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ARTICLE

Investigation of sperm telomere length as a potential marker of paternal genome integrity and semen quality

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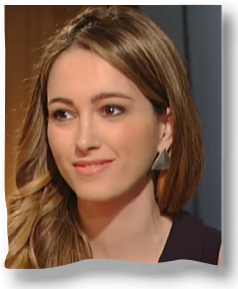

Federica Cariati obtained her Masters degree in Biotechnology of Reproduction Magna cum Laude from University of Teramo (2009), Masters in Medicine of Reproduction from University of Padua (2011) and her PhD in Biotechnology of Human Reproduction (2014) from the University of Rome "Sapienza", Italy. Since 2015, she has specialized in clinical pathology at the University of Naples "Federico II". Currently, she is working as a research assistant at CEINGE-Biotecnologie Avanzate, Naples. Dr Cariati's current research is on the effect of chromosome abnormalities on human reproduction. Her work also involves the development of new methods for sperm DNA analysis.

Abstract Recent studies have reported shorter sperm telomere length (STL) in men with idiopathic infertility. The aim of this study was to measure STL in semen samples from men to evaluate whether STL variation is associated with chromosomal abnormality, DNA fragmentation, traditional semen parameters, IVF outcome, or all four factors. A significant correlation between telomere length and diploidy was observed ($P = 0.037$). Additionally, STL was found to be positively associated with sperm count ($P = 0.006$); oligospermic samples had particularly short telomeres (0.9 ± 0.1 versus 1.4 ± 0.1; $P = 0.0019$). The results confirmed a link between sperm DNA fragmentation and aneuploidy, previously proposed ($P = 0.009$). A negative relationship was demonstrated between sperm concentration and aneuploidy and Sperm DNA framentation ($P = 0.03$, $P < 0.0001$, respectively). For a subset of 51 of the 73 sperm samples used for fertilization, IVF outcomes were known. A total of 17.6% of these samples had atypical STLs. None of these samples produced an ongoing pregnancy. In contrast, the pregnancy rate for samples that had STLs in the normal range was 35.7% ($P = 0.044$). In conclusion, STL has potential as a fast and inexpensive form of sperm quality assessment.

KEYWORDS: aneuploidy, human sperm, male infertility, sperm DNA damage, telomere length

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Introduction

Telomeres are structures composed of non-coding tandem repeats of a TTAGGG DNA sequence, located at the end of each chromosome arm (Moyzis et al., 1988). Together with numerous telomere-associated proteins, including the six-member Shelterin complex, telomeres play a key role in the maintenance of chromosome stability and genome integrity (O'Sullivan and Karlseder, 2010). Additionally, telomeres serve important functions in meiosis, helping to facilitate chromosomal alignment, pairing, synapsis and crossing over, which are critical steps during gamete formation (O'Sullivan and Karlseder, 2010).

Telomere length is maintained by telomerase, a reverse transcriptase, that is maximally expressed in a few types of highly proliferative cells, such as germ and neoplastic cells (Blackburn, 1991). In normal somatic cells, telomeres shorten with each mitotic division, eventually reaching a critical length associated with the induction of senescence, cell cycle arrest and apoptosis (Harley et al., 1990).

Three telomere-specific differences between somatic cells and sperm are presently known. First, in contrast to the telomeres of somatic cells, those in sperm do not shorten with age, ensuring the transmission of intact chromosomes over generations. Indeed, several studies have reported that increasing paternal age is actually associated with longer telomeres in sperm and in the leukocytes of offspring (Aston et al., 2012; Eisenberg et al., 2012; Ferlin et al., 2013; Kimura et al., 2008; Unryn et al., 2005). The length of telomeric DNA in human spermatoza is 10–20 kb, about twice the 5–10 kb typically observed in somatic cells (Kozik et al., 1998). Second, different telomere binding proteins have been isolated in spermatoza, and in-vitro studies have confirmed their involvement in telomere DNA recognition (Gineitis et al., 2000). Third, during spermatogenesis, telomeres migrate towards the nuclear membrane where they form telomere associations (Gineitis et al., 2000).

A relationship between telomere function and aspects of semen quality is an intriguing possibility, of potential clinical importance. Studies into the relationship between telomere length and different sperm parameters, however, have yielded contradictory data. In the case of sperm DNA fragmentation, a marker often assessed in fertility clinics to shed light on the genetic integrity of a sample, discordant results have prevented a consensus being reached on the association of telomere length and DNA damage (Moskovtsev et al., 2010; Thilagavathi et al., 2013). It is conceivable that fragmentation, or other types of DNA damage, could result in delocalization of telomeres, with consequences for chromosome segregation and nuclear architecture. The arrangement of chromosomes within the nucleus has been proposed to be important for fertilization, appropriate gene expression, and early embryo development. Therefore, correct positioning of telomeres is likely to be of significance for viability (Ward and Zalensky, 1996).

A few studies have indicated that measurement of telomere length in somatic cells may provide useful information concerning reproductive potential. Shorter telomeres in the leukocytes of both men and women seem to be associated with some cases of idiopathic recurrent pregnancy loss (Thilagavathi et al., 2013). At this time, however, little evidence supports the notion that variation in the length of sperm telomeres is related to reproductive capacity. Indirect data, suggesting that sperm telomere length (STL) may be important, comes from research showing that shorter telomeres can be detected in some samples from men with idiopathic infertility (Thilagavathi et al., 2013). Few studies have been published on STL in relation to semen parameters, and the small amount of information that is available is contradictory. Thilagavathi et al. (2013) did not find any correlation with conventional sperm parameters and telomere length, whereas other studies reported a positive association between STL and sperm count in young donors (aged 18–19 years) (Ferlin et al., 2013) and in infertile patients (Yang et al., 2015).

The biological reasons for variation in STL in samples from different men and its potential correlations with fertility, outcome of assisted reproductive treatment and other clinical features remain poorly understood. Moreover, no data have been reported on STL in relation to chromosome abnormality in human sperm. Therefore, the aim of this study was to provide a detailed assessment of STL in men at ages typically encountered at fertility centres, and to evaluate whether telomere length variation is associated with chromosomal abnormality, DNA fragmentation, traditional semen parameters, IVF outcome, or all four factors. Additionally, this paper provides information on the potential utility of STL assessment in the evaluation of male infertility, considering the extent to which the information can add to, complement, or replace that conveyed by other forms of semen assessment.

Material and methods

Participants

In this study, 73 samples from men aged between 31 and 52 years, who had requested sperm DNA fragmentation and aneuploidy analyses, were assessed. Participants were divided in normozoospermic (total sperm count ≥15 million/ml; $n = 54$) and oligozoospermic (sperm count <15 million/mL; $n = 19$). The study was approved in 2013 by the Institutional Ethics Committee of the University of Naples Federico II by protocol No. 42/13, and a written informed consent form was signed by all the participants involved in the study. The study was conducted in accordance with the principles expressed in the Declaration of Helsinki.

Sperm preparation

Standard semen analysis was carried out according to World Health Organization protocol (World Health Organization, D.o.R.H.a.R, 2010) after 2–4 days of sexual abstinence. An aliquot of semen sample was assessed using the Sperm Chromatin Dispersion Test (SCDt) for sperm DNA fragmentation (SDF) analysis, whreas another was used for the analysis of chromosome numerical abnormalities. Multi-colour fluorescence in-situ hybridization with probes specific to chromosomes 13, 18, 21, X and Y was used. This has previously been described (Enciso et al., 2013). Additionally, a maximum of 10 x 10^6/ml of sperm was centrifuged using Percoll gradient (PureSperm, Nidacon International AB, Sweden) and checked

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Table 1 Patient characteristics and semen quality parameters[a] in the oligozoospermic and normozoospermic groups.

	Normozoospermic group	Oligozoospermic group	*P*-value[b]
Number of patients	54	19	
Age (years)	39.4 ± 5.5	39.3 ± 5.3	
Sperm count (x10^6/ml)	43.1 ± 3.4	7.2 ± 1.1	<0.001
Sperm motility (%)	60.6 ± 1.8	53.2 ± 4.9	NS
Normal sperm morphology (%)	23.7 ± 0.5	17.4 ± 2.0	0.0027
Relative STL	1.4 ± 0.1	0.9 ± 0.1	0.0024
SDF	29.7 ± 0.6	40.9 ± 1.3	0.0005
Aneuploidy rate	5 ± 0.3	6.4 ± 0.6	0.047

[a]According to World Health Organization (WHO, 2010).
[b]Mann–Whitney test, $P < 0.05$.
Age values are mean ± SD. All other values are mean ± SEM.
NS, not statistically significant; SDF, sperm DNA fragmentation index; STL, sperm telomere length.

under the microscope to confirm the absence of round cell and contaminating somatic cells.

Telomere length measurement

Sperm DNA was extracted using the QIAamp DNA Mini Kit according to manufacturer's recommendations. The STL was measured as the average telomere length from all the spermatozoa present in each sample using quantitative polymerase chain reaction. The average amount of telomere DNA relative to a single copy gene (*36B4*) was assessed using the StepOnePlus quantitative polymerase chain reaction (Q-PCR) platform (Life Technologies, UK). Briefly, triplicate DNA samples were amplified in parallel reactions that included 1.5 ng/μl per well of sample DNA, Power SYBR® Green Master Mix (Life Technologies, UK) and 0.05 μM of specific primers for the telomere (tel 1: 5ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT3; tel2 5TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA3) and for the 36B4 (36B4u, 5CAGCAAGTGGGAAGGTGTAATCC3; 36B4d, 5CCCATTCTATCATCAACGGGTACAA3) (Cawthon, 2002). The thermal cycling conditions were as follows: one cycle at 95°C for 10 min, followed by 30 cycles at 95°C for 15 s and 60°C for 1 min. Melting curve analysis was used to check for primer specificity and the PCR was optimized by developing standard curves using serial dilutions from a reference DNA, carried out in each assay. The linear correlation coefficient (r2) was greater than 0.99 for both the telomere and *36B4* standard curves. Each sample's STL was calculated using the mean Ct for telomere and the *36B4* single-copy gene according to the formula: ΔCtsample = Cttelomere – Ctcontrol. The relative STL was then calculated by normalizing the values against a common reference sample run within each experiment.

Statistical analysis

Kolmogorov–Smirnov test was used for evaluating the normality of the data. Mann–Whitney U-test or chi-squared test was used as appropriate. Spearman's rho was calculated for correlation analysis. $P < 0.05$ was considered statistically significant. All analyses were carried out using the SPSS Statistics software, version 22.0 (IBM Corps., Armonk, NY, USA).

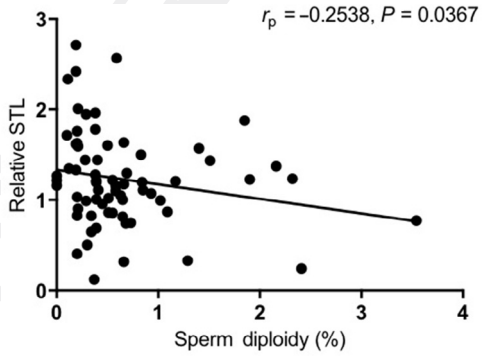

Figure 1 Correlation between relative sperm telomere length (STL) and diploid sperm. Relative STL is a normalized measure of the ratio of telomere copy number to the *36B4* gene. The Pearson's correlation coefficient (rp) and *P*-value are shown.

Results

Patient characteristics and semen quality parameters are shown in **Table 1**. The analysis of STL in relation to genetic complement showed that shorter telomeres are correlated with an elevated percentage of diploidy (determined by the detection of two copies of each of the chromosomes assessed within the same sperm nucleus) (R = –0.254; $P = 0.037$) (**Figure 1**). No direct significant correlation was found between STL and overall aneuploidy rate or SDF (R = –0.071 and R = –0.105, respectively).

Analysis of the data produced during this study also showed that samples with elevated aneuploidy rates show high levels of spermatozoa displaying DNA fragmentation, confirming the findings of a previous study (Enciso et al., 2013) (R = 0.312; $P = 0.009$) (**Figure S1**). Interestingly, correlation of SDF results with the aneuploidy data obtained for each chromosome revealed a strong association between chromosome 18 abnormality and DNA fragmentation (R = 0.297; $P = 0.013$) (**Figure S1**). This relationship was not observed for other chromosomes.

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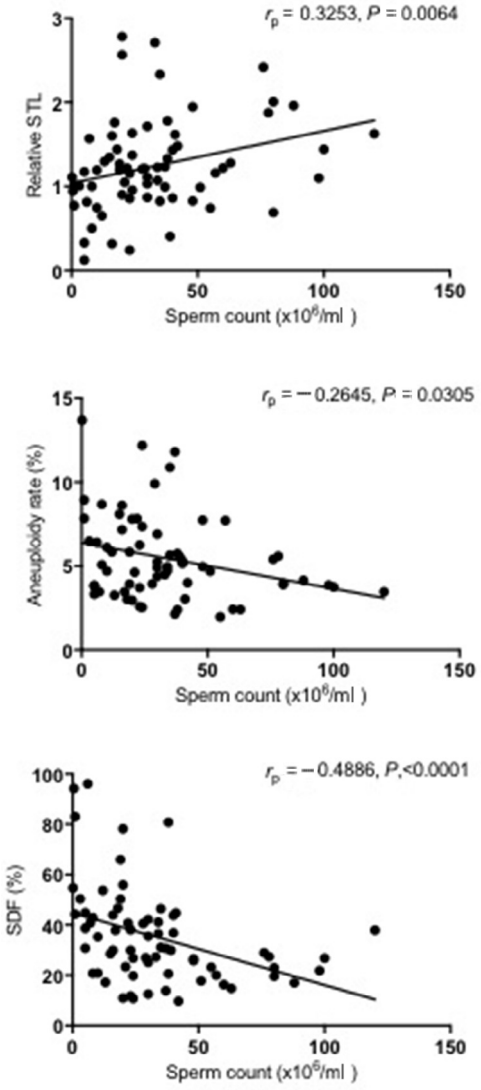

Figure 2 Correlation between relative sperm telomere length (STL) and sperm count (upper panel), sperm aneuploidy rate and sperm count (middle panel) and between sperm DNA fragmentation (SDF) and sperm count (bottom panel). Relative STL is a normalized measure of the ratio of telomere copy number to the *36B4* gene. The Pearson's correlation coefficient (rp) and *P*-value are shown.

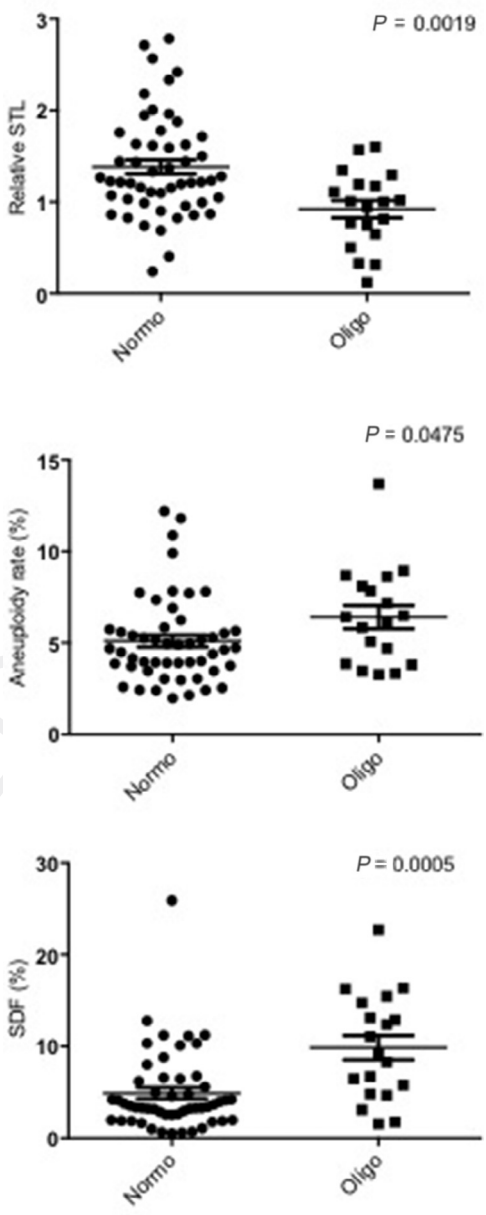

Figure 3 Relative sperm telomere length (STL) (upper panel), aneuploidy rate (middle panel) and sperm DNA fragmentation (SDF) (lower panel) in participants with normozoospermia (sperm count ≥ 15x10⁶/ml; normo; $n = 54$) and oligozoospermia (sperm count ≤ 15 x 10⁶/ml; oligo, $n = 19$). The Mann–Whitney test was carried out and the *P*-value is shown.

Results from the analysis of STL in relation to conventional semen parameters revealed a significant positive correlation between telomere length and sperm count (R = 0.325; $P = 0.006$) (Figure 2). Additionally, sperm count had a significant negative relationship with aneuploidy rate and SDF participants were grouped based on sperm concentration, patients with isolated oligozoospermia, i.e. all semen parameters normal with the exception of concentration showed significantly shorter STL ($P = 0.0019$) and higher rates of aneuploidy and SDF compared with normozoospermic men ($P = 0.048$; $P = 0.0005$) (Figure 3).

Most of the 73 samples assessed had relative telomere lengths that were distributed around the mean value, which was 1.25 (84% of STL measurements fell between 0.5 and 2.0). Sixteen per cent of samples, however, had STL that displayed a more obvious deviation from the average than the other samples examined, evenly split between those with unusually high STL measurements, i.e. long telomeres, and those with low values, i.e. short telomeres.

A Venn diagram was drawn, for samples with abnormal parameters only, to highlight the extent to which elevated levels of aneuploidy, increased amounts of DNA fragmentation and atypical telomere lengths (unusually short or long) overlap in sperm samples. Cut-offs for these parameters, used to distinguish "normal" samples from "abnormal", have been validated internally in our laboratory (30% for SDF, 6% for

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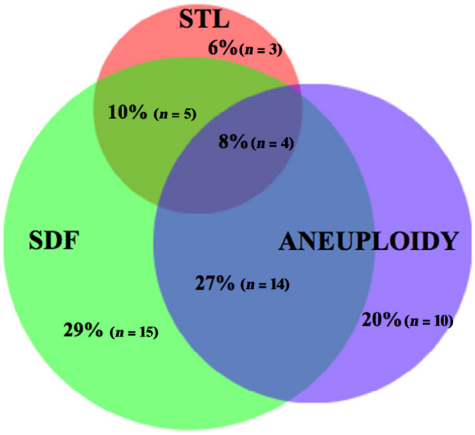

Figure 4 Sperm telomere length (STL), aneuploidy rate and sperm DNA fragmentation (SDF) analysis for patients with atypical sperm telomere length (STL) < 0.5 and >2, aneuploidy rate >6% and sperm DNA fragmentation (SDF) > 30%. The cut-offs have been validated internally.

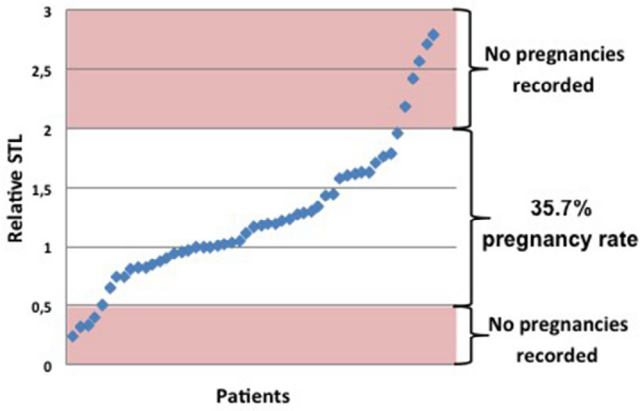

Figure 5 Distribution of relative sperm telomere length (STL) related to the occurrence of an ongoing pregnancy. Relative STL is a normalized measure of the ratio of telomere copy number to the *36B4* gene.

aneuploidy rate) (Figure 4). The "normal" range for relative STL (0.5–2) was based on the observed data distribution in this study. Atypical STL was less common than elevated DNA fragmentation or increased aneuploidy and in most cases overlapped with one or both types of anomaly. Seventy-five per cent of samples with atypical telomere length were also compromised for DNA fragmentation, aneuploidy, or both.

The outcomes of fertility treatments were known for 51 sperm samples used for fertilization (35 intracytoplasmic sperm injection and 16 IVF). Forty-two of these samples had relative STLs in the normal range (0.5–2.0) and the ongoing pregnancy rate (more than 12 weeks) for assisted reproductive cycles in which they had been used was 35.7% (15/42). Nine of the samples had relative STLs outside this range (four <0.5 and five >2.0), and none of the treatments using these samples resulted in a pregnancy (0.0% pregnancy rate). The difference in pregnancy rate between the two classes of sample, defined by STL, was statistically significant ($P = 0.04$), suggesting that this parameter may be predictive of the clinical outcome (Figure 5).

Discussion

The results of this study indicate that unusually short telomeres in sperm are likely associated with chromosomal abnormality, specifically failed meiotic division leading to the production of diploid sperm. Previous studies focusing on somatic cells have demonstrated that excessive telomere shortening results in chromosome instability (Blackburn, 2000), but the underlying mechanism remains unclear and discordant findings have been reported. A few studies, on amniocytes and peripheral blood from newborns, have shown a higher rate of telomere shortening in individuals with trisomy 21 (Vaziri et al., 1993; Wenger et al., 2014). Additionally, some in-vitro experiments have indicated that hematopoietic stem cells under proliferative stress exhibit telomere. In another model, a link between aneuploidy and ultra-short telomeres was not observed, although there was a relationship with carcinogenesis (Friis-Ottessen et al., 2014).

Analysis of the female gamete has revealed a significant reduction in the amount of telomeric DNA associated with aneuploidy. This has bvee shown in studies in which telomere DNA was quantified in aneuploid polar bodies and chromosomally normal polar bodies from oocytes of the same patient and within the same IVF treatment cycle (the polar bodies mirroring the telomere lengths present in the oocytes from which they were derived) (Treff et al., 2011). After fertilization, the relationship between telomere length and aneuploidy seems to persist, leading some researchers to suggest that shorter telomeres predispose to chromosome malsegregation in preimplantation embryos as well as gametes (Simpson and Wells, 2014; Treff et al., 2011). In mosaic cleavage-stage embryos, aneuploid cells were reported to have shorter telomeres compared with chromosomally normal cells from the same embryo. Additionally, shorter telomeres were observed in the embryos of patients of advanced reproductive age who had a history of repeated spontaneous abortion, hinting at a potential role for telomeres in viability at later stages of development (after establishment of a pregnancy) (Mania et al., 2014).

The association of telomere length and simple aneuploidy, reported in oocytes and preimplantation embryos, was not observed in spermatoza in the present study. An association with diploidy, however, was detected. Although statistically significant, the effect was relatively subtle, rates of diploidy rarely exceeding 1% even in samples with the shortest telomeres. The increase in diploid sperm in samples in which the average telomere length was relatively short might be a consequence of reduced meiotic recombination and impaired chromosome pairing and synapsis, features previously described in the sperm of telomerase deficient mice (Franco et al., 2002; Hemann et al., 2001; Lee et al., 1998). Anything that disrupts the capacity of telomeres to organize homologous chromosomes in the nucleus could conceivably increase the likelihood of meiotic errors occurring, ultimately resulting in aneuploid, and possibly diploid, sperm (Emery and Carrell, 2006). An increased frequency of

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diploidy has also been reported in the sperm of infertile patients with altered semen parameters such as concentration, motility and morphology (Pang et al., 1999; Younan et al., 2015).

In accordance with other studies using Q-PCR for the assessment of telomere length, no correlation between STL and SDF was found (Thilagavathi et al., 2013a, 2013b). A positive correlation between levels of sperm DNA-damage and aneuploidy rates, however, was detected, in agreement with recent published research (Enciso et al., 2013). Interestingly, increased SDF was particularly apparent in samples with elevated rates of aneuploidy involving chromosome 18, suggesting that abnormal copy number of genes situated on that chromosome may be especially likely to be associated with DNA fragmentation or abortive apoptosis in spermatozoa.

When samples were grouped according to sperm concentration, those that were oligozoospermic showed significantly shorter telomere lengths compared with samples produced by normozoospermic men. Reduced telomere length was recently reported in oligozoospermic samples from young patients (aged 18–19 years) (Ferlin et al., 2013). The research presented here shows that this correlation is a general feature of sperm and is not restricted to a particular age group.

A relationship between oligozoospermia and aneuploidy rate was also noted. An elevated frequency of sperm chromosome abnormality is well known to be related to infertility in some men (Piomboni et al., 2014; Ryu et al., 2001). Progressively higher levels of aneuploidy were seen with declining semen quality, as assessed based upon traditional parameters (e.g. the higher the incidence of morphologically abnormal forms the greater the rate of aneuploidy) (Carrell et al., 2004; Vegetti et al., 2000). In line with published data, our results show a negative correlation between sperm count and aneuploidy rate and also a higher incidence of chromosome abnormality in oligozoospermic men when compared with those with normozoospermia (Durak Aras et al., 2012; Fes'kov et al., 2013; Omran et al., 2013). Additionally, an inverse correlation between aneuploidy rate and sperm motility has also been demonstrated (Levron et al., 2013). Sperm samples with a high rate of aneuploidy have been associated with poor IVF treatment outcome when used for fertilization (Burrello et al., 2003; Carrell et al., 2003).

Patients with oligozoospermia also displayed elevated SDF, in agreement with previous studies reporting significant negative correlations between sperm DNA damage and abnormal semen parameters, including low concentration (Fei et al., 2013; Novotny et al., 2013). The fact that patients with oligozoospermia frequently display reduced STL, high SDF and elevated aneuploidy rates suggests that diminished sperm concentration is symptomatic of meiotic and spermatogenic processes that are compromised on multiple levels. Even when spermatozoa from such patients can be found and used for fertilization, an increased risk that the embryos produced will not be viable is likely. It is possible that SDF and aneuploidy may have effects on sperm that are independent of telomere length. It seems, however, that STL is predictive of semen quality and identifies poor samples (with too long or too short STL) that could be missed by current SDF and aneuploidy assessment.

Interestingly, 17.6% of samples from group of 51 patients with known outcome of fertility treatment had atypical STLs, appearing as outliers (**Figure 5**), with quantities of telomere DNA that were either exceptionally large or unusually small. No pregnancies were achieved when these samples were used during assisted reproductive treatments. This compares with a pregnancy rate of 35.7% for samples with STLs falling within the typical range, a statistically significant difference. Within that range, samples with higher STLs were associated with higher pregnancy rates (24%, 44% and 55% for STLs between 0.51.0, >1.0–1.5 and >1.5–2.0, respectively). The sample size, however, was not sufficient for robust statistical analysis of so many subdivisions of the data. It should also be noted that the "atypical" group of samples was relatively small and consequently it would be valuable to confirm these observations in a larger, independent series of samples. Nonetheless, this is an extremely interesting and potentially important finding.

In conclusion, it is clear that defects in the genetic constitution of the paternal germ line can influence the course of embryonic development and the outcome of assisted reproductive treatments. Methods exist for the analysis of sperm DNA integrity and aneuploidy, and such tests are already widely used by fertility clinics. Evaluation of sperm telomere length represents another form of genetic assessment, which can be carried out rapidly (<4 h) and economically (a fraction of the costs of aneuploidy testing and sperm DNA fragmentation analysis). Substantial overlap exists between sperm samples found to have atypical telomere lengths and those displaying increased aneuploidy and excessive DNA fragmentation. Consequently, the implementation of low-cost STL measurement, as a form of preliminary assessment, may assist in reducing the number of more expensive tests required. For example, 75% of samples with atypical telomere length also displayed compromised DNA integrity (elevated SDF) and aneuploidy. Furthermore, evidence shows that measurement of telomere length provides additional clinical predictive information, independent of SDF and aneuploidy. Samples with telomere lengths that were unusually long or short were associated with greatly reduced pregnancy rates when used in assisted reproductive treatments. Indeed, within this set of samples, atypical STL was associated with 100% failure to establish a viable pregnancy. This suggests that STL determination may actually have far more predictive power than measurement of either sperm DNA fragmentation or aneuploidy. Clearly, this finding now needs to be verified in an independent series of sperm samples. Combined together, the three tests (aneuploidy, SDF and STL) provide a detailed assessment of paternal genome integrity, independent of traditional sperm parameters. Clinical pathways for men found to have elevated SDF include treatment with antibiotics to clear any urogenital tract infections, repair of varicocele if present in the testis, administration of antioxidants, or retrieval of epididymal sperm. For men with increased aneuploidy rates in their sperm, preimplantation genetic screening of any embryos produced using IVF or intracytoplasmic sperm injection can be considered, allowing the transfer of chromosomally abnormal embryos to the uterus to be avoided. It remains to be seen whether atypical STL can be addressed by any alteration of clinical treatment. Nonetheless, if the predictive value of STL measurement is confirmed, identification of individuals who have abnormal sperm telomere lengths may assist in counselling, especially in terms of managing patient expectations.

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Uncited references

Thomay et al, 2014

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.rbmo.2016.06.006.

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