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ORIGINAL ARTICLE ***Reproductive genetics***

In young men sperm telomere length is related to sperm number and parental age

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STUDY QUESTION: What are the relationships between telomere lengths in leukocytes and sperm, sperm count and parents' age at conception in a group of apparently healthy subjects of the same age?

SUMMARY ANSWER: Sperm telomere length (STL) is related to sperm count, it is lower in oligozoospermic than in normozoospermic men and it is directly related to parents' age at conception.

WHAT IS KNOWN ALREADY: Leukocyte telomere length (LTL) decreases with age but STL increases and offspring of older fathers tend to have longer leukocyte telomeres. Only one study analyzed STL in relation to male fertility, and reported shorter telomeres in infertile versus fertile men. No data have been reported on STL in relation to parents' age at conception.

STUDY DESIGN, SIZE, DURATION: Prospective study conducted from January to December 2012 of 18–19-year-old high school students.

PARTICIPANTS/MATERIALS, SETTING AND METHODS: The volunteers were 81 apparently healthy subjects, including 61 with normozoospermia and 20 with idiopathic oligozoospermia. Leukocyte and sperm telomere length were measured by real-time PCR. Data were analyzed for determining the relationships between LTL, STL, sperm count and parents' age at conception.

MAIN RESULTS AND THE ROLE OF CHANCE: Sperm and leukocyte telomere length were strongly correlated, but STL was significantly longer. A significant positive correlation between STL and total sperm number was found. STL was significantly lower in oligozoospermic than in normozoospermic men. Finally, we found a significant positive relationship between maternal age and both leukocyte and sperm telomere length and a significant positive relation between paternal age and STL in the offspring. The relative contributions of mothers' and fathers' ages to their offspring's telomere length could not be determined because of the high correlation between paternal and maternal ages.

LIMITATIONS AND REASONS FOR CAUTION: Although consistent with previous findings, this is the first study on telomere length in oligo- and normozoospermic men and included a relatively low number of subjects. Our study was also restricted to young (18–19 year old) men, so future studies should determine whether our findings can be generalized to men at ages typically encountered at fertility centers. Future studies should also try to determine the possible effect of abstinence time and frequency of ejaculation with STL.

WIDER IMPLICATIONS OF THE FINDINGS: Our study sheds new light on the association between STL and sperm count and on the inheritance of telomere length (in leukocytes and sperm) in relation to the parents' age at conception. Additional studies are needed to confirm these observations, to clarify if the association between shorter STL and damaged spermatogenesis represents a pathophysiological link, and to determine the effect on offspring telomere length of assisted reproduction techniques performed on couples of advanced age or where the man is oligozoospermic.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by the Italian Ministry of University and Research (grant no. 2009AMPA9C to C.F.) and Padova University (grant 2010 to A.D.R.). The authors have no competing interests to declare.

Key words: male infertility / oligozoospermia / paternal age at conception / spermatogenesis / telomere length

Introduction

Telomeres are non-coding DNA sequences, composed of highly conserved hexameric tandem nucleotide repeats (TTAGGG), which cap the ends of eukaryote chromosomes. Telomeres confer stability on the chromosome and preserve genomic stability. Their dysfunction has been implicated in different conditions, such as cancer and ageing (de Lange, 2002; Jiang *et al.*, 2007; Calado and Young, 2009). They 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). When telomeres reach a critical minimum length, cells cannot divide and the cell enters cell-cycle arrest or undergoes apoptosis (Blackburn *et al.*, 2006). Telomere length (TL) is maintained by telomerase, a ribonucleoprotein complex that is maximally expressed in highly proliferative cells such as germ and neoplastic cells (Dolcetti and De Rossi, 2012).

Although the role of sperm telomeres and sperm TL (STL) is not clearly known, recent studies documented intriguing findings in different aspects of male reproduction that merit further investigation. In fact, although STL and leukocyte telomere length (LTL) tend to be correlated in the same individual (Aston *et al.*, 2012), LTL decreases and STL increases with age (Allsopp *et al.*, 1992; Baird *et al.*, 2006; Kimura *et al.*, 2008; Aston *et al.*, 2012). This finding is not fully understood, but it has been related to the high activity of the telomerase reverse transcriptase (TERT), the catalytic subunit of the telomerase, in germ cells (Wright *et al.*, 1996; Zalenskaya and Zalensky, 2002; Riou *et al.*, 2005) or to a selective cellular attrition leading to death of sperm stem cells with shortened TL and therefore selection of a subset of sperm with longer telomeres (Kimura *et al.*, 2008; Eisenberg *et al.*, 2012). Consistent with the elongation of STL with age, a positive correlation has been found between paternal age at birth and offspring LTL (Nawrot *et al.*, 2004; Nordfjäll *et al.*, 2005, 2010; Unryn *et al.*, 2005; Akkad *et al.*, 2006; De Meyer *et al.*, 2007; Njajou *et al.*, 2007; Kimura *et al.*, 2008; Arbeev *et al.*, 2011; Prescott *et al.*, 2012). The paternal age contribution to offspring LTL is stronger than the maternal contribution (Broer *et al.* 2013), and the paternal age effect is cumulative across multiple generations (Eisenberg *et al.*, 2012).

No data have been published on the inheritance of STL in relation to paternal and maternal age and the role of STL in spermatogenesis and/or fertility potential of sperm is unknown. Only one recent study (Thilagavathi *et al.*, 2013) analyzed STL (but not LTL) in small groups of fertile and infertile subjects with normal sperm counts and found lower TL in sperm in the latter group.

The aim of the present study was to analyze LTL and STL in the same individual in relation to spermatogenic activity and parents' age at birth. Age is the most important confounding factor when dealing with TL and most previous studies conducted on LTL and STL recruited subjects of different ages. To eliminate the age variable and better define the role of STL in male fertility and the parental age effect, we selected subjects of the same age (18–19 years) with well-defined andrological and seminological evaluation.

Materials and Methods

Subjects

From January to December 2012 we enrolled 81 volunteers aged 18–19 years among students participating in a screening protocol for prevention of andrological disorders performed in the high schools of Padova and surroundings (Veneto Region, the North-East of Italy), described in detail elsewhere (Foresta *et al.*, 2013).

Standard semen analysis was performed according to World Health Organization protocol (WHO, 2010) after 2–4 days of sexual abstinence. For this study, subjects were divided in normozoospermic (total sperm count $\geq$39 million/ejaculate, 61 subjects) and oligozoospermic (sperm count $<$39 million/ejaculate, 20 subjects). Selected subjects had no evident causes of spermatogenic impairment [seminal infection, sperm autoantibodies, varicocele, history of cryptorchidism and orchitis, Y chromosome microdeletions (Ferlin *et al.*, 2007), karyotype anomalies, medication, fever in the previous month, systemic diseases or endocrine disorders], and were selected among 155 subjects initially screened, randomly selected among the 776 subjects who agreed to semen analysis (Foresta *et al.*, 2013). Age of parents was recorded from a questionnaire.

The study has been approved by the Health Service of the Veneto Region and 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.

TL measurement

Genomic DNA was extracted from peripheral blood leukocytes by QIAamp® DNA Blood Midi Kit (QIAGEN, Milan, Italy) according to manufacturer's recommendations. Sperm were isolated by using Percoll gradient centrifugation and DNA was extracted using the QIAamp® DNA Mini Kit according to manufacturer's recommendations.

TL was determined by real-time polymerase chain reaction (PCR) as previously described (Rampazzo *et al.*, 2010, 2012). Briefly, two PCRs were performed for each sample, one to determine the cycle threshold (Ct) value for telomere (T) amplification with the primer pair TEL1B and TEL2B (O'Callaghan *et al.*, 2008) and the other one to determine the Ct value for the amplification of a single-copy (S) control gene [acidic ribosomal protein P0, RPLP0 (also known as 36B4)] with the primer RPLP01 and RPLP02 (Boulay *et al.*, 1999). Each sample was run in triplicate and each PCR was performed using 10 μl of DNA sample (1 ng of DNA per μl) in 50 μl final reaction volume. A reference curve, consisting of reference DNA from the RAJI cell line (Nishikura *et al.*, 1985) serially diluted from 10 to 0.041 ng/μl, was generated at each PCR run. All PCRs were carried out in 96-well plates using the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Milan, Italy). Intra- and inter-assay reproducibility of both telomere and RPLP0 PCR results was evaluated initially in a series of experiments using dilutions of the reference curve. The standard deviation (SD) of Ct values was $\leq$0.189 (% coefficient of variation $\leq$1.13) in six replicates of samples amplified in the same PCR run, and $\leq$0.251 (% coefficient of variation $\leq$1.58) among mean values of triplicates in different PCR runs. Variation of Ct values in the sample was $<$0.3 Ct (SD $\leq$0.212; % coefficient of variation $\leq$1.25) in both telomere and RPL0 PCR runs. Mean Ct values were used to calculate the relative TL using the telomere/single copy gene ratio (T/S) 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 $T/S = 2^{-\Delta\Delta Ct}$ (O'Callaghan *et al.* 2008). The mean of the coefficient of variation of samples' T/S values was 3.17%.

Statistical analyses

Data are reported as mean $\pm$ SD for continuous variables and as count and percentages for categorical ones. The normality of the variables was evaluated with Shapiro–Wilks test. The association of LTL with STL, sperm count with LTL and STL, and maternal and paternal age at birth of offspring with LTL and STL was analysed with Pearson correlation (r_p) or Spearman rank correlation (r_s) according to the normal distribution of the variables and the results are reported as *P*-value and correlation coefficient. The

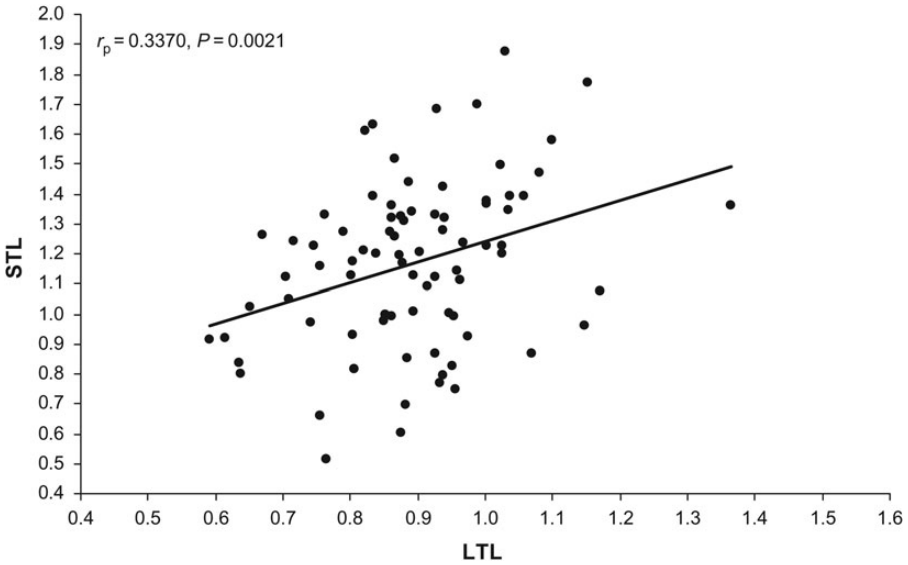

Figure 1 Correlation between telomere length in leukocytes (LTL) and sperm (STL). The Pearson's correlation coefficient (r_p) and *P*-value are shown.

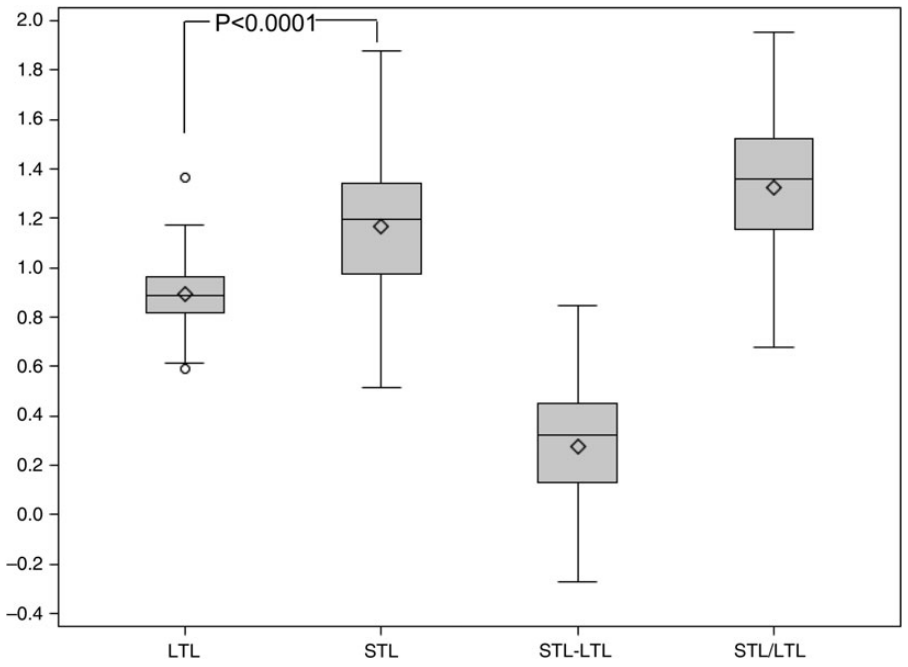

Figure 2 Telomere length in leukocytes (LTL) and sperm (STL), expressed also as STL/LTL ratio and the difference between STL and LTL. The boxes represent the first and third quartiles; the whiskers extend from the end of the box for a distance = 1.5 × the interquartile range; the line that bisects the boxes represents the median; the diamond in the boxes represents the mean; circles outside the boxes represent the outlier values.

statistical significance of the difference STL-LTL was analysed with paired Student's *t*-test. LTL and STL were compared between normo- and oligozoospermic subjects with Wilcoxon rank sum and Student's *t*-test, respectively. The number of subjects in which STL was shorter than LTL was analyzed with Fisher exact test. Statistical analyses were performed with SAS version 9.2 (SAS Institute, Cary, NC, USA) for Windows.

Results

Sperm and leukocyte TL, although with high inter-individual variations, were strongly correlated ($r_p = 0.34$; $P = 0.0021$) (Fig. 1). The mean ($\pm$ SD) STL was significantly longer than LTL (1.17 $\pm$ 0.28 versus 0.89 $\pm$ 0.14, $P < 0.0001$) and STL was shorter than LTL in only 14/81

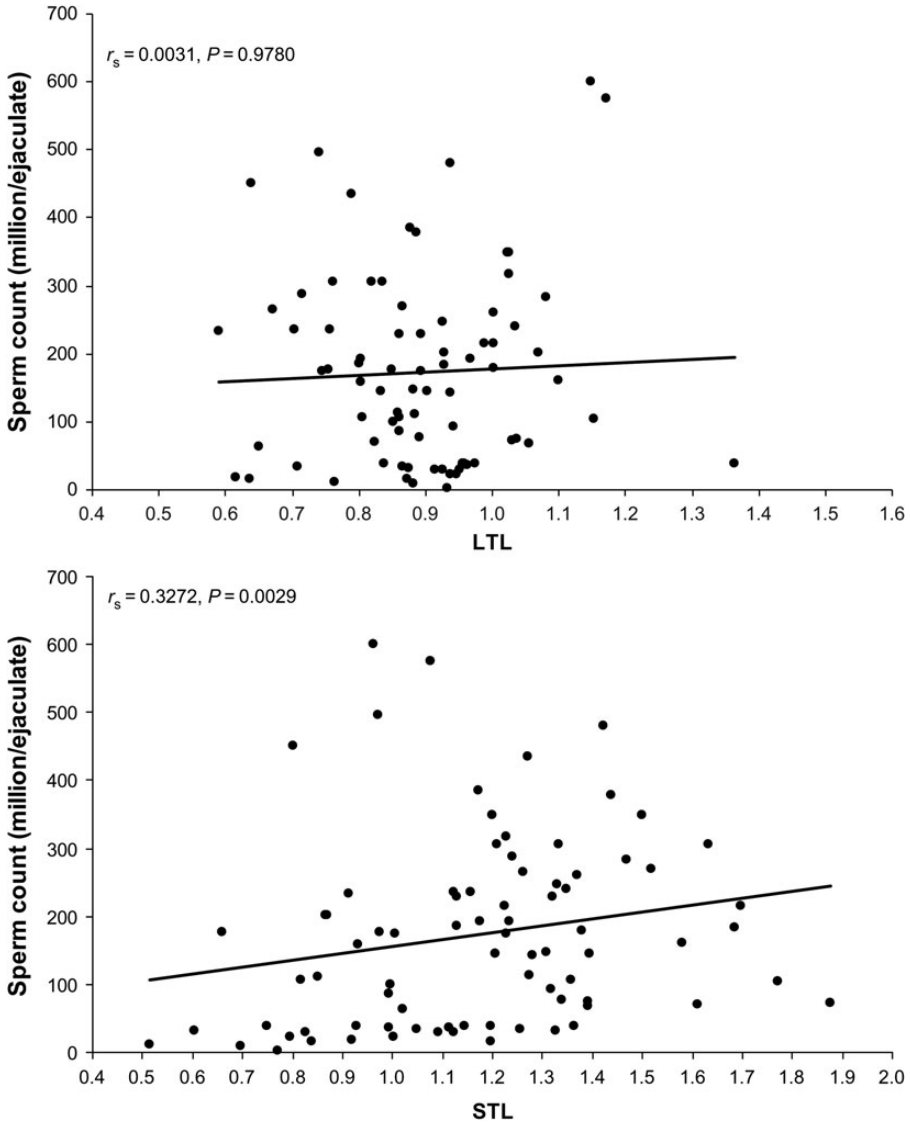

Figure 3 Correlation between telomere length in leukocyte (LTL) and total sperm count (upper panel) and between telomere length in sperm (STL) and total sperm count (lower panel). Spearman's correlation coefficient (r_s) and *P*-value are shown.

cases (17.3%). This is evident also from the analysis of the difference between STL and LTL (mean value 0.28 ± 0.26) and the STL/LTL ratio (mean value 1.32 ± 0.30) (Fig. 2).

The analysis of the relationship between STL, LTL and sperm count demonstrated a significant positive correlation between STL and total sperm number ($r_S = 0.33$; $P = 0.0029$) (Fig. 3), but not between LTL and sperm count ($r_S = 0.003$; $P = 0.9780$). A residual analysis does not show a quadratic pattern suggesting the need to add a quadratic term. We then analyzed TL in subjects with normozoospermia ($n = 61$) and oligozoospermia ($n = 20$). No difference was observed in LTL between the two groups, whereas STL was significantly lower in oligozoospermic men with respect to normozoospermic men (0.95 ± 0.22 versus 1.24 ± 0.25, $P < 0.0001$) (Fig. 4). In the group of oligozoospermic men, 8/20 subjects (40.0%) had STL shorter than LTL compared with 7/61 (11.5%) subjects in the normozoospermic group ($P = 0.0080$).

Analysis of TL in relation to father's and mother's age at the time of conception showed significant positive relation between maternal age and both LTL ($r_s = 0.34$; $P = 0.0028$) and STL ($r_s = 0.33$; $P = 0.0031$) of offspring and significant positive relation between paternal age and STL of offspring ($r_S = 0.25$; $P = 0.0277$) (Fig. 5). The relative contribution of mother's and father's age on offspring TL is, however, not clear because paternal and maternal ages are highly significantly correlated ($r_S = 0.61$; $P < 0.0001$).

Discussion

By studying a group of apparently healthy subjects of the same age (18–19 years old), we obtained interesting and novel information on the relation between TL in leukocyte and sperm measured in the same individual, spermatogenic activity and parents' age at birth. We found that, although with high inter-individual variations, STL and LTL are

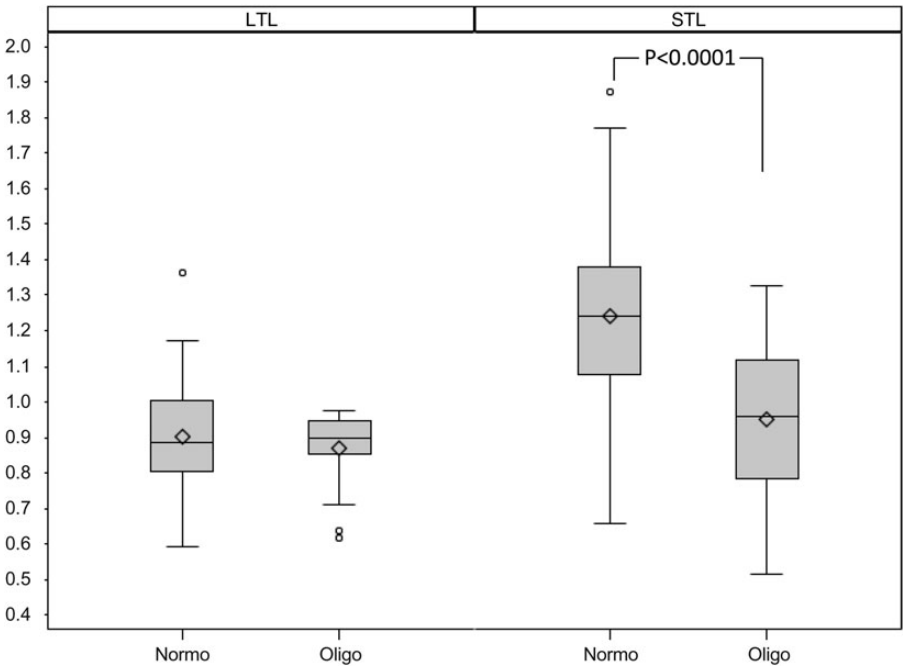

Figure 4 Telomere length in leukocytes (LTL, left) and sperm (STL, right) in subjects with normozoospermia (total sperm count ≥39 million/ejaculate; normo, *n*. 61) and oligozoospermia (total sperm count <39 million/ejaculate; oligo, $n = 20$). The boxes represent the first and third quartiles; the whiskers extend from the end of the box for a distance =1.5 × the interquartile range; the line that bisects the boxes represents the median; the diamond in the boxes represents the mean; circles outside the boxes represent the outlier values.

positively correlated, but sperm telomeres are longer than leukocyte telomeres. This is consistent with previous results (Aston *et al*., 2012) and probably related to the reported higher activity of telomerase in male germline cells with respect to hematopoietic stem cells (Wright *et al*., 1996; Riou *et al*., 2005). For the first time, we found a significant positive correlation between STL and sperm count, and significantly shorter STL in oligozoospermic men with respect to normozoospermic men. Finally, we showed for the first time a parental age effect on offspring STL.

The role of sperm telomeres and their length in sperm function, spermatogenic activity and fertility is largely unknown. Only one recent study (Thilagavathi *et al*., 2013) addressed this question. By analyzing STL in men with idiopathic infertility and controls, the authors found shorter TLs in infertile men, suggesting a possible contribution of shorter TL in unexplained male infertility. However, this study did not consider leukocyte TL and included low numbers of subjects (32 infertile men and 25 fertile controls) with unknown age and normal mean sperm count. In our study performed on subjects of the same age and with total sperm count ranging from 3 to 600 million cells, we found a linear correlation between STL and sperm count and shorter STL in oligozoospermic subjects with respect to normozoospermic subjects. This finding suggests at least two alternative interpretations. Firstly, since telomeres play a critical role in meiosis and maintenance of genome integrity (Siderakis and Tarsounas, 2007), one could hypothesize that shorter telomeres might impair spermatogenesis through segregation errors, apoptosis of germ cells and finally reduced sperm count. This is consistent with the dynamics of telomerase enzyme in testis whose expression peaks in meiosis I primary spermatocytes (Siderakis and Tarsounas, 2007). In this light, shorter telomeres in germ cells might be regarded as a novel putative cause of spermatogenic impairment and male infertility, although this hypothesis should be confirmed with additional studies. Secondly, shorter telomeres in ejaculated sperm might be a marker of damaged spermatogenesis, i.e. short STL could be regarded as a consequence rather than the cause of altered spermatogenesis. It is well known that many factors commonly implicated in spermatogenic impairment and male infertility, including environmental factors, infections, oxidative stress, smoking and obesity, might be implicated in telomere shortening (Thilagavathi *et al*., 2012). Therefore, studies are needed to clarify the possible role of these factors also on STL, and this might be performed for example also by comparing STL in idiopathic and non-idiopathic oligozoospermic men. Nevertheless, the finding of shorter telomeres in sperm of infertile (Thilagavathi *et al*., 2013) and oligozoospermic men have implications for assisted reproduction techniques, as in these cases offspring will tend to inherit shorter telomeres.

Of particular interest is the relation between parents' age at birth and offspring STL found in our study. Previous studies highlighted a direct relation between parents' age on leukocyte TL, showing a stronger effect for father's age with respect to mother's age (Nawrot *et al*., 2004; Nordfjäll *et al*., 2005, 2010; Unryn *et al*., 2005; Akkad *et al*., 2006; De Meyer *et al*., 2007; Njajou *et al*., 2007; Kimura *et al*., 2008; Arbeev *et al*., 2011; Eisenberg *et al*., 2012; Prescott *et al*., 2012; Broer *et al*. 2013), but no data have been published on sperm TL. We found longer STL in offspring of older fathers and mothers, but were unable to determine the relative contribution of father's and mother's age as they were highly correlated. In any case, because of the previous reports and our larger biological understanding, it is presumable that paternal, not maternal, age is the likely causal effect. We did not find significant relation between father's age and offspring LTL, but this might be simply due to the relative low number of subjects

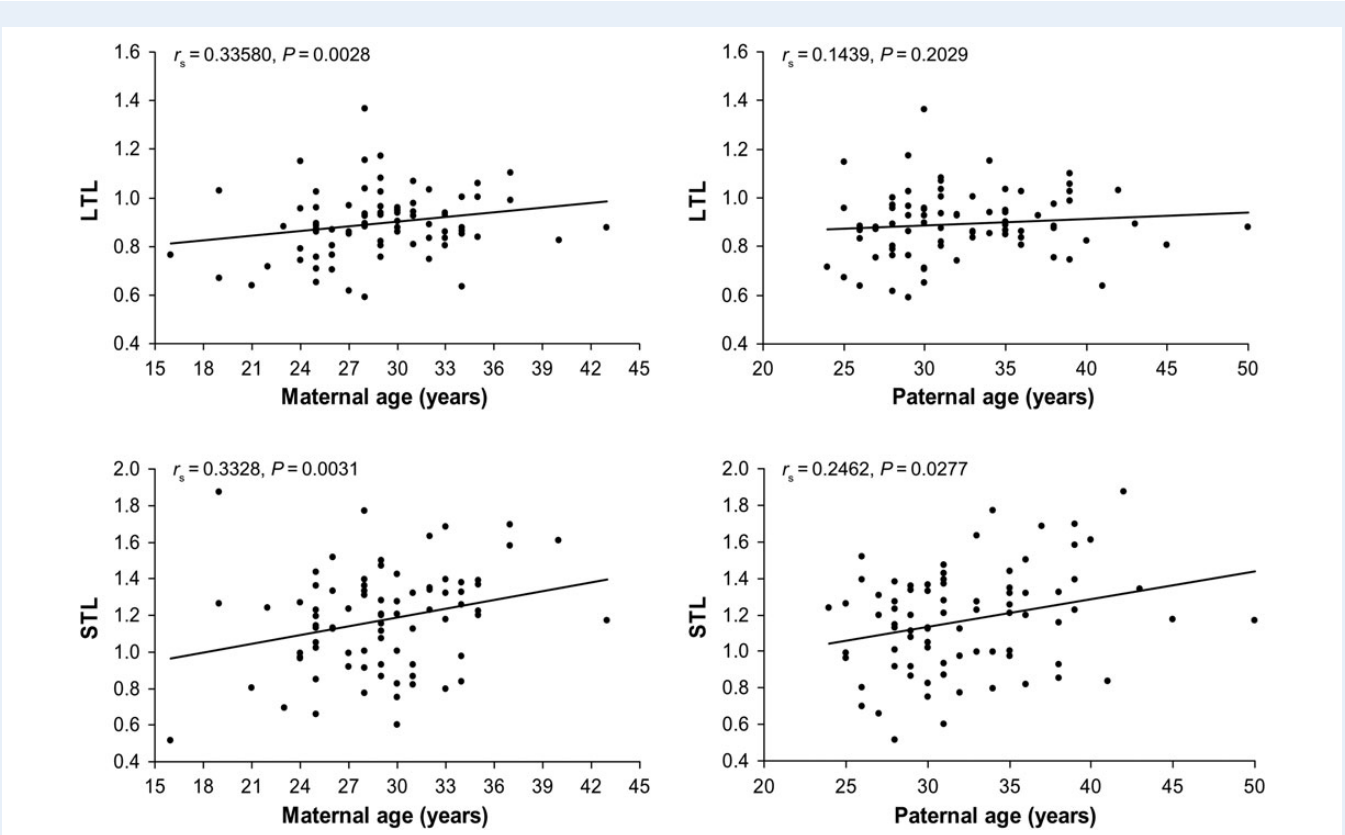

Figure 5 Correlation between offspring telomere length in leukocyte (LTL) and maternal age at conception (upper panel, left), LTL and paternal age at conception (upper panel, right), telomere length in sperm (STL) and maternal age at conception (lower panel, left), and STL and paternal age at conception (lower panel, right). Spearman's correlation coefficient (r_s) and *P*-value are shown.

($n = 81$, when compared, for example, with 2023 in the study of Eisenberg (Eisenberg *et al.*, 2012)). Regardless, this is consistent with data showing longer STL in older men (Allsopp *et al.*, 1992; Baird *et al.*, 2006; Kimura *et al.*, 2008; Aston *et al.*, 2012) and might be regarded as a potentially beneficial effect of older paternal age on the health of the offspring. Since delayed reproduction is a feature of contemporary society and assisted reproduction techniques increasingly are performed on couples of advanced age, and because we studied TL only in young men, future studies should determine whether our findings can be generalized to older men.

In conclusion, although preliminary, our study shed new light on the possible role of sperm telomeres in spermatogenesis and male infertility and on the inheritance of TL in relation to parents' age at conception. Additional studies are needed to confirm these observations, to extend them to older men, who represent the majority of patients undergoing ART, to clarify if the association between shorter STL and damaged spermatogenesis represents a pathophysiological link and to determine the effect on offspring TL of performing assisted reproduction techniques on couples of advanced age and/or where the man is oligozoospermic.

Authors' roles

A.F. performed clinical analysis, selected the subjects, analyzed the data and wrote the manuscript. M.S.R. extracted the DNA, analyzed the data and contributed to the writing of the manuscript. E.R. and S.K. performed the analysis of TL. A.C.F. performed the statistical analysis. A.D.R. supervised the experimental procedures and results of telomere determination, and contributed to the writing of the manuscript. C.F. supervised the project, critically analyzed the data and contributed to the writing of the manuscript.

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Conflict of interest

None declared.

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