9	101.9	123.8	147.1	164.9	222.7	119.1
%Tail	9.9	16.2	19.9	25.6	40.1	48.1	64.4	27.4
TDM (μm)	13.8	20.2	24.1	28.5	32.1	36.8	59.5	27.7

VisComet image analysis software was used to measure comet extent, a measure of total comet length; %Tail: percentage DNA in tail, a measure of the proportion of total DNA present in the comet tail, and TDM: tail distributed moment, an integrated measure of length and intensity.

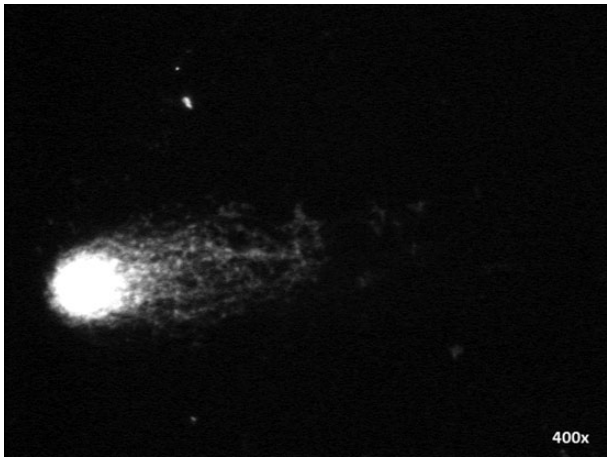

Figure 1 DNA fragmentation visualized by the comet assay. A single sperm processed using the comet assay; ×400 magnification, excitation 490 nm, emission 510 nm. In the comet assay, fragmented DNA is allowed to migrate out of the cell nucleus by means of enzymatic decondensation. After gel electrophoresis, a fluorescent dye is added that allows for visualization of the DNA. The length of the comet tail and the percentage of DNA found in the tail are measures of DNA fragmentation.

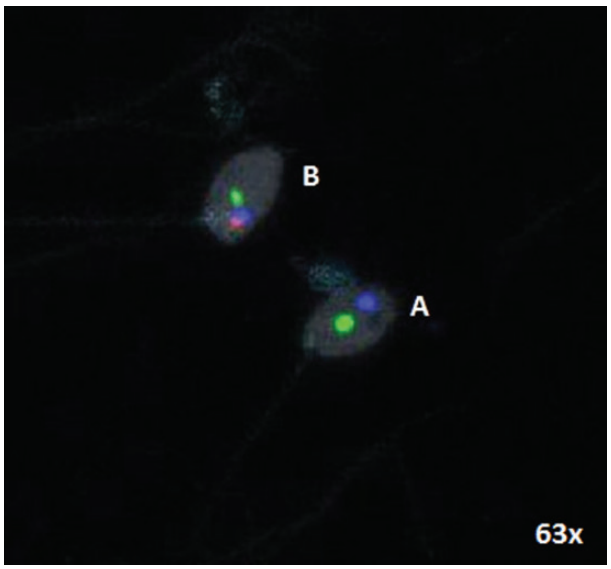

Figure 2 Fluorescence *in situ* hybridization results for chromosomes 18, X and Y. Composite image (×63 magnification) of channels acquired for each of the four signals: SpectrumAqua (18), SpectrumGreen (X), SpectrumOrange (Y) and the nuclear stain. Cell A is a normal sperm cell (X18). Cell B is a disomic sperm cell (XY18).

($r = 0.41$, $P = 0.05$). The %Tail was weakly correlated with TDM ($r = 0.10$, $P = 0.17$). None of the correlation coefficients for aneuploidy and DNA damage measures were statistically significant.

Smoking was found to be the only significant predictor of DNA damage measures in models adjusted for age. Men who had ever smoked showed a significant increase in comet extent and TDM when compared with those whom had never smoked, but not for %Tail.

Table IV presents the age and smoking adjusted regression coefficients and 95% confidence intervals (CI) for linear regression models exploring the relationships between sperm sex chromosome disomy and sperm DNA damage parameters. Regression coefficients for age and previous history of smoking are also presented. For each 0.1% increase in XY disomy, the mean comet extent increased by 0.58 μm (95% CI: 0.01, 1.14), after adjusting for age and smoking status. For an interquartile range change in percentage XY disomy (e.g. from 0.56 to 1.47%), the mean comet extent increased by 5.08 μm. No other statistically significant findings were observed between sex chromosome disomy and DNA damage. Additionally, no effect modification by abnormal semen parameters was detected.

Discussion

The question of whether sperm sex chromosome disomy is associated with sperm DNA damage has not been adequately addressed previously, and this is the largest study to date to examine the question using multivariate analyses. Only the relationship between XY disomy and mean comet extent reached statistical significance in our regression model adjusted for relevant covariates; we found that for each 0.1% increase in XY disomy, mean comet extent increased by 0.58 μm. However, we did not observe other statistically significant associations. There may be other true associations, but a larger sample size may be needed in order to better evaluate them statistically.

Endogenous or exogenous factors can impact both meiotic segregation and sperm DNA integrity. For example, oxidative stress has been linked to DNA damage (Aitken and Krausz, 2001; Mahfouz *et al.*, 2010; Aktan *et al.*, 2013) and diminished sperm parameters (Kao *et al.*, 2008; Benedetti *et al.*, 2012). A number of exposures can induce oxidative stress in the testis, including environmental chemicals, infection, heavy metals, radiation and the spermatogenic process itself (Turner and Lysiak, 2008). Additionally, markers of oxidative stress are associated with disruptions in chromatin remodeling in human sperm (De Iuliis *et al.*, 2009; Henkel *et al.*, 2010), a process that plays a role in the prevention of DNA damage and aneuploidy (Papamichos-Chronakis and Peterson, 2013). Correlations between DNA damage and aneuploidy may be indicative of a suboptimal testicular environment (Mroz *et al.*, 1999) where spermatocytes undergoing various maturation processes may be impacted differentially.

Of the few previously published studies on this topic, evidence for an association has been mixed. Muriel *et al.* (2007) used the SCD test and FISH to simultaneously assess DNA damage and reported a mean ± SD 5.9 ± 3.5-fold increase in disomy rates in sperm with fragmented DNA compared with sperm without fragmented DNA. Overall, they found significant differences for XX, YY and XY disomy in sperm cells with fragmented DNA compared with cells without fragmented DNA. Enciso *et al.* (2013) replicated these findings in a study of 45 men undergoing assisted reproduction cycles. In contrast, Balasuriya *et al.* (2011) reported no association between aneuploidy and DNA fragmentation when using the combined SCD-FISH assay. They also reported no associations when DNA fragmentation and aneuploidy were assessed separately using SCD and FISH analyses, respectively. Balasuriya *et al.* (2011)

Table III Number of sperm nuclei scored and percentage disomy for a subset of men seeking fertility evaluation at the Massachusetts General Hospital Fertility Clinic ($n = 190$).

Sperm outcomes	Mean ± (SD)	Median (*n*)	25th percentile	75th percentile
Nuclei (*n*)	6961 ± (4644)	5895	3248	9960
1818	0.13 ± (0.24)	0.04 (2)	0.00	0.15
XX18	0.41 ± (0.40)	0.27 (16)	0.16	0.50
YY18	0.38 ± (0.33)	0.30 (18)	0.19	0.46
XY18	1.11 ± (0.83)	0.89 (52)	0.56	1.46
Total disomy[a]	1.90 ± (1.26)	1.56 (92)	1.10	2.47

[a]Total sex chromosome disomy = ∑(XX18 + YY18 + XY18).

Table IV Linear regression coefficients (95% confidence intervals) for the relationship between DNA damage and sperm sex chromosome disomy adjusted* for smoking status and age for 190 men seeking fertility evaluation at the Massachusetts General Hospital Fertility Clinic.

	DNA damage parameters from neutral comet assay		
Disomy	**Comet extent (μm)**	**%Tail**	**TDM (μm)**
XX18	−0.15 (−1.32, 1.01)	0.16 (−0.31, 0.62)	−0.06 (−0.30, 0.18)
Age	0.41 (−0.55, 1.38)	0.23 (−0.15, 0.62)	0.08 (−0.12, 0.28)
Smoker	15.17 (1.98, 28.36)**	2.21 (−3.02, 7.44)	3.01 (0.30, 5.72)**
YY18	−0.64 (−2.07, 0.79)	−0.06 (−0.63, 0.51)	−0.04 (−0.33, 0.26)
Age	0.43 (−0.53, 1.40)	0.24 (−0.14, 0.62)	0.08 (−0.12, 0.28)
Smoker	14.68 (1.50, 27.86)**	2.25 (−2.99, 7.49)	2.95 (0.24, 5.67)**
XY18	0.58 (0.01, 1.14)**	0.07 (−0.16, 0.29)	0.11 (−0.01, 0.23)
Age	0.50 (−0.46, 1.46)	0.25 (−0.14, 0.63)	0.10 (−0.10, 0.29)
Smoker	15.41 (2.37, 28.45)**	2.32 (−2.91, 7.55)	3.04 (0.36, 5.72)**
Total sex chromosome disomy[a]	0.19 (−0.19, 0.57)	0.04 (−0.11, 0.19)	0.04 (−0.04, 0.12)
Age	0.43 (−0.53, 1.40)	0.24 (−0.14, 0.63)	0.08 (−0.11, 0.28)
Smoker	15.23 (2.09, 28.38)**	2.31 (−2.91, 7.54)	3.01 (0.31, 5.71)**

Regression coefficients: change in comet assay parameter per 0.1% change in disomy (XX18, YY18, XY18 and Total).
*Smoking status (never versus ever (past, present)), Age (continuous).
[a]Total sex chromosome disomy = XX18 + YY18 + XY18.
**$P < 0.05$

suggest that current DNA fragmentation methods, especially when combined with FISH analysis, may not be reliable assays for determining DNA fragmentation in sperm.

Other studies investigating this association relied on the TUNEL assay to assess DNA fragmentation. Carrell *et al.* (2003) reported a significant association between DNA damage and the percentage of aneuploid sperm among men whose partners had recurrent pregnancy loss. Conversely, among a similar population of men whose partners were experiencing recurrent pregnancy loss or implantation failure, Bronet *et al.* (2012) reported no association between DNA fragmentation and aneuploidy in sperm.

The end-point measurement of the SCD test consists of determining the percentage of sperm with non-dispersed or dispersed nuclei. The presence or absence of very small halos determines DNA dispersion, and therefore DNA fragmentation (Fernandez *et al.*, 2003). DNA fragmentation is caused by breaks in the DNA double helix. As reported here, the end-points of the comet assay are %Tail, TDM and comet extent. Although these two methods use different end-points, it has been suggested that both provide an overall measure of DNA integrity, allowing for potential comparisons (Baumgartner *et al.*, 2009). Unlike the comet assay, the modified SCD-FISH test uses the same sperm to measure DNA fragmentation and aneuploidy. It may be the case that within disomic sperm there are higher frequencies of DNA damage because of increased apoptosis leading to increased DNA damage as part of a genetic screening mechanism. Our methods did not allow for measurement in the same sperm, and therefore, we measured comet tails in a population with a large number of normal sperm. Further validation of the Muriel *et al.* (2007) and Enciso *et al.* (2013) findings is needed, as well as an exploration of the potential apoptotic genetic screening mechanism hypothesized as a possible explanation for their findings.

These findings highlight uncertainty about whether DNA damage and aneuploidy in sperm are causally linked or associated by a common mechanism of damage. The current lack of standardized methods for determining sperm DNA damage makes it difficult to compare across studies and may lead to the use of DNA damage biomarkers that are weak or invalid (Barratt *et al.*, 2010). Continued work is needed to optimize and thus standardize sperm DNA damage biomarkers and further studies are needed to explore potential biological mechanisms to better understand the relationship between sex chromosome disomy and DNA damage.

Acknowledgements

The authors thank Dr. Russ Hauser for instrumental support.

Authors' roles

M.E.M. collaborated in the design of the study and analysis; performed FISH, imaging and nuclei scoring analyses; and led the manuscript writing. P.L.W. collaborated in the design of the study and analysis, provided guidance on statistical programming and collaborated on the interpretation of the results and manuscript writing. S.A.K. collaborated in the design of the study and analysis, and collaborated on the interpretation of the results and writing the manuscript. F.M. collaborated on the design of the study, and on the interpretation of results and writing the manuscript. R.D. performed semen analysis, sample retrieval for FISH, assisted with the Comet Assay and reviewed the manuscript. S.E.M. contributed to the interpretation of results and writing of the manuscript. M.J.P. collaborated in the design of the study and analysis, directed the laboratory that performed FISH, and collaborated on the interpretation of the results and writing the manuscript.

Funding

This work was supported by NIOSH Grant T42 OH008416, and NIH/NIEHS Grants ES 009718, ES 000002 and R01 ES017457.

Conflict of interest

At the time this work was conducted and the initial manuscript written, M.E.M. was affiliated with the Environmental Health Department at the Harvard School of Public Health. Currently, M.E.M. is employed by Millennium: The Takeda Oncology Company.

References

Aitken RJ, De Iuliis GN. On the possible origins of DNA damage in human spermatozoa. *Mol Hum Reprod* 2010;**16**:3–13.

Aitken RJ, Koppers AJ. Apoptosis and DNA damage in human spermatozoa. *Asian J Androl* 2011;**13**:36–42.

Aitken RJ, Krausz C. Oxidative stress, DNA damage and the Y chromosome. *Reproduction* 2001;**122**:497–506.

Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z, Irvine DS. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod* 1998;**59**:1037–1046.

Aktan G, Doğru-Abbasoğlu S, Küçükgergin C, Kadıoğlu A, Ozdemirler-Erata G, Koçak-Toker N. Mystery of idiopathic male infertility: is oxidative stress an actual risk? *Fertil Steril* 2013;**99**:1211–1215.

Balasuriya A, Speyer B, Serhal P, Doshi A, Harper JC. Sperm chromatin dispersion test in the assessment of DNA fragmentation and aneuploidy in human spermatozoa. *Reprod Biomed Online* 2011;**22**:428–436.

Barratt CLR, Aitken RJ, Bjorndahl L, Carrell DT, de Boer P, Kvist U, Lewis SEM, Perreault SD, Perry MJ, Ramos L *et al.* Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications-a position report. *Hum Reprod* 2010;**25**:824–838.

Baumgartner A, Van Hummelen P, Lowe XR, Adler ID, Wyrobek AJ. Numerical and structural chromosomal abnormalities detected in human sperm with a combination of multicolor FISH assays. *Environ Mol Mutagen* 1999;**33**:49–58.

Baumgartner A, Cemeli E, Anderson D. The comet assay in male reproductive toxicology. *Cell Biol Toxicol* 2009;**25**:81–98.

Benedetti S, Tagliamonte MC, Catalani S, Primiterra M, Canestrari F, De Stefani S, Palini S, Bulletti C. Differences in blood and semen oxidative status in fertile and infertile men, and their relationship with sperm quality. *Reprod Biomed Online* 2012;**25**:300–306.

Bronet F, Martínez E, Gaytán M, Liñán A, Cernuda D, Ariza M, Nogales M, Pacheco A, San Celestino M, Garcia-Velasco JA. Sperm DNA fragmentation index does not correlate with the sperm or embryo aneuploidy rate in recurrent miscarriage or implantation failure patients. *Hum Reprod* 2012;**27**:1922–1929.

Brugnon F, Van Assche E, Verheyen G, Sion B, Boucher D, Pouly JL, Janny L, Devroey P, Liebaers I, Van Steirteghem A. Study of two markers of apoptosis and meiotic segregation in ejaculated sperm of chromosomal translocation carrier patients. *Hum Reprod* 2006;**21**:685–693.

Carrell DT, Wilcox AL, Lowy L, Peterson CM, Jones KP, Erickson L, Campbell B, Branch DW, Hatasaka HH. Elevated sperm chromosome aneuploidy and apoptosis in patients with unexplained recurrent pregnancy loss. *Obstet Gynecol* 2003;**101**:1229–1235.

Castedo M, Perfettini JL, Roumie T, Andreau K, Medema R, Kroemer G. Cell death by mitotic catastrophe: a molecular definition. *Oncogene* 2004;**23**:2825–2837.

De Iuliis GN, Thomson LK, Mitchell LA, Finnie JM, Koppers AJ, Hedges A, Nixon B, Aitken RJ. DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodeling and the formation of 8-hydroxy-2′-deoxyguanosine, a marker of oxidative stress. *Biol Reprod* 2009;**81**:517–524.

Dobles M, Liberal V, Scott ML, Benezra R, Sorger PK. Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2. *Cell* 2000;**101**:635–645.

Duty SM, Singh NP, Ryan L, Chen Z, Lewis C, Huang T, Hauser R. Reliability of the comet assay in cryopreserved human sperm. *Hum Reprod* 2002;**17**:1274–1280.

Duty SM, Singh NP, Silva MJ, Barr DB, Brock JW, Ryan L, Herrick RF, Christiani DC, Hauser R. The relationship between environmental exposures to phthalates and DNA damage in human sperm using the neutral comet assay. *Environ Health Perspect* 2003;**111**:1164–1169.

Enciso M, Alfarawati S, Wells D. Increased numbers of DNA-damaged spermatozoa in samples presenting an elevated rate of numerical chromosome abnormalities. *Hum Reprod* 2013;**28**:1707–1715.

Evan G, Littlewood T. A matter of life and cell death. *Science* 1998;**281**:1317–1322.

Fernandez JL, Muriel L, Rivero MT, Goyanes V, Vazquez R, Alvarez JG. The sperm chromatin dispersion test: A simple method for the determination of sperm DNA fragmentation. *J Androl* 2003;**24**:59–66.

Gagnon C. The dark and bright sides of reactive oxygen species in male fertility. *Hum Reprod* 1997;**12**:O28–O28.

Gandini L, Lombardo F, Paoli D, Caponecchia L, Familiari G, Verlengia C, Dondero F, Lenzi A. Study of apoptotic DNA fragmentation in human spermatozoa. *Hum Reprod* 2000;**15**:830–839.

García-Peiró A, Oliver-Bonet M, Navarro J, Abad C, Amengual MJ, López-Fernández C, Gosálvez J, Benet J. Differential clustering of sperm subpopulations in infertile males with clinical varicocele and carriers of rearranged genomes. *J Androl* 2012;**33**:361–367.

Gorczyca W, Traganos F, Jesionowska H, Darzynkiewicz Z. Presence of DNA strand breaks and increased sensitivity of DNA *in situ* to denaturation in abnormal human sperm cells—analogy to apoptosis of somatic-cells. *Exp Cell Res* 1993;**207**:202–205.

Hassold T, Hunt P. To ERR (meiotically) is human: the genesis of human aneuploidy. *Nat Rev Gen* 2001;**2**:280–291.

Hauser R, Chen Z, Pothier L, Ryan L, Altshul L. The relationship between human semen parameters and environmental exposure to polychlorinated biphenyls and p,p'-DDE. *Environ Health Perspect* 2003;**111**:1505–1511.

Henkel R, Bastiaan HS, Schüller S, Hoppe I, Starker W, Menkveld R. Leucocytes and intrinsic ROS production may be factors compromising sperm chromatin condensation status. *Andrologia* 2010;**42**:69–75.

Hughes CM, Lewis SEM, McKelveyMartin VJ, Thompson W. Reproducibility of human sperm DNA measurements using the alkaline single cell gel electrophoresis assay. *Mutat Res* 1997;**374**:261–268.

Johannisson R, Leuschner E, Huppe M, Hinrichs F, Al-Hasani S, Diedrich K, Schwinger E, Mennicke K. Increased frequency of X-bearing sperm in males from an infertility clinic: analysis by two-color fluorescence *in situ* hybridization. *Cytogenet Genome Res* 2002;**98**:240–244.

Kao S-H, Chao H-T, Chen H-W, Hwang TIS, Liao T-L, Wei Y-H. Increase of oxidative stress in human sperm with lower motility. *Fertil Steril* 2008; **89**:1183–1190.

Kovanci E, Kovacs T, Moretti E, Vigue L, Bray-Ward P, Ward DC, Huszar G. FISH assessment of aneuploidy frequencies in mature and immature human spermatozoa classified by the absence or presence of cytoplasmic retention. *Hum Reprod* 2001;**16**:1209–1217.

Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Oehninger S. Predictive value of abnormal sperm morphology in *in vitro* fertilization. *Fertil Steril* 1988;**49**:112–117.

Liu CH, Tsao HM, Cheng TC, Wu HM, Huang CC, Chen CI, Lin DPC, Lee MS. DNA fragmentation, mitochondrial dysfunction and chromosomal aneuploidy in the spermatozoa of oligoasthenoteratozoospermic males. *J Assist Reprod Genet* 2004;**21**:119–126.

Mahfouz R, Sharma R, Thiyagarajan A, Kale V, Gupta S, Sabanegh E, Agarwal A. Semen characteristics and sperm DNA fragmentation in infertile men with low and high levels of seminal reactive oxygen species. *Fertil Steril* 2010;**94**:2141–2146.

Manicardi GC, Bianchi PG, Pantano S, Azzoni P, Bizzaro D, Bianchi U, Sakkas D. Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin a(3) accessibility. *Biol Reprod* 1995;**52**:864–867.

Marchetti F, Wyrobek AJ. Mechanisms and consequences of paternally-transmitted chromosomal abnormalities. *Birth Defects Res C Embryo Today* 2005;**75**:112–129.

Martin RH. Meiotic errors in human oogenesis and spermatogenesis. *Reprod Biomed Online* 2008;**16**:523–531.

Martin RH, Spriggs E, Rademaker AW. Multicolor fluorescence *in situ* hybridization analysis of aneuploidy and diploidy frequencies in 225846 sperm from 10 normal men. *Biol Reprod* 1996;**54**:394–398.

McPherson SMG, Longo FJ. Nicking of rat spermatid and spermatozoa DNA—possible involvement of DNA topoisomerase-II. *Dev Biol* 1993; **158**:122–130.

Moustafa MH, Sharma RK, Thornton J, Mascha E, Abdel-Hafez MA, Thomas AJ, Agarwal A. Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. *Hum Reprod* 2004;**19**:129–138.

Mroz K, Hassold TJ, Hunt PA. Meiotic aneuploidy in the XXY mouse: evidence that a compromised testicular environment increases the incidence of meiotic errors. *Hum Reprod* 1999;**14**:1151–1156.

Muriel L, Goyanes V, Segrellesj E, Gosalvez J, Alvarez JG, Fernandez JL. Increased aneuploidy rate in sperm with fragmented DNA as determined by the sperm chromatin dispersion (SCD) test and FISH analysis. *J Androl* 2007;**28**:38–49.

Ollero M, Gil-Guzman E, Lopez MC, Sharma RK, Agarwal A, Larson K, Evenson D, Thomas AJ, Alvarez JG. Characterization of subsets of human spermatozoa at different stages of maturation: implications in the diagnosis and treatment of male infertility. *Hum Reprod* 2001;**16**:1912–1921.

Papamichos-Chronakis M, Peterson CL. Chromatin and the genome integrity network. *Nat Rev Genet* 2013;**14**:62–75.

Perrin A, Caer E, Oliver-Bonet M, Navarro J, Benet J, Amice V, De Braekeleer M, Morel F. DNA fragmentation and meiotic segregation in sperm of carriers of a chromosomal structural abnormality. *Fertil Steril* 2009;**92**:583–589.

Perry MJ, Chen X, Lu X. Automated scoring of multiprobe FISH in human spermatozoa. *Cytometry A* 2007;**71A**:968–972.

Perry MJ, Chen X, McAuliffe ME, Maity A, Deloid GM. Semi-automated scoring of triple-probe FISH in human sperm: methods and further validation. *Cytometry A* 2011;**79A**:661–666.

Sakkas D, Alvarez JG. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil Steril* 2010; **93**:1027–1036.

Sakkas D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG, Bianchi U. Origin of DNA damage in ejaculated human spermatozoa. *Rev Reprod* 1999;**4**:31–37.

Schmid TE, Kamischke A, Bollwein H, Nieschlag E, Brinkworth MH. Genetic damage in oligozoospermic patients detected by fluorescence *in situ* hybridization, inverse restriction site mutation assay, sperm chromatin structure assay and the Comet assay. *Hum Reprod* 2003; **18**:1474–1480.

Sega GA, Alcota RPV, Tancongco CP, Brimer PA. Acrylamide binding to the DNA and protamine of spermiogenic stages in the mouse and its relationship to genetic-damage. *Mut Res* 1989;**216**:221–230.

Shen HM, Dai J, Chia SE, Lim A, Ong CN. Detection of apoptotic alterations in sperm in subfertile patients and their correlations with sperm quality. *Hum Reprod* 2002;**17**:1266–1273.

Singh NP, Stephens RE. X-ray-induced DNA double-strand breaks in human sperm. *Mutagenesis* 1998;**13**:75–79.

Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low-levels of DNA damage in individual cells. *Exp Cell Res* 1988;**175**:184–191.

Tiido T, Rignell-Hydbom A, Jonsson B, Giwercman YL, Rylander L, Hagmar L, Giwercman A. Exposure to persistent organochlorine pollutants associates with human sperm Y : X chromosome ratio. *Hum Reprod* 2005;**20**:1903–1909.

Trisini AT, Singh NP, Duty SM, Hauser R. Relationship between human semen parameters and deoxyribonucleic acid damage assessed by the neutral comet assay. *Fertil Steril* 2004;**82**:1623–1632.

Turner TT, Lysiak JJ. Oxidative stress: a common factor in testicular dysfunction. *J Androl* 2008;**29**:488–498.

World Health Organization [WHO]. *WHO Laboratory Manual for the Examination and Processing of Human Semen*, 5th edn. Geneva: WHO Press, 2010.

Xu DX, Zhu QX, Zheng LK, Wang QN, Shen HM, Deng LX, Ong CN. Exposure to acrylonitrile induced DNA strand breakage and sex chromosome aneuploidy in human spermatozoa. *Mutat Res* 2003; **537**:93–100.