Running title: Telomere length and sperm quality

Title: Is telomere length a biomarker of sperm quality? A systematic review and meta-analysis of observational studies

Authors:

María Fernández de la Puente, MSc,[1,2,3]*, Albert Salas-Huetos, PhD, [2,3,4,5]*#, Cristina Valle-Hita, MSc, [1,2,3], Nancy Babio, PhD, [1,2,3], Michelle M. Murphy, PhD, [1,4], Silvia Canudas, PhD, [6,7]#, Jordi Salas-Salvadó, MD, PhD, [1,2,3]

Affiliations:

[1] Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Unitat de Nutrició Humana, Reus, Spain.
[2] Consorcio CIBER, M.P. Fisiopatología de la Obesidad y Nutrición (CIBERObn), Instituto de Salud Carlos III (ISCIII), Madrid, Spain.
[3] Institut d'Investigació Sanitària Pere Virgili (IISPV), Hospital Universitari San Joan de Reus, Reus, Spain
[4] Unit of Preventive Medicine and Public Health, Faculty of Medicine and Health Sciences, IISPV, Universitat Rovira i Virgili, Reus, Spain.
[5] Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, MA, USA.
[6] Department of Nutrition, Food Sciences and Gastronomy, School of Pharmacy and Food Sciences, Food Torribera Campus, University of Barcelona, Santa Coloma de Gramenet, Spain.
[7] Institute of Nutrition and Food Safety of the University of Barcelona (INSA-UB), Santa Coloma de Gramenet, Spain.

* Should be considered similar in author order.

Corresponding authors. Should be considered similar in author order.

Corresponding authors contact information: Albert Salas-Huetos. Unit of Preventive Medicine and Public Health, Faculty of Medicine and Health Sciences, IISPV, Universitat Rovira i Virgili, Reus, Spain. Email: albert.salas@urv.cat. Tel: 977759379

Silvia Canudas, Department of Nutrition, Food Sciences and Gastronomy, School of Pharmacy and Food Sciences, Food Torribera Campus, University of Barcelona, E-08921 Santa Coloma de Gramenet, Spain. Email: silvia.canudas@ub.edu Tel: +34934020862

ORCID corresponding authors: 0000-0001-5914-6862 (AS-H) and 0000-0002-5630-1588 (SC)

Emails of all the authors:

María Fernández de la Puente, maria.fernandezdelapuente@urv.cat

Albert Salas-Huetos, albert.salas@urv.cat

Cristina Valle-Hita, cristina.valle@urv.cat

Nancy Babio, nancy.babio@urv.cat

Michelle M. Murphy, michelle.murphy@urv.cat

Silvia Canudas, silvia.canudas@ub.edu

Jordi Salas-Salvadó, jordi.salas@urv.cat

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Data sharing statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

ABSTRACT

Background: Telomeres are essential for the integrity of chromosome ends during cell division and their involvement in different processes linked to aging has been established. These chromosome components are involved in spermatogenesis and seem to play an important role in fertilization and embryo development. Telomere length is shortened with each cell division. Recently, short sperm telomere length has been proposed as a potential biomarker of male infertility.

Objectives: To conduct a systematic review and meta-analysis of studies exploring the association between sperm and/or leukocyte telomere length with sperm quality parameters and different infertility conditions.

Material and methods: A systematic review and meta-analysis was conducted with studies from Medline-PUBMED and Cochrane Library databases until May 2022. Eligible studies included cohort, cross-sectional and case-control studies, and telomere length in sperm and/or leukocytes cells was defined as the exposure. Semen quality parameters or infertility conditions (e.g., oligozoospermia, asthenozoospermia, teratozoospermia, or other spermatogenic impairment combinations) were defined as the outcomes.

Results: Twenty-four observational studies were included. In the qualitative analysis, high heterogeneity was observed between studies regarding the associations between telomere length and semen parameters in different normozoospermic/fertile and oligozoospermic/infertile populations. In the meta-analysis, sperm and leukocyte telomere length were shorter in infertile compared to fertile individuals (mean difference, MD; 95% confidence interval, CI) (-1.43; -1.66 to -1.21; P-value<0.001, and -1.67; -2.02 to -1.31; P-value<0.001, respectively). Moreover, in terms of sperm telomere length, these differences were also significant between individuals with a normal seminogram and individuals with low quantity of spermatozoa in the ejaculate (-0.97; -1.32, -0.61; P-value<0.001).

Conclusion: The current systematic review and meta-analysis suggest the potential role of sperm or leukocyte telomere length as a reliable biomarker of semen quality, which may help distinguish between infertility conditions beyond the routine semen analysis.

Registration number: PROSPERO CRD42021227690.

1. Introduction

Infertility is a disease of the reproductive system leading to the failure to achieve pregnancy after 12 months or more of regular unprotected sexual intercourse, according to the World Health Organization (WHO) (World Health Organization (WHO) 2018). Currently, infertility affects 8-12% of the world's population and over the last years this condition has increased [2]. Globally, of couples affected by infertility, male factors are responsible for 40-50% of cases [3]. Research on infertility is therefore important and quality of life and mental health of populations may also benefit [4].

Oxidation and inflammation processes have a recognized influence on sperm quality parameters and consequently to male fertility due to the susceptibility of spermatozoa to oxidative stress (OS) [5]. The pathological characteristics of oxidative molecules, such as reactive oxygen species (ROS), can lead to impairments in the male reproductive system resulting from imbalance between oxidant production and antioxidant capacity. A comparative study showed that seminal ROS concentrations were higher in infertile men compared to healthy sperm donors and in infertile men with all abnormal sperm parameters compared to infertile men with normal sperm parameters [6]. Therefore, investigating the biological mechanisms implicated in infertility beyond conventional sperm parameter analysis and identifying reliable biomarkers of infertility is of clinical relevance.

Telomeres are repetitive DNA sequences and specialized proteins in the end of the eukaryote chromosome, whose main function is to maintain genome integrity [7]. With each cell division under normal conditions, a small fragment of telomeric DNA is lost, leading to the activation of a DNA damage response that induces replicative senescence anticipating the onset of age-related diseases [8]. Telomeric structures are susceptible to oxidation processes due to their high guanine content, thus leading to accelerated telomere shortening [9]. Telomere length (TL) is mainly regulated by a reverse transcriptase, called telomerase, that adds 5'-TTAGGG-3' sequences in tandem [10] and whose activity decreases progressively during embryonic differentiation in somatic

tissues. However, in male germ cells, this activity is maintained until spermatogenesis, resulting in longer telomeres in spermatozoa than other cell types [10].

TL has been consolidated as a hallmark of processes linked to aging as oxidation, inflammation, epigenetic regulation, or mitochondrial dysfunction, among others [11]. Furthermore, in recent decades, telomere function has been investigated as a potential biomarker of infertility due to their apparently important role in fertilization and embryo development [12]. A recent systematic review and meta-analysis of observational studies evaluating sperm telomere length TL as a biomarker of embryonic development revealed that higher sperm TL was associated with higher probability of pregnancy, but not with fertilization rate [13]. However, the study has design limitations in the evaluation of the associations between sperm TL and sperm quality.

Recently, observational studies measuring telomere length as a marker of infertility in men [14,15] or evaluating the relationship between telomere length and sperm-related parameters [16,17] have increased. The main aim of the present study is to conduct a systematic review and meta-analysis of: a) cross-sectional studies exploring the association between sperm and/or leukocyte telomere length with sperm quality parameters, and, b) case-control studies comparing telomere length in populations with different semen abnormalities or (in)fertility. Our main hypothesis is that telomere length is a biomarker of semen quality and male infertility and can complement the analysis of other parameters predicting semen quality.

2. Methods

2.1 Protocol and registration

The protocol of the systematic review and meta-analysis was registered in the international prospective register of systematic reviews PROSPERO (https://www.crd.york.ac.uk/prospero) with the code CRD42021227690.

2.2 Literature search strategy

A literature search of human studies published in English was carried out in both MEDLINE-PubMed and Cochrane Library databases from the earliest available indexing year until May 2022. In order to obtain a reference list of the articles we performed a systematic search of two subsets of Medical Subject Heading terms and keywords: the first subset comprised telomere-related terms [telomere OR telomere shortening OR telomere homeostasis OR telomerase OR telomere length OR telomerase activity OR telomere maintenance] and the second subset comprised keywords related to seminogram alterations or infertility [spermatozoa OR spermatogenesis OR sperm motility OR sperm count OR sperm maturation OR sperm capacitation OR semen OR semen analysis OR infertility, male OR oligospermia OR aspermia OR asthenozoospermia OR azoospermia OR teratozoospermia OR sperm OR semen quality OR oligozoospermia OR oligoasthenozoospermia OR oligoasthenoteratozoospermia OR male fertility OR sperm dysfunction OR spermatogenesis OR protamine deficiency OR sperm parameters OR sperm DNA fragmentation OR sperm DNA damage OR varicocele OR non obstructive azoospermia OR erectile dysfunction OR sperm DNA extraction OR spermatozoa abnormality OR sperm chromosomal abnormalities]. In **Supplemental Information 1** the complete search strategy is available.

2.3 Eligibility criteria and study selection

In a preliminary screening, two independent researchers screened titles and abstracts for eligibility (MF, CVH), and the discrepancies were re-evaluated by two other authors (AS-H and SC). Eligible studies included in the systematic review and meta-analysis were those with a cohort design, cross-sectional and case-control studies. Articles defining telomere length in sperm and/or leukocyte cells as exposure, and semen quality parameters (volume, ejaculate pH, total sperm count or concentration, sperm vitality, sperm motility, sperm morphology) or seminogram alterations (oligospermia, aspermia, asthenozoospermia, azoospermia, teratozoospermia, oligozoospermia, oligoasthenozoospermia, oligoasthenoteratozoospermia, varicocele, non-

obstructive azoospermia) as outcomes were included in this systematic review and meta-analyzed when the number of studies were three or more. Studies including data regarding sperm DNA fragmentation, sperm DNA damage or protamine deficiency were included in this systematic review with these endpoints as secondary outcomes. Study exclusion criteria were as follows: ecological, retrospective, methodological or case report studies; review or meta-analysis articles; animal or *in vitro* studies; studies without describing telomere length as exposure and without semen quality parameters or fertility outcomes as endpoints. Finally, non-original articles (letters, commentaries, viewpoints, summaries, editorials), abstracts, symposium presentations or invited lectures, guidelines or scientific statements, and special articles were also excluded.

2.4 Data extraction

A standardized model was used to extract the information from each study: title, type of publication, first author, journal and year of publication, study design and period, sample size and participant's disease status, age, country of origin and city or place of recruitment, endpoint data, statistical analysis performed and main conclusions.

The main exposure of this study, TL, can be reported in relative (telomere (T) to single-copy gene (S) sequence (T/S) ratio) or absolute (bp) units. TL was mainly reported in relative units, so we contacted the corresponding authors of studies reporting absolute units to transform them into relative units, to standardize data.

2.5 Study quality assessment

The National heart, Lung and Blood Quality Assessment Tool for Observational cohort and Cross-Sectional and for Case-Control studies was used to evaluate the risk of bias of the articles included in this systematic review and meta-analysis (https://www.nhlbi.nih.gov/health-topics/study-quality-assessment-tools; accessed on 6 March, 2023). Two independent researchers (MF, CVH) assessed the risk of bias, and the discrepancies were re-evaluated by two other authors (AS-H and SC). The studies were categorized as good, fair or poor depending on the overall

quality score. Cross sectional studies with a score between 0-5 points and case-control studies with a score between 0-4 were considered low-quality and therefore excluded.

2.6 Statistical and sensitivity analysis

When three or more articles analyzed the same exposure and outcome, the results were meta-analyzed. Meta-analyses were conducted using the *meta* package for R 4.2.2 statistical software and Review Manager 5.4 in accordance with Cochrane guidelines [18]. The mean difference (MD) and 95% confidence interval (CI) were computed from the mean and standard deviation (SD) extracted in each study included. If other data distribution values (e.g., median, SEM, or IQR) were presented in the original studies, they were recalculated to mean and SD. Values were obtained by two authors (MF and CVH) and checked by another author (ASH). Fixed effect models were used to obtain summary MD and 95% CI of the studies analyzed. Statistical significance level was set at $p<0.05$ (two-tailed). Chi-square tests and the I^2 index were used to evaluate heterogeneity between studies and in this case the significance level was set at $p<0.1$. I^2 values <50% were considered moderate, ≥50% to <75% were considered substantial, and ≥75% were considered of considerable heterogeneity. To evaluate the robustness of our findings, sensitivity analyses using random effect models were performed.

3. Results

3.1 Article selection

Study selection, identification, screening, inclusion and exclusion processes are summarized in **Figure I**. Following a primary search, screening of the titles and abstracts of 681 articles led to 37 studies that were eligible for inclusion. At this point, two studies not in English or unavailable for download were excluded. Following full-text screening, 12 studies were excluded for a) only including processed sperm data (density gradient centrifugation and/or swim-up); b) being *in vitro*

experiments; c) including a specific selected population highly exposed to pollutants; or d) being reviews or hypothesis analysis articles. Finally, 23 cross-sectional or case-control studies were included for analyses.

3.2 Study characteristics

The characteristics of the cross-sectional and case-control studies included in the analyses are presented in **Table 1** and **Table 2**, respectively. The studies were conducted mainly in China (n=3), the United Kingdom (n=1), India (n=3), Iran (n=5), Israel (n=1), Italy (n=5), France (n=1), and Spain (n=4) with sample sizes ranging from 20 to 239 participants in the case of cross-sectional studies, and from 20 to 866 for case-control studies. Fertile and/or infertile populations were included with ages ranging from 18 to 52 years.

3.3 Qualitative analysis

3.3.1 Results of cross-sectional studies

A total of 17 cross-sectional studies were selected for the qualitative analysis. Four articles included fertile men only, four included infertile men, and ten included both.

3.3.1.1 Fertile or normozoospermic men

The study of the relationship between sperm TL and sperm parameters in fertile or normozoospermic populations revealed contradictory results among the papers. In relation to sperm vitality and motility, Rocca et al. reported positive correlations between sperm TL and vitality and progressive motility in 100 normozoospermic subjects [19]. No associations between sperm TL and motility were reported in the other 2 studies including semen samples from 65 normozoospermic, and from 60 donor participants, respectively [16,20]. A positive correlation between sperm TL and sperm concentration was reported by Torra-Massana et al., [20] but this association was not reported in the other two studies [16,19]. Regarding other sperm-related parameters, no associations were observed between sperm TL and volume, count or morphology

in any of the three aforementioned studies. A study by Balmori et al., only observed a positive correlation between sperm TL and sperm count, as well as total progressive motility, in the group of 20 normozoospermic participants under 25 years of age [21].

Concerning secondary outcomes, a negative correlation between sperm TL with sperm DNA fragmentation, and a positive correlation with normal sperm protamination was reported [19].

3.3.1.2 Infertile population

In relation to semen parameters, the study performed by Lafuente et al. showed in 42 infertile participants, a positive correlation between sperm TL measured before sperm selection and percentage of immotile spermatozoa [17]. The investigators also reported a negative correlation between sperm TL and progressive motility [17]. However, Zhao and collaborators found a significant but positive correlation between sperm TL and progressive motility in unprocessed semen samples of 150 normozoospermic infertile patients [22]. Lafuente et al. reported a negative correlation between sperm TL and sperm concentration [17]. Nevertheless, this association was not significant in the case of sperm concentration or morphology in the analysis performed by Zhao et al. [22]. A positive correlation between sperm TL and sperm count was reported in two of the four studies that have evaluated this endpoint [22,23].

On the other hand, Thilagavathi et al. did not report any significant association between leukocyte TL and sperm count or motility in 25 men from couples with a history of idiopathic recurrent pregnancy loss (iRPL) [24].

Three studies investigated the associations between sperm TL and secondary outcomes in infertile populations. While Zhao and collaborators reported a negative correlation between sperm TL and sperm DNA fragmentation index [22], no association between these two parameters was reported by Lafuente et al. [17]. Besides, no significant correlations between leukocyte TL and sperm DNA fragmentation index were observed [24].

3.3.1.3 Populations including a mix of fertile and infertile or normozoospermic and oligozoospermic subjects

Ten studies have analysed the association between sperm TL and sperm quality parameters in populations including fertile and infertile or normozoospermic and oligozoospermic subjects. A positive correlation between sperm TL and sperm count was found in 81 subjects (61 normozoospermic and 20 idiopathic oligozoospermic) [14]. This was also true in a similar population including 54 normozoospermic and 19 oligozoospermic adults [15], as well as in the study performed by Amirzadegan et al., in which 10 fertile and 10 oligozoospermic men were included [25]. On the contrary, in a group of 32 idiopathic infertile men and 25 fertile controls, sperm TL and sperm count were not correlated [26]. No associations were reported between sperm TL and motility nor morphology in the aforementioned studies [15,25,26]. Two studies reported a positive correlation between sperm TL and sperm concentration [25,27], but in the case of Darmishonnejad et al. (2018), they did not observe correlations between sperm TL and sperm count, motility or abnormal morphology in 10 fertile and 10 infertile subjects [27]. In Darmishonnejad et al.'s study (2020) no associations between relative sperm TL and different semen-related parameters (concentration, motility and abnormal morphology) in 38 infertile and 19 fertile participants mixed together were found [28]. Besides, Gentiluomo et al's study, performed in 239 participants, revealed no associations between sperm TL and semen-related parameters (concentration, total number, motility and morphology) [29]. Only Tahamtan et al. reported a positive correlation between sperm TL and spermatozoa motility in 20 fertile and 18 infertile men with grade II or III varicocele. In relation to leukocyte TL, positive correlations with concentration and sperm count were also shown in two studies [25,30]. Amirzadegan et al. also reported negative associations between leukocyte TL and abnormal morphology [25]. In contrast, leukocyte TL was not significantly related with sperm count [14] or with motility [25].

With respect to secondary outcomes, one study did not observe a significant relationship between sperm TL and sperm DNA fragmentation [26]. However, in three other articles, negative correlations

between sperm TL with DNA fragmentation [25,28,30] were observed. In addition, two studies reported negative correlations between sperm or leukocyte TL and sperm protamination deficiencies [25,30].

3.3.2 Results of case-control studies

Sixteen studies evaluated the differences in sperm and leukocyte TL between controls and cases.

3.3.2.1 Differences in sperm TL between normozoospermic (controls) and oligozoospermic (cases) subjects

Three studies clearly showed that the normozoospermic men had significantly longer telomeres than the oligozoospermic patients [14,15,25]. Similarly, Balmori et al., reported significant differences (in the same direction) in sperm TL between older normozoospermic and older oligozoospermic participants, between younger normozoospermic and older normozoospermic participants and between younger oligozoospermic and older normozoospermic participants [21].

3.3.2.3 Differences in sperm TL between fertile (controls) and infertile (cases) subjects

Four studies reported significantly longer sperm TL in fertile compared to infertile men [26–28,31], even after adjustment for age [32]. Besides, Rocca et al. observed longer sperm TL in 30 controls compared to 35 men who underwent their first assisted reproductive procedure [33]. On the contrary, Berby et al. reported no differences between control men and infertile men [34]. While significant differences in sperm TL were observed between 18 infertile men with grade II or III varicocele and 20 fertile men [30], no differences were reported by Lara-Cerrillo et al. between 12 fertile donors and 20 patients before microsurgical varicocelectomy (MV) and after MV [35].

3.3.2.4 Differences in leukocyte TL between fertile and infertile or normozoospermic and oligozoospermic subjects

Regarding TL in leukocytes, three studies reported lower leukocyte TL in infertile patients compared to fertile men [24,28,36]. Similarly, longer leukocyte TL in 10 fertile men compared to 10 oligozoospermic patients was reported by Amirzadegan and collaborators [25]. On the other hand, Yang et al., reported shorter leukocyte TL in 349 non-obstructive azoospermia patients than in 247 obstructive azoospermia patients and 270 normospermic men [37], and results from the Tahamtan et al. study showed significant lower leukocyte TL in men with infertility resulting from varicocele compared to fertile controls [30]. Only two studies found no differences in leukocyte TL neither between normozoospermic and oligozoospermic men [14] nor between fertile and infertile subjects [27].

3.4 Quantitative analysis

The relatively high number of case-control studies providing sufficient data and the homogeneity between them led us to conduct three meta-analyses to test the associations between telomere length in male sexual cells (spermatozoa) and somatic cells (leukocytes) and different case and control populations. No quantitative analysis was carried out with the cross-sectional studies due to the heterogeneity and insufficient data from these.

Infertile vs. Fertile populations

Sperm telomere length

Data from five studies were meta-analyzed to test the associations between sperm telomere length and fertility group. In summary, the infertile group of patients had significantly shorter sperm telomere length than the fertile group (MD; 95% CI) (-1.43; -1.66, -1.21; P-value<0.001). However, there was evidence of considerable interstudy heterogeneity (I^2=94%, P-value<0.001) (**Figure 2A**). Sensitivity analysis was consistent with the primary analysis (**Supplemental Figure 1A**).

Leukocyte telomere length

Analyzing data from four different studies testing the associations between leukocyte telomere length and fertility group, showed that the infertile group had significantly shorter leukocyte telomere length than the fertile group (MD; 95% CI) (-1.67; -2.02, -1.31; P-value<0.001). This comparison displayed a considerable interstudy heterogeneity (I^2=86%, P-value<0.001) (**Figure 2B**). The results were consistent with those of the sensitivity analysis (**Supplemental Figure 1A**).

Oligozoospermic vs. Normozoospermic populations

Sperm telomere length

Data form three studies were meta-analyzed to test associations between sperm telomere length and the WHO 2010 reference limits in sperm concentration or total sperm count; oligozoospermic vs. normozoospermic patients. In summary, the oligozoospermic group of patients had shorter sperm telomere length than the normozoospermic group (MD; 95% CI) (-0.97; -1.32, -0.61; P-value<0.001). Interstudy heterogeneity was nonsignificant in this evaluation (I^2=0, P-value=0.51) (**Figure 3**). The sensitivity analysis result was identical to the primary analysis (**Supplemental Figure 2**).

Discussion

This systematic review and meta-analysis of observational studies provides the most comprehensive and up-to-date analysis of the associations between sperm and leukocyte TL and sperm quality parameters and male fertility, to date. Our qualitative results showed high heterogeneity in methodology evaluation and contradictory outcomes, making the identification of clear patterns difficult. However, when data was comparable, the quantitative analysis revealed that sperm and leukocyte TL are shorter in infertile compared to fertile men. Moreover, these differences, in terms of sperm TL, are also significant between men with a normal seminogram and those with a low quantity of ejaculate spermatozoa. This systematic review and meta-analysis suggest the potential role of sperm and leukocyte telomere length as a biomarker of semen quality which may help distinguish between different spermatogenic alterations beyond the routine semen analysis. These results suggest that sperm and leukocyte TL may also be relevant biomarkers to help discriminate fertility potential in cases with sperm quality parameters that are within or close to the threshold values established by the WHO.

To the best of our knowledge, only Yuan et al. [13] performed a systematic review and meta-analysis evaluating the role of sperm telomere length as biomarker of male infertility and embryonic development. However, only the data from studies that they meta-analyzed was summarized, the qualitative results were omitted, and important limitations to their meta-analysis should be highlighted. For example, in the main analysis, all normozoospermic individuals were considered as fertile population and all oligozoospermic men as the infertile population without considering their fertility status. However, in a secondary analysis, they appropriately compared fertile men versus unexplained infertile men and reported similar results. Although the MD and 95% CI were completely different, they also concluded that sperm TL is shorter in infertile compared to fertile men.

In our study, we also compared sperm TL between normozoospermic and oligozoospermic men, and it was shorter in the oligozoospermic population. It is important to mention that in the study

performed by Yuan et al., on some occasions, untransformed data to mean and SD was incorrectly included in their meta-analysis. However, in our study we carefully estimated for each meta-analyzed study the mean and SD values using the original data. Finally, in our study, we have not only analyzed studies measuring sperm TL, but also those measuring TL in leukocytes, and our results also revealed that infertile men had shorter TL in leukocytes compared to fertile men.

Although this was not the scope of our study, in the Yuan et al. systematic review and meta-analysis, the capacity of sperm TL to predict the success of pregnancy outcome and embryo development was also explored. A relationship between longer sperm TL and clinical pregnancy was reported, whereas higher fertilization probabilities were not observed in men with higher sperm TL. We recognize that in light of medical and social concerns, identifying biomarkers not only of sperm quality but also of infertility and reproductive outcomes is essential. This will provide valuable insights into possible clinical implications.

Two other studies not included in Yuan et al.'s meta-analysis should also be mentioned. Firstly, in normozoospermic male partners of couples undergoing ART treatments, Berneau et al. reported positive significant associations between sperm TL and the percentage of fertilization rate [16]. Secondly, regarding embryological parameters, sperm TL was positively associated with good embryo quality and transplantable embryo rates in the men of couples undergoing IVF [38]. On the contrary, no associations between sperm TL and embryological parameters (e.g. good embryo cleavage, implantation rates or biochemical, clinical and ongoing pregnancy rates) were found [16]. Likewise, no associations were observed with clinical pregnancy and fertilization rates [38]. These contradictory results might be explained by differences in the study design, population studied, and/or unconsidered factors related to female partners, suggesting that evidence exploring the associations between sperm TL and clinical and embryological outcomes is still needed.

The effect of parental age at conception has been studied since offspring conceived by older parents have been found to have longer telomeres. Kimura et al. confirmed that paternal age was positively associated with leukocyte TL from the offspring in four cohorts, and this may be related

to telomere characteristics in paternal sperm. Longer sperm TL was observed in older than younger men [39]. Moreover, when comparing the effect of maternal and paternal age at conception, the major determinant of offspring leukocyte TL was paternal age, with longer leukocyte TL observed in the offspring of older fathers [40]. Ferlin et al. also explored the relationship between maternal and paternal age with offspring sperm TL and reported longer sperm TL in the offspring of older fathers and mothers. However, since there was a high correlation between paternal and maternal ages, which contributed the most could not be determined [14]. The fact that paternal age may act as a determining factor of human telomere dynamics, due to the inheritance of telomere length to the offspring, demonstrates the important role of these structures in reproduction. Therefore, sperm selection procedures of spermatozoa with longer telomeres in ART practices may play a role in IVF success, but this must be extensively explored. Unfortunately, we could not evaluate this because the studies did not report maternal and paternal age at conception.

Several methods are currently available for TL measurement and quantification, each with its own advantages and limitations. These methods can be broadly categorized into four groups: hybridization, Polymerase Chain Reaction (PCR), Fluorescence In Situ Hybridization (FISH), and mixed methods (e.g. hybridization/PCR combination), each providing different information about TL. Terminal restriction fragment (TRF) analysis, the "gold standard", is based on hybridization techniques, and it is considered more accurate in providing absolute telomere length measurements. However, it requires large amounts of genomic DNA and laborious processes, making it less practical for large-scale population studies. PCR-based methods have become the most popular method for large-scale studies due to their high-throughput capability, simplicity, and because small amounts of DNA are required. These methods determine the ratio between the telomere (T) and single-copy gene (S) signals, providing a proportional measure of relative average TL. Nevertheless, these PCR-based methods often exhibit difficulties in standardization. Methods based on Fluorescence In Situ Hybridization (e.g. Quantitative-FISH, Flow FISH) are also used to determine TL. Because its low detection limit capacity, these techniques are particularly useful for detecting short telomeres, which are highly indicative of cellular

429 senescence. These procedures can be suitable for large-scale studies to determine TL on fixed
430 lymphocytes but are time consuming and limited to specialized laboratories. Additionally, other
431 techniques, such as Single Telomere Length Analysis (STELA) or Telomere Shortest Length
432 Assay (TeSLA) are also used to quantify the shortest telomeres, but with low throughputs [41].

433 This study has several strengths that should be highlighted. This is the first comprehensive
434 systematic review and meta-analysis that has meta-analyzed sperm and leukocyte TL
435 differentiating between fertility conditions and seminogram alterations: fertile vs. infertile, and
436 normozoospermic vs. oligozoospermic individuals, separately. Secondly, in the meta-analysis, we
437 only included studies measuring telomere length by quantitative PCR technique as a mode of
438 standardizing the results. Thirdly, in this systematic review and meta-analysis we did not include
439 studies using sperm selection procedures as we understand that TL measurement would not be
440 representative of the whole sperm population. However, our work has also some limitations that
441 should be considered. Relatively few studies evaluating sperm and leukocyte TL as a biomarker
442 of infertility have been carried out. Besides, we could not meta-analyze cross-sectional data due
443 to the lack of information provided despite trying to obtain this through contact with the
444 corresponding authors. Unfortunately, due to the limited number of published studies we cannot
445 conduct a sensitivity analysis to consider differences between the populations of the studies
446 included with regard to age, sample size, or other factors that could affect TL. Finally, it is worth
447 mentioning that although positive associations were observed between TL and conventional
448 sperm quality parameters, its translation into better reproductive outcomes (e.g., clinical
449 pregnancy or live birth) remains unanswered and deserves further studies.

450 In conclusion, our study presents a global review of the evidence available regarding the
451 associations between telomere length and semen quality parameters and differences in TL
452 between different populations based on their seminogram abnormalities or fertility. Our results
453 suggest that telomere length has the potential to be used as a biomarker of sperm quality and male
454 infertility. However further and larger studies are warranted in the future to increase the certainty
455 of evidence of a potential relationship between TL, semen quality and fertility. Finally, if TL is a

proxy of sperm quality or fertility potential (or vice versa) should be tested using molecular and *in-vitro* experiments.

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TABLES

TABLE 1. Summary of the cross-sectional studies investigating the association between sperm and/or leukocyte telomere length and sperm quality parameters or fertility outcomes

Reference	Location	Population studied	Age (years)	Cell type	Primary outcomes	Secondary outcomes
Fertile or normozoospermic populations						
Rocca et al. 2016 [19]	Italy	100 normozoospermic fertile participants	34.0 (8.6)	STL	Semen parameters (volume, total count, concentration, progressive motility, vitality and morphology)	Sperm DNA fragmentation and normal protamination
Berneau et al. 2020 [16]	United Kingdom	66 normozoospermic participants	35.5 (4.5); [25-45]	STL	Semen parameters (volume, total count, concentration, progressive and grade-A motility and immotile sperm)	No
Torra-Massana et al. 2018 [20]	Spain	60 donor participants	24.3 (5.0); [18-35]	STL	Sperm parameters (concentration and motility)	No
Balmori et al. 2021[21]	Spain	20 normozoospermic subjects	21.2 (2.4)	STL	Semen parameters (total count and total progressive motility)	No
Infertile populations						
Lafuente et al. 2017 [17]	Spain	42 infertile patients	NR	STL	Sperm parameters (concentration, progressive motility and immotile sperm)	Sperm DNA fragmentation
Thilagavathi et al. 2013 [24]	India	25 iRPL subjects	33.2 (5.2)	LTL	Sperm parameters (pH, total count and motility)	Sperm DNA fragmentation
Zhao et al. 2016 [22]	China	150 normozoospermic infertile subjects	31.8 (6.1)	STL	Semen parameters (volume, total count, concentration, progressive motility and normal morphology)	Sperm DNA fragmentation
Sun et al. 2015[23]	China	105 infertile subjects	31.2 (6.1)	STL	Semen parameters (total count)	No
Fertile and infertile or normozoospermic and oligozoospermic populations						
Ferlin et al. 2013 [14]	Italy	61 normozoospermic and 20 oligozoospermic subjects	[18-19]	STL and LTL	Semen parameters (total count)	No
Cariati et al. 2016 [15]	Italy	54 normozoospermic and 19 oligozoospermic	Normozoospermic 39.4 (5.5); oligozoospermic 39.3 (5.3); [31-52]	STL	Semen parameters (total count, motility and normal morphology)	No

(Continued)

577

578

TABLE 1. *Continued*

Reference	Location	Population studied	Age (years)	Cell type	Primary outcomes	Secondary outcomes
Thilagavathi et al. 2013 [26]	India	32 idiopathic infertile and 25 fertile participants	NR	STL	Semen parameters (pH, volume, total count, motility and normal morphology)	Sperm DNA fragmentation
Amirzadegan et al. 2021 [25]	Iran	10 fertile and 10 oligozoospermic subjects	Fertile: 35.5 (5.6); oligozoospermic: 40.3 (3.8)	STL and LTL	Semen parameters (total count, concentration, motility, abnormal morphology)	Sperm DNA fragmentation, protamine deficiency
Darmishonnejad et al. 2018 [27]	Iran	10 fertile and 10 infertile subjects	Fertile: 40.1 (3.1); infertile: 38.1 (4.2)	STL and LTL	Semen parameters (total count, concentration, motility and abnormal morphology)	No
Darmishonnejad et al. 2020 [28]	Iran	19 fertile and 38 infertile subjects	Fertile: 40.5 (3.8); infertile 32.6 (6.6); [20-50]	STL	Semen parameters (concentration, motility and abnormal morphology)	Sperm DNA fragmentation, protamine deficiency
Mishra et al. 2016 [32]	India	102 fertile participants and 112 infertile	Fertile: 32.2 (4.0); infertile:31.7 (4.4); [18-45]	STL	No	No
Tahamtan et al. 2019 [30]	Iran	20 fertile and 18 infertile varicocele patients	Fertile: 41.4 (3.6), varicocele: 28.5 (5.5)	STL and LTL	Semen parameters (sperm count, concentration, motility and abnormal sperm morphology)	Sperm DNA fragmentation, protamine deficiency
Getiluomo et al. 2021 [29]	Italy	239 participants	34.8 (7.5)	STL	Semen parameters (total count, concentration, motility, morphology)	No

The studies are ordered as: fertile population, infertile population, and fertile and infertile population. Age is given as mean (SD) or [range] where such data are available.
Abbreviations: iRPL, idiopathic recurrent pregnancy loss; LTL, leukocyte telomere length; NR, not reported; STL, sperm telomere length; PBMCs, peripheral blood mononuclear cells

TABLE 2. Summary of case-control results investigating the differences on sperm and/or leukocyte telomere length between normozoospermic (controls) and oligozoospermic (cases) or fertile (controls) and infertile (cases) subjects.

Reference	Location	Population studied	Age (years)	Cell type	Main analyses
Normozoospermic (controls) and oligozoospermic (cases) subjects					
Ferlin et al. 2013 [14]	Italy	61 normozoospermic and 20 oligozoospermic subjects	[18-19]	STL and LTL	Differences in STL and LTL between normozoospermic and oligozoospermic men
Cariati et al. 2016 [15]	Italy	54 normozoospermic and 19 oligozoospermic subjects	Normozoospermic 39.4 (5.5); oligozoospermic 39.3 (5.3); [31-52]	STL	Differences in STL between normozoospermic and oligozoospermic men
Amirzadegan et al. 2021 [25]	Iran	10 fertile and 10 oligozoospermic subjects	Fertile: 35.5 (5.6); oligozoospermic: 40.3 (3.8)	STL and LTL	Differences in STL and LTL between fertile and oligozoospermic men
Balmori et al. 2021[21]	Spain	Younger group: 20 normozoospermic and 17 oligozoospermic Older group: 20 normozoospermic and 20 oligozoospermic	Younger group: 20 normozoospermic 21.2 (2.4) and 17 oligozoospermic 21.4 (2.3) Group ≥40 years: 20 normozoospermic 43.3 (3.4) and 20 oligozoospermic 43.6 (4.0)	STL	Differences in STL between normozoospermic and oligozoospermic men under 25 years and between normozoospermic and oligozoospermic men over 40 years
Fertile (controls) and infertile (cases) populations					
Mishra et al. 2016 [32]	India	102 fertile participants and 112 infertile patients	Fertile: 32.2 (4.0); infertile:31.7 (4.4); [18-45]	STL	Differences in STL between fertile and infertile men
Darmishonnejad et al. 2020 [28]	Iran	19 fertile and 38 infertile subjects	Fertile: 40.5 (3.8); infertile 32.6 (6.6); [20-50]	STL and LTL	Differences in STL between fertile and infertile men
Berby et al. 2021[34]	France	20 control men and 30 infertile patients	Control: 35.1 (5.7); infertile: 35.2 (8.0)	STL	Differences in STL between control and infertile men
Rocca et al. 2021 [33]	Italia	30 healthy controls and 35 men undergoing ART	Control: 36.1 (6.8); ART group: 39.6 (5.4)	STL	Differences in STL between control and ART men

Continued

TABLE 2. *Continued*

Reference	Location	Population studied	Age	Cell type	Main analyses
Thilagavathi et al. 2013 [26]	India	32 idiopathic infertile and 25 fertile men	NR	STL	Differences in STL between fertile and idiopathic infertile men
Darmishonnejad et al. 2018 [27]	Iran	10 fertile and 10 infertile patients	Fertile: 40.1 (3.1); infertile: 38.1 (4.2)	STL and LTL	Differences in STL between fertile and infertile men
Biron-Shental et al. 2017 [31]	Israel	10 fertile and 16 sub-fertile subjects requiring ICSI	Fertile: 36.5 (7.0); sub-fertile: 37.4 (5.0)	STL	Differences in STL between fertile and sub-fertile subjects
Yang et al. 2018 [37]	China	270 normal men, 247 with obstructive azoospermia and 349 with nonobstructive azoospermia patients	Normal: 33.0 (29.0-38.0); OA: 27.0 (25.0-31.0); NOA: 27.0 (25.0-31.0)	LTL	Differences in LTL between normal, OA and NOA men
Thilagavathi et al. 2013 [24]	India	20 fertile controls and 25 iRPL patients	Controls: 31.5 (5.3); iRPL subjects: 33.2 (5.2)	LTL	Differences in STL between fertile and iRPL patients
Tahamtan et al. 2019 [30]	Iran	20 fertile controls and 18 infertile subjects with grade II or III varicocele	Fertile: 41.4 (3.6); varicocele: 28.5 (5.5)	STL and LTL	Differences in STL between fertile and varicocele patients
Lara-Cerillo et al. 2020 [35]	Spain	12 fertile donors and 20 patients with unilateral or bilateral grade II or higher varicocele	17-44	STL	Differences in STL between fertile controls, before and after surgery in varicocele patients
Heidary et al. 2018 [36]	Iran	30 fertile participants and 30 idiopathic non-obstructive azoospermic patients	35.4 (4.5)	LTL	Differences in LTL between fertile controls and idiopathic non-obstructive azoospermic men

Age is given as mean (SD), [range] or median (25th-75th percentile) where such data are available. Abbreviations: ART, assisted reproductive technology; DGC, density or discontinuous gradient centrifugation; ICSI, intracytoplasmic sperm injection; iRPL, idiopathic recurrent pregnancy loss; IVF, *in* vitro fertilization; LTL, leukocyte telomere length; NOA, nonobstructive azoospermia; NR, not reported; OA, obstructive azoospermia; STL, sperm telomere length; SU, swim-up.

579

FIGURE CAPTIONS

Figure 1. Flowchart of the literature search and selection process.

Figure 2. Forest plot of mean differences (MD) and 95% of confidence intervals (CI) for studies evaluating the association between **(A)** Sperm Telomere Length and **(B)** Leukocyte Telomere Length in infertile vs. fertile participants. Red squares for each study indicate the MD, the size of the boxes indicates the weight of the study, and the horizontal lines indicate the 95% CI. The data in bold and diamond represents the pooled MD and 95% CI. Overall estimates were obtained using fixed-effect models. A MD value <0 indicates a negative association between telomere length and fertility group; infertile group had lower telomere length than fertile group.

Figure 3. Forest plot of mean differences (MD) and 95% of confidence intervals (CI) for studies evaluating the association between Sperm Telomere Length in oligozoospermic vs. normozoospermic participants. Red squares for each study indicate the MD, the size of the boxes indicates the weight of the study, and the horizontal lines indicate the 95% CI. The data in bold and diamond represents the pooled MD and 95% CI. Overall estimates were obtained using fixed-effect models. A MD value <0 indicates a negative association between telomere length and normal seminogram result; oligozoospermic group had lower telomere length than normozoospermic group.

SUPPLEMENTAL INFORMATION

Supplemental Information 1. Complete search strategy for the literature published between the earliest available online indexing year and May 2022 in the MEDLINE-Pubmed database.

Search terms:

"(""telomere""[MeSH Terms] OR ""telomere shortening""[MeSH Terms] OR ""telomere homeostasis""[MeSH Terms] OR ""telomerase""[MeSH Terms] OR (""telomere""[All Fields] OR ""telomere shortening""[All Fields] OR ""telomere homeostasis""[All Fields] OR ""telomer*""[All Fields] OR ""telomere length""[All Fields] OR ""telomerase""[All Fields] OR ""telomerase activity""[All Fields] OR ""telomere maintenance""[All Fields])) AND (""spermatozoa""[MeSH Terms] OR ""spermatogenesis""[MeSH Terms] OR ""sperm motility""[MeSH Terms] OR ""sperm count""[MeSH Terms] OR ""sperm maturation""[MeSH Terms] OR ""sperm capacitation""[MeSH Terms] OR ""semen""[MeSH Terms] OR ""semen analysis""[MeSH Terms] OR ""infertility, male""[MeSH Terms] OR ""oligospermia""[MeSH Terms] OR ""aspermia""[MeSH Terms] OR ""asthenozoospermia""[MeSH Terms] OR ""azoospermia""[MeSH Terms] OR ""teratozoospermia""[MeSH Terms] OR (""sperm""[All Fields] OR ""sperm*""[All Fields] OR ""sperm motility""[All Fields] OR ""sperm count""[All Fields] OR ""semen""[All Fields] OR ""semen""[All Fields] OR ""semen analysis""[All Fields] OR ""semen quality""[All Fields] OR ""oligospermia""[All Fields] OR ""aspermia""[All Fields] OR ""azoospermia""[All Fields] OR ""asthenozoospermia""[All Fields] OR ""teratozoospermia""[All Fields] OR ""oligozoospermia""[All Fields] OR ""oligoasthenozoospermia""[All Fields] OR ""oligoasthenoteratozoospermia""[All Fields] OR ""male fertility""[All Fields] OR ""sperm dysfunction""[All Fields] OR ""spermatogenesis""[All Fields] OR ""protamine deficiency""[All Fields] OR ""sperm parameters""[All Fields] OR ""sperm dna fragmentation""[All Fields] OR ""sperm dna damage""[All Fields] OR ""varicocele""[All Fields] OR ""non obstructive azoospermia""[All Fields] OR ""erectile

dysfunction""[All Fields] OR ""sperm dna extraction""[All Fields] OR ""spermatozoa abnormality""[All Fields] OR ""sperm chromosomal abnormalities""[All Fields]))"

Supplemental Figure 1. Sensitivity analysis. Forest plot of mean differences (MD) and 95% of confidence intervals (CI) for studies evaluating the association between **(A)** Sperm Telomere Length and **(B)** Leukocyte Telomere Length in infertile vs. fertile participants. Red squares for each study indicate the MD, the size of the boxes indicates the weight of the study, and the horizontal lines indicate the 95% CI. The data in bold and diamond represents the pooled MD and 95% CI. Overall estimates were obtained using random-effect models. A MD value <0 indicates a negative association between telomere length and fertility group; infertile group had lower telomere length than fertile group.

Supplemental Figure 2. Sensitivity analysis. Forest plot of mean differences (MD) and 95% of confidence intervals (CI) for studies evaluating the association between Sperm Telomere Length in oligozoospermic vs. normozoospermic participants. Red squares for each study indicate the MD, the size of the boxes indicates the weight of the study, and the horizontal lines indicate the 95% CI. The data in bold and diamond represents the pooled MD and 95% CI. Overall estimates were obtained using random-effect models. A MD value <0 indicates a negative association between telomere length and normal seminogram result; oligozoospermic group had lower telomere length than normozoospermic group.