Sperm telomere length in donor samples is not related to ICSI outcome

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Abstract

Purpose Variations in sperm telomere length (STL) have been associated with altered sperm parameters, poor embryo quality, and lower pregnancy rates, but for normozoospermic men, STL relevance in IVF/ICSI is still uncertain. Moreover, in all studies reported so far, each man's STL was linked to the corresponding female partner characteristics. Here, we study STL in sperm donor samples, each used for up to 12 women, in order to isolate and determine the relationship between STL and reproductive outcomes.

Methods Relative STL was determined by qPCR in 60 samples used in a total of 676 ICSI cycles. Univariable and multivariable statistical analyses were used to study the STL effect on fertilization rate; embryo morphology; biochemical, clinical, and ongoing pregnancy rates; and live birth (LB) rates.

Results The average STL value was 4.5 (relative units; SD 1.9; range 2.4–14.2). Locally weighted scatterplot smoothing regression and the rho-Spearman test did not reveal significant correlations between STL and the outcomes analyzed. STL was not different between cycles resulting or not in pregnancy and LB (Mann-Whitney *U* test, $p > 0.05$). No significant effect of STL on reproductive outcomes was found, with the OR for each unit increase in STL (95% CI) of 0.94 (0.86–1.04), 0.99 (0.9–1.09), 0.98 (0.89–1.09), and 0.93 (0.8–1.06) for biochemical, clinical, and ongoing pregnancy and LB, respectively. The multilevel analysis confirmed that the effect of STL on fertilization; biochemical, clinical, and ongoing pregnancy; and LB was not significant ($p > 0.05$).

Conclusion After addressing STL independently from female variables, results show that STL measurement is not useful to predict reproductive outcomes in ICSI cycles using donor semen.

Keywords IVF/ICSI · Embryo morphology · Sperm telomere length · Live birth

Introduction

Telomeres are evolutionary conserved structures composed of non-coding hexameric tandem repeats (5'-TTAGGG-3') of

genomic DNA. Located at the ends of eukaryotic chromosomes, telomeres play a role in maintaining genomic integrity, prevent chromosome end joining, and facilitate homologue pairing and synapsis in meiosis [1]. In most cell types, telomere length decreases with each cell division, as DNA replication mechanisms are unable to replicate the end of their 3' sequence [2]. In fact, when telomeres reach a critical length, chromosome uncapping, apoptosis, or cell-cycle arrest occur, thus affecting genome stability, cell division, and meiosis [3, 4].

In male germ cells, telomeres progressively increase in length during spermatogenesis, although further research is necessary to understand the molecular background and regulatory systems involved in this process [5]. Telomere length in spermatozoa increases with a man's age and positively correlates with parental age at conception [6–8]. Conversely, telomeres in oocytes decrease with a woman's age [4] and shorten during oocyte maturation [9].

Human sperm telomere length (STL) is around 10–20 kb [10], and considerable variability in STL exists among

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individuals and even among spermatozoa from the same ejaculate [11]. Telomere length can be affected by oxidative stress, which can lead to DNA damage and telomere shortening [12]; environmental chemicals [13]; smoking; obesity; stress; and diet [14]. Additionally, there seem to be ethnical differences in STL [15].

STL has been proposed as a novel marker of IVF outcomes, and some studies indicate that shorter telomeres are concurrent with altered sperm parameters. For example, oligozoospermic or asthenozoospermic samples have lower STL than normozoospermic ones [8, 11, 16]. Additionally, recent studies indicate a positive correlation between STL, progressive sperm motility, vitality, and protamination, as well as a negative correlation between STL, DNA fragmentation, and diploidy [17, 18]. Other authors did not find correlation between STL and sperm DNA fragmentation, concentration, or motility [9].

Moreover, STL has been positively correlated with embryo morphology in IVF cycles [16]. On the other hand, the role of normozoospermic men's STL in pregnancy and live birth rates is uncertain, and it remains to be established if it can be used as a reliable marker in fertility clinics.

Inevitably, a significant limitation in all studies reported so far is that each man's STL is necessarily linked to his female partner's characteristics, making it impossible to assess STL independently from these uncontrolled for variables. This is a significant flaw of current studies, given the relevance of some variables such as a woman's age for ART outcomes [19, 20]. The aims of this study were to analyze the STL of sperm donors, whose samples have been used for several female patients in different cycles, and assess its relationship with reproductive outcomes.

Materials and methods

Ethical considerations

Permission to conduct this study was obtained from the local Ethical Committee for Clinical Research. All procedures performed were in accordance with the ethical standards of the institutional research committees and with the 1964 Helsinki declaration, as revised in 2013.

Study population

In the present study, 60 sperm donor samples were included after being used for fertility treatments. All the samples came from external sperm banks. Demographic data is shown in Table 1. Semen was thawed, and motility and concentration were determined. The samples used were representative of the whole sperm sample, as they were collected by pooling the swim-up fraction (after ICSI) with the pelleted cells. All the

samples included were assigned to between 6 and 12 ICSI cycles. ICSI cycles were performed using donor sperm with either donor oocytes or the patient's own oocytes. All embryo transfers were fresh and on D2–3 of development. A total of 631 women (either oocyte donors or patients) performed the 676 ICSI cycles included in the study (i.e., 93% of the women were included once). However, only in 2 cases did an oocyte donor or a patient repeat the cycle with the same sperm donor, representing little to no repetition in the database (99.7% independent cycles/gamete mix). Demographic and clinical variables and reproductive outcomes were collected (Tables 1 and 2 and Supp. Table 1).

Sperm telomere length determination

Genomic DNA (gDNA) was extracted from 3 to 10 million sperm using QIAMP DNA Blood Mini Kit (QIAGEN, Germany) following the manufacturer's instructions with minor modifications, specifically increasing lysis time up to 2 h; gDNA was quantified by measurement of absorbance at $\lambda = 260$ nm (Quawell). Relative STL (relative units, RU) was determined by real-time quantitative PCR (qPCR), using a method previously described [21], with minor modifications. Briefly, amplification of the telomeric region (*T*) was expressed relative to the amplification of a single-copy gene (*36B4*, which encodes for RPLP0, S). Then, to obtain the relative STL, the *T/S* ratio from each sperm sample was compared with the *T/S* ratio from a common DNA sample for the whole study (HeLa DNA). Primers used to amplify the telomeric regions were 5'-CGGTTTGGTTTGGGTTTGGGT TTGGGTTTGGGTTTGGGTT-3' (forward) and 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3' (reverse), while those used to amplify the single-copy gene were 5'-CCCATTCTATCATCAACGGG TACAA-3' (forward) and 5'-CAGCAAGTGGAAG GTGTAATCC-3' (reverse) [21]. Each qPCR included 1 ng of gDNA, 10 μ L $2\times$ SsoAdvanced Universal SYBR Green Supermix (BioRad, Hercules, CA, USA), and 0.1 μ L of each primer (100 μ M), in a 20- μ L final reaction volume. The program used in each qPCR run consists of an initial denaturalization step of 30 s at 95 °C and 40 cycles of 95 °C for 5 s and 60 °C for 30 s.

All qPCRs were carried out in triplicate in 96-well plates using CFX96 (BioRad). Inter-assay reproducibility (mean $C_q = 20.69$) and error measurement ($SD = 0.5$) were assessed by adding a reference curve in each qPCR plate, consisting of the same gDNA sample from the HeLa cell line serially diluted from 10 to 0.01 ng/well. Analysis was performed using CFX Manager software (BioRad) and corrected, taking into account both telomere and *36B4* primer pair efficiencies, which were evaluated using sequential dilutions of the HeLa gDNA, as well as for sperm gDNA samples (Supp. Table 2).

Table 1 Baseline characteristics of the sperm donor population ($n = 60$) and variables included within the cycle level ($n = 676$)

	Variable, units	Overall
Sperm donor level ($n = 60$)	Sperm donor age, years	24.3 ± 5 [18–35]
	Sperm concentration, million/mL	66.9 ± 33.2 [15.9–173]
	Sperm motility, %	24.5 ± 14.1 [5.8–78]
	Sperm telomere length, RU	4.5 ± 1.9 [2.4–14.2]
Cycle level ($n = 676$)	Oocyte age, years	32.7 ± 7.5 [18–49]
	Female patient age, years	40.1 ± 4.7 [23–50]
	Female patient BMI, kg/m ²	24.3 ± 4.5 [16.8–41.4]
	Oocyte donor BMI, kg/m ²	22.9 ± 3.4 [17–33.7]
	MII, number	6.4 ± 3.1 [1–24]
	2PN, number	4.5 ± 2.6 [0–21]
	Fertilization rate, %	69.6 ± 23.9 [0–100]
	Abnormal fertilization rate, %	7.6 ± 12.9 [0–100]
	Mean embryo morphological score, AU	7 ± 1.54 [0–10]
	Embryos obtained, number	4.3 ± 2.4 [0–20]

Values are presented as mean $\pm$ standard deviation [range]

RU relative units, BMI body mass index, MII metaphase II oocyte, 2PN 2 pronuclei zygote, AU arbitrary units

Univariable statistical analysis

Univariate differences in STL between pregnancies with and without biochemical (positive bHCG in serum), clinical (ultrasound evidence of a gestational sac with fetal heartbeat), and ongoing pregnancy (normally proceeding pregnancy at 10–12 weeks), and live birth were tested by the Mann-Whitney U test. Non-parametric correlation between STL

and sperm concentration, sperm motility, sperm donor age, the average score of embryo morphology, fertilization rate, and abnormal fertilization rate (defined as the percentage of oocytes with 1PN and 3PN in each ICSI cycle) was tested by the rho-Spearman test. The embryo morphological score system used was based on the system described by Coroleu and colleagues [22], which takes into account the number and symmetry of cells and percentage of fragmentation.

Table 2 Mean values of oocyte age within study groups, variables included in the embryo transfer level ($n = 626$), and reproductive outcomes: pregnancy (biochemical, clinical, ongoing) and live birth rates

Variable, units	Overall	Cycles w/ own oocytes	Cycles w/ donor oocytes
Total ICSI cycles	676	351	325
Oocyte age, years	32.7 ± 7.5 [18–49]	38.3 ± 4.4 [23–49]	26.5 ± 5 [18–35]
Total cycles with transfer	626	316	310
Transferred embryo average morphological score, RU	8 ± 1.3 [4–10]	7.9 ± 1.4 [4–10]	8.2 ± 1.2 [4–10]
Transferred embryos, n			
1	107 (17.1)	65 (20.6)	42 (13.5)
2	461 (73.6)	194 (61.4)	267 (86.1)
3	58 (9.3)	57 (18)	1 (0.3)
Transfer day			
D2	247 (39.5)	167 (52.8)	80 (25.8)
D3	379 (60.5)	149 (47.2)	230 (74.2)
Biochemical pregnancy, %	305/625 (48.8)	123/316 (38.9)	182/309 (58.9)
Clinical pregnancy, %	240/619 (38.8)	103/313 (32.9)	137/306 (44.8)
Ongoing pregnancy, %	187/605 (30.9)	80/305 (26.2)	107/300 (35.7)
Live birth, %	162/586 (27.6)	67/295 (22.7)	95/291 (32.6)
Multiple pregnancy, %	52/619 (8.4)	19/313 (6.1)	33/306 (10.8)

Values are presented as mean $\pm$ standard deviation [range] or number (%) when appropriate

RU relative units

Fig. 1 Distribution of sperm telomere length (STL) among sperm donors by real-time quantitative PCR (qPCR). To obtain the relative STL (relative units, RU), the T/S ratio for each sperm sample ($n = 60$) was normalized against the T/S ratio from a common gDNA sample for the whole study (HeLa gDNA). Data is presented as mean values (black columns) and SD (error bars) of triplicate measurements. T , amplification value for the telomeric region; S , amplification value for a single-copy gene (*36B4*)

According to this system, a viable embryo has a value between 4 and 10, while 0 was assigned to arrested embryos.

Embryo morphology, fertilization, and abnormal fertilization rate, as well as the probability of pregnancy and live birth across STL, were further evaluated by means of locally weighted scatterplot smoothing (LOWESS) regression, with a fit to 50% of the points and an Epanechnikov weight function (Kernel) (data near the current point receive higher weights than extreme data received). The advantage of this regression is that it does not require the specification of a function to fit a model to all of the data in the sample, thus fitting to the data accurately across the whole range of time.

Multivariable statistical analysis

A logistic multilevel regression was used to investigate the effect of STL on the reproductive outcomes (biochemical, clinical, and ongoing pregnancy and live birth). To investigate the effect of sperm telomere length on the embryo morphology score, linear multilevel regression was performed, while general linear modeling (logit link function and robust estimation of the standard errors) was employed to investigate the effect of sperm telomere length on fertilization rates.

Multilevel analysis allows addressing hierarchical data structures where each donor has several recipients, by decomposing the variance into two levels: cycles (level 1) nested within donors (level 2). We included sperm concentration (million/mL) and sperm motility (%AB) as level-2 covariates. We included oocyte origin (heterologous vs. autologous), oocyte age (years), female patient BMI (kg/m^2), number of injected oocytes (MII), average morphological score of the transferred embryos, transfer day (2 vs. 3), and number of transferred embryos (2–3 vs. 1) as level-1 covariates. Analyses were performed using SPSS version 22.0 and MLwiN 2.31 and Stata 13.0/SE (Stata Corp. LT). A p value < 0.05 was set as statistically significant.

Results

A database including a total of 676 ICSI cycles grouped with 60 different donors was generated. Baseline clinical characteristics of the population are shown in Table 1, while individualized data for each donor (STL, number of ICSI cycles performed, and reproductive outcomes) is indicated in Supp. Table 3. A total of 676 ICSI cycles were performed using

Table 3 Sperm telomere length (STL) by occurrence of pregnancy (biochemical, clinical, and ongoing) and live birth. In this univariate statistical analysis, the effects of maternal age, oocyte origin, and other variables were not included as confounding factors

	Negative	Positive	p^*	Mean difference	95% CI	
					Lower	Upper
Biochemical	4.43 ± 1.8	4.27 ± 1.54	0.23	0.16	-0.10	0.43
Clinical	4.35 ± 1.7	4.33 ± 1.6	0.86	0.02	-0.24	0.3
Ongoing	4.35 ± 1.7	4.3 ± 1.6	0.75	0.05	-0.24	0.34
Live birth	4.34 ± 1.7	4.11 ± 1.26	0.08	0.23	-0.027	0.48

Values are presented as mean $\pm$ standard deviation. STL is expressed in relative units (RU)

CI confidence interval

*Mann-Whitney U test

Fig. 2 Forest plot of sperm telomere length (STL) association with reproductive outcomes. A forest plot for STL and reproductive outcomes is shown using rates for pregnancy (biochemical, clinical, and ongoing) and live birth. Adjusted odds ratios (95% CIs) for reproductive outcomes by each relative unit increase in STL are denoted by black diamonds (black lines)

either donor oocytes ($n = 325$) or the patient's own oocytes ($n = 351$). On D2 (39.5%) or D3 (60.5%) of development, 1 (17.1%), 2 (73.6%), or 3 (9.3%) embryos were transferred.

Overall outcomes were 48.8, 38.8, and 30.9% for biochemical, clinical, and ongoing pregnancy rates respectively, while live birth rate was 27.6% (Table 2). As expected, we found some significant differences when comparing those cycles using donor oocytes with cycles using the patient's own oocytes, for example, in a woman's age or number of MII oocytes (Supp. Table 1).

Mean STL was 4.50 RU, with considerable individual variation (SD = 1.9; range 2.4–14.2; Fig. 1). No relevant correlations were found between STL and sperm motility (rho-Spearman coefficient 0.13; $p = 0.34$) and a man's age (rho-Spearman coefficient -0.007 ; $p = 0.96$), while significant correlation was found between STL and sperm concentration (rho-Spearman coefficient 0.28; $p = 0.03$) (Supplementary Fig. 1).

The Mann-Whitney U test revealed that cycles ending in pregnancy and live birth did not differ in STL with those which did not ($p > 0.05$; Table 3). Additionally, no significant effect of STL was found on reproductive outcomes, with ORs for each unit increase in STL of 0.94 (95% CI 0.86–1.04), 0.99 (95% CI 0.9–1.09), 0.98 (95% CI 0.89–1.09), and 0.93 (95% CI 0.8–1.06) for biochemical, clinical, and ongoing pregnancy and livebirth, respectively (Fig. 2).

There was no significant correlation between STL and the average score of embryo morphology (rho-Spearman coefficient -0.07 ; $p = 0.08$), fertilization rate (rho-Spearman coefficient -0.04 ; $p = 0.35$), and abnormal fertilization rate (rho-Spearman coefficient 0.02; $p = 0.65$).

On the other hand, LOWESS regression did not suggest correlation (linear or non-linear) between STL and embryo morphology, fertilization rate, pregnancy rates (biochemical, clinical, and ongoing), and live birth rate (Supplementary Fig. 2).

A logistic multilevel regression analysis showed that the effect of STL on reproductive outcomes analyzed remained

non-significant after adjustment for potential confounders and addressing the hierarchical data structure (different cycles within each donor), with p values of 0.411, 0.986, 0.769, and 0.595 for biochemical, clinical, and ongoing pregnancy rates and LB rate, respectively (Table 4). General linear modeling showed no significant effect of STL on fertilization rate ($p = 0.528$) and abnormal fertilization rate ($p = 0.575$) (Table 5). Multilevel analysis showed a statistically significant effect of STL on embryo morphological score ($p = 0.003$, regression coefficient -0.246 ; Table 5), although this does not seem to be clinically relevant.

Discussion

We present one of the largest studies on sperm donor STL to date, and the only one addressing STL independently from the female factor and other variables associated with ICSI outcomes. Although the relative STL mean value (4.5 RU) obtained in this study was different from the values found in other similar studies, the variation in relative STL found in the samples analyzed is comparable [17, 18]. Differences in relative STL mean value between studies could be explained by different PCR reagents and conditions (e.g., Taq polymerase) and in the reference DNA used (HeLa gDNA in our case). In order to validate our results, we have performed southern blot, showing that HeLa cells present telomere lengths shorter than those of sperm samples (data not shown), suggesting that telomere length measurements by qPCR should be independently validated on each lab. In addition, it is important to note that a whole sperm sample might contain cells other than sperm. In sperm donor samples, which follow WHO guidelines, this amount represents no more than 1% which could be considered negligible when qPCR is used (2 somatic telomere copies per 98 sperm telomere copies).

Although our objective was to study STL effect on reproductive outcomes in donors, some information about other

Table 4 Logistic multilevel regression analysis of the association between STL and reproductive outcomes (first level: female patient; second level: sperm donor). STL effect on pregnancy (biochemical, clinical, and ongoing) and live birth is adjusted by sperm parameters (concentration and motility), female characteristics (oocyte age, oocyte origin, and female BMI), and ICSI cycle variables (number of MII, transferred embryo average morphological score, transfer day, number of transferred embryos). Oocyte origin means that the oocytes may come from either a donor or the patient. Oocyte age is defined by the age of the patient or the oocyte donor when oocytes are retrieved after hormonal stimulation. The number of MII represents the number of injected oocytes for each cycle

		<i>p</i>	OR	Upper 95% CI	Lower 95% CI
Biochemical pregnancy	Sperm telomere length (RU)	0.411	0.95	0.85	1.068
	Sperm concentration (million/mL)	0.023	0.99	0.99	0.999
	Sperm motility (%)	0.321	0.99	0.97	1.009
	Oocyte age (years)	0.022	0.95	0.91	0.991
	Female patient BMI (kg/m ²)	0.456	0.99	0.95	1.024
	Oocyte origin	< 0.001	2.38	1.56	3.620
	Number of MII	0.276	1.04	0.97	1.130
	Transferred embryo average morphological score (AU)	< 0.001	1.37	1.18	1.601
	Transfer day (2+ vs. 3+)	0.038	1.57	1.04	2.379
	Number of transferred embryos (2–3 vs. 1)	0.173	1.44	0.86	2.407
Clinical pregnancy	Sperm telomere length (RU)	0.986	1.00	0.90	1.12
	Sperm concentration (million/mL)	0.023	0.99	0.99	1.00
	Sperm motility (%)	0.508	0.99	0.98	1.01
	Oocyte age (years)	0.025	0.95	0.91	0.99
	Female patient BMI (kg/m ²)	0.395	0.98	0.94	1.02
	Oocyte origin	0.018	1.70	1.11	2.61
	Number of MII	0.053	1.08	1.00	1.17
	Transferred embryo average morphological score (AU)	< 0.001	1.35	1.15	1.58
	Transfer day (2+ vs. 3+)	0.140	1.39	0.90	2.12
	Number of transferred embryos (2–3 vs. 1)	0.033	1.87	1.07	3.30
Ongoing pregnancy	Sperm telomere length (RU)	0.769	0.98	0.87	0.87
	Sperm concentration (million/mL)	0.085	0.99	0.99	0.99
	Sperm motility (%)	0.372	0.99	0.97	0.97
	Oocyte age (years)	0.005	0.93	0.89	0.89
	Female patient BMI (kg/m ²)	0.786	0.99	0.95	0.95
	Oocyte origin	0.012	1.83	1.16	1.16
	Number of MII	0.106	1.07	0.99	0.99
	Transferred embryo average morphological score (AU)	0.002	1.34	1.13	1.13
	Transfer day (2+ vs. 3+)	0.087	1.50	0.95	0.95
	Number of transferred embryos (2–3 vs. 1)	0.216	1.47	0.81	0.81
Live birth	Sperm telomere length (RU)	0.321	0.99	0.97	1.01
	Sperm concentration (million/mL)	0.050	1.00	1.00	1.00
	Sperm motility (%)	0.619	1.00	1.00	1.00
	Oocyte age (years)	0.012	0.99	0.98	1.00
	Female patient BMI (kg/m ²)	0.803	1.00	0.99	1.01
	Oocyte origin	0.035	1.10	1.01	1.19
	Number of MII	0.066	1.02	1.00	1.03
	Transferred embryo average morphological score (AU)	0.006	1.04	1.01	1.08
	Transfer day (2+ vs. 3+)	0.130	1.07	0.98	1.16
	Number of transferred embryos (2–3 vs. 1)	0.372	1.05	0.95	1.15

RU relative units, *AU* arbitrary units, *BMI* body mass index, *OR* odds ratio, *MI* metaphase II oocyte, *CI* confidence interval

Table 5 Multilevel regression analysis of the association between STL and the embryo morphological score, fertilization, and the abnormal fertilization rate (first level: female patient; second level: sperm donor). The STL effect on the embryo morphological score, fertilization rate, and abnormal fertilization rate is adjusted by sperm parameters (concentration and motility) and female characteristics (oocyte age, oocyte origin, and female BMI) and number of MII. Oocyte origin means that the oocytes may come from either a donor or the patient. Oocyte age is defined by the age of the patient or the oocyte donor when oocytes are retrieved after hormonal stimulation. The number of MII is the number of injected oocytes for each cycle

		Coefficient	<i>p</i>	Upper 95% CI	Lower 95% CI
Embryo morphological score	Sperm telomere length (RU)	−0.246	0.003	0.671	0.911
	Sperm concentration (million/mL)	0.009	0.077	0.999	1.019
	Sperm motility (%)	−0.014	0.248	0.963	1.010
	Oocyte age (years)	0.078	0.068	0.996	1.174
	Female patient BMI (kg/m ²)	−0.034	0.292	0.908	1.029
	Oocyte origin	−0.16	0.647	0.431	1.686
Fertilization rate	Number of MII	0.237	<0.001	1.127	1.426
	Sperm telomere length (RU)	0.176	0.528	−0.370	0.722
	Sperm concentration (million/mL)	−0.011	0.131	−0.026	0.003
	Sperm motility (%)	0.013	0.648	−0.044	0.070
	Oocyte age (years)	−0.086	0.324	−0.258	0.085
	Female patient BMI (kg/m ²)	0.034	0.597	−0.091	0.158
Abnormal fertilization rate	Oocyte origin	−0.674	0.556	−2.920	1.572
	Number of MII	1.107	<0.001	0.622	1.591
	Sperm telomere length (RU)	0.045	0.575	−0.113	0.203
	Sperm concentration (million/mL)	0.003	0.613	−0.008	0.014
	Sperm motility (%)	−0.010	0.391	−0.031	0.012
	Oocyte age (years)	0.009	0.794	−0.060	0.079
	Female patient BMI (kg/m ²)	−0.033	0.260	−0.092	0.025
	Oocyte origin	0.074	0.814	−0.545	0.694
	Number of MII	0.363	<0.001	0.221	0.505

RU relative units, *BMI* body mass index, *MI* metaphase II oocyte, *CI* confidence interval

variables was also assessed. We found a slight but significant positive association between sperm concentration and STL, as in previous studies [8]. An explanation for the absence of significant correlation between STL and a man's age could be found in the small range of age of our population (18–34 years old).

Given the role of telomeres in chromosomal orientation, synapsis, and segregation, STL could affect fertilization and the first mitotic division. Sperm telomeres are the first region in the sperm genome to respond to oocyte signals for pronucleus formation [14], and oocytes fertilized with sperm from telomerase-null (*TR*^{−/−}) mice exhibit high rates of abnormally fertilized oocytes, with increasing percentages of oocytes with one pronucleus [3]. However, we did not find an association between STL and fertilization rates and the percentage of zygotes with one or three pronuclei. Our samples came from sperm donors with high fertilization rates (around 70% on average), and further research using samples from patients with recurrent abnormal fertilization could help understand if there is a role for STL in fertilization.

It has been proposed that sperm with short telomeres may not respond to oocyte signals to form a pronucleus, leading to impaired cleavage, poor-morphology embryos, and implantation failure [3]. In fact, STL has been reported as the factor that determines the telomere length of early embryos before telomerase is expressed [23], and short telomeres in the human embryo itself have been associated with higher fragmentation and worse morphology [24]. However, our results from a database of 2993 embryos at D2–3 of development did not support a clinically relevant effect of STL on embryo morphology.

Few studies have been performed addressing the effect of STL on IVF outcomes up to live birth. In 2013, Turner and Hartshorne did not find differences in the average STL between cycles ending in pregnancy and those that did not in 50 patients [9], while recently, Cariati et al. proposed that abnormal levels of STL may alter pregnancy rates in normozoospermic patients [18]. However, in both studies, the samples included were used to fertilize oocytes from the partner, making it impossible to isolate the effect of STL from both

female and cycle variables. Another limitation of the described reports is that confounding variables such as a woman's age, BMI, number of MII collected, or embryo morphology were not included in the analysis. After adjusting for these potential confounders, we did not find any effect of STL.

Telomeres lengthen during preimplantation development in both mice and humans [3, 9, 23]; perhaps, telomere lengthening in the embryo might compensate for the effect of short STL, and reduce its effect on IVF outcomes. It is also possible that systemic telomere length rather than STL has some role in pregnancy rates, as couples experiencing idiopathic recurrent pregnancy seem to have shorter leukocyte telomeres [14]. Moreover, telomere length in oocytes could also play a role in predicting IVF outcomes [25].

Telomere length measuring in other tissues could be used as a marker to be applied in assisted reproduction. Pathologies like endometriosis and reproductive cancers have been linked to telomere length abnormalities [4], and telomere length in cumulus cells seems to be associated with oocyte and embryo quality [26]. Recently, Xu et al. found that patients with primary ovarian insufficiency had shorter telomeres in granulosa cells, although they did not find an association with the quality of the generated embryos [27].

We recognize some limitations of our study: Although, following Spanish law and according to the World Health Organization (WHO), the semen sample from sperm donors must present an outstanding quality (i.e., normozoospermic), sperm motility is reduced after thawing. As it was performed in fertile donor men, associations between STL and reproductive outcomes cannot be discarded in other groups of patients. Only the first fresh embryo transfer was studied; thus, no information is available on cumulative pregnancy rates. Although the use of sperm donor samples enabled us to include a high number of ICSI cycles and analyze STL independently (up to 12 women per donor), STL values were determined from a single sample per donor, making it impossible to control possible variations between ejaculates.

In conclusion, although STL might be altered in some groups of patients with clear clinically defined infertility, caution should be exerted when connecting STL results to laboratory or clinical outcomes after ICSI treatment.

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Compliance with ethical standards

Ethical approval Permission to conduct this study was obtained from the local Ethical Committee for Clinical Research. All procedures

performed were in accordance with the ethical standards of the institutional research committees and with the 1964 Helsinki declaration, as revised in 2013.

Conflict of interest The authors declare that they have no conflict of interest.

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