

Sperm Telomere Length and Its Correlation with Semen Parameters and Chromatin Protamination Across Semen Phenotypes in Infertile Men

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Abstract

Purpose

To correlate absolute sperm telomere length with conventional semen parameters and chromatin protamination.

Methods

This interim analysis from a prospective observational study included 50 male patients who attended a tertiary care centre for semen analysis as a part of fertility assessment. Semen was evaluated based on WHO 2021 guidelines. Chromatin maturation was determined using the aniline blue staining method, and absolute telomere length (ATL in kb/genome) was quantified via real-time PCR technique. Statistical analysis, like bivariate Spearman rank correlation and multiple linear regression model was employed to evaluate the correlation. The ROC curve was generated to define the protamination cut-off to predict a normal ATL. The ATL was classified into clinical cohorts of Critical (< 3 kb), Low (3–6 kb), Healthy (6–12 kb), and Fertile (> 14 kb).

Results

Bivariate analysis revealed no significant correlation between conventional parameters and ATL ($p > 0.05$). Although protamine maturation exhibited the strongest positive trend, it failed to achieve statistical significance ($r = 0.28$, $p = 0.124$). In multiple linear regression, protamine maturation was identified as the sole significant and independent predictor of ATL ($B = 0.0516$, $p = 0.047$). The ROC confirmed that percentage of protamination can predict a normal telomere length of ≥ 16.63 kb/genome with a moderate diagnostic performance ($AUC = 0.679$), with an optimal cut-off of > 75% yielding a 62.5% sensitivity and 73.9% specificity (Youden's $J = 0.364$). Median protamine maturation increased progressively across the ATL cohorts Critical (64%), Low (69%), Healthy (70%), and Fertile (75.5%).

Conclusion

Protamine maturation is the most consistent independent predictor of sperm telomere length. Hidden molecular telomeric defects occur even in normozoospermic phenotypes, highlighting the diagnostic utility of nuclear protamination as a functional biomarker for paternal genomic integrity in ART.

Introduction

Male factor infertility accounts for nearly half of the burden of infertility^{1,2}. Standard diagnostic procedures rely primarily on conventional semen analysis, such as sperm concentration, motility, and

morphology, according to World Health Organization (WHO) guidelines³. However, these conventional parameters fail to explain the cause of idiopathic male infertility, poor embryo development, or recurrent implantation failure, as they do not reflect on the sub-microscopic structural and functional integrity of paternal chromatin^{4,5}.

During spermiogenesis, somatic histones are replaced by protamines, which in turn causes the DNA to hyper-condense and form a transcriptionally silent structure that shields the genome against reactive oxygen species⁶. Incomplete protamination leaves nuclear DNA loosely packed and more susceptible to DNA breaks⁷. Concurrently, the telomeres, which are tandem repeats of TTAGGG nucleoproteins present at the end of the chromosomes, play a vital role in genomic stability and chromosomal positioning during fertilization⁸. Guanine-rich telomeric ends are susceptible to oxidative stress, which in turn disrupts the protamine cross-link, thus inducing a dual molecular lesion⁹.

While preliminary studies suggested an interplay between telomeres and chromatin quality^{8,10}, the multivariate relationship between absolute sperm telomere length (ATL), conventional semen parameters, and protamination status across diverse clinical semen phenotypes remains poorly defined. This study evaluates the independent predictive power of protamine maturation for ATL and investigates the distribution of telomere defects across classical semen phenotypes.

MATERIALS AND METHODS

Study Cohort and Design

This prospective observational study was conducted in a tertiary care centre in southern India, where patients attended the fertility clinic for fertility evaluation. Semen samples were recruited from 50 patients over a period of 8 months; all phenotypes were included in the study except severe oligo-azoospermia and surgically retrieved sperm.

Semen Analysis and Phenotypic Classification

Semen samples collected after 2–7 days of abstinence were allowed to liquify at 37°C. Conventional semen parameters like volume (mL), concentration ($10^6/\text{mL}$), total motility (%), progressive motility (%), non-progressive motility (%), morphology (%), and vitality (%) were assessed based on WHO 2021 manual³. Samples were further categorized into standard semen phenotypes: normozoospermia, asthenoteratozoospermia, oligoteratozoospermia, and teratozoospermia.

Assessment of Sperm Protamination.

Protamine maturation was evaluated using the Aniline Blue (AB) staining method, which targets the lysine-rich residual histones. The centrifuged pellet was incubated with 4% paraformaldehyde, and a smear was made after dispersing the pellet. The smears were stained with aniline blue, and a minimum

of 200 spermatozoa were assessed under oil immersion³. They were classified as: light blue stained sperms– fully protaminated, and dark blue stained sperms – immature.

DNA Extraction and Absolute Telomere Length Assessment

DNA Extraction and Absolute Telomere Length Assessment

Following seven optimisation iterations, sperm DNA was extracted using the QIAGEN QIAamp DNA blood mini kit modified with dithiothreitol (DTT), sodium dodecyl sulfate (SDS), and Triton X-100. DNA yield and purity were quantified using a NanoDrop spectrophotometer. Quantitative real-time PCR (qPCR) was conducted based on the protocol by Cawthon et al., utilising specific primers, SYBR Green chemistry and target DNA across 30 thermal cycles¹¹.

Relative telomere length (T/S ratio) was calculated post amplification, and absolute telomere length was calculated based on this T/S ratio using the validated formula¹². Patients were stratified into four clinical telomere tiers: Critical (< 3 kb), Low (3–6 kb), Healthy (6–12 kb), and Fertile (> 14 kb)^{10,13}.

Statistical Analysis

Statistical analysis was conducted using Python and R statistical environments. Bivariate relationships were evaluated using Spearman rank correlation coefficients (r or ρ). Multiple linear regression was performed using log(ATL) as the continuous dependent variable and age, BMI, concentration, total motility, morphology, vitality, and protamine maturation as independent predictors. ROC curve analysis assessed the diagnostic performance of protamination in predicting normal telomere length (ATL $\geq$ 16.63 kb/genome). Cross-tabulation with Pearson's Chi-square (χ^2) and Cramer's V was utilised for phenotypic distribution analysis. $p < 0.05$ was considered statistically significant.

RESULTS

Baseline Demographics and Semen Characteristics

The cohort of 50 patients exhibited a mean age of 34.43 ± 3.62 years (median 34.20, IQR 30.06–38.52) and a mean BMI of 26.06 ± 3.62 kg/m² (median 28.15, IQR 24.20–30.85). Distribution of semen parameters and genomic metrics are presented in Table 1. The mean absolute telomere length was 11.61 ± 2.97 kb/genome (median 11.61, IQR 8.61–18.66).

Table 1

Demographics, conventional semen parameters, protamination status, and absolute telomere length (ATL) in the analytical cohort (n = 50).

Variable (n = 50)	Mean $\pm$ SD	Median	Interquartile Range (Q1–Q3)
Age (years)	34.433 $\pm$ 3.623	34.200	30.06–38.52
BMI (kg/m ²)	26.058 $\pm$ 3.623	28.150	24.20–30.85
Volume (mL)	3.011 $\pm$ 1.626	2.500	2.14–3.73
Concentration (10 ⁶ /mL)	34.581 $\pm$ 25.341	30.100	20.50–57.50
Total Motility (%)	61.742 $\pm$ 13.122	64.000	52.00–70.00
Progressive Motility (%)	35.097 $\pm$ 10.737	37.000	25.00–45.00
Non-progressive Motility (%)	26.645 $\pm$ 7.842	25.000	23.50–30.00
Morphology (% normal)	2.065 $\pm$ 1.237	2.000	1.00–2.00
Vitality (%)	76.613 $\pm$ 15.405	80.000	65.00–85.00
Protamination (%)	66.968 $\pm$ 11.610	70.000	66.00–76.50
Absolute Telomere Length (kb/genome)	11.610 $\pm$ 2.97	11.610	8.61–18.66

Bivariate and Multiple Regression Analysis

Among all evaluated parameters, none demonstrated a statistically significant bivariate correlation with log(ATL) at $p < 0.05$. However, protamine maturation exhibited the strongest positive trend ($r = 0.28$, $p = 0.124$). Figure 1.

To control the confounding factors, a multiregression model was performed. As shown in Table 2, protamine maturation was identified as the sole statistically significant independent predictor of log(ATL) (Coefficient B = 0.0516, Standard Error = 0.0147, $p = 0.047$). Conventional parameters—including sperm concentration ($p = 0.3331$), total motility ($p = 0.6710$), morphology ($p = 0.2220$), and vitality ($p = 0.9811$)—failed to predict ATL independently.

*Protamine maturation had the strongest positive trend ($r = 0.28$, $p = 0.124$)

Table 2
Multiple linear regression analysis predicting log(ATL) from clinical, semen, and chromatin variables

Predictor Variable	Coefficient (B)	Std Error	t-value	p-value
Intercept	0.5260	3.1399	0.167	0.8680
Concentration	0.0156	0.0158	0.990	0.3331
Total Motility	0.0067	0.0158	0.426	0.6710
Morphology	0.3227	0.2588	1.247	0.2220
Vitality	-0.0034	0.1403	-0.024	0.9811
Protamine Maturation	0.0516	0.0147	3.510	0.0470*

*Protamine maturation was the only parameter that could significantly and independently predict ATL with a p-value of <0.05

Protamination Gradient across Telomere Tiers and Diagnostic ROC Performance

Evaluation of the median across stratified ATL tiers demonstrated a clear positive gradient – Critical (< 3 kb) = 64%, Low (3–6 kb) = 69%, Healthy (6–12 kb) = 70%, and Fertile (> 14 kb) = 75.5%. ROC analysis confirmed that protamination predicts normal telomere length (≥ 16.63 kb/genome) with an AUC of 0.679. An optimal cut-off of 75% protamination yielded a sensitivity of 62.5% and specificity of 73.9% (Youden's J = 0.364)Fig. 2

*An optimal cut-off of 75% protamination yielded a sensitivity of 62.5% and specificity of 73.9% (Youden's J = 0.364)

Distribution of Telomere Length Categories Across Semen Phenotypes

Analysis of ATL categories across clinical semen phenotypes showed no statistically significant overall association ($\chi^2 = 11.55$, $p = 0.240$, Cramer's V = 0.358, $n = 50$). Table 3 details the percentage distribution, with nearly 10% of normozoospermic men harbouring critically short telomeres (< 3 kb), while 26.3% of teratozoospermic men were in the critical tier.

Table 3

Distribution of absolute sperm telomere length (ATL) categories across clinical semen phenotypes

Semen Phenotype	Critical (< 3 kb)	Low (3–6 kb)	Healthy (6–12 kb)	Fertile (> 14 kb)
Normozoospermia	10.0%	30.0%	60.0%	0.0%
Asthenoteratozoospermia	11.3%	0.0%	75.6%	13.1%
Oligoteratozoospermia	0.0%	33.3%	66.7%	0.0%
Teratozoospermia	26.3%	10.5%	15.8%	47.4%

DISCUSSION

The paternal genetic and epigenetic material transmitted to the oocyte is crucial for fertilisation, syngamy, and embryonic development. While the conventional semen analysis evaluates macroscopic cellular characteristics, it provides limited information about chromatin stability or telomere preservation. In this study we assessed the relationship between sperm telomere length (ATL) and chromatin protamination in infertile men.

During late spermiogenesis, spermatids undergo substantial nuclear remodeling, with 85–90% of histones being replaced by protamines. This compaction produces toroidal chromatin structures, which protect the DNA against oxidative stress^{6,7}. The positive correlation between protamine maturation and absolute sperm telomere length (Spearman $\rho = +0.308$ overall and $\rho = +0.353$ across stratified clinical tiers; $r = 0.55$ between Critical and Fertile cohorts) suggests that abnormal spermiogenesis impairs protamination while also accelerating telomeric erosion. Reactive oxygen species (ROS) target the guanine-rich telomeric repeats to create 8-hydroxy-2'-deoxyguanosine (8-OHdG) and disrupt disulphide cross-linking between protamines^{14,6}. Poor protamination and shorter telomeres could be the result of increased oxidative stress the chromatin has endured at the time of spermatogenesis⁸.

The ROC analysis showed that protamination of 75% is a moderate diagnostic predictor for normal telomere length (≥ 16.63 kb/genome). Because determination of telomere length necessitates specialised and labour-intensive laboratory procedures, aniline blue staining for protamination could be used as a simple screening technique to identify men with short telomere lengths.

An important clinical takeaway point from this study is that there is no association between traditional semen parameters – concentration, motility, and morphology, and sperm telomere length ($p > 0.27$). Most importantly, this study also reveals that nearly 10% of the normozoospermic men possess critically short telomeres < 3 kb. This explains why normozoospermic men frequently experience unexplained ART failure or embryo arrest.

STUDY LIMITATIONS

The limitations include the sample size $n = 50$, which restricts statistical power in rare phenotypic subgroups, and the minimal data available on the lifestyle oxidative stress, hence it could not be included. Multi-centre trials with clinical ART follow-up data (fertilisation and live birth rates) are warranted.

CONCLUSION

This study identified protamine maturation as the sole significant and independent predictor of absolute telomere length (ATL). Conventional semen parameters like concentration, motility and morphology failed to independently predict or correlate with telomere length. Interestingly hidden telomeric defects were identified even in normozoospermic men with a notable proportion of morozoospermic men harbouring critically short telomeres (< 3 kb). This demonstrates that routine microscopic evaluation fails to reflect the underlying sub-microscopic genomic integrity, also these findings offer a biological explanation for cases with unexplained ART failures and early embryonic arrest.

Declarations

Declaration of Interest

The authors declare no conflicts of interest related to this study

Author Contribution

Dr. Swathy Yuvaraj: Conceptualization, methodology, investigation, data curation, formal analysis, interpretation of results, and writing—original draft and review and editing. Dr. Radha V: Supervision, conceptualization, methodology, critical review and revision of the manuscript, and overall guidance of the research. Dr. Teena Koshy: Co-supervision, methodology, interpretation of results, and critical review and revision of the manuscript. Dr. Yamini Asokan: Research guidance, critical review of the study methodology and results, and critical review of the manuscript.

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Figures

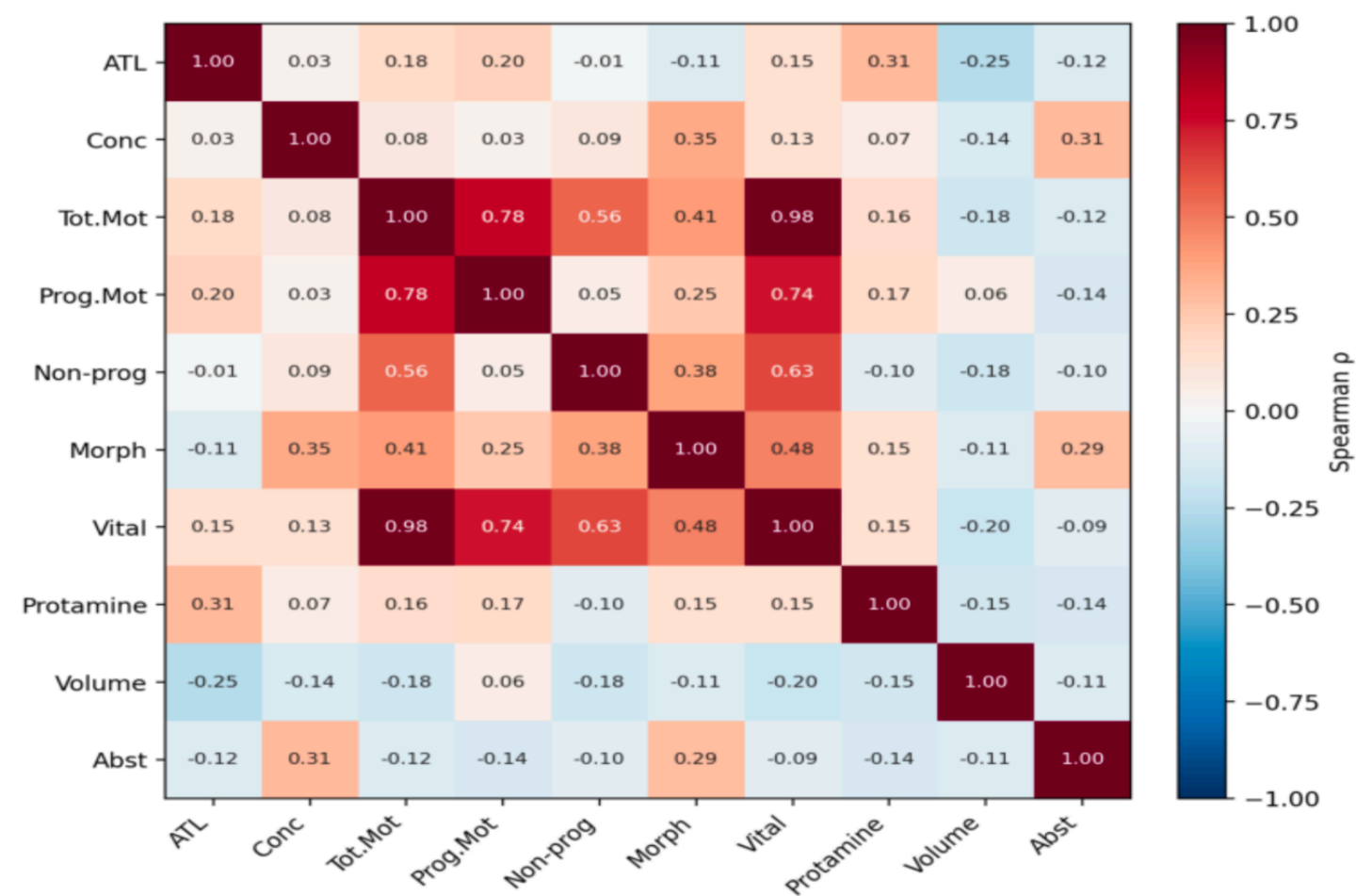


Figure 1

Heatmap depicting Spearman rank correlation coefficients (ρ) across patient demographics, semen parameters, protamination and absolute telomere length (ATL). Values represent ρ , and colour indicates the strength and direction of correlation (orange = positive, blue =negative) *

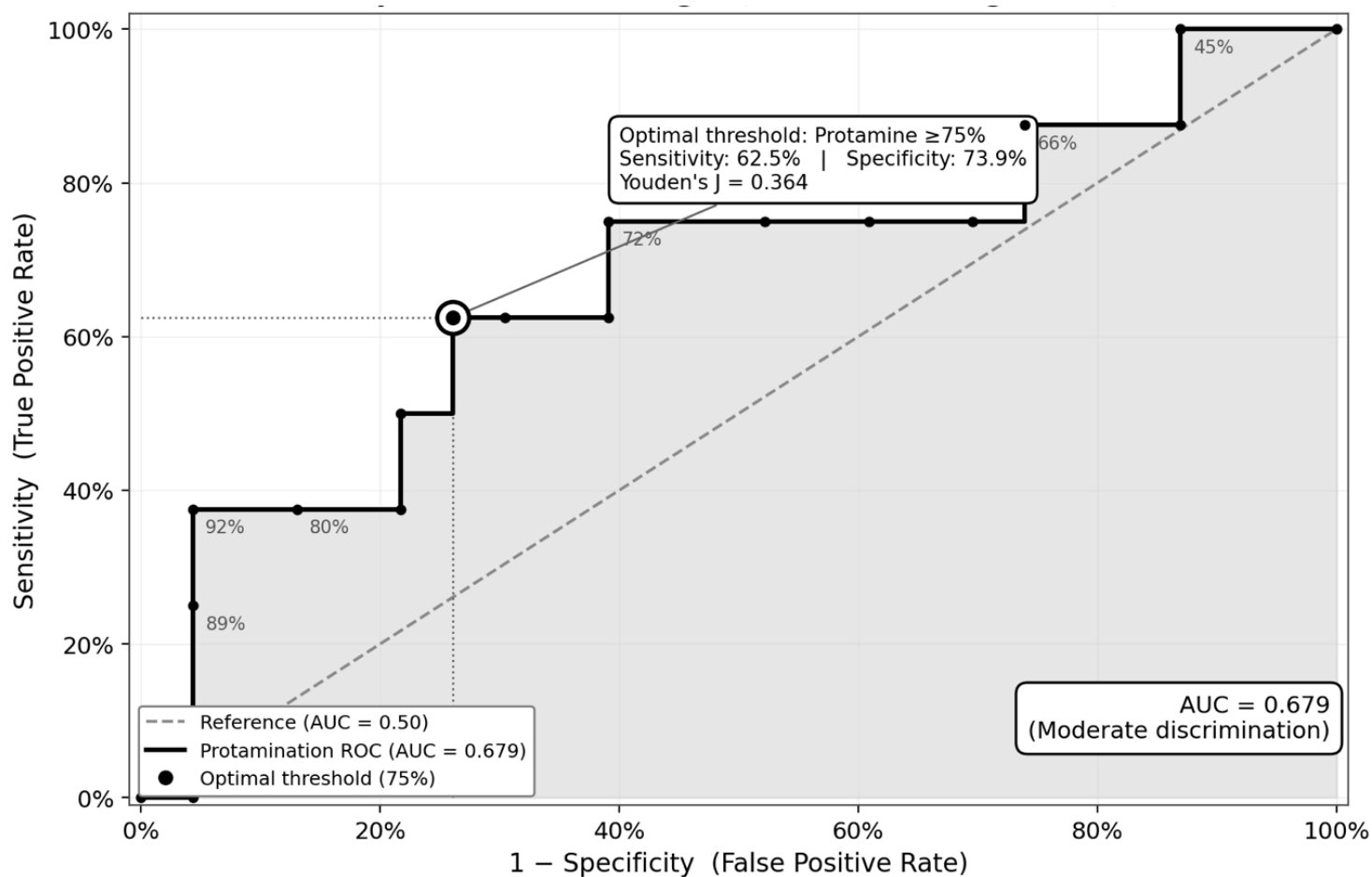


Figure 2

ROC Curve – Protamine Maturation Predicting Normal Sperm Telomere Length (ATL ≥ 16.63 kb/genome)