Human Reproduction

Elemental composition of human semen is associated with motility and genomic sperm defects among older men.

Running title: PIXE Analyses of Sperm of Old Men

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Abstract

BACKGROUND: Older men tend to have poorer semen quality and are generally at higher risks for infertility and abnormal reproductive outcomes.

METHODS: We employed proton induced x-ray emission (PIXE, 3 MeV proton beam) to investigate the concentrations of zinc, copper, calcium, sulfur, chlorine, potassium, titanium, iron, and nickel in washed sperm and seminal plasma from a group of ten older men (65-80 years old) and ten younger men (22-28 years old) who were concurrently assayed for sperm function and genetically defective sperm.

RESULTS: The older group showed elevated zinc, copper and calcium in sperm and elevated sulfur in seminal plasma compared to the younger men. The older group also showed reduced motility as well as increased sperm DNA fragmentation, achondroplasia mutations, DNA strand breaks, and chromosomal aberrations. Sperm calcium and copper were positively associated with sperm DNA fragmentation ($p<0.03$). Seminal sulfur was positively associated with sperm DNA fragmentation and chromosomal aberrations ($p<0.04$), and negatively associated with sperm motility ($p<0.05$). Sperm calcium was negatively associated with sperm motility, independent of male age ($p=0.01$).

CONCLUSIONS: We identified major differences in elemental concentrations between sperm and seminal plasma, and that higher sperm copper, sulfur, and calcium are quantitatively associated with poorer semen quality and increased frequencies of genomic sperm defects.

Key words: male aging, calcium, copper, semen, zinc, human sperm

Introduction

There is an increasing trend for older men to father children (Rolf and Nieschlag 2001, Sloter, et al. 2004). Advancing male age has been associated with increased frequencies of sperm with chromosomal defects (Sloter, et al. 2004, Sloter, et al. 2007), with mutations in genes associated with achondroplasia and Apert syndromes (Glaser, et al. 2003, Tiemann-Boege, et al. 2002, Wyrobek, et al. 2006) and with DNA damage measured by Sperm Comet and the sperm chromatin structure assay (SCSA) (Morris, et al. 2002, Schmid, et al. 2007, Singh, et al. 2003, Wyrobek, et al. 2006). Interestingly, male aging was not associated with an increase in the frequencies of aneuploid sperm (Wyrobek, et al. 2006). Also, ~60% of male infertility has been attributed to genetic causes (de la Rochebrochard and Thonneau 2002), but the underlying mechanisms and roles of male aging are poorly understood (Ventura, et al. 1997). The age-associated increases in sperm defects support the epidemiological findings that fathering children at older ages is associated with a greater risk of abnormal reproductive outcomes and children carrying specific gene mutations of paternal origin (see review in Wyrobek et al 2006). Understanding the effects of male age on sperm quality is especially relevant for older men who are attending reproductive clinics because they are marginally fertile and seek assisted reproduction technologies in order to father children.

It is well known that certain elements are involved in normal spermatogenesis, sperm function and male fertility (Figa-Talamanca, et al. 2001). Elements such as zinc, magnesium, copper, and calcium are important for the maintenance of normal spermatogenesis, sperm maturation, DNA metabolism and repair, and gene expression in germ cells (Benoff, et al. 2000, Fenech 2002, Schrag and Dixon 1985, Wong, et al.

2001, Wong, et al. 2002, Wong, et al. 2000, Yuyan, et al. 2008). However, little is known about the relative concentrations of elements in sperm versus seminal plasma, whether concentrations of elements in either compartment are related to semen quality or genomic defects in sperm, and how these processes may be modified in the aging male.

Proton induced X-ray emission (PIXE), which is based on the analyses of the energy spectra of X-rays emitted from samples bombarded with protons, is the preferred method for trace element detection in biological tissue (Bench, et al. 1996, Forslind, et al. 1991). The advantage of the PIXE technique over the more common methods of atomic absorption spectroscopy (Henkel, et al. 1999) and inductively coupled argon plasma with mass spectrometry (ICP-MS) is its ability to detect trace elements with high sensitivity due to low background interference. PIXE is also 100 times more sensitive than electron micro-analysis systems for the identification and quantification of trace elements. PIXE is capable of detection sensitivity for trace elements generally down to a few parts per million (Forslind, et al. 1991) but can be as sensitive to tens or hundreds of ppb with extended collection times and detectors with large collection efficiencies (Ryan 2011). When ions pass through matter, they interact with the electrons in the atoms and occasionally, a vacancy is produced by an excited electron. When this occurs in an inner shell, the vacancy is filled by an electron from an outer shell, and an x-ray photon of characteristic energy is emitted. The PIXE method measures the energy of this photon in order to identify the atomic number and the amount of that element present. PIXE requires very small biological mass (<1 μg) compared to other methods, which is advantageous for semen analysis (Bench, et al.

1998). In a previous application to semen samples, PIXE showed that the protein and DNA contents of sperm differed between fertile and infertile individuals and infertile men had elevated levels of sulfur within sperm heads compared to fertile individuals (Bench, et al. 1998, Bench, et al. 1996).

The current study uses a subset of samples from the California Age and Genetic Effects on Sperm (AGES) population. Analyses of genetic (numerical aneuploidy, structural chromosome aberrations, DNA strand breakage, DNA fragmentation (DFI) and achondroplasia mutations) and physiological endpoints (count, various motility parameters including Computer-Assisted Semen Analysis (CASA)) of this study population were previously described (Eskenazi, et al. 2003, Schmid, et al. 2007, Sloter, et al. 2006, Sloter, et al. 2007, Tiemann-Boege, et al. 2002, Wyrobek, et al. 2006). The AGES study has shown that male aging was associated with reduced semen quality (Eskenazi, et al. 2003), and increased frequencies of sperm carrying structural aberrations (Sloter, et al. 2007), DNA strand damage (Schmid, et al. 2007), and DNA fragmentation and specific gene mutations (Wyrobek, et al. 2006). In the AGES population, we also found that micronutrient intake was associated with improved semen quality, in particular, motility (Eskenazi, et al. 2005) and lower frequencies of aneuploidy sperm (Young, et al. 2008).

The aims of the present study are to apply PIXE to semen samples collected in the well-characterized AGES study to: (1) measure the concentrations of specific elements and metals within isolated sperm and seminal plasma (zinc, copper, calcium, sulfur, chlorine, potassium, titanium, iron, and nickel); (2) determine whether older men had significantly different concentrations of these elements in their sperm or seminal

plasma compared to younger men; and (3) determine whether changes in elemental concentrations could explain previously determined associations between male age and semen quality and genomic defects in sperm.

Materials and Methods

Participants

A group of 20 healthy male volunteers, 10 men aged 22-28 years and 10 men aged 65-80 years, were selected from the AGES study (N = 97), which aimed to investigate whether there were differences in semen quality, and genomic defects in sperm associated with male aging and nutritional factors (Eskenazi, et al. 2003). These men represented the ten youngest and the ten oldest participants of the AGES study. Men who participated in the AGES study were recruited from a national laboratory in California. Men were eligible to participate if they currently worked or were retired from that workplace; had no current fertility or reproductive problems; had not smoked cigarettes in the last six months; had no vasectomy; and no history of an undescended testicle, prostate cancer, or azoospermia. The AGES study was approved by the Institutional Review Boards of all participating institutions, and written consent to participate was obtained from all participants.

Men were mailed a semen collection container with instructions and a questionnaire covering medical and reproductive histories, sociodemographic characteristics (age, race, education), occupation, other possible exposures, as well as diet and lifestyle habits. Semen samples were analyzed for count and motility upon collection (Eskenazi, et al. 2003, Sloter, et al. 2006) and immediately stored at -80°C until later analysis. The majority of sperm samples from both age groups were collected during fall and winter (80%).

Procedure for preparing sperm for PIXE

Semen aliquots were thawed and washed twice in 0.15 M ammonium acetate buffer (pH 7.4; Amereso, Solon, USA) to separate sperm from seminal plasma (Bench, et al. 1996). Samples were centrifuged (4124 g for 5 min) to sediment sperm and the pellets were resuspended in a small volume of 0.15 M ammonium acetate, pH 7.4. Seminal plasma and washed sperm (about 0.5 µL) were micro-pipetted onto separate ultraclean, 1-µm thick, transparent nylon foils stretched over a 15-mm diameter hole in a plastic support frame. The sperm and the seminal plasma were allowed to settle onto the nylon surface for 5 min, and the excess buffer was removed with a Pasteur pipette and blotting paper.

Measurements of sperm motility

Computer-assisted semen analysis (CASA) was performed using the HTM-Ceros semen analyzer (Hamilton Thorne Research, USA) according to the manufacturer's operation guidelines (Eskenazi, et al. 2003, Sloter, et al. 2006). Semen samples were maintained at room temperature until analyzed. Fifty microlitres of each sample were diluted 1:1 using Dulbecco's phosphate-buffered saline solution (DPBS) with 1 g/L of glucose and 0.3 g/l of bovine serum albumin (BSA). All samples with >70 × 10^6/mL sperm were diluted to a standardized sperm concentration of ~35 × 10^6/mL sperm. About 3–4 µL of diluted semen was pipetted into one side of a 2X-CEL 20-µm-depth chambered microscope slide (Hamilton Thorne Research) maintained at 37°C by a MiniTherm slide warmer (Hamilton Thorne Research). After 1 min, multiple microscope fields spanning the entire slide preparation were analysed on an Olympus CH30 microscope equipped with a 10× negative phase objective for 0.5 s per field using a

video frame rate of 60 Hz. When possible, at least 150 motile sperm were evaluated per drop of semen. Between 2 and 4 drops of diluted semen were evaluated per donor, and the median value was used for data analyses. Motile sperm includes rapid sperm plus progressive sperm. Per cent rapid sperm equals the number of sperm with time-average velocity ≥ 25 μm/s divided by the total number of sperm analyzed × 100. Per cent progressive sperm equals the number of sperm with time-average velocity ≥ 25 μm/s and straightness of trajectory ≥ 80% divided by the total number of sperm analyzed × 100 (Eskenazi, et al. 2003, Sloter, et al. 2006).

Scanning Transmission Ion Microscopy (STIM) for measurement of mass

Sperm and seminal plasma were examined in the nuclear microprobe facility located at the Lawrence Livermore National Laboratory. A beam of 3.0 MeV protons was used to quantitate the mass of thin samples using a beam of no more than 2000 ions per second. These ions interact with the electron density of the sample causing a loss in energy of the traversing ions. The energy loss was accurately measured with a particle detector behind the sample specimen (Bench, et al. 1996). An initial image was taken without any material in front of the detector to check the detector for defects and to provide for the zero energy loss data. Mylar foils of known areal density (mg/cm^2) were used to check the calibration. The data were reduced by selecting a region of interest (ROI) that contains the sample deposit and a ROI of the mylar that was free from containing any sample. The area of the sample-free ROI allowed the calculation of the mass of mylar that was within the sample region, which was then subtracted from the sample mass.

Proton Induced X-ray Emission (PIXE) for elemental analysis

The PIXE methods for measuring elemental concentrations in sperm were previously described in detail (Bench, et al. 1996). Briefly, a beam of 3.0 MeV protons was used to eject inner-shell electrons from atoms within the specimen. As resulting electron vacancies are filled by outer-shell electrons, characteristic x-rays are emitted whose energies identify the particular element. X-rays were detected simultaneously for elements with atomic numbers > 12 using solid-state energy dispersive detectors. The element limits of detection (LOD) in sperm and seminal plasma were determined by integrating the energy window corresponding to the background signal under the element peak.

The laboratory quality assurance procedure for using the nuclear microprobe at Lawrence Livermore National Laboratory has been previously described in detail (Bench et al., 1996). Background levels of the elements on the mylar foils were typically below minimum detectable limits of 5 ng/cm^2 for each sample. Data were reduced off-line and analyzed with X-ray spectrum codes (Bench et al., 1996). A series of thin film standards containing the elements were used to calibrate the X-ray detection system, and elemental masses of the sperm were calculated with the thin film approximation. The measurement of elemental masses using this calibration procedure has an accuracy that is better than 95% (Bench et al., 1996).

Statistical analysis

Descriptive demographic statistics were calculated for this sample of 20 men, including duration of abstinence (≤ 5 days versus > 5 days); season of sample collection (fall/winter versus spring/summer); body mass index (≤25 kg/m^2 versus >25 kg/m^2); and histories of previous tobacco use, regular alcohol use, vitamin supplement use in the last year, high blood pressure and kidney, bladder or urinary tract infection. The number and percent of participants in each category by age group was compared using the Fisher's exact test. Spearman correlations were performed to detect associations between levels of elements in washed sperm versus in seminal plasma. We summarized each elemental concentration as the median and interquartile range by age group and tested their differences using Mann-Whitney rank sum test. We used unadjusted and age-adjusted linear regression to determine whether these elements were associated with semen quality and genomic defects in sperm.

We constructed multivariable linear regression models to determine whether changes in elemental concentrations could explain previously determined associations between age and the various sperm/semen endpoints. These models focused on elemental measures that differed by age (Ca, Cu, and Zn measured in sperm and S measured in seminal plasma). The main independent variables were age group and the specific element, and the dependent variables were the various sperm/semen endpoints. To approximate a normal distribution, we square-root transformed sperm count, percent progressively motile, and total progressively motile sperm, and log-transformed DNA fragmentation, aneuploidy, comet, achrondroplasia and structural aberration variables. We also log-transformed all elemental concentrations. Separate models were run for each element and each outcome. We compared the coefficients

and p-values for age group in the crude linear regression models (without the element in the model) against the element-adjusted model to determine if the age associations persisted. If the age variable was no longer significant or if the coefficients had changed more than 10% after adjusting by an element, we concluded that the element may play a role in the poorer genetic integrity or semen quality of older men. P-values ≤ 0.05 were considered statistically significant.

Results

Characteristics of study population

Our study was performed on samples collected from the well-characterized AGES population, a non-clinical group of non-smoking men that included a younger group of 10 men with a median age of 23 years (age range= 22-28) and an older group of 10 men with a median age of 69 years (age range= 65-80). The older men tended to have slightly longer durations of sexual abstinence (p=0.06) and were more likely to report prior tobacco use (p=0.04) (Supplemental Table I).

Elements detected in sperm and seminal plasma

Nine elements of the PIXE spectrum were included in the analysis of isolated sperm: zinc, copper, calcium, sulfur, chlorine, potassium, titanium, iron and nickel (Figure I). An additional two elements (bromine and rubidium) were below the LOD in sperm and were measured only in seminal plasma. The concentrations of three elements were positively correlated between sperm and seminal plasma: sulfur ($r = 0.54$, $p = 0.01$), titanium ($r = 0.50$, $p = 0.02$) and zinc ($r = 0.47$, $p = 0.04$) (Supplemental Table II).

Elemental concentrations in washed sperm and seminal plasma of young and old men

Table I summarizes the median and interquartile range of element concentrations measured by PIXE in sperm and seminal plasma of the young and old age groups. The sperm of the old group had significantly higher concentrations of calcium, copper and zinc compared to the young group; the median levels in the old group were 2.2 times

higher for calcium (81 vs. 38 fg per sperm; p=0.02), 1.6 times higher for copper (0.08 vs. 0.05 fg per sperm; p=0.03) and 1.8 times higher for zinc (20 vs. 11 fg per sperm; p = 0.05). No statistically significant differences between age groups were found for the five other elements: titanium, iron, nickel, chlorine, and potassium, even though in all cases, concentrations were higher in the old group.

Sulfur was the only element out of 11, that showed an age-related difference in seminal plasma concentrations (median of 4600 ppm in old group and 3400 ppm in young group, p = 0.01).

Age associations with elemental concentrations, semen quality, and genomic defects in sperm of young and old men

Table II lists the unadjusted associations between the elements found to differ by age and sperm outcomes. Higher sperm calcium was associated with lower sperm motility (by CASA and conventional analyses), and with increased frequency of DNA fragmentation in sperm and sperm Comet damage (under alkaline but not neutral conditions). Higher sperm copper was also associated with lower sperm motility and higher DNA fragmentation. No statistically significant associations were found between sperm zinc and any of the semen quality and sperm endpoints used in this study (Table II). The concentration of sulfur in seminal plasma was associated with lower sperm motility, increased sperm DNA fragmentation, and increased frequencies of sperm with structural aberrations. When controlled for age (Supplemental Table III), the following associations were significant: sperm calcium and decreased sperm motility (p=0.01) and seminal sulfur and increased sperm count (p=0.04). Figure II shows the age-adjusted

and unadjusted relationships between sperm calcium concentration and sperm motility (using visual assessment of sperm motility as the example of the motility effect).

Associations between male age and semen/sperm endpoints adjusted for elemental measurements

As shown in Supplemental Table IV, we generally confirmed in our subgroup of 20 men the associations we previously reported between male age and various measures of semen quality and sperm damage assessed in the total AGES cohort (Eskenazi, et al. 2003, Schmid, et al. 2007 , Sloter, et al. 2007, Wyrobek, et al. 2006). Using the younger men, as reference, the group of older men had: (a) lower sperm motility (conventional medians: 12 vs. 47%, $p = 0.02$); (b) higher sperm DNA fragmentation measured by sperm chromatin structure analysis (SCSA) (4.1 vs. 2.4, $p < 0.01$); (c) a trend to higher single DNA strand breaks as measured by sperm Comet (1.7 vs. 1.6%, $p = 0.06$); and (d) higher frequencies of sperm with achrondroplasia mutations and with structural aberrations (1.7 vs. 1.1, $p = 0.01$ and 1.5 vs. 1.2, $p = 0.01$, respectively). In Table III, we control for the elements that were related to these endpoints. After adjusting for calcium in sperm, we found that the associations between age and sperm motility and between age and alkaline Comet were markedly reduced. Similarly, when we controlled for seminal sulfur, the associations between age and motility decreased while the associations between age and alkaline Comet increased. The other age-related differences in sperm endpoints changed by 10% or less after controlling for sperm calcium, sperm copper, sperm zinc, or sulfur in seminal plasma.

Discussion

Our study is the first to measure the concentrations of various elements in both sperm and seminal fluid using the sensitive PIXE method to investigate the effects of these elements on previously reported associations between male aging and the deterioration of certain semen quality and sperm genomic damage endpoints. In a pilot study of 20 men from our larger AGES study, we found that older men (≥65 years) had significantly higher levels of zinc, copper, and calcium in their sperm and higher levels of sulfur in their seminal fluid than younger men (≤28 years). We also found that higher sperm calcium was associated with lower sperm motility, increased frequency of DNA fragmentation in sperm, and a marginal increase in sperm Comet damage; higher sperm copper was also associated with lower motility and higher DNA fragmentation. In addition, seminal plasma sulfur was negatively associated with sperm motility and structural aberrations, and positively associated with DNA fragmentation. When we controlled for male age, only the negative association between sperm calcium and sperm motility remained, suggesting that sperm calcium concentrations are significantly associated with sperm motility, independent of male age. When we adjusted for calcium in sperm, the associations between overall aging and sperm motility and alkaline Comet damage were markedly reduced, implicating sperm calcium in motility as well as sperm single strand DNA breaks. However, when we controlled for sulfur in seminal plasma, although the associations between age and motility markedly decreased, the association between age and alkaline Comet increased.

Calcium (Ca^{2+}) has a broad role in male reproduction and sperm development. It is important for sperm physiology including motility, metabolism, acrosome reaction and

fertilization, but only a small portion (2%–4%) of the calcium in semen is present in ionized form (Meseguer, et al. 2004). Ca^{2+} influxes through the Ca^{2+} channels of the sperm plasma membrane for the acrosome reaction in the vicinity of the egg and plays a key role in mediating sperm fertility (Chung, et al. 2011). Motility is also dependent on intracellular free calcium concentration in the principal piece of the flagellum (Marquez, et al. 2007). The quantitative relationship between sperm intracellular Ca^{2+} levels and sperm motility is still controversial (Marquez, et al. 2007). It was shown that spermatozoa modulate their movement in response to an alteration in the intracellular calcium concentration which is dependent on the pH of the medium (Giroux-Widemann, et al. 1991, Serres, et al. 1991) Furthermore, Ca^{2+} apparently switches on hyperactivation a swimming pattern characterized by asymmetric flagellar beating and the development of high-amplitude flagellar waves, that is essential for fertilization (Bedu-Addo, et al. 2008). Calcium plays a central role in both acrosome reaction and sperm chemotaxis, with spermatozoa failing to perform either of these crucial functions in the absence of extracellular Calcium. The chemotaxis of sperm towards eggs is a widespread phenomenon that occurs in most forms of life from lower plants to mammals and plays important roles in ensuring fertilization (Yoshida and Yoshida 2011). However, the relationship between Ca^{2+}_{i} and the chemotactic response of a sperm flagellum is not well known (Bedu-Addo, et al. 2008).

Our findings are consistent with those of Arver and Sjöberg, who reported that low concentrations of ionized calcium in sperm were associated with better progressive motility (Arver and Sjoberg 1982, Arver and Sjoberg 1983). In addition, average sperm path velocities (straight line velocity (VSL) and linearity (LIN)) measured by CASA

showed significant inverse correlations to total calcium concentration in sperm (Meseguer, et al. 2004). VSL expresses the forward linear progress that a sperm travels in a defined amount of time, and is one of the most important clinical CASA parameters for motility. Marquez and colleagues showed that (a) adding calcium to demembranated human sperm suppressed motility, and (b) intracellular calcium concentrations following cryopreservation were negatively correlated with sperm motility and fertilizing ability (Marquez, et al. 2007). These findings suggest that intracellular calcium concentrations regulate sperm motility and that influx of extracellular calcium is required for successful fertilization.

Copper plays an essential role in spermatogenesis and male infertility (Aydemir, et al. 2006). The older men in our study had higher concentrations of copper in their sperm than the younger men, while seminal copper levels were similar between the two groups. Our analysis shows an association between higher concentrations of sperm copper and increased DNA fragmentation and poorer sperm motility. High levels of sperm copper are known to be detrimental to sperm maturation, motility and fertility (Massanyi, et al. 2004, Massanyi, et al. 2003, Skandhan 1992) and blood serum copper was also negatively associated with motile and viable sperm (Telisman, et al. 2000). Similarly, Aydemir and colleagues found higher copper levels in seminal plasma of subfertile males than in healthy males (Aydemir, et al. 2006), yet we found no age-related differences in seminal copper in our study. These results suggest that sperm copper may be a more sensitive indicator than seminal copper for age-related effects on sperm motility.

Zinc is broadly involved in male reproduction and fertility (Ebisch, et al. 2007, Evenson, et al. 1993, Henkel, et al. 1999, Henkel, et al. 2005, Lee, et al. 2009, Telisman, et al. 2007, Telisman, et al. 2000, Yuyan, et al. 2008, Zhao and Xiong 2005) and we observed that the older age group had increased sperm zinc. Our prior studies using PIXE analysis found zinc to be bound stoichiometrically with protamine 2 in sperm nuclei (Bench, et al. 2000). In our current study we found no significant associations between zinc concentrations in sperm or seminal plasma and any of the sperm or semen quality endpoints used in our study.

Our group of older men showed higher concentrations of sulfur in seminal plasma, with significant associations between concentrations of seminal sulfur and poorer sperm motility, more DNA fragmentation, and increased frequencies of sperm with chromosomal aberrations. After adjusting for age, these effects were no longer significant, but age-adjustment produced a minor association between sulfur in seminal plasma and sperm count. After adjusting for sulfur in sperm, there was an increased association between age and DNA breaks in the sperm. Prior data using PIXE (Bench, et al. 1998) showed that sperm from infertile men were highly deficient in both sulfur and protamines. Our study did not find a change is sperm sulfur between the old and young men, which suggests that the semen quality of the older men was not like that of infertile men.

Our study had several methodological strengths and limitations. Its major strength is that we were able to perform elemental analyses on the same semen samples for which we obtained extensive measurements of semen quality and genetically defective sperm, and the study cohort's demographic characteristics, habits

and medical and reproductive history were well documented. Another strength is that our sample was comprised of relatively healthy non-smokers with no evidence of reproductive problems. However, these characteristics may also limit the generalization of study findings to sick persons, clinical groups and ethnically diverse populations (Jha, et al. 1995). In the AGES study, only a single semen sample was provided by each man; thus, we cannot be sure that our single measurement represents the time-average element levels in the sperm and seminal plasma of these men. Our sample size of 20 men may be insufficient to detect small changes between the age groups, and did not allow us to consider multiple confounders of the association between elements and age or to determine whether the measured elements modified the relationships between age and the sperm endpoints. In addition, multiple tests were performed between our various elements and various outcomes; therefore, it is possible that some findings may be due to chance alone.

In summary, we found that older men have significantly higher elemental concentrations of copper, calcium and zinc in their sperm and higher levels of sulfur in their seminal plasma. These findings demonstrate major differences in elemental concentrations of whole sperm and seminal plasma between younger and older men, and that these elements are quantitatively associated with increased risks for poorer semen quality and genomic defects in sperm of older men.

Authors' roles: T.E. Schmid and A.J. Wyrobek conceived of this study, organized its execution, and drafted the manuscript. P.G. Grant was responsible for the PIXE analyses. R.H. Weldon performed statistical analyses and manuscript preparation. B. Eskenazi, F. Marchetti, and A.J. Wyrobek designed and conducted the original AGES study that provided the reference data for sperm quality and sperm genomic damage, and were vital to the interpretations and revisions of this manuscript.

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Tables

Table I. Elemental concentrations in washed sperm (fg per sperm) and seminal plasma (ppm) in the young and old groups of men.

	Washed sperm (fg/sperm)				Seminal Plasma (ppm)			
	Young Group 22-28 years N=10		Old Group 65-80 years N=10		Young Group 22-28 years N=10		Old Group 65-80 years N=10	
Element	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Sulfur	66.78	(47.96)	144.53	(128.03)	3400.9	(619.8)	4624.9	(1801.2)*
Chlorine	18.14	(23.05)	21.82	(27.22)	19219.6	(15480.9)	25715.3	(5731.1)
Potassium	29.01	(30.19)	31.69	(100.08)	49993.8	(24773.9)	52335.1	(22459.0)
Calcium	37.74	(43.25)	81.18	(40.53)*	25086.0	(10784.1)	25489.7	(13114.9)
Titanium	1.03	(0.51)	1.67	(2.81)	63.7	(82.4)	69.4	(109.0)
Iron	1.21	(1.25)	1.74	(1.79)	28.7	(26.2)	29.0	(24.0)
Nickel	0.08	(0.05)	0.10	(0.15)	18.1	(7.2)	18.8	(7.8)
Copper	0.05	(0.05)	0.08	(0.05)*	4.2	(3.0)	4.3	(2.7)
Zinc	10.98	(7.96)	19.66	(21.44)*	3314.6	(1448.6)	4417.4	(2175.2)
Bromine	N/A	N/A	N/A	N/A	26.7	(22.8)	44.2	(13.7)
Rubidium	N/A	N/A	N/A	N/A	22.7	(6.8)	24.7	(15.1)

*$p \leq 0.05$ by Mann-Whitney test

Table II. Unadjusted associations between concentrations of sperm calcium, sperm copper and sperm zinc and seminal plasma sulfur and semen and sperm quality.[a]

	Calcium in sperm				Copper in sperm				Zinc in sperm				Sulfur in seminal plasma			
Sperm/Semen Outcomes[b]	Coef.	(95% CI)		p	Coef.	(95% CI)		p	Coef.	(95% CI)		p	Coef.	(95% CI)		p
Sperm Count	-10.0	(-20.8,	0.8)	0.07	-2.2	(-13.6,	9.3)	0.70	-2.8	(-16.5,	10.9)	0.67	30.7	(-16.7,	78.1)	0.19
Motility																
Visual: % motile	-34.6	(-53.0,	-16.3)	<0.005	-16.8	(-39.7,	6.1)	0.14	-17.9	(-45.7,	9.9)	0.19	-102.9	(-195.0,	-10.8)	0.03
Visual: % progressively motile	-3.1	(-4.9,	-1.4)	<0.005	-2.1	(-4.1,	0.0)	0.05	-1.7	(-4.3,	0.8)	0.18	-7.8	(-16.7,	1.1)	0.08
Visual: Total progressively motile sperm	-9.1	(-14.2,	-4.0)	<0.005	-3.0	(-9.4,	3.4)	0.34	-3.8	(-11.5,	3.8)	0.31	-8.9	(-36.9,	19.2)	0.52
CASA: % motile	-38.6	(-61.2,	-15.9)	<0.005	-22.2	(-48.8,	4.4)	0.10	-22.9	(-55.4,	9.6)	0.16	-126.3	(-233.6,	-18.9)	0.02
CASA: % progressively motile	-22.0	(-33.7,	-10.3)	<0.005	-11.3	(-25.8,	3.2)	0.12	-13.3	(-30.7,	4.2)	0.13	-63.0	(-122.5,	-3.5)	0.04
CASA: % sperm with velocity >25um/s	-27.8	(-42.2,	-13.5)	<0.005	-13.5	(-31.6,	4.6)	0.14	-16.6	(-38.2,	5.0)	0.12	-78.0	(-152.0,	-4.1)	0.04
Sperm DNA Fragmentation																
SCSA: DNA fragmentation	1.33	(0.53,	2.12)	<0.005	0.94	(0.14,	1.74)	0.02	0.93	(-0.19,	2.05)	0.10	4.61	(1.32,	7.90)	0.01
SCSA: mean DFI	0.60	(0.22,	0.99)	<0.005	0.39	(-0.00,	0.78)	0.05	0.38	(-0.15,	0.92)	0.15	2.14	(0.59,	3.69)	0.01
SCSA: high DNA sustainability	-0.13	(-0.75,	0.48)	0.65	0.03	(-0.52,	0.58)	0.91	-0.42	(-1.10,	0.27)	0.22	-1.98	(-4.15,	0.19)	0.07
Sperm Aneuploidy																
X-Y-21 FISH: Hyperhaploidy	-0.14	(-0.29,	0.01)	0.06	-0.04	(-0.20,	0.12)	0.62	-0.16	(-0.33,	0.01)	0.07	-0.45	(-1.10,	0.20)	0.16
X-Y-21 FISH: Hypohaploidy	-0.10	(-0.27,	0.07)	0.22	0.02	(-0.15,	0.19)	0.83	0.00	(-0.21,	0.20)	0.96	-0.20	(-0.93,	0.53)	0.57
X-Y-21 FISH: Total hyper- & hypo- haploidy	-0.13	(-0.27,	0.01)	0.07	-0.01	(-0.16,	0.14)	0.87	-0.08	(-0.26,	0.09)	0.32	-0.33	(-0.95,	0.30)	0.29
X-Y-21 FISH: Diploidy	-0.07	(-0.44,	0.30)	0.71	0.22	(-0.12,	0.56)	0.20	-0.07	(-0.50,	0.36)	0.74	0.02	(-1.54,	1.57)	0.98
Sperm Comet																
Alkaline Comet: Tail DNA	0.13	(-0.00,	0.26)	0.05	0.08	(-0.04,	0.20)	0.18	0.06	(-0.11,	0.22)	0.48	0.22	(-0.34,	0.77)	0.41
Neutral Comet: Tail DNA	0.01	(-0.12,	0.13)	0.91	-0.02	(-0.13,	0.09)	0.74	-0.07	(-0.21,	0.06)	0.26	0.05	(-0.43,	0.53)	0.82
Sperm Gene Mutation																
Achrondroplasia	0.11	(-0.44,	0.67)	0.67	0.40	(-0.25,	1.05)	0.21	0.25	(-0.34,	0.83)	0.37	1.50	(-0.66,	3.65)	0.15
Sperm Chromosomal Aberrations																
ACM: breaks	0.04	(-0.15,	0.23)	0.64	0.10	(-0.08,	0.27)	0.27	0.10	(-0.11,	0.32)	0.32	0.78	(0.09,	1.47)	0.03
ACM: Duplications & deletions	0.04	(-0.28,	0.36)	0.80	0.26	(-0.03,	0.55)	0.07	0.17	(-0.20,	0.54)	0.34	1.17	(-0.06,	2.40)	0.06
ACM: structural aberrations	0.02	(-0.21,	0.24)	0.88	0.16	(-0.04,	0.37)	0.11	0.13	(-0.12,	0.38)	0.29	0.92	(0.09,	1.75)	0.03

[a] elemental concentrations were log-transformed; Count, % progressively motile sperm and total progressively motile sperm were square-root transformed and SCSA, aneuploidy, ACM, comet and achrondroplasia outcomes were log transformed; [b] Abbreviations:Computer Assisted Semen Analysis (CASA), Sperm Chromatin Structure Assay (SCSA), DNA Fragmentation Index (DFI), Fluorescence in situ hybridization assay for chromosomes X, Y and 21 (X-Y-21 FISH), Fluorescence in situ hybridization assay used to detect structural and numerical aberrations of chromosome 1 (ACM).

Table III. Associations between male aging and semen/sperm endpoints adjusting for sperm calcium, sperm copper, sperm zinc and sulfur in seminal plasma.

		Adjusted by Calcium in sperm			Adjusted by Copper in sperm			Adjusted by Zinc in sperm			Adjusted by Sulfur in seminal plasma		
Sperm endpoints[a]	N	Coef.	(95% CI)	% change in coefficient	Coef.	(95% CI)	% change in coefficient	Coef.	(95% CI)	% change in coefficient	Coef.	(95% CI)	% change in coefficient
% motile[b]	20	-13.77	(-31.26, 3.73)	-45%	-23.39	(-44.93, -1.85)	-7%	-24.01	(-45.08, -2.95)	-4%	-19.27	(-43.26, 4.72)	-23%
% progressively motile[c]	20	-10.14	(-21.04, 0.76)	-40%	-15.92	(-29.22, -2.61)	-7%	-15.80	(-28.76, -2.83)	-7%	-14.64	(-29.62, 0.34)	-14%
DNA fragmentation[d]	19	1.43	(0.99, 1.88)	-8%	1.50	(1.09, 1.92)	-3%	1.54	(1.16, 1.93)	0%	1.57	(1.10, 2.03)	1%
Alkaline COMET[e]	17	0.08	(-0.06, 0.21)	-32%	0.10	(-0.03, 0.23)	-10%	0.12	(-0.01, 0.25)	5%	0.25	(0.06, 0.43)	115%

[a] % progressively motile sperm was square-root transformed; SCSA and comet outcomes were log-transformed to satisfy model assumptions; [b] conventional semen quality analysis; [c] Computer-Assisted Semen Analysis (CASA); [d] Sperm Chromatin Structure Assay (SCSA); [e] Alkaline Comet analysis

Figure I. PIXE spectrum of elemental concentrations in washed human sperm.

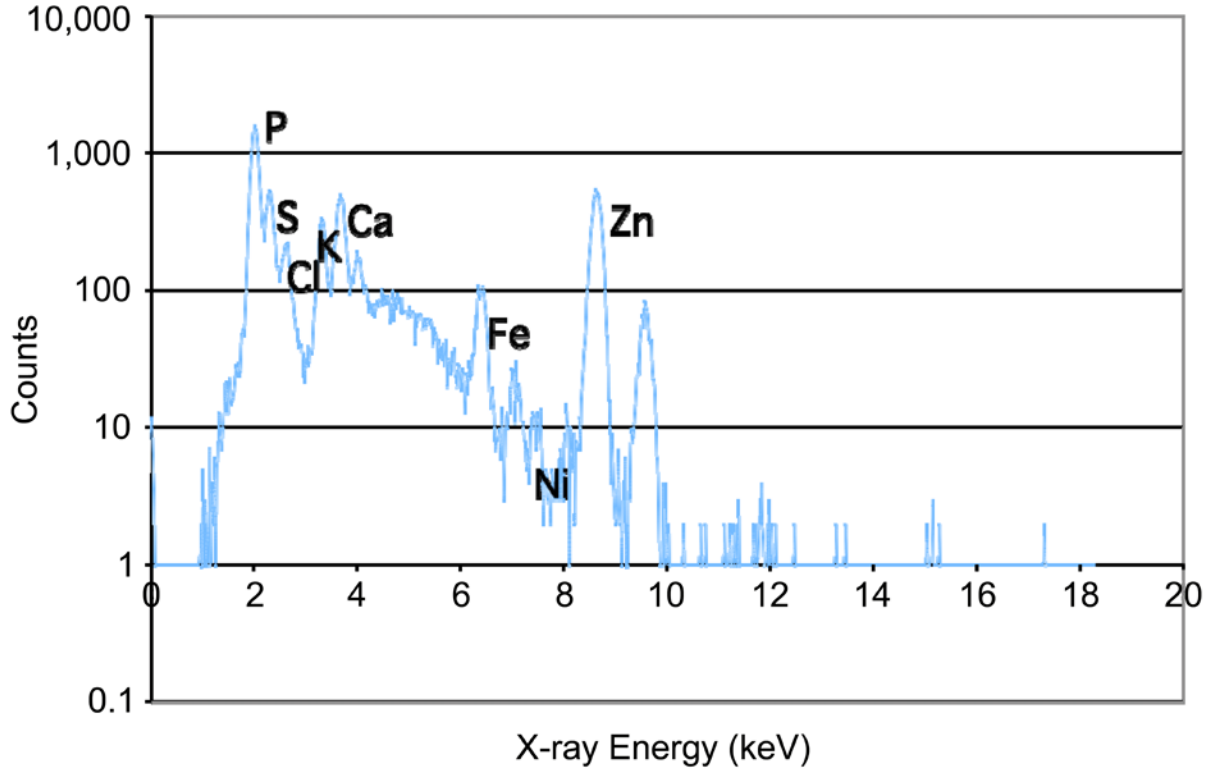
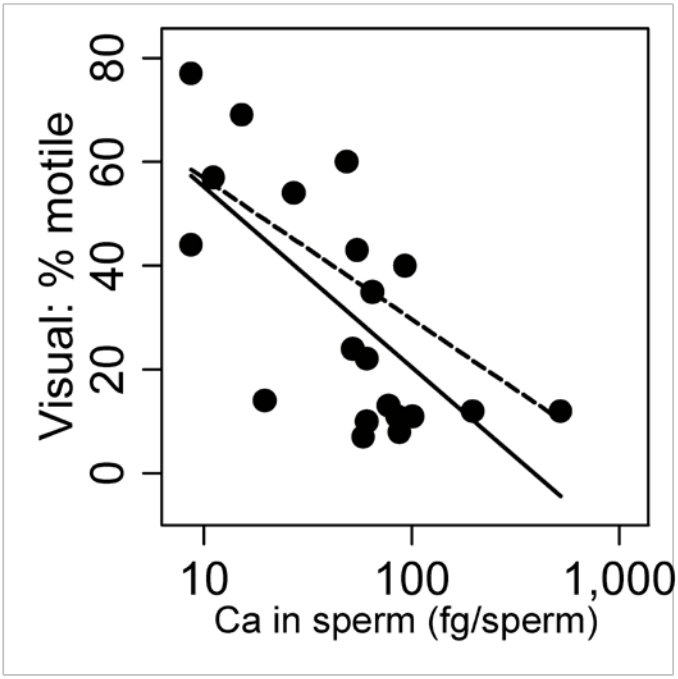


Figure II. Age-adjusted (dashed) and unadjusted (solid) associations between the concentration of calcium in sperm and sperm motility (% motile sperm).



Supplemental Material

Supplemental Table I. Characteristics of the young and old age groups selected for the PIXE analyses from the California AGES* study.

Characteristic	Young Group (22-28 years) N = 10		Old Group (65-80) N=10		Test statistic	p-value
Age, median (IQR)	23	(4)	69	(6)	N/A	N/A
Abstinence, median (IQR)	4	(1)	5	(6)	-1.88	0.06
Abstinence, n (%)						
≤ 5 days	8	(80)	6	(60)	0.63	0.31
> 5 days	2	(20)	4	(40)		
Previous tobacco use, n (%)						
No	10	(100)	6	(60)	0.09	0.04
Yes	0	(0)	4	(40)		
Regular alcohol use, n (%)						
No	2	(20)	3	(30)	1.00	0.50
Yes	8	(80)	7	(70)		
Vitamin supplement use in the last year, n (%)						
No	6	(60)	4	(40)	0.66	0.33
Yes	4	(40)	6	(60)		
Season of sample collection (2 category), n (%)						
Fall & winter	8	(80)	8	(80)	1.00	0.71
Spring & summer	2	(20)	2	(20)		
Body mass index (kg/m^2), n (%)						
≤ 25	4	(40)	5	(50)	1.00	0.50
> 25	6	(60)	5	(50)		
Ever had high blood pressure, n (%)						
No	10	(100)	7	(70)	0.21	0.11
Yes	0	(0)	3	(30)		
Ever had kidney, bladder, urinary tract infection, n (%)						
No	10	(100)	9	(90)	1.00	0.50
Yes	0	(0)	1	(10)		

[a] Ranksum test used for continuous covariates and Fisher's exact test used for categorical covariates.

* AGES: Age and Genetic Effects on Sperm study (see methods)

Supplemental Table II. Spearman correlations of elemental concentrations in sperm versus seminal plasma.

Element	Correlation	p-value
Sulfur	0.54	0.01
Chlorine	0.26	0.27
Potassium	0.02	0.92
Calcium	0.37	0.11
Titanium	0.50	0.02
Iron	0.08	0.75
Nickel	0.12	0.62
Copper	0.02	0.94
Zinc	0.47	0.04

Supplemental Table III. Age-adjusted associations between log-transformed calcium, copper and zinc concentrations measured in sperm and sulfur concentrations measured in seminal plasma and transformed semen/sperm endpoints.[a]

	Calcium in sperm				Copper in sperm				Zinc in sperm				Sulfur in seminal plasma			
Semen/sperm endpoints[b]	Coef.	(95%	CI)	p	Coef.	(95%	CI)	p	Coef.	(95%	CI)	p	Coef.	(95%	CI)	p
Sperm Count	-11.52	(-24.10,	1.07)	0.07	-1.41	(-15.08,	12.26)	0.83	-2.01	(-17.97,	13.95)	0.79	62.96	(4.36,	121.56)	0.04
Motility																
% motile	-27.10	(-47.10,	-7.10)	0.01	-3.79	(-27.68,	20.11)	0.74	-3.12	(-31.09,	24.86)	0.82	-43.42	(-158.44,	71.60)	0.44
% progressively motile	-2.50	(-4.40,	-0.61)	0.01	-1.09	(-3.27,	1.10)	0.31	-0.44	(-3.07,	2.19)	0.73	-1.62	(-12.61,	9.37)	0.76
Total progressively motile sperm	-8.14	(-14.02,	-2.26)	0.01	-0.22	(-7.32,	6.87)	0.95	-0.83	(-9.11,	7.45)	0.84	11.89	(-22.24,	46.02)	0.47
CASA % motile	-26.45	(-49.74,	-3.16)	0.03	-4.98	(-31.39,	21.43)	0.70	-3.18	(-34.15,	27.79)	0.83	-40.64	(-168.60,	87.33)	0.51
CASA % progressively motile	-16.47	(-28.93,	-4.01)	0.01	-2.47	(-17.23,	12.30)	0.73	-3.54	(-20.77,	13.69)	0.67	-17.79	(-89.62,	54.04)	0.61
CASA % sperm with velocity >25um/sec	-21.42	(-36.83,	-6.01)	0.01	-2.62	(-21.20,	15.96)	0.77	-4.94	(-26.57,	16.68)	0.64	-24.17	(-114.36,	66.01)	0.58
DNA Fragmentation																
SCSA: DNA fragmentation	0.22	(-0.32,	0.77)	0.40	0.10	(-0.35,	0.54)	0.65	0.02	(-0.53,	0.57)	0.94	-0.12	(-2.29,	2.05)	0.91
SCSA: mean DFI	0.11	(-0.20,	0.41)	0.46	-0.01	(-0.26,	0.24)	0.95	-0.03	(-0.34,	0.27)	0.83	0.05	(-1.16,	1.26)	0.94
SCSA: high DNA sustainability	0.35	(-0.37,	1.07)	0.32	0.38	(-0.18,	0.95)	0.17	-0.19	(-0.91,	0.54)	0.59	-1.08	(-3.92,	1.77)	0.43
Aneuploidy																
Hyperhaploidy	-0.15	(-0.33,	0.02)	0.08	-0.02	(-0.21,	0.17)	0.82	-0.17	(-0.37,	0.03)	0.09	-0.56	(-1.43,	0.31)	0.20
Hypohaploidy	-0.15	(-0.34,	0.04)	0.12	0.01	(-0.19,	0.21)	0.94	-0.02	(-0.26,	0.21)	0.84	-0.46	(-1.41,	0.50)	0.33
Total hyper- & hypo-haploidy	-0.16	(-0.32,	0.00)	0.05	-0.01	(-0.19,	0.17)	0.90	-0.10	(-0.31,	0.10)	0.30	-0.52	(-1.35,	0.31)	0.20
Diploidy	0.02	(-0.40,	0.45)	0.91	0.41	(0.05,	0.77)	0.03	0.03	(-0.46,	0.52)	0.89	0.85	(-1.15,	2.84)	0.38
Sperm Comet																
Comet: Tail DNA, alkaline	0.07	(-0.09,	0.24)	0.35	0.02	(-0.11,	0.16)	0.73	-0.02	(-0.18,	0.15)	0.83	-0.71	(-1.55,	0.12)	0.09
Comet: Tail DNA, neutral	-0.02	(-0.18,	0.14)	0.79	-0.05	(-0.18,	0.09)	0.46	-0.11	(-0.26,	0.03)	0.12	-0.18	(-1.08,	0.72)	0.67
Gene Mutation																
Achrondroplasia	-0.06	(-0.44,	0.32)	0.73	0.11	(-0.38,	0.61)	0.62	0.01	(-0.42,	0.44)	0.96	-2.12	(-4.31,	0.07)	0.06
Chromosomal Aberrations																
ACM: breaks	-0.07	(-0.25,	0.12)	0.45	0.00	(-0.19,	0.19)	1.00	0.00	(-0.22,	0.21)	0.97	0.42	(-0.46,	1.31)	0.33
ACM: Duplications & deletions	-0.14	(-0.47,	0.18)	0.37	0.15	(-0.18,	0.47)	0.36	0.00	(-0.38,	0.39)	0.99	0.54	(-1.05,	2.13)	0.48
ACM: structural aberrations	-0.12	(-0.34,	0.10)	0.28	0.07	(-0.15,	0.29)	0.52	0.01	(-0.25,	0.28)	0.93	0.53	(-0.55,	1.60)	0.31

[a]Count, % progressively motile sperm and total progressively motile sperm were square-root transformed and SCSA, aneuploidy, ACM, comet and achrondroplasia outcomes were log transformed. Age was a categorical variable (young vs. old); [b] Abbreviations:Computer Assisted Semen Analysis (CASA), Sperm Chromatin Structure Assay (SCSA), DNA Fragmentation Index (DFI), Fluorescence in situ hybridization assay for chromosomes X, Y and 21 (X-Y-21 FISH), Fluorescence in situ hybridization assay used to detect structural and numerical aberrations of chromosome 1 (ACM).

Supplemental Table IV. Median and interquartile range for sperm biomarkers of physiological and genomic damage in young and old age groups and the significance of the difference between the groups[a]

Sperm Biomarkers of Physiological and Genomic Damage (Method used: endpoint)[b]	Young men N	Young men Median	Young men IQR	Old men N	Old men Median	Old men IQR	ranksum p-value
Visual: Sperm Count	10	19.5	(10.4, 26.4)	10	15.2	(5.9, 24.3)	0.50
Visual: % motile	10	47.0	(24.0, 60.0)	10	12.0	(11.0, 22.0)	0.02
Visual: % progressively motile	10	5.7	(3.0, 6.6)	10	2.2	(2.0, 3.0)	0.01
Visual: Total progressively motile sperm	10	8.8	(6.0, 16.3)	10	4.8	(0.9, 8.8)	0.07
CASA: % motile	10	56.0	(13.0, 66.0)	10	6.8	(3.0, 23.0)	0.00
CASA: % progressively motile	10	26.8	(6.5, 32.5)	10	2.5	(1.0, 10.0)	0.01
CASA: % sperm with velocity >25um/sec	10	35.0	(8.5, 41.5)	10	3.8	(1.5, 15.0)	0.01
SCSA: DNA fragmentation	10	2.4	(2.0, 2.7)	9	4.1	(3.8, 4.2)	0.00
SCSA: mean DFI	10	5.3	(5.2, 5.4)	9	6.1	(5.9, 6.1)	0.00
SCSA: high DNA sustainability	10	1.8	(1.6, 2.2)	9	1.5	(0.9, 1.8)	0.12
Sperm FISH X-Y-21: Hyperhaploidy	10	1.5	(1.4, 1.6)	10	1.4	(1.3, 1.6)	0.50
Sperm FISH X-Y-21: Hypohaploidy	10	1.4	(1.3, 1.5)	10	1.4	(1.3, 1.5)	0.65
Sperm FISH X-Y-21: Total hyper- & hypo-haploidy	10	1.7	(1.6, 1.9)	10	1.7	(1.6, 1.8)	0.82
Sperm FISH X-Y-21: Diploidy	10	1.1	(0.8, 1.4)	10	1.0	(0.8, 1.3)	0.33
Sperm Comet: Tail DNA, alkaline	10	1.6	(1.6, 1.7)	7	1.7	(1.7, 1.8)	0.06
Sperm Comet: Tail DNA, neutral	10	1.5	(1.3, 1.6)	7	1.5	(1.5, 1.5)	0.92
PCR: Achrondroplasia	9	1.1	(1.0, 1.2)	4	1.7	(1.4, 2.0)	0.01
Sperm FISH ACM: breaks	10	1.0	(1.0, 1.1)	10	1.3	(1.1, 1.4)	0.03
Sperm FISH ACM: Duplications & deletions	10	0.8	(0.5, 0.8)	10	1.1	(1.0, 1.1)	0.01
Sperm FISH ACM: structural aberrations	10	1.2	(1.2, 1.3)	10	1.5	(1.3, 1.6)	0.01

[a] Sperm endpoints were measured within the same samples measured by PIXE. Sperm count (10^6), % progressively motile sperm and % total progressively motile sperm were square-root transformed and SCSA, aneuploidy, ACM, comet and achrondroplasia outcomes were log transforme; [b] Abbreviations:Computer Assisted Semen Analysis (CASA), Sperm Chromatin Structure Assay (SCSA), DNA Fragmentation Index (DFI), Fluorescence in situ hybridization assay for chromosomes X, Y and 21 (X-Y-21 FISH), Fluorescence in situ hybridization assay used to detect structural and numerical aberrations of chromosome 1 (ACM).d.