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Associations of sperm telomere length with semen parameters, clinical outcomes and lifestyle factors in human normozoospermic samples

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Abstract

Background: Many studies have demonstrated that lifestyle factors can affect sperm quality and fertility. Sperm telomere length (STL) has been reported as potential biomarker or sperm quality. However, no studies have investigated how lifestyle factors can affect STL and associated clinical outcomes.

Objectives: The purpose of this manuscript is to investigate any association between STL with lifestyle factors, semen parameters and clinical outcomes.

Materials and methods: Sperm telomere length was measured using real-time PCR in normozoospermic male partners ($n = 66$) of couples undergoing ART treatment. Each participant also completed a detailed questionnaire about general lifestyle. Linear regression univariate analysis and ANCOVA were performed to respectively determine correlations between STL and study parameters or identify statistically significant differences in STL while controlling for age, BMI and other factors.

Results: Using a linear regression model, STL is positively correlated with in vitro fertilization success ($n = 65$, $r = 0.37$, $P = .004$) but not with embryo cleavage rates and post-implantation clinical outcomes including gestational age-adjusted birth weight. No associations were observed between STL and sperm count, concentration or progressive motility. We further found that STL did not associate age, BMI, health or lifestyle factors.

Discussion: In somatic cells, the rate of telomere shortening is influenced by a number of lifestyle factors such as smoking, diet and occupation. However, little is known about how lifestyle factors affect STL and subsequently reproductive outcome. Our data suggest that STL might have an important role mechanistically for fertilization rate regardless of sperm parameters and lifestyle factors.

Conclusion: The results of this study demonstrate that STL is associated with in vitro fertilization rates, but not with semen parameters nor lifestyle factors. Further

investigations are warranted to identify the potential variation of STL overtime to clarify its significance as a potential biomarker in ART.

KEYWORDS

fertilization, lifestyle factors, male infertility, semen parameters, sperm telomere length

1 | INTRODUCTION

Telomeres are complex ribonucleoprotein structures with repetitive DNA sequences (5'-TTAGG-3') that function as chromosomal stabilizing elements.^{1,2} Repetitive cell divisions lead to telomere attrition.³ Once telomeres reach a critical short length, genome stability and cell division are affected, which can result in an increase in DNA damage and cell apoptosis.⁴ Furthermore, the rate of telomere shortening can be accelerated by the impact of lifestyle factors, environmental agents and increased activity of reactive oxygen species (ROS).^{5,6,7,8}

In male germ cell development, telomere length increases from spermatogonia to spermatozoa and is inversely correlated with the expression of telomerase activity.^{9,10} In human sperm cells, sperm telomere length (STL) increases with age and positively correlates with male parental age at conception.^{11,12} In a young student population, Ferlin et al first demonstrated a shorter STL in men having a low sperm count (threshold = 39 million), following WHO guidelines¹³ compared with normozoospermic subjects and a significant and positive correlation between STL and sperm count.¹⁴ In another study from the same research group, a positive correlation between STL and sperm count in a normozoospermic selection of the population was demonstrated.¹⁵ Additionally, men with idiopathic infertility or having varicocele have lower sperm counts and shorter STLs compared with fertile men who recently fathered a child.^{16,17} Furthermore, Lafuente et al demonstrated that STL in infertile men was negatively correlated with sperm concentration and progressive motility.¹⁸ An increasing number of studies have found that STL may play a role in reproduction as a molecular marker of sperm quality.

Investigating the link between STL and fertility outcomes, Yang et al showed STL was positively associated with the quality of embryos generated in clinical assisted reproductive technologies (ART). Moreover, there was no significant association between STL and the fertilization rate.¹⁹ Additionally, the reduction in STL was associated with an increased DNA fragmentation index.¹⁷ DNA-damaged spermatozoa give rise to reduced embryo quality and development, but fertilization is not impaired, and pregnancy outcomes are not strongly affected.²⁰ In another study, the occurrence of an ongoing pregnancy after in vitro fertilization (IVF) treatment was null in a limited number of patients with abnormal relative STL.²¹ Therefore, STL measurement is suggested to predict clinical outcomes.²²

Several studies have demonstrated an impact of lifestyle factors on telomere lengths, mainly in leucocytes (for reviews, see.²³ Lifestyle factors have also been stated to potentially affect sperm quality and fertility.²⁴ However, no studies have investigated the

association of STL and lifestyle with sperm quality and ART outcomes. The aim of this study was to investigate any association between human STL with normozoospermic sperm parameters and clinical ART outcomes, and its potential link to participants' lifestyles.

2 | MATERIALS AND METHODS

2.1 | Study subjects and sample preparation

Between 2013 and 2015, male partners of couples undergoing ART treatment at St Mary's Hospital Manchester were recruited for research with fully informed consent in writing with Local Ethics Committee Approval (Central Manchester REC ERP/91/078) and Human Fertility and Embryology Authority research licence R0026. Ninety-four subjects' sample and data were randomly allocated for this study (including 3 that had to be discarded due to confounding anomalies or failure of oocyte culture).

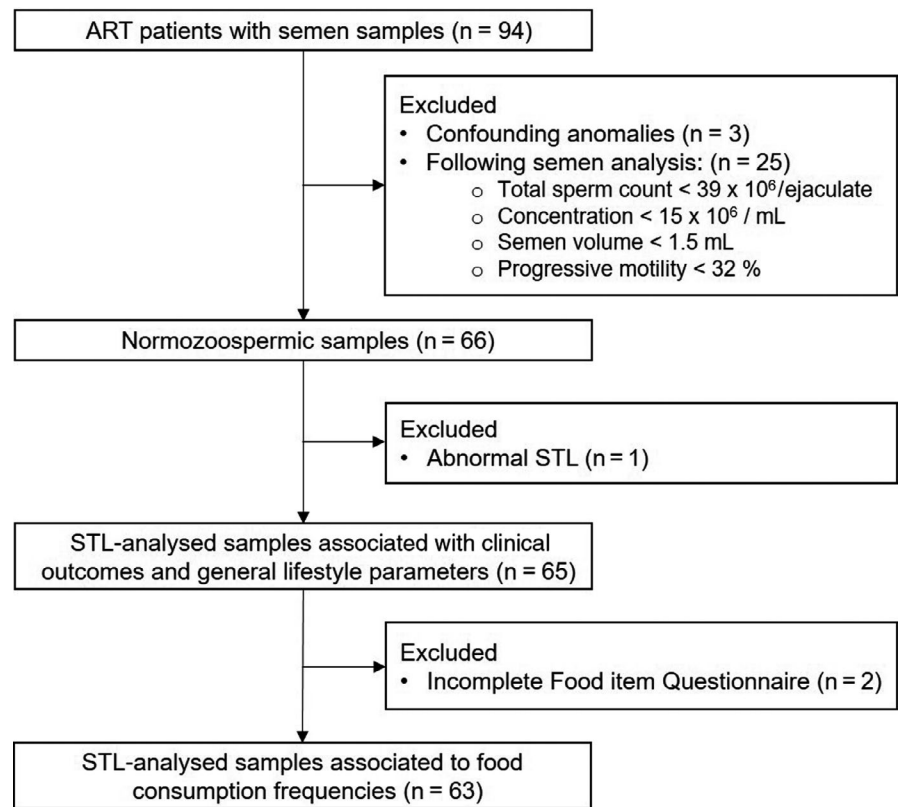
Semen samples were produced by masturbation after 2-5 days of abstinence. Semen analysis was carried out according to World Health Organization¹³ guidelines. Briefly, following semen liquefaction after 30-60 min, semen volume was calculated by subtracting the weight of a pre-weighted container from the weight of the container with the semen. Sperm concentration was measured using a haemocytometer, and total count was calculated by multiplying the sperm concentration by the volume of the whole ejaculate. Sperm motility was assessed by measuring the percentages of progressive, non-progressive and immotile spermatozoa, according to the WHO guidelines.

For this study, only subjects with a normozoospermic semen sample (total sperm count $\geq 39 \times 10^6$ /ejaculate, concentration $\geq 15 \times 10^6$ /mL, progressive motility $\geq 32\%$, semen volume ≥ 1.5 mL, 66 participants) were included, leading to a total study population of 66 male patients for STL analysis Figure 1. After collecting the remaining of the sample from the hospital, the neat semen was centrifuged and washed to separate the spermatozoa from the seminal plasma. Sample sperm concentration was measured (see above), and the sample was pelleted and frozen at -80°C until sperm DNA extraction.

2.2 | Sperm DNA extraction

DNA was extracted from up to 25×10^6 spermatozoa using the Qiagen Blood and Tissue Midi Kit (Qiagen, UK). Sperm pellets were

FIGURE 1 Repartition of samples. Flow chart showing exclusion criteria for repartition of samples. ART: assisted reproductive technologies, STL: sperm telomere length; WHO: World Health Organization



incubated at 4°C overnight in the lysis buffer supplemented with RNase (10 mg/mL, Sigma, UK) and Proteinase K (1 mg/mL, Sigma, UK). To complete sperm lysis, a dithiothreitol solution (5 mM, Sigma, UK) was added and first incubated at 4°C for 1 h on a rotator and second placed at 37°C for 1 hour. Following the manufacturer's instructions, the samples were added into gravity-flow columns. Eluted from the columns, DNA was isopropanol- and ethanol-precipitated and rapidly air-dried to remove alcohol residual. DNA was quantified in TE buffer using a Synergene HT nucleic acid plate (BioTek, UK).

2.3 | Sperm telomere length measurement

The STL of the DNA samples was analysed using real-time PCR, as previously described,²⁵ with some adjustments. Amplification of the telomeres was performed using the primers Tel O Forward (5'-CGGTTTGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and Tel O Reverse (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'), and the two control genes were amplified using Col6a1 Forward (5'-TCACTCCTCTCCCTGTGGTTAT-3'), Col6a1 Reverse (5'-GGTTGACCTCGGTGGATGTG-3'), Col3a1 Forward (5'-ATGCACGTCTACATTAAGGAATC-3') and Col3a1 Reverse (5'-TGCCCTCAGTGTCAGTATG-3') using iTaq™ Universal SYBR® Green SuperMix (Bio-Rad). All of the samples were run on 96-well plates using a 7500 Real-Time PCR System (Applied Biosystems, USA). Amplification of the telomeric repeat region (T) was expressed relative to the amplification of the two control genes Col3a1 and Col6a1, single-copy genes (S) located respectively on chromosome

2 and chromosome 21. The telomere-to-single copy (T/S) ratio is proportional to the average telomere length of the sample as the amplification is proportional to the number of primer-binding sites in the first cycle of the PCR. Standard curves were used to ensure no great experimental variation between plates. Each sample's STL was calculated using a formula: $\Delta Ct_{\text{sample}} = ((Ct_T - Ct_{\text{Col3a1}}) + (Ct_T - Ct_{\text{Col6a1}})) / 2$. A significant positive correlation was observed between ΔCt values of Telomere-Col3a1 and Telomere-Col6a1 ($r = 0.491$, $P < .0001$, $n = 66$). Relative STLs were obtained by normalizing the values against the mean of all STL measures. The coefficient of variation of samples' STL was 8.3%. One STL measure was determined to be an outlier (more than 2 standard deviation variable from STL mean) and excluded from the data set Figure 1.

2.4 | ART procedures

Ovulation induction was achieved using conventional down-regulation involving pituitary desensitization. Recombinant follicle-stimulating hormone (FSH) was administered by a step-down protocol. When three or more follicles reached ≥ 17 mm diameter, beta-human chorionic gonadotrophin (β -hCG) hormone was administered and oocytes were recovered 36 hour later by ultrasound-guided retrieval. In in vitro fertilization (IVF) treatment, metaphase II (MII) oocytes were inseminated with $\sim 1 \times 10^5$ prepared spermatozoa/ml in IVF culture medium (G-IVF, Vitrolife) at 37°C , 6% CO_2 . In intracytoplasmic sperm injection (ICSI) treatment, a single motile spermatozoon with apparent normal morphology

was microinjected into each MII oocyte. Oocytes were examined 16–18 h after insemination for the presence of two pronuclei, which is indicative of successful fertilization. Thus, the fertilized oocytes were in vitro-cultured and their development was monitored. After 3 or 5 days, one or two good quality graded embryos were transferred into the uterus. After a fresh ART treatment, any spare embryos could be stored for future use, depending on their quality. Embryo implantation was assessed 12–14 days after fertilization by measuring the level of β -hCG hormone in the woman blood. Clinical pregnancy was confirmed 5–7 weeks after embryo transfer by the presence of gestational sac (foetal heart) detected by ultrasound scan. Biochemical pregnancy (early miscarriage) and late miscarriages were also recorded and were respectively identified as the absence of a foetal heart after a positive β -hCG and a pregnancy loss occurring after the detection of foetal heart. Births were reported including gestational age at delivery, birthweight and gestational age-adjusted birthweight.

2.5 | Clinical ART outcome analysis

Clinical ART data were extracted from the current integrated data management system (AcuBase) at St Mary's Hospital, Manchester. Each clinical ART outcome was analysed combining data from both IVF and ICSI treatments. Fertilization rates were calculated using the number of embryos at the pronuclei stage divided by the total number of either microinjected or inseminated oocytes. Embryo cleavage rates were calculated as the number of cleaved embryos 2–3 days after fertilization divided by the total number of fertilized oocytes. Ectopic pregnancy was counted as a successful pregnancy. To analyse the gestational age at birth and birthweights, these outcomes were referred to live birth events and only birthweights from singleton pregnancies were analysed due to the major biological characteristics of twin pregnancies. Gestational age-adjusted birthweights were calculated for using the Gestation-Related Optimal Weight (GROW) formula as described in Castillo et al.²⁶

2.6 | Lifestyle and diet questionnaire analysis

Participants completed a detailed questionnaire on general lifestyle exposures in the last 24 hours and over the past 3 months prior to ART treatment. The questionnaire was always completed on the day eggs were recovered from the female partner, with the sperm samples produced on the same day and added to the eggs for fertilization that day, and the remainder donated to research. The questionnaire composed of 23 questions and 1 food frequency table to determine the participants' parameters: biometrics (height and weight), ethnicity, education, general health (flu and work illnesses), lifestyle factors (physical activity, caffeine/ alcohol consumption and smoking history) and dietary factors (patterns and food consumption frequencies). In relation to STL, all lifestyle factors, except education, were analysed using a binary categorization such as 'yes/no' or applying a selected threshold.

All factors containing continuous numerical data were also analysed to their respective STL values using direct correlation. In this cohort, 64 males out of 65 were White Europeans. To examine biometric factors, thresholds for age and BMI were respectively applied at 35 years (starting age for male fertility decline²⁷; and 25 kg/m² (to separate underweight/normal and overweight/obese). Weekly caffeine intake and alcohol consumption were calculated and reported respectively in mg of caffeine and alcohol units of alcohol following guidelines.^{28,29} Physical activity was analysed using both a binary score linked to the number of exercise hours and the Godin-Shephard Leisure Score Index (LSI) threshold defined at 24.³⁰ The unit had a strict policy to not treat smokers, hence, no participants identified themselves as being a current smoker. Regarding the food frequency analysis (units per week), all single food items were grouped into food groups such as red meat, poultry and cruciferous vegetables. Food supercategories were created from food groups following similar biological classification (ie vegetables include cruciferous, leafy green and other vegetables, presented with a '1' exponent in Table 6. Two patients had minor missing information about consumption frequencies in the questionnaire and were excluded from the STL and food frequency analysis Figure 1' which was carried out on 63 males of the cohort in Table 6.

2.7 | Statistical analysis

Data are represented as mean $\pm$ the standard deviation (SD) or median with the interquartile range (IQR) using GraphPad Prism (La Jolla, CA) and IBM SPSS (Chicago, IL) software. Data normality was assessed using the Shapiro-Wilk normality test. Depending on normality results, either parametric Welch's t test or non-parametric Wilcoxon-Mann-Whitney tests were performed to determine differences between two study categories for biometric, health and education/job data (expect for education level, Bonferroni's one-way ANOVA; Table 5. ANCOVA tests were performed to test differences while justifying for male age and BMI between study categories for ART outcomes Table 4 and lifestyle/dietary factors Table 5. Linear regression analyses were performed using a general linear model univariate analysis to determine correlations between STL and study parameters while adjusting for age, BMI and sporadically any other appropriate factors (sexual abstinence for sperm parameters in Table 3 and number of oocytes or embryos for ART outcomes in Table 4. *P*-values < .05 were considered statistically significant. *P*-values < .08 were reported as non-significant trends.

3 | RESULTS

3.1 | General semen characteristics and clinical outcomes

Male biometrics and basic semen characteristics are shown in Table 1 and are reported as the mean $\pm$ standard deviation (SD) or as the median (interquartile range, IQR) and minimal-maximal values. All normozoospermic semen parameters are based on the WHO

TABLE 1 Clinical characteristics of the participants

Biometrics (n = 65)		min-max
Age (years) ^{a,a}	35.53 ± 4.48	25.0-45.0
Body mass index (kg/m ²) ^{a,a}	25.08 ± 3.16	17.5-33.3
Semen parameters (n = 65)		
Total sperm count (× 10 ⁶) (≥39 × 10 ⁶) ^{b,a}	191.2 (127-329.6)	46.8-600.0
Sperm concentration (× 10 ⁶ /mL) (≥15 × 10 ⁶ /mL) ^{b,a}	63 (39-94.5)	15.0-362.0
Semen volume (mL) (≥1.5 mL) ^b	3.3 (2.2-4)	1.5-7.2
Progressive motility (%) (≥32%) ^{a,a}	55.31 ± 10.95	34.0-83.0
Grade A motility (%) ^{a,a}	35.59 ± 13.52	9.0-80.0
Immotile spermatozoa (%) ^{a,a}	35.11 ± 9.48	13.0-63.0
Sexual abstinence (day) ^{a,a}	3.43 ± 0.75	2.0-5.0

Note: Continuous data are presented as mean ± SD. The World Health Organization lower references for human semen characteristics are notified with a star.¹³ n, number of participants.

^aMedian (IQR).

^bWith minimum and maximum values (min-max).

lower reference values, with values greater than the lower reference values established by¹³ guidelines.¹³ ART clinical outcomes are presented in Table 2, showing the 65 couples achieving fertilization using either IVF (n = 48) or ICSI treatment (n = 17), with 62 of them obtaining embryo cleavage. Two couples had their embryos frozen and transferred during a later cycle. Out of 60 fresh embryo transfers, 23 pregnancies (including 1 ectopic pregnancy and 3 early miscarriages prior 6-week scan) occurred resulting in 21 live births and 1 still birth. From the 62 couples with cleaved embryos, 33 had embryos frozen for future embryo transfer. Subsequently, 25 couples had their embryos thawed and 24 underwent a single or multiple frozen embryo transfers resulting in an additional 14 pregnancies and 9 live births. In total, 23 singletons and 4 sets of twins were born from 97 cumulative embryo transfers. Only one couple achieved two live birth events from fresh and frozen embryo transfers.

3.2 | Sperm telomere length and normozoospermic semen parameters

No direct associations were observed between STL and semen parameters including total sperm count, concentration, semen volume, progressive motility, grade A motility, percentage of immotile spermatozoa and sexual abstinence in a normozoospermic population Table 3.

3.3 | Sperm telomere length and clinical ART outcomes

STL, in vitro fertilization procedures and clinical outcomes are presented in Table 4. When comparing STL in the ART procedures, no

TABLE 2 Clinical ART outcomes of the participants

Clinical outcomes	Full sample	Fresh ET	Frozen ET
Number of couples	65	65 (100%)	25 (38.5%)
Fertilization types	65 (100%)	–	–
IVF	48 (73.8%)	–	–
ICSI	17 (26.2%)	–	–
Fertilization rate (%)	69.2 ± 20.4, n = 65	–	–
Embryo cleavage	62 (95.4%)	–	–
Embryo cleavage Rate (%)	100 (62.5-100), n = 62	–	–
Number of ET	97	60 (61.9%)	37 (38.1%)
Number of pregnancies	37 (38.1%)	23 (38.3%)	14 (37.8%)
Singleton pregnancy	23 (62.2%)	16 (69.6%)	7 (50%)
Twin pregnancy	4 (10.8%)	3 (13%)	1 (7.2%)
Early miscarriage	6 (16.2%)	3 (13%)	3 (21.4%)
Late miscarriage	3 (8.1%)	0 (0%)	3 (21.4%)
Ectopic	1 (2.7%)	1 (4.4%)	0 (0%)
Births	31	22 (71%)	9 (29%)
Singleton live births	23 (74.2%)	16 (72.3%)	7 (77.8%)
Twin live births	7 (22.6%)	5 (22.7%)	2 (22.2%)
Stillbirth	1 (3.2%)	1 (5%)	0 (0%)
Gestational age (weeks)	38 (37-39), n = 23	38 (37-39), n = 16	39 (38-40), n = 7
Birthweight (kg)	3.345 (2.860-3.765), n = 23	3.331 (2.569-3.556), n = 16	3.765 (3.118-4.451), n = 7
GA-adjusted	3.695 (3.337-4.266), n = 23	3.596 (3.360-4.170), n = 16	3.695 (3.337-4.622), n = 7

Note: Continuous variables are presented as mean ± SD

Abbreviations: ET, embryo transfer; ICSI, intracytoplasmic sperm injection; n, number of events. IVF, in vitro fertilization.

^aMedian (IQR).

^bCategorical variables are presented as row total (percentage).

^cOutcomes referring to live birth singleton events are notified.

significant differences were observed between IVF and ICSI, and as such for the further analyses of STL with clinical ART outcomes, we used the combined data from both treatments. This revealed that STL positively correlated with the rate of fertilization Figure 2: $r = .32$, $P = .0097$, adjusted for BMI: $r = .36$, $P = .004$). In a limited number of cases where all embryos failed to develop, these embryos tended to have been fertilized from lower STL spermatozoa. Nevertheless, for the couples who had cleaved embryos, there were no significant associations between STL and embryo cleavage rate. In cumulating outcomes from fresh and frozen cycles, we observed a non-significant trend where STL was higher in couples who had successfully implanted

TABLE 3 Linear correlations between semen parameters and STL

Semen parameters (n = 65)	GLM univariate analysis, <i>r</i> (P-values)
Total sperm count ($\times 10^6$)	0.01 (0.970)
Sperm concentration ($\times 10^6/\text{mL}$)	0.07 (0.579)
Semen volume (mL)	-0.12 (0.371)
Progressive motility (%)	-0.07 (0.622)
Grade A motility (%)	-0.14 (0.291)
Immotile spermatozoa (%)	-0.05 (0.680)

Note: A general linear model (GLM) univariate analysis was used to identify correlations between sperm telomere length and sperm parameters while adjusting for age, body mass index and sexual abstinence duration. n, number of semen samples; *r*, correlation coefficient.

embryos compared to couples with failed implantation ($P = .051$). However, STL was not found significantly different or associated with post-implantation events including clinical pregnancy, successful live birth event and gestational age-adjusted birthweight.

3.4 | Sperm telomere length, biometrics, education and general health

Table 5 shows the correlation between STL and biometric factors. No significant difference in STL was identified between threshold-defined groups for age or BMI. The STL of the normozoospermic men demonstrated no correlation with age ($r = -.10$, $P = .4528$) and BMI ($r = .06$, $P = .6575$). Additionally, no associations were found with the degree of education or between those who had worked in the last 24 h prior to ART treatment. Approximately 12% of participants had suffered from flu and fever, but no difference in STL was

found ($P = .8180$). Participants suffering from an illness caused or made worse by work tended to have a lower STL ($P = .0575$).

3.5 | Sperm telomere length, lifestyle and dietary factors

When investigating common lifestyle factors such as smoking, drinking caffeine and exercise, which are known to influence leucocyte telomere length (for reviews, see³¹, we found no differences in STL for each lifestyle factor Table 5 and no direct associations with STL (data not shown): consumption of caffeine or alcohol (respectively $r = -.06$, $P = .6235$, and $r = -.07$, $P = .6518$), exercising ($r = -.09$, $P = .4894$, LSI: $r = .02$, $P = .1150$) and smoking (non-smokers vs ex-smokers: $P = .4117$) or being exposed to cigarette smoke ($P = .9426$).

Dietary patterns including meat consumption in the last 24 h were also investigated together with STL Table 6. All male participants had a meat-inclusive diet. The difference in STL was not significantly linked to either dietary pattern (meat only vs meat + fish) or meat consumption. Overall food consumption frequencies of the last three months prior ART treatment were analysed for food categories such as meat, dairy, fruit, vegetables and more Table 6. None of the food categories and single food items (data not shown) were significantly associated with STL. The consumption of processed meat tended to be inversely correlated with STL ($r = -.24$, $P = .068$), however, the correlation coefficient remains low.

4 | DISCUSSION

Exploring the role of STL in semen quality, male fertility and reproductive clinical outcomes is a growing area of interest in reproductive medicine. In this study, we identified a positive correlation between the STL and the fertilization rate in normozoospermic samples and a very close trend towards higher STL in successful embryo implantation rates. However, we found no association between STL and sperm parameters. Furthermore, occupational health, diet and lifestyle factors were not correlated with STL. Altogether, our data suggest that STL might have an important role mechanistically for fertilization rate regardless of sperm parameters and lifestyle factors.

Recent studies demonstrated that STL decreased in ICSI patients with previous low fertilization rate ($n = 10$)³² and positively correlated with embryo quality,¹⁹ which is in agreement with data presented in the present study. In assisted conception practice, it is accepted that implantation and pregnancy outcomes are improved by the transfer of good quality embryos compared with low-quality ones.^{33,34} However, in a recent study, Cariati and colleagues noted that no ongoing pregnancies were observed from patients having an atypical STL following IVF.²¹ In another study, it was demonstrated that spermatozoon with shorter STL was associated with lower natural pregnancy rates compared with couples who did achieve pregnancy naturally with longer STL.¹⁸ Herein, a trend was observed in which

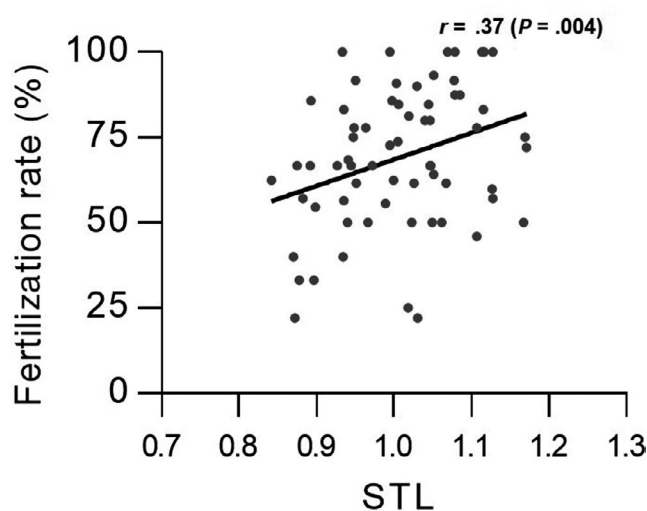
**FIGURE 2** Positive correlation between STL and fertilization rate

TABLE 4 Correlation between clinical outcomes and STL. (A) ANCOVA tests were performed to test differences in STL between groups while controlling for the number of embryos used, male age and BMI. (B) Direct correlations between STL and continuous values of ART outcomes were assessed using Pearson's correlation tests

Clinical outcomes (n = 65)		STL mean $\pm$ SD, n	ANCOVA P-values	r (P-values)	
(A)				(B)	
Procedures	IVF	1.013 $\pm$ 0.087, n = 48	0.306	–	–
	ICSI	0.991 $\pm$ 0.074, n = 17			
Fertilization	Yes	1.007 $\pm$ 0.084, n = 65	–	Fertilization (%)	0.32 (<0.010)
	No	–		(adjusted for male age, oocyte number and male BMI)	0.37 (0.004)
Embryo cleavage	Yes	1.010 $\pm$ 0.084, n = 62	0.079	Embryo cleavage (%)	0.00 (0.981)
	No	0.956 $\pm$ 0.029, n = 3		(adjusted for male age, embryo number and male BMI)	0.07 (0.611)
Cumulative pregnancy				Gestational age (weeks)	0.14 (0.514)
Embryo implantation	Yes	1.030 $\pm$ 0.012, n = 32	0.051		
	No	0.988 $\pm$ 0.017, n = 30			
Clinical pregnancy	Yes	1.026 $\pm$ 0.013, n = 29	0.188		
	No	0.995 $\pm$ 0.016, n = 33			
Birth	Yes	1.029 $\pm$ 0.013, n = 27	0.119		
	No	0.995 $\pm$ 0.016, n = 35			
Birth weight (singleton)	<2.5 kg	1.022 $\pm$ 0.023, n = 5	0.586	GA-Adjusted Birth weight (singleton, kg)	0.32 (0.137)
	$\geq$ 2.5 kg	1.038 $\pm$ 0.016, n = 18			

Note: General linear model univariate analyses for fertilization and embryo development rates were performed to identify associations with STL and adjust for male age, male body mass index and number of oocytes or embryos.

Abbreviations: STL, sperm telomere length; BMI, body mass index; GA, gestational age; n, number of events; r, correlation coefficient.

sperm samples with higher STLs tend to have an increased embryo implantation rate. However, in the present study, we observed live birth rates occurred with some of the lowest and highest STL values in our cohort. However, there was no STL association with birth-weight or gestational age. Therefore, it could be hypothesized that a higher STL may be associated with increased fertilization rates and subsequent increased embryo development/ quality, leading to improved implantation rates and pregnancy outcomes. Decreased STL was repeatedly observed in patients with oligozoospermia compared with normozoospermic samples, indicating that shorter STL may be associated with impaired spermatogenesis through segregation errors during meiosis.^{14,16,18,21}

Studies investigating the association between STL and sperm quality have shown a positive correlation with total sperm count and STL.^{14,21} In other studies using normozoospermic samples, STL was found to be positively linked to sperm progressive motility/ vitality and negatively linked to sperm DNA fragmentation¹⁵ but no association was detected with male age, sexual abstinence, total sperm count, concentration and morphology. However, in another study, there was no association noted between STL and sperm parameters.¹⁶ In the present study, we found no correlation between sperm motility, count or concentration with STL. The current assessment of sperm quality as using the WHO guidelines¹³ does not take molecular andrology pathways (such as STL and sperm DNA integrity) into account.³⁵ Therefore, the quality of spermatozoa and its fertilization

capacity may be dependent on molecular integrity in addition to the conventional sperm parameters.

In humans, lifestyle and environmental factors are known to influence telomere length in leucocytes.^{31,36,37} This telomere attrition is associated with smoking cigarettes, low physical activity and stress exposure^{38–40} though a recent large-scale study with repeated measures did not support any effect of alcohol consumption on leucocyte telomere length.⁴¹ Importantly, however, these parameters have never been investigated in relation to STL. The results presented here suggest that neither occupational nor lifestyle factors associated with STL.

Similar to the other lifestyle factors, dietary patterns and food frequencies have been associated negatively and positively to leucocyte telomere lengths. Shorter telomeres have been associated with the level of consumed total fat and saturated fatty acid⁴² whereas, consumption of seeds, nuts, legumes and coffee correlated with increase telomerases. Further recent studies support that a diet with more plant-based food items and reduced processed meat might be beneficial for increased telomeres and longer life, according to research on the Mediterranean diet.^{43,44} Interestingly, the effect of diet interventions has been shown to have only minor to no effect on telomeres.^{45,46} In our cohort, STL was not associated with any food categories (such as meats, vegetables and fruits) or specific food item consumption. Processed meat consumption had a non-significant, but mild negative

TABLE 5 Correlation between STL and biometric parameters/education/health. Continuous data are presented as mean $\pm$ SD (a) or median (IQR) (b). (A, B, C) Differences in STL between groups were statistically analysed using either t test with Welch's correction or Mann-Whitney test (expect for education level, Bonferroni's one-way ANOVA). (D) ANCOVA tests were performed to test differences in STL between groups while controlling for male age and BMI

A. Biometrics (n = 65)		STL, n	p-values	D. Lifestyle and dietary factors (n = 65)		STL, n	ANCOVA p-values
Age ^a (years)	< 35	1.016 $\pm$ 0.0795, n = 26	0.4919	Caffeine intake ^a	Yes	1.007 $\pm$ 0.08411, n = 64	-
	$\geq$ 35	1.001 $\pm$ 0.0868, n = 39			No	1.049, n = 1	
BMI ^a (kg/m ²)	< 25	1.007 $\pm$ 0.0927, n = 35	0.9384	Alcohol consumption ^a	Yes	1.002 $\pm$ 0.07566, n = 44	0.611
	$\geq$ 25	1.008 $\pm$ 0.0732, n = 30			No	1.018 $\pm$ 0.09952, n = 21	
B. Health n = 65)				Exercising ^a	Yes	1.004 $\pm$ 0.08545, n = 55	0.424
					No	1.025 $\pm$ 0.07428, n = 10	
Fever/ Flu ^a	Yes	1.013 $\pm$ 0.09121, n = 7	0.8180	Leisure Score Index ^a	Inactive (< 24)	1.004 $\pm$ 0.08525, n = 31	0.918
	No	1.006 $\pm$ 0.08234, n = 58			Active ($\geq$ 24)	1.010 $\pm$ 0.08307, n = 34	
Illness (job) ^a	Yes	0.967 $\pm$ 0.06435, n = 9	0.0575	Smoking ^a	Ex-smoker	1.016 $\pm$ 0.07442, n = 31	0.335
	No	1.022 $\pm$ 0.08140, n = 56			Non-smoker	0.999 $\pm$ 0.09155, n = 34	
Stress (job) ^b	Yes	0.9368 $\pm$ 0.07545, n = 4	0.0820	Dietary patterns ^a	Meat	1.010 $\pm$ 0.09249, n = 15	0.749
	No	1.0170 $\pm$ 0.08155, n = 61			Meat + Fish	1.006 $\pm$ 0.08177, n = 50	
C. Education and Job (n = 65)				Meat consumption (last 24 hours) ^a	Yes	1.006 $\pm$ 0.08577, n = 60	0.285
					No	1.018 $\pm$ 0.05677, n = 5	
Education level ^a	Secondary school	0.966 $\pm$ 0.09748, n = 9	0.1094				
	GCSE or equivalent	1.040 $\pm$ 0.07605, n = 20					
	A-Level	0.976 $\pm$ 0.07377, n = 14					
	Undergraduate Level	1.011 $\pm$ 0.08701, n = 13					
	Postgraduate Level	1.019 $\pm$ 0.07738, n = 9					
At work	Yes	1.006 (0.9409-1.079), n = 47	0.8820				
No	1.017 (0.9384-1.051), n = 16						

Abbreviations: n, number of participants; STL, sperm telomere length.

^aContinuous data are presented as mean $\pm$ SD

^bMedian (IQR).

TABLE 6 Correlation between lifestyle and dietary factors and STL

Dietary categories (n = 63)	GLM univariate analysis, <i>r</i> (P-values)
Meat ¹	-0.15 (0.263)
Poultry	0.12 (0.337)
Red meat	-0.07 (0.574)
Processed meat	-0.24 (0.068)
Fish	0.17 (0.202)
Dairies ¹	-0.08 (0.543)
High-fat dairy products	-0.08 (0.543)
Low-fat dairy products	-0.04 (0.790)
Fruits	-0.18 (0.175)
Vegetables ¹	-0.06 (0.628)
Cruciferous vegetables	-0.03 (0.828)
Leafy green vegetables	0.08 (0.524)
Other vegetables	-0.22 (0.085)
Nuts	0.19 (0.138)
Cereals and derived	-0.04 (0.742)
Cereals	-0.03 (0.804)
Confectionery	-0.12 (0.367)
Caffeinated no sugar beverages	-0.07 (0.570)
Caffeinated sugar beverages	-0.18 (0.159)
Vitamins	0.01 (0.956)

Note: General linear model univariate analyses were performed to identify associations between STL and frequencies of food categories while adjusting for male age and male body mass index. Supercategories are notified by a '1' in exponent; n, number of participants; *r*, correlation coefficient.

association, correlation with STL ($P = .068$). Therefore, it seems that dietary factors do not impair telomere lengths in spermatozoa to the degree in leucocytes.

Our data concur with other reports demonstrating no significant link between BMI and STL.¹⁹ This may be due to in part by the complex lifestyle and biological confounding factors.⁴⁷ In patients with high BMI ($>28 \text{ kg/m}^2$) compared with normal BMI, increased reactive oxygen species (ROS) activity in semen and sperm DNA fragmentation were found, these are factors linked to poorer sperm quality that may account for poorer IVF treatment outcomes.^{48,49} STL attrition is linked to ROS exposure in relation to environmental and lifestyle factors such as alcohol consumption and smoking.⁵⁰⁻⁵² However, Mishra and colleagues demonstrated that a mild increase in oxidative stress is beneficial for STL maintenance.⁵³

Age is a well-investigated parameter for telomere lengths, and advanced age is associated with a decrease in leucocytes⁵⁴ and increase in spermatozoa.⁵⁵ The ageing protection mechanism of telomere length in leucocytes is well defined in the literature, whereas the mechanism of STL elongation remains currently unclear. This elongation phenomenon is inherited from the father to offspring independently to the offspring sex⁵⁶ and seems to be conserved

across species.^{57,58} Interestingly, this paternal inheritance is also evident in leucocytes.⁵⁹ Older parental age at conception has been shown to have a negative effect on both offspring viability in mice⁶⁰ and the overall fitness of offspring in humans.^{61,62} As a confounding factor, ageing is linked to fertility issues, such as the decline in male hormones and sperm quality.⁶³ Therefore, an age threshold is required to investigate the effect of age on STL (such as < 30 years vs > 50 years, 0.^{27,64} Herein, the age threshold has matched the age considered the start of the male fertility decline (35 years).²⁷ In the present study, age did not show any significant correlation with STL. However, the age group of the men in this study (25 to 45 years) is within the 'middle adulthood' group (25 to 44 years).⁶⁵ Nevertheless, our study population with a small age and BMI range highly represents current male patients, as men with outlying ages and BMI, which can be found in other studies, are simply not treated.

This study sought to investigate any association with STL, lifestyle factors, sperm parameters and clinical outcome. However, we recognize some limitations of our study. We selected normozoospermic samples, which limited the number of samples available. Moreover, as the samples were only provided at a single time point—we were unable to control for variations between ejaculates or test possible causal interactions to changes in lifestyle factors, STL and sperm parameters. In addition, as sperm DNA extraction was performed in pelleted spermatozoa sampled, we appreciate possible contamination of leucocyte and round cells (RCs). However, as these samples were used for clinical treatment there was minimal contamination noted. Furthermore, a recent study reported that specific markers indicate that seminal RCs are mostly immature post-meiotic germ cells.⁶⁶ Therefore, given the low number of possible leucocyte or RC relative to sperm cell numbers in our study samples, we are confident that the STL results are representative of the spermatozoa.

This study demonstrates a positive association between STL and the fertilization rate in a normozoospermic population. However, we did not find any association between the sperm parameters and the individual biometric and lifestyle factors. The data presented in this present study set the scene for larger longitudinal studies to explore the dynamics of STL in humans in relation to lifestyle influences and to clarify its significance as a potential biomarker in ART, and the inheritance effect of a longer STL on ART offspring's fertility.

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CONFLICT OF INTEREST

None declared.

AUTHORS CONTRIBUTIONS

MC, CM, DRB and ACP designed the study. CN, BA, GP and GH acquired the samples and clinical outcomes. CN, G. P. and JS carried out the experimental work. SCB carried out the analysis. SCB and MC wrote the paper, which was edited by CM, DRB and ACP

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