Human Reproduction, Vol.31, No.6 pp. 1158–1163, 2016
Advanced Access publication on April 6, 2016 doi:10.1093/humrep/dew061

human reproduction

ORIGINAL ARTICLE *Andrology*

Sperm telomere length as a parameter of sperm quality in normozoospermic men

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Submitted on November 20, 2015; resubmitted on February 24, 2016; accepted on March 1, 2016

STUDY QUESTION: Could sperm telomere length (STL) represent a novel parameter and biomarker of sperm quality?

SUMMARY ANSWER: STL is associated with standard semen quality parameters and, more importantly, it is significantly associated with levels of DNA fragmentation and sperm protamination.

WHAT IS KNOWN ALREADY: Telomeres are fundamental for genome integrity. Recent studies have demonstrated that STL increases with age and men with oligozoospermia have shorter sperm telomeres than normozoospermic men.

STUDY DESIGN, SIZE, DURATION: Cohort study conducted from September 2014 to June 2015 on 100 subjects with normal standard semen parameters.

PARTICIPANTS/MATERIALS, SETTING, METHODS: STL was measured indirectly by quantitative polymerase chain reaction using telomere/single-copy gene ratio, sperm DNA fragmentation by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling assay and protamination by aniline blue staining. Data were analyzed for determining the relationships between STL, standard semen parameters and DNA fragmentation and protamination.

MAIN RESULTS AND THE ROLE OF CHANCE: Among standard semen parameters, STL was positively associated with progressive motility ($P = 0.004$) and vitality ($P = 0.007$). STL was significantly and negatively associated with sperm DNA fragmentation ($P = 0.001$) and significantly and positively associated with protamination ($P = 0.002$). The role of chance was limited and the findings have biological relevance and a pathophysiological explanation.

LIMITATIONS, REASONS FOR CAUTION: For the present study, we deliberately selected only men with normozoospermia to better analyze whether STL might represent a biomarker of sperm quality beyond traditional sperm parameters. Additional studies in proven fertile men with normal sperm parameters are needed.

WIDER IMPLICATIONS OF THE FINDINGS: The measurement of STL is a simple and rapid method that offers further information about the quality of sperm. The results of this study demonstrate that STL could be considered as an additional sperm parameter and opens new perspectives in the evaluation of the infertile male. Additional studies will clarify the significance of this parameter also as a prognostic biomarker in assisted reproduction.

STUDY FUNDING/COMPETING INTEREST(S): No external funding was either sought or obtained for this study. There are no conflicts of interest to be declared.

Key words: DNA fragmentation / male infertility / protamination / sperm quality / sperm telomere length

Introduction

The evaluation of male (in)fertility is commonly based on standard semen analysis, which, however, cannot clearly distinguish fertile from infertile populations and fails to detect any abnormality in many cases. Therefore, a large proportion of infertile men do not receive a clear diagnosis, and thus they are reported as idiopathic. This is particularly evident in cases of infertility or repeated assisted reproduction failure with

normal semen parameters. Abnormal sperm function, aneuploidy or specific molecular defects can be hypothesized in these cases (Ferlin and Foresta, 2014; Pizzol *et al.*, 2014).

A number of methods have been proposed in the diagnostic work-up of the infertile male in addition to traditional semen parameters to better assess sperm quality and function, as well as DNA integrity, such as tests to quantitate protamination and DNA packaging, DNA fragmentation, chromosome aneuploidy and molecular karyotyping (Patassini *et al.*, 2013; Ferlin and Foresta, 2014). A major interest is the use of these tests is in men with normal standard semen parameters, in order to identify new diagnostic and prognostic biomarkers of sperm function and quality (Pizzol *et al.*, 2014).

Moreover, studies in recent years focused on the role of sperm telomeres in reproduction (Ferlin and Foresta, 2014). Telomeres are non-coding highly conserved tandem repeat DNA sequences (TTAGG)*n*, that cap the ends of eukaryote chromosomes, extended from 5 to 15 kb in human (Schmidt and Cech, 2015). These structures preserve the ends of chromosomes and confer stability to genome (Blackburn, 1991; de Lange, 2005). Telomere length is maintained by the enzyme telomerase, a reverse transcriptase that is maximally expressed in highly proliferative cells such as germ cells, stem and progenitor cells and neoplastic cells (Flores *et al.*, 2006). Telomerase activity is high in germ cells and in most tumors, whereas it is absent in normal human somatic cells after the birth, so that telomeres undergo progressive shortening with each cell division because of the inability of the normal DNA replication machinery to fully replicate at the 3′-end of chromosomes (Harley *et al.*, 1990). This is probably the reason why telomere length in sperm increases with age in contrast with somatic cells (Allsopp *et al.*, 1992; Baird *et al.*, 2006; Kimura *et al.*, 2008; Aston *et al.*, 2012). Telomere shortening induces proliferation arrest, promotes senescence or apoptosis (Blackburn *et al.*, 2006), and triggers mechanisms inducing cancer progression or suppression (Martien and Abbadie, 2007). Telomeres also play key roles in movement, localization and anchoring of the chromosomes to the nuclear membrane and mediate the pairing of homologous chromosomes, synapsis formation and homologous recombination during cell division (Counter, 1996; Scherthan, 2007).

Although the role of sperm telomeres and sperm telomere length (STL) is not completely known, recent studies documented intriguing findings in different aspects of male reproduction. Sperm of infertile men have shorter telomeres when compared with controls (Thilagavathi *et al.*, 2013a,b). We previously reported that STL is related to sperm count, it is lower in oligozoospermic than in normozoospermic men and it is related to parents' age at conception (Ferlin *et al.*, 2013), a finding recently confirmed in men undergoing assisted reproduction (Yang *et al.*, 2015a,b). In addition, a correlation between STL and embryo quality has been demonstrated (Yang *et al.*, 2015a) as well as longer STL in sperm selected with density gradient (Yang *et al.*, 2015b) or swim-up procedures (Santiso *et al.*, 2010). This evidence suggests that the measurement of STL might be of interest also in the prognostic evaluation during assisted reproductive technologies to increase the possibility of a successful outcome.

Apart from the relation with sperm concentration and fertility status, nothing is known about the correlation of STL with standard and second step semen parameters such as DNA fragmentation and protamination. In this study, we investigated these relationships in a cohort of men with normozoospermia, in order to clarify whether STL could represent an additional novel parameter in the evaluation of sperm quality.

Materials and Methods

Ethical approval

The study has been approved by the Ethics Committee of the University-Hospital of Padova and each participant gave his written informed consent. The study has been conducted in accordance with the principles expressed in the Declaration of Helsinki.

Subjects

From September 2014 to June 2015, we consecutively recruited 100 normozoospermic men (total number and concentration of spermatozoa, and percentages of progressively motile and morphologically normal spermatozoa, equal to or above the lower reference limits) (WHO, 2010) (mean ± SD age 34.0 ± 8.6 years), who were referred to the Unit of Andrology and Reproduction Pathology of the University of Padova for semen analysis. Selected subjects had no seminal infection, sperm autoantibodies, varicocele, history of cryptorchidism and orchitis, nor did they have systemic diseases or endocrine disorders.

Sperm preparation

Standard semen analysis was performed according to the World Health Organization protocol (WHO, 2010) after 2–7 days of sexual abstinence.

An aliquot of semen sample was assessed for DNA fragmentation and for the evaluation of histone replacement. Additionally, an amount of 10×10^6/ml sperm was isolated using Percoll (Sigma-Aldrich, Germany) 45 and 90% gradients in order to measure STL. Although STL in density gradient selected sperm might be different, we used this strategy to remove somatic cells that could bias the results.

TUNEL assay

Evaluation of DNA fragmentation was performed using the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay carried out with the Cell Death Detection Kit (Roche Diagnostics, Milan, Italy) and FACScan (Becton Dickinson, Oxford, UK). The Cellquest software (Becton Dickinson, Oxford, UK) was used to acquire and analyze the data, as previously reported (Garolla *et al.*, 2015). Negative controls were obtained by incubating sperm cells without enzyme. Results are expressed as the percentage of fragmented sperm.

Aniline test

For the evaluation of histone replacement by sperm-specific nuclear protamines, semen samples were washed in 0.2 M phosphate-buffered saline (pH 7.2) and fixed in 3% glutaraldehyde for 30 min before air drying sperm smears on glass slides. The sperm smears were then stained for 5 min in 5% aniline blue (Sigma, St Loius, MO, USA) in 4% acetic acid (pH 3.5) as previously described (Hofmann and Hilscher, 1991). Spermatozoa were classified as either blue stained (immature nucleus due to histone persistence), or unstained (mature nucleus with complete histone-protamine replacement), by counting 200 spermatozoa per slide. Results are expressed as the percentage of unstained spermatozoa.

STL measurement

STL was measured, as previously reported with minor modifications (Ferlin *et al.*, 2013), on sperm isolated from Percoll gradients following DNA extraction using the QIAamp DNA Mini Kit (QIAGEN, Milan, Italy).

STL was determined by real-time quantitative PCR where one PCR is used to determine the cycle threshold (Ct) value for telomere (T) amplification with the primer pair TEL1B and TEL2B (O'Callaghan *et al.*, 2008) and a

second PCR to determine the Ct value for the amplification of a single-copy (S) control gene with the primers 36B4u and 36B4d (Cawthon, 2002).

Each sample was run in triplicate and each reaction well of 50 μl contained 10 μl of DNA sample (10 ng DNA), 40 μl of mixture containing 2× SYBR Green PCR Master Mix (Life Technologies, UK), as well as primer pair TEL1B 300 nM and TEL2B 900 nM or primer pair 36B4u 300 nM and 36B4d 500 nM, and H_2O. A standard curve of a positive control DNA (1301 human cell line) serially diluted from 10 to 0.625 ng/μl was included in each run.

All PCRs were carried out in 96-well plates using the StepOnePlus Q-PCR platform (Life Technologies, UK). The SD of Ct values was <1 [coefficient of variation (CV <5%)]. Mean Ct values were used to calculate the relative STL using the telomere/single-copy gene (T/S) ratio according to the formula: $\Delta Ct_{sample} = Ct_{telomere} - Ct_{control}$, $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference\ curve}$ (where $\Delta Ct_{reference\ curve} = Ct_{telomere} - Ct_{control}$) and then $T/S = 2^{-\Delta\Delta Ct}$ (25, 26). The mean of the CV of sample T/S values was 2.8%.

Statistical analysis

Statistical analyses were performed with SAS version 9.2 (SAS Institute, Cary, NC, USA) for Windows. Data are reported as mean ± SD. The normality of the variables was evaluated with the Shapiro–Wilks test. The association of STL with semen parameters, including DNA fragmentation and protamination, was analyzed with a Pearson correlation (r_p) or Spearman rank correlation (r_s) according to the normal distribution of the variables and the results are reported as a *P*-value and correlation coefficient. The significance level was set to $P < 0.05$.

Results

Table I shows the basic characteristics, semen parameters, DNA fragmentation and protamination of the 100 normozoospermic men. Table II shows the correlations between STL and semen parameters, including sperm DNA fragmentation and protamination. Among standard semen parameters, STL was positively and significantly associated with progressive motility ($P = 0.004$) and vitality ($P = 0.007$). Interestingly, STL was significantly and negatively associated with sperm DNA fragmentation ($P = 0.001$), and significantly and positively associated with protamination ($P = 0.002$). Figure 1 shows the correlations between significantly associated variables. Among other correlations, DNA fragmentation was negatively associated with protamination ($P = 0.0003$) and progressive motility ($P < 0.0001$) (data not shown).

Table I Basic characteristics, semen analysis, sperm DNA fragmentation and protamination levels of the 100 normozoospermic subjects.

Parameter*	Mean ± SD
Age (years)	34.0 ± 8.6
Sexual abstinence (days) (2–7 days)	4.2 ± 1.8
Semen volume (ml) (>1.5 ml)	3.6 ± 1.5
Sperm concentration (×10^6/ml) (>15 × 10^6/ml)	66.6 ± 46.6
Total sperm count (×10^6) (>39 × 10^6)	205.9 ± 139.6
Progressive motility (%) (>32%)	61.3 ± 14.7
Sperm vitality (%) (>58%)	83.0 ± 8.1
Normal sperm morphology (%) (>4%)	10.2 ± 5.7
Sperm DNA fragmentation (TUNEL assay) (%) (<35%)	26.5 ± 18.5
Normal sperm protamination (aniline blue test) (%) (>60%)	72.8 ± 12.5

TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling.
*The normal values of the World Health Organization (WHO, 2010) are in parenthesis.

Discussion

This is the first study analyzing in detail the relation between STL and conventional semen parameters, as previous studies focused mainly on sperm number (Ferlin *et al.*, 2013) or on the effect of age (Allsopp *et al.*, 1992; Baird *et al.*, 2006; Kimura *et al.*, 2008; Aston *et al.*, 2012). Furthermore, we used two validated methods to analyze DNA fragmentation and protamination to better characterize the relation between STL and sperm quality, also in the light or reports describing a correlation between STL and embryo quality (Yang *et al.*, 2015a,b) and longer STL in sperm selected with density gradient (Yang *et al.*, 2015a,b) or swim-up procedures (Santiso *et al.*, 2010).

The positive correlation found in the present study between STL and progressive motility and vitality is well in agreement with previous data showing a relation with total sperm count, and shorter telomeres in oligozoospermic and infertile men compared with normozoospermic (Ferlin *et al.*, 2013) and fertile men (Thilagavathi *et al.*, 2013a,b). Taken together, these findings suggest that STL is a marker that can distinguish between normal and abnormal spermatogenesis and it is associated with the most important standard semen parameters. Whether this association reflects a cause-effect relationship is actually unknown. In fact, telomeres play a critical role in meiosis and maintenance of genome integrity (Siderakis and Tarsounas, 2007), and therefore one could hypothesize that shorter telomeres might impair spermatogenesis through segregation errors and/or apoptosis of germ cells. Alternatively, shorter telomeres in ejaculated sperm might be a consequence rather than the cause of altered spermatogenesis, as many factors commonly implicated in spermatogenic impairment and male infertility (environmental factors, infections, oxidative stress, smoking and obesity), might be implicated also in telomere shortening (Thilagavathi *et al.*, 2013a,b). Whatever

Table II Correlation of human sperm telomere length with age, semen parameters, sperm DNA fragmentation and protamination.

Parameter	ρ^a (*P*-values)
Age (years)	−0.081 (0.443)
Sexual abstinence (days)	−0.140 (0.146)
Semen volume (ml)	−0.098 (0.363)
Sperm concentration (×10^6/ml)	−0.024 (0.823)
Total sperm count (×10^6)	−0.132 (0.218)
Progressive motility (%)	0.46 (0.004)
Sperm vitality (%)	0.340 (0.007)
Normal sperm morphology (%)	−0.063 (0.547)
Sperm DNA fragmentation (TUNEL assay) (%)	−0.44 (0.001)
Normal sperm protamination (aniline blue test) (%)	0.405 (0.002)

[a]A Pearson correlation or Spearman rank correlation according to the distribution of the variables.

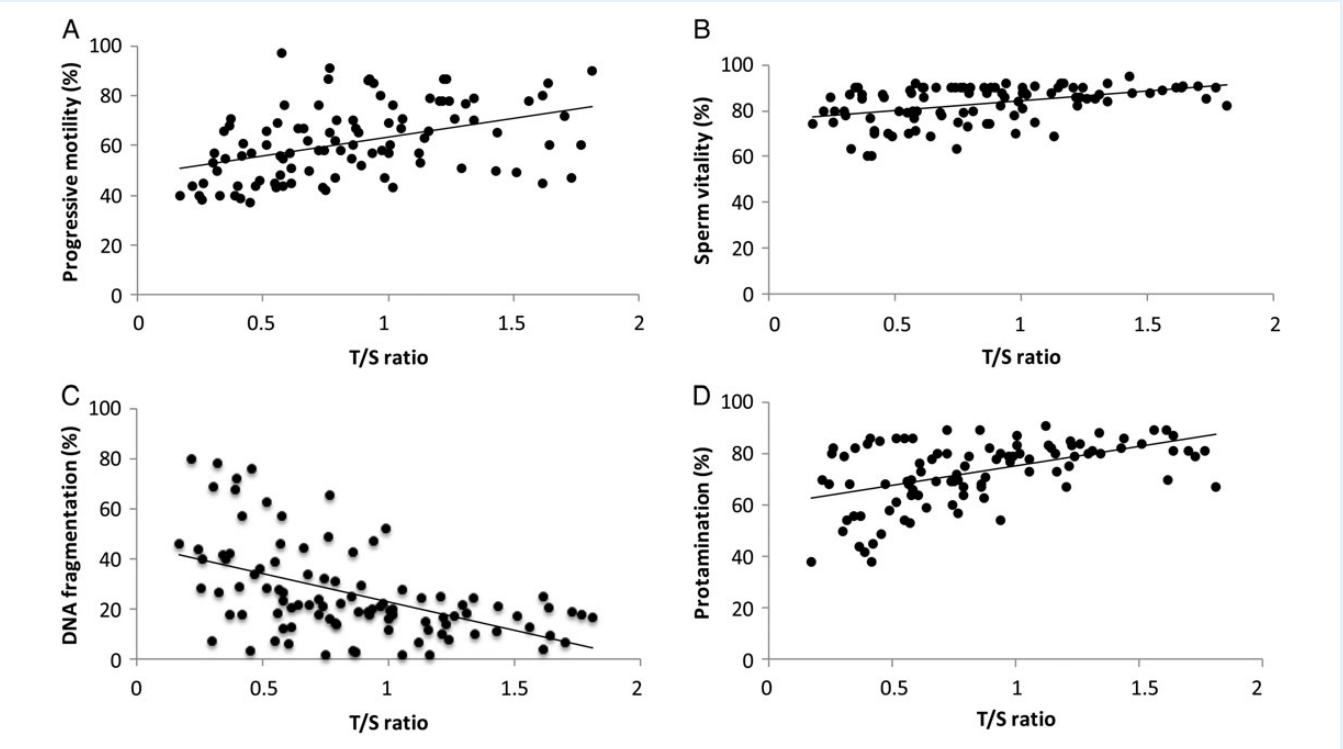

Figure 1 Correlation between T/S ratio* (an indirect measure of telomere length) and progressive motility (**A**), sperm vitality (**B**), DNA fragmentation evaluated by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling assay (**C**) and protamination evaluated by aniline blue test (**D**). $n = 100$ samples. *The quantity of telomere repeats (T) and the single copy reference gene (S) in each experimental sample ($n = 100$) was measured by quantitative PCR.

the pathophysiological links between shorter STL, low-sperm count, motility and vitality are, these observations, although needing additional studies, are also important in the setting of assisted reproductive techniques, where these parameters are the main characteristics used to select sperm and to provide prognostic information.

The association between STL and sperm DNA fragmentation and protamination is particularly intriguing, as this correlation might underlie interesting pathophysiological relations. Telomeres consist of tandem repeat DNA sequences and telomere-associated proteins, with the latter playing a key role in preserving DNA integrity within these repeated sequences. Telomere proteins have in fact an essential role in the maintenance of telomere length, regulating telomerase access and preventing telomeres from being recognized as damaged DNA (Mattern *et al.*, 2004; Longhese, 2008). Furthermore, these proteins protect the ends of chromosomes from endogenous or environmental agents that might cause DNA degradation (Von Zglinicki, 2002; Hussain *et al.*, 2003). In fact, telomeres are G-rich sequences and defects in the telomere structure might contribute to making chromosome ends more accessible to oxidative radicals normally produced during the maturation process of sperm. As is well-known, oxidative radicals induce sperm DNA breaks and, as telomeres are more susceptible to breaks than non-telomeric DNA due to their high content of guanine residues (Coluzzi *et al.*, 2014), dysfunctional telomeres might be recognized as a target for oxidative radicals (Raha and Robinson, 2000; Liu *et al.*, 2002), that, in turn, might contribute to an increase in the percentage of sperm with fragmented DNA and to the loss of telomeric repeats. Indeed, a previous study using quantitative fluorescence *in situ* hybridization found a negative correlation between STL and DNA fragmentation (Turner and Hartshorne, 2013).

In the light of our results, one hypothesis is therefore that sperm DNA fragmentation, that can be induced by several factors including oxidative stress, might trigger telomere shortening independently from the normal shortening that occurs at each cell cycle. Ramírez *et al.* (2003) have in fact observed that telomere shortening may be considered both the result and the cause of DNA damage, although previous studies have suggested that DNA damage is exclusively the result of telomere shortening (Multani *et al.*, 2000; Lovejoy *et al.*, 2012). Based on the finding of Ramírez *et al.* (2003), shortened telomeres might be considered the consequence of sperm DNA fragmentation. However, we cannot definitively assess, which is the cause and which the effect (telomere shortening or DNA fragmentation), and there is also the possibility that certain factors trigger both events.

Furthermore, since high fragmentation might be both the cause and the consequence of a decreased percentage of motile and viable cells, and cells with shorter telomeres have more fragmented DNA, we could also speculate that STL, sperm motility and sperm vitality might depend on sperm DNA integrity. Accordingly, we found a positive correlation between STL and sperm motility and vitality rates.

Further studies are clearly needed to better understand the contribution of oxidative stress to the post-meiotic regulation of STL (when telomerase cannot restore telomere length). Recently, Thilagavathi *et al.* (2013a) have analyzed the relation between STL and sperm DNA fragmentation and between STL and reactive oxygen species levels in infertile

men. However, in contrast to our findings, no correlation was found between STL and these parameters, or with standard semen characteristics, probably due to the lower number of men.

Finally, we observed a strong positive correlation between STL and sperm DNA maturity, as showed by degree of protamination evaluated by aniline blue staining. The substitution of histones by protamines is essential to pack the DNA, protect it from chemical and physical damage (Barone *et al.*, 1994; Braun, 2001) and to avoid sperm DNA fragmentation (Aoki *et al.*, 2005, 2006; Nasr *et al.*, 2005; Torregrosa *et al.*, 2006). Therefore, we might suppose that an altered packaging in the chromatin might make spermatozoa more vulnerable to all those damaging factors (endonucleases, free radicals etc.) that trigger DNA fragmentation and consequently telomere shortening. In the other words, a normal protamine status most likely reflects normal chromatin packaging and, therefore, the spermatozoa, whose development has occurred normally, have longer telomeres. However, this hypothesis need further investigation to better understand whether short telomeres might be the collateral effect of defective chromatin packaging during spermatogenesis.

Unlike our previous study (Ferlin *et al.*, 2013), we found no correlation between STL and sperm count, or between STL and age, as in other reports (Allsopp *et al.*, 1992; Baird *et al.*, 2006; Kimura *et al.*, 2008; Aston *et al.*, 2012). However, for the present study, we deliberately selected only men with normozoospermia and the age range was limited, and this is probably the reason why these associations were not seen. Indeed, the demonstration that STL is an additional sperm parameter that may add information about DNA damage in normozoospermic men, opens new perspectives in the evaluation of the infertile males. Additional studies on men of proven fertility will clarify the significance of this biomarker also as prognostic parameter in assisted reproduction.

Authors' roles

M.S.R. performed the analysis of TL, critically analyzed the data, drafted and revised the manuscript. E.S. performed the analysis of TL, contributed to interpretation of data and to the writing of manuscript. M.M. performed sperm analyses. A.G. performed clinical analysis. C.F. supervised the project and revised manuscript critically. A.F. selected the subjects, performed clinical analysis, supervised the experimental procedures and results and wrote the manuscript.

Funding

No external funding was either sought or obtained for this study.

Conflict of interest

None declared.

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