Asian Journal of Andrology (2018) 20, 425–431
www.asiaandro.com; www.ajandrology.com

Open Access

ORIGINAL ARTICLE

Semen Analysis

# Increased expression of PELP1 in human sperm is correlated with decreased semen quality

**Izabela Skibinska[1], Miroslaw Andrusiewicz[1], Michal Soin[1], Magdalena Jendraszak[1], Paulina Urbaniak[1], Piotr Jedrzejczak[2], Malgorzata Kotwicka[1]**

**Proline-, glutamic acid-, and leucine-rich protein 1 (PELP1) is a scaffolding protein involved in both genomic and nongenomic estrogen signal transduction pathways. To date, the role of PELP1 protein has yet to be characterized in human sperm and has not been associated with sperm parameters. To confirm the presence of PELP1 in human sperm, fresh semen samples were obtained from 178 donors. The study was designed to establish both mRNA and protein presence, and protein cellular localization. Additionally, the number of PELP1-positive spermatozoa was analyzed in men with normal and abnormal semen parameters. Sperm parameters were assessed according to the World Health Organization (WHO) 2010 standards. The presence of PELP1 in spermatozoa was investigated using four precise, independent techniques. The qualitative presence of transcripts and protein was assessed using reverse transcription-polymerase chain reaction (RT-PCR) and western blot protocols, respectively. The cellular localization of PELP1 was investigated by immunocytochemistry. Quantitative analysis of PELP1-positive cells was done by flow cytometry. *PELP1* mRNA and protein was confirmed in spermatozoa. Immunocytochemical analysis identified the presence of PELP1 in the midpieces of human sperm irrespective of sperm parameters. Becton Dickinson fluorescence-activated cell sorting (FACSCalibur™) analysis revealed a significantly lower number of PELP1-positive cells in males with normal semen parameters versus abnormal samples (42.78% ± 11.77% *vs* 61.05% ± 21.70%, respectively; $P = 0.014$). The assessment of PELP1 may be a time-saving method used to obtain information about sperm quality. The results of our study suggest that PEPL1 may be utilized as an indicator of sperm quality; thereby, PELP1 may be an additional biomarker useful in the evaluation of male infertility.**

*Asian Journal of Andrology* (2018) **20**, 425–431; doi: 10.4103/aja.aja_11_18; published online: 20 April 2018

**Keywords:** male infertility; proline-, glutamic acid-, and leucine-rich protein 1; semen parameters; sperm quality; spermatozoa

## INTRODUCTION

Infertility affects over 50 million couples, and potentially as many as 186 million people worldwide suffer from fertility issues. Due to limitations regarding precise numbers, the male factor is expected to contribute toward approximately half of all infertility cases.[1] The current diagnostic approaches focus on analysis of standard semen parameters: motility, morphology, and sperm count. Regardless, up to one-third of infertile males are considered as possessing idiopathic sperm abnormalities.[2,3] However, this issue becomes difficult to address as some sperm donors who do not show abnormalities in semen parameters display issues with conception. It is suggested that, in some cases, molecular defects leading to conception failure may be the root of the problem. Increasing evidence indicates that basic semen analysis does not fully encompass the potential of male fertility.[3,4]

For this reason, plausible sperm biomarkers, such as the expression patterns of sperm RNAs and protein, need to be evaluated instead of reliance on the standard semen parameters. These factors could play a functional role after delivery to the oocyte, or they may play a part in modulation of gene expression processes. The amount of particular RNAs and/or proteins may be a distinguishing factor between fertile and infertile men, even when the latter present normal semen parameters.[3] In this manner, molecular biomarkers are thought to be a promising area of specific and suitable therapeutic tools in fertility issues' exploration.[4]

Sperm viability parameters are essential for successful conception, and they depend on complex orchestration of biological systems. It is postulated that steroid hormones play a crucial role in the spermatogenesis process.[5–8] Previously, attention was focused on estrogens, androgens, and their cognate receptors. Steroid hormones can affect target cells via genomic and/or nongenomic mechanisms, creating a network of proteins involved in signal transduction.[9,10] Estrogen plays a critical role in the male reproductive system as it is essential for its proper development as well as the process of spermatogenesis.[6,11–13] It has been suggested that the target cells for estrogen can also be human sperm cells. Recent studies demonstrated that estrogens modulate sperm motility, capacitation, and acrosome reaction.[14–18] Estrogens affect target cells via specific estrogen receptors (ESRs): ESR1 and ESR2. Spermatozoa possess both forms of the ESRs as well as aromatase, the enzyme that converts testosterone to estrogen, indicating that these proteins are key factors in the male reproductive system.[19–22] Our previous studies showed the presence of ESRs in the midpiece

[1]Department of Cell Biology, Faculty of Health Sciences, Poznan University of Medical Sciences, Rokietnicka 5D, Poznan 60-806, Poland; [2]Division of Infertility and Reproductive Endocrinology, Faculty of Medicine I, Poznan University of Medical Sciences, Polna 33, Poznan 60-535, Poland.
Correspondence: Dr. I Skibinska (iskibinska@ump.edu.pl)
Received: 12 August 2017; Accepted: 20 December 2017

region of sperm, suggesting their importance in the context of mitochondrial function.[21,23]

Novel findings indicate the primary role of proline-, glutamic acid-, and leucine-rich protein 1 (PELP1) in crosstalk between cellular processes mediated via ESR. Additionally, the mechanistic relationship between G protein-coupled estrogen receptor (GPER30) and the estrogen-related signaling pathway has been reported.[24] Because many environmental factors are described as endocrine estrogen disruptors, which may bind to ESR, GPER30, and the androgen receptor,[25] it is essential to trace the molecular pathway of estrogen action in males. As a result, PELP1 would be placed among the molecules involved in signal modulation of rapid estrogen signaling, particularly in sperm cells.

PELP1 is present both within the cell nucleus and cytoplasm. The genomic action of PELP1 may influence gene expression by binding to H3 and H4 histones and, as a consequence, remodels chromatin structure. Nongenomically, PELP1 is engaged in estrogen-dependent signal transduction based on a social network of phosphorylated protein complexes.[26–28] It also participates in ribosome biogenesis and serves as a protein–protein mediator in various cellular processes.[29,30] *PELP1* RNA levels and protein expression in mature sperm cells are under scrutiny.[31]

Taking into consideration the influence of environmental estrogen-like compounds on the biology of male reproductive system and sperm cells, the aim of our study was to determine a PELP1 expression pattern and compare it with sperm parameters. As postulated, this protein is essential for estradiol-mediated, extranuclear response, and thus plays a crucial role in signal transduction in spermatozoa. We analyzed *PELP1* mRNA and protein concentrations in pooled semen samples. First of all, we confirmed its presence in sperm, and then we estimated its cellular localization in the spermatozoa and determined the proportion of PELP1 in subjects presenting normal and abnormal semen parameters.

## MATERIALS AND METHODS

### *Ethical approval statement*

The Institutional Review Board at the Poznan University of Medical Sciences, Poznan, Poland, approved this study (No. 553/13). Written informed consent was obtained from each participant.

### *Materials*

Semen samples were obtained from 178 men at the Division of Infertility and Reproductive Endocrinology of Poznan University of Medical Sciences. All participants were of Caucasian descent. All patients have been undergoing the diagnostic procedures due to infertility of unknown cause. Varicocele, prostate dysfunction, vas deferens obstruction, as well as metabolic diseases were excluded.

Semen samples of 43 men, aged 18–45 years (median age 33 years), were evaluated according to the World Health Organization (WHO) guidelines for normozoospermia: concentration above $15 \times 10^6$ sperm ml$^{-1}$, at least 58% live sperm, >32% sperm showing progressive movement, more than 4% spermatozoa with normal morphology, and a leukocyte content of $<10^6$ cells ml$^{-1}$.[32] Seventeen samples were classified as normozoospermic according to the WHO standards and 26 samples which did not meet anyone of above-mentioned criteria, thereby, were qualified as abnormal (**Supplementary Table 1**).

All 43 semen samples used for Becton Dickinson fluorescence-activated cell sorting (FACSCalibur™; Becton-Dickinson, Franklin Lakes, NJ, USA) analyses were obtained from each patient. Only one sample from each patient was used for the analysis. Sperm smears were prepared using 10 μl of the whole semen volume, and the remaining part was examined by FACSCalibur™ flow cytometry (Becton-Dickinson). Further investigations were performed at the Cell Biology Department of Poznan University of Medical Sciences. A schematic diagram describing semen sample acquisition is shown in **Figure 1**.

### *mRNA and protein presence validation*

Due to the small amount of RNA in spermatozoa, nine separate trials of pooled semen samples were obtained from 12–18 donors ($n = 135$). Half of the pooled sample volume was used for RNA extraction and the other half for protein isolation. Swim-up technique was used to obtain a pure fraction of sperm cells from each sample according to the WHO guidelines.[32]

### *Analysis of PELP1 mRNA expression*

Total RNA was isolated using TriPure isolation reagent (Roche, Basel, Switzerland) according to a modified Chomczynski and Sacchi method.[33] The modifications included repeated TriPure isolation step and RNA precipitation at −80°C instead of room temperature (RT) incubation, followed by nucleic acid concentration using a silica matrix column system (ZymoResearch, Irvine, CA, USA). Additionally, the repeated TriPure step during RNA extraction prevented DNA contamination. RNA sample quantity and purity was assessed by measuring absorbance (NanoDrop ND-1000 spectrophotometer; Thermo Fisher Scientific, Waltham, MA, USA) at A260 nm, A230 nm, and A280 nm; and the A230/260 and A260/280 ratios showed that all samples met the criteria for purity (both ratios ranged from 1.9 to 2.0). To evaluate sample integrity, the ribosomal RNA bands were analyzed by 0.8% agarose gel electrophoresis in denaturing conditions of 1 μg RNA in 1 × FA buffer (pH 7.0, 1 mmol l$^{-1}$ ethylenediaminetetraacetic acid, 5 mmol l$^{-1}$ sodium acetate, 20 mmol l$^{-1}$ 3-(N-morpholino)-propanesulfonic acid [free acid; Sigma-Aldrich, Saint Louis, MO, USA]) and 0.8% paraformaldehyde (Avantor Performance Materials Poland S.A.,

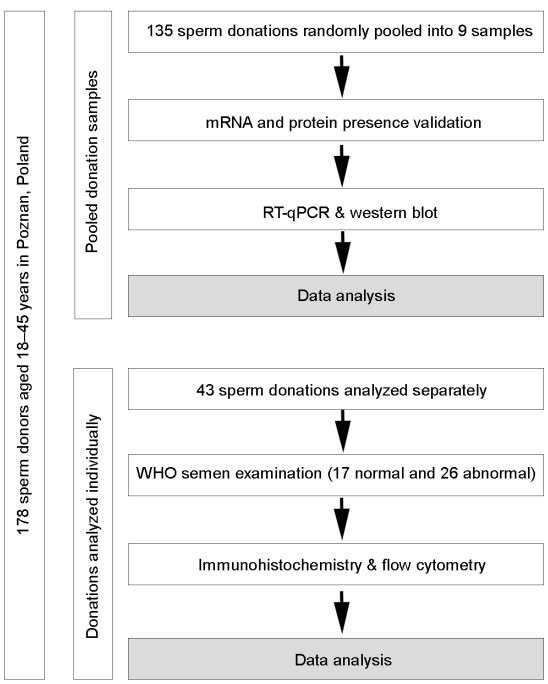


**Figure 1:** Schematic diagram of the sperm analysis procedure. Analysis of pooled semen samples (top) and individually analyzed donations (bottom). RT-qPCR: reverse transcription-quantitative polymerase chain reaction.

Gliwice, Poland) providing denaturing conditions in the presence of ethidium bromide (Sigma-Aldrich).

The reverse transcription reaction was performed according to the Transcriptor reverse transcriptase manufacturer protocol (Roche). The total reaction volume was 10 µl. In the first step, 500 ng of total cellular RNA, DNase, RNase, pyrogen-free water, and 5 pmol $\mu l^{-1}$ universal oligo $(dT)_{10}$ primer were combined, and the samples were denatured at 65°C for 10 min then cooled on ice. Next, each sample was complemented with 5 pmol $\mu l^{-1}$ of each deoxynucleotide triphosphates (dNTPs), 10U for reaction (rxn) ribonuclease inhibitor, 1 × buffer (Transcriptor RT buffer; Roche, Basel, Switzerland), and 10U for rxn of reverse transcriptase. The following thermal profile was applied: 25°C for 10 min, 55°C for 60 min, then 5 min at 85°C. cDNA was immediately used for quantitative polymerase chain reaction (qPCR) or stored at −20°C until further analysis (but no longer than 1 week).

To establish RNA expression, the LightCycler® 2.0 carousel glass capillary-based system was applied (Roche). Primer sequences and TaqMan® hydrolysis probe position for the gene of interest (GOI) were determined using Roche Universal Probe Library Assay Design Center (https://lifescience.roche.com/en_pl/brands/universal-probe-library.html, last accessed on September 28, 2017). The hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) gene encoding hypoxanthine-guanine phosphoribosyltransferase was served as an internal control (Roche assay N° 05046157001). The reaction mixture in a total volume of 20 µl contained 5 µl template cDNA (obtained in the reverse transcription reaction step), 1 × LightCycler® FastStart TaqMan® Probe Master (Roche), 0.1 µmol $l^{-1}$ hydrolysis probe (0.2 µmol $l^{-1}$ in the case of GOI), and 0.5 µmol $l^{-1}$ gene-specific primers. The Universal Probe Library (UPL) probe and the sense and antisense primers for the GOI were as follows: probe #62 (Roche cat. N°: 04688619001) and 5'-GCACTGTGTGTCTTGGCTTC-3' and 5'-GAGGAGGTCCCTCAGGACA-3'. The primers were designed so that they annealed in two different exons spaced by a 7.9-kb intron. We used the standard cycling and acquisition reaction profile for UPL probes.[34] Each reaction was performed in duplicate on independently synthetized cDNA. Samples were sequenced to confirm their identity with those deposited in the Nucleotide Database of National Center for Biotechnology Information (GenBank N°: NM_014389.2).

***PELP1 protein detection***

Sperm cells were suspended in an appropriate volume of isolation buffer (80% phosphate-buffered saline [PBS; PAN-biotech, Aidenbach, Germany]); 10% protein extraction and immunoprecipitation 1 × radio-immunoprecipitation assay (RIPA) buffer (Merck Millipore, Darmstadt, Germany); and 10% protease inhibitor cocktail (Sigma-Aldrich). The samples were incubated for 15 min at 4°C with gentle shaking, then cells were centrifuged (Centrifuge 5812R; Eppendorf, Hamburg, Germany) at 12 000 *g*, 4°C, l5 min., and the supernatants containing protein extracts were used for western blot analysis. Quick Start Bradford Dye Reagent (BioRad, Hercules, CA, USA) and spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific) were used for colorimetrical measurement of extracted proteins (595 nm wavelength) and referred to decimal dilution series of bovine serum albumin fraction V (BSA V; Roche).

Thirty micrograms of extracted proteins were separated using 12% SDS-PAGE running buffer (25 mmol $l^{-1}$ Tris, 192 mmol $l^{-1}$ glycine, 0.1% SDS, pH 8.3 [LabEmpire, Rzeszow, Poland]; 200V, 40 min, RT) and subsequently transferred to activated Hybond-P polyvinylidene fluoride membrane (GE Healthcare Europe, Little Chalfont, UK) in transfer buffer (1 × SDS-PAGE running buffer supplemented with 20% methanol; 300 mA, 90 min, RT). After blotting, membranes were incubated for 1 h at 4°C in Tris-buffered saline with Tween 20 (TBST) buffer (5% Blotto blocking reagent solution [BioRad]; 1 × TBST). Protein detection was evaluated using primary polyclonal anti-PELP1 rabbit antibody (Bethyl Laboratories, Montgomery, TX, USA) followed by incubation with polyclonal anti-rabbit alkaline phosphatase-conjugated goat secondary antibody (Sigma-Aldrich), both at a 1:2500 dilution (200 rpm orbital shaker, 1 h, 4°C). Each time, unbounded antibodies were rinsed three times in TBST buffer (200 rpm, 10 min, RT). Glyceraldehyde 3-phosphate dehydrogenase protein (GAPDH) extracted from spermatozoa, as well as from the ovarian cancer cell line OVCAR-3 (ATCC, Manassas, VA, USA), was used as internal and external positive control for PELP1 expression. Visualization was performed using the BCIP®/NBT-Blue liquid substrate system for membranes (Sigma-Aldrich). Membranes were digitized using G:BOX Chemi system (Syngene, Cambridge, UK). Western blots were repeated in triplicate for all 9 pooled samples.

***Immunocytochemistry***

Smears were fixed on glass microscope slides in a 4% buffered paraformaldehyde solution (Avantor Performance Materials Poland S.A.) for 10 min at RT. In order to retrieve antigens, the slides were boiled in a microwave oven twice (2 × 10 min, 200W) in citrate buffer (pH 6.0, 0.1 mol $l^{-1}$ citric acid solution, 0.1 mol $l^{-1}$ sodium citrate solution; Avantor Performance Materials Poland S.A.) and then cooled at RT. In order to block endogenous peroxidase activity, slides were incubated for 3 min in 3% hydroxyperoxide solution (Avantor Performance Materials Poland S.A.). To avoid nonspecific antibody binding, the slides were placed in TBST buffer (containing 3% BSA) for 1 h at RT. To evaluate PELP1 protein localization, the same primary antibodies (Bethyl Laboratories) as for western blot were used at a 1:500 dilution. Incubation was carried out overnight at 4°C.

Subsequently, the slides were rinsed three times for 15 min in TBST buffer. In order to identify cellular localization of the antigen, the slides were processed with commercial Dako EnVision+ System HRP (DAB) kit (Dako, Glostrup, Denmark) containing a horseradish peroxidase-conjugated anti-rabbit secondary antibody. The detection procedure was provided by the manufacturer. As positive controls, 3 µm dewaxed and rehydrated tissue specimen sections of ovarian cancer were used. Evaluation was performed under the Axiophot light microscope (Zeiss-Opton, Oberkochen, Germany).

***Flow cytometry***

Cells were separated using a gradient isolation system (Sil-Select Plus, FertiPro N.V., Beernem, Belgium) according to the manufacturer's protocol. Sperm cells from the lower phase were harvested and pelleted (300 *g*, 10 min, RT), and spermatozoa were fixed in an appropriate volume of 4% buffered paraformaldehyde solution (1 h, RT) followed by centrifugation 10 min, 200 *g*, 4°C (Centrifuge 5415D, Eppendorf). Next, cells were permeabilized on ice for 20 min in 500 µl 0.1% saponin solution in PBS supplemented with 0.5% BSA. Samples were then centrifuged again under the same conditions, and 100 µl of PELP1 primary polyclonal antibodies were applied at a 1:500 dilution. The incubation period was 1 h on ice. Next, the solution was filled up with saponin (to the maximum volume of the 1.5 ml eppendorf tube), centrifuged (200 *g*, 5 min, 4°C), and washed with PBS. The next step was a 30-min incubation on ice in darkness with secondary goat anti-rabbit antibody conjugated with fluorescein (Santa Cruz Biotechnology Inc., Dallas, TX, USA) at a 1:100 dilution.

Samples were then filled with saponin solution, centrifuged, and suspended in 500 µl PBS. In the negative control, samples omitted the primary antibody. For each experiment performed using BD FACSCalibur™ flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA), 10 000 cells were examined. The fluorescence dye was excited by argon laser (488 nm) and emission was measured in the FL1 channel (515–545 nm). All data were gathered and analyzed using BD CellQuest™ Pro software (version 5.2.l; Becton Dickinson). The FACS operator was blinded for the results of the semen analyses. The intensity of fluorescence of PELP1-positive cells derived from patients presenting abnormal semen parameters was compared to the values obtained from patients fulfilling the criteria for normozoospermia.

### *Statistical analysis*

Statistical analysis was performed using Statistica version 12 software for Windows (StatSoft Inc., Tulsa, OK, USA). To describe experimental results, mean ± standard deviation was used. The Shapiro–Wilk test was used for the normality of continuous variables distribution assessment. The Welch's *t*-test, as well as Mann–Whitney U-test, was applied. The correlation coefficient (*r*) between sperm parameters and mean fluorescent intensity was determined by the Spearman's rank correlation tests. Using the Medical plugin version 4.0 for Statistica software (StatSoft), the receiver operating characteristic (ROC) curve was created. Subsequently, Hanley and McNeil methods for ROC comparison were used for further analysis of the data and for calculation of the sensitivity and specificity of PELP1 quantification in relation to normal/abnormal semen quality. Data were considered statistically significant at $P < 0.05$.

## RESULTS

### *PELP1 mRNA and protein validation*

*PELP1* amplification was confirmed in all analyzed samples using specific primers and TaqMan® probes (Roche, assay N° 05046157001 for *HPRT*, and probe #62 Roche cat. N°: 04688619001 for *PELP1*). The reaction product specificity was evaluated by electrophoretic separation in agarose gel, sequencing, and alignment analysis. Based on comparison with the molecular size marker Nova l00 (Novazym, Poznan Poland), bands were observed at a size corresponding to the *PELP1* mRNA fragment, in length of 100 bp (**Figure 2**). The consensus sequence of the PCR product was confirmed by sequencing analysis and compared to the mRNA of human *PELP1*, transcript variant 1 (GenBank N°: NM_014389.2).

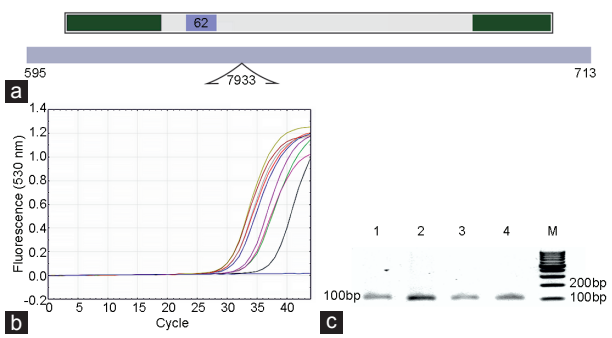


**Figure 2:** Analysis of the *PELP1* transcripts in human spermatozoa. (**a**) Relative position of primers and #62 TaqMan® probe in *PELP1* coding sequence (7933 bp intron is indicated). (**b**) *PELP1* RT-qPCR amplification curves. (**c**) qPCR products visualized in agarose gel. 1, 3, 4: human spermatozoa; 2: ovarian cancer (positive control); M: DNA ladder. PELP1: proline-, glutamic acid-, and leucine-rich protein 1; RT-qPCR: reverse transcription-quantitative polymerase chain reaction.

Western blot analysis confirmed the presence of the PELP1 protein in sperm cells. Immunoreactive bands were observed at approximately 170 kDa, corresponding to the molecular weight of human PELP1. A weak signal was detected in the case of total cellular proteins isolated from swim-up prepared spermatozoa, while a stronger signal was observed in the positive control samples (proteins isolated from ovarian cancer cell line OVCAR-3). The 37 kDa bands of GAPDH reference protein were strongly immunoreactive in all cases (**Figure 3**).

### *Expression pattern analysis*

Immunocytochemical staining confirmed the presence of PELP1 protein in human sperm. In cells with normal morphology, strong immunostaining was observed in the midpieces. Likewise, in cells with disturbed morphology (of head, midpiece, and/or tail), PELP1 protein was mostly presented within midpieces. Nuclear PELP1 localization was observed in ovarian cancer tissue samples (positive control) (**Figure 4**).

FACS analysis quantified sperm with PELP1 expression. For each sample, 10 000 cells were analyzed. The intensity of fluorescence of PELP1-positive sperm was lower in normozoospermic semen in comparison to abnormal samples (42.78% ± 11.77% *vs* 61.05% ± 21.70% respectively; $P = 0.014$; **Figure 5**). In all analyzed parameters, with the exception of viability and total motility, there were differences between mean fluorescence intensity ($P \leq 0.001$). The mean expression level was higher in samples with abnormal semen parameters. In the analysis presented in **Supplementary Table 2**, the parameters described in each row were taken under consideration as the major subgroup determinant ($P < 0.01$). A negative correlation was found between all sperm parameters and PELP1 expression. The correlation coefficients varied from –0.41 to –0.59 ($P < 0.01$; **Supplementary Table 3** and **Figure 6**). ROC curve analyses for the "normal/abnormal" semen categories revealed areas under the curve (AUC) for PELP1 quantification (0.781; $P = 0.0001$). The cutoff value of 42.15 made it possible to predict the abnormality in semen quality with a sensitivity of 88% and specificity of 65%.

## DISCUSSION

Our analysis of mRNA and protein expression revealed, for the first time, the presence of PELP1 in human spermatozoa using four precise,

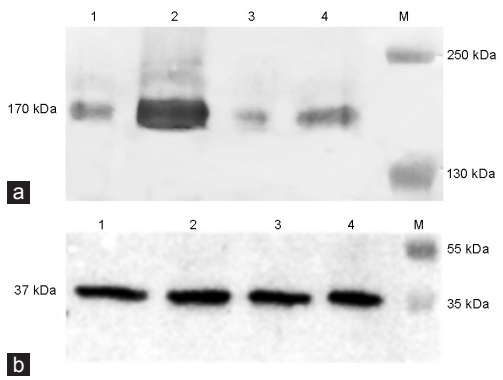


**Figure 3:** Western blot evaluation of PELP1 in human sperm. (**a**) Thirty micrograms of proteins were separated using 12% SDS-PAGE; lines 1, 3, 4: proteins isolated from sperm cells, line 2: external positive control derived from ovarian cancer cell line (OVCAR-3) shows the strongest immunoreaction. (**b**) Immunoreactive bands of GAPDH protein samples (referring to PELP1 western blot) were used as loading control. Lanes 1, 3, 4: proteins isolated from sperm cells; lane 2: proteins derived from OVCAR-3; M: protein molecular weight marker. GAPDH: glyceraldehyde 3-phosphate dehydrogenase protein; PELP1: Proline-, glutamic acid-, and leucine-rich protein 1; SDS-PAGE: sodium dodecylsulfate-polyacrylamide gel electrophoresis.

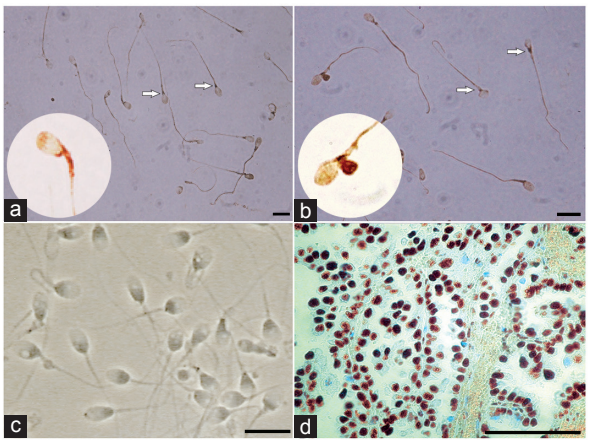


**Figure 4:** PELP1 immunolocalization in human sperm (light microscope, original magnification ×100). (**a**) PELP1 within midpieces of human spermatozoa with normal morphology (indicated by arrows). (**b**) PELP1 in sperm cells with disturbed morphology. (**c**) Nonprimary antibody negative control. (**d**) Positive control reaction in ovarian cancer tissue. Scale bars = 5 μm in **a**–**c**, and 100 μm in **d**. PELP1: proline-, glutamic acid-, and leucine-rich protein 1.

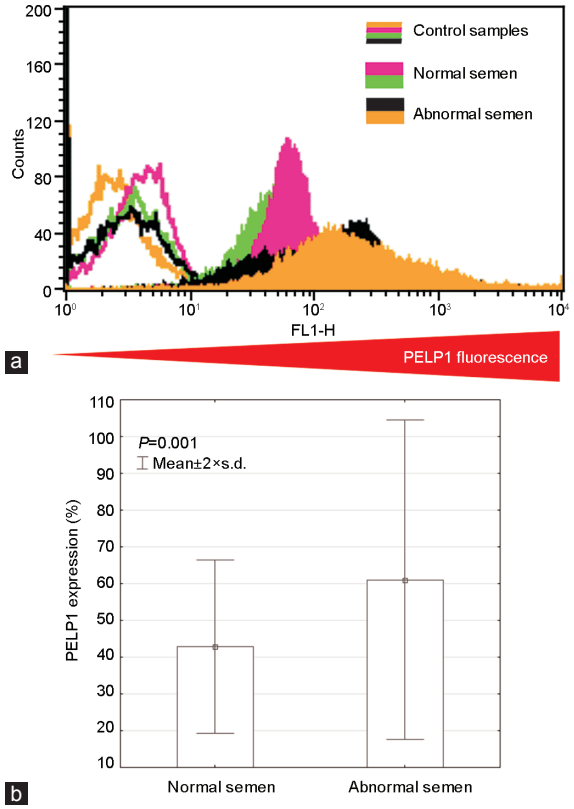


**Figure 5:** Flow cytometry analysis of the PELP1 in human spermatozoa of normal and abnormal semen samples. (**a**) Representative histogram of PELP1-positive sperm cells. Colors indicate samples obtained from different patients. (**b**) Bar graph of the fluorescence intensity of PELP1-positive cells. PELP1: proline-, glutamic acid-, and leucine-rich protein 1; FL1-H: fluorescence relative intensity (height) of PELP1 in channel 1 measured by flow cytometry.

independent techniques. The qualitative presence of transcripts and protein was established by reverse transcription-polymerase chain reaction (RT-PCR) and western blot protocols, respectively. The cellular localization of PELP1 was investigated immunocytochemically. Quantitative analysis of PELP1-positive cells was performed using flow cytometry (FACSCalibur™; Becton Dickinson Biosciences).

Until this point, no studies have indicated the presence of PELP1 in spermatozoa. We suspect that PELP1 contributes to the biology of sperm cells. This protein is involved in both genomic and nongenomic pathways of estrogen signal transduction in somatic cells.[35–39]

Besides the presence of RNA, the protein inheritance in mature sperm was confirmed by the western blot analysis. Detection of PELP1 protein can be supported by the presence of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) in the midpiece region of spermatozoa.[40] PI3K is involved in estrogen action, transduced via ESRs. PI3K can influence the regulation of calcium channels through phosphatidylinositol (3,4,5)-trisphosphate and diacylglycerol. Those findings are interesting especially in the context of increased $Ca^{2+}$ concentrations observed in the sperm in a quick/nongenomic response to estrogens.[41] The PELP1 expression pattern observed using Axiophot light microscopy indicated its localization in the same region as PI3K in sperm with normal and abnormal parameters (**Figure 4**). Considering studies reporting on different cellular localization of PELP1 (cytoplasmic *vs* nuclear), its function is compartment dependent.[42]

Moreover, it has been reported that one ESR isoform can interact with a G-protein-coupled estrogen receptor located in the midpiece.[19,43,44] Due to PELP1 interaction with ESR, such localization seems to be justified. The presence of ESRs and PELP1 in the same cellular region could be related to a potential role of estrogens in energy production for motility, capacitation reaction, and sperm fertilizing ability.

In this study, we did not observe significant differences in the PELP1 expression pattern regarding total motility and viability parameters. Surprisingly, in the case of other analyzed parameters, the percentage of PELP1-positive spermatozoa was higher in sperm samples with abnormal parameters. A negative correlation was observed between decreasing PELP1 expression and increasing sperm quality. This may be due to an excess of residual cytoplasm in disturbed sperm morphology or protein redistribution. Moreover, PELP1 was reported to induce nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway, which is related to inflammatory signals that modulate macrophage activation.[42] Taking all of these into account, this could lead to decrease of sperm parameters.

For the first time, our study demonstrates the presence of PELP1 in spermatozoa and its increased expression in sperm with abnormal parameters.

## CONCLUSIONS

This study demonstrates the presence of mRNA, protein, and cellular localization of PELP1 in ejaculated spermatozoa. Flow cytometry analysis showed that the percentage of sperm cells stained with PELP1 antibody is negatively correlated with increasing sperm quality. Thus, it might be a marker of impaired semen quality. To evaluate the exact role of PELP1 in male germ cell development, as well as in mature sperm physiology and pathology, further studies need to be conducted.

## AUTHOR CONTRIBUTIONS

IS was responsible for conception and design of the study, acquisition, analysis and interpretation of data, and drafting the article. MA, MK, and PJ participated in the study design, its coordination, as well as in analysis and interpretation of data, and revising the article critically

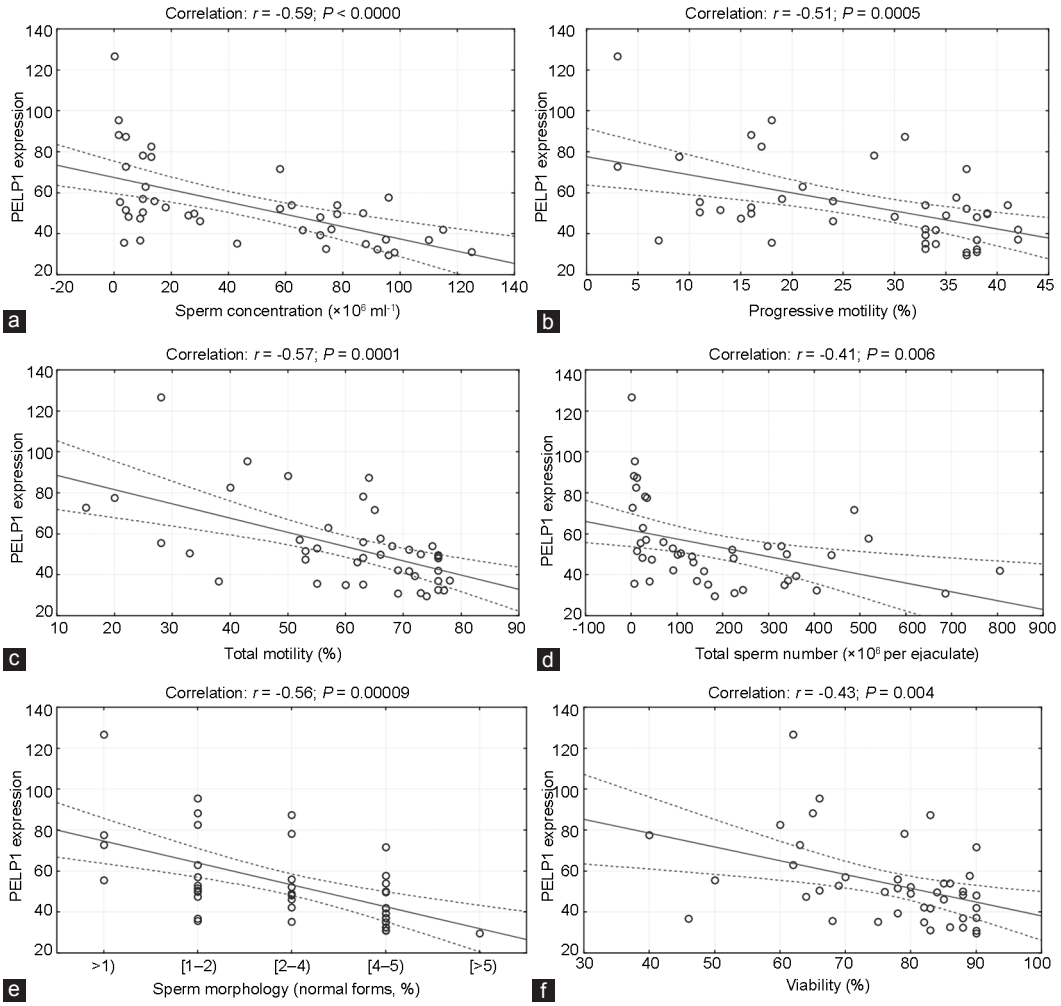


**Figure 6:** Correlation scatter plots of PELP1 fluorescence intensity and various semen parameters. Scatter plots display the correlation between PELP1 fluorescence intensity and (**a**) sperm concentration, (**b**) progressive motility, (**c**) total motility, (**d**) total sperm number, (**e**) sperm morphology, and (**f**) viability. PELP1: proline-, glutamic acid-, and leucine-rich protein 1.

for important intellectual content. IS, MA, MS, MJ, and PU carried out study analyses, including protein immunolocalization analyses, PCR, flow cytometry, and are also responsible for revising the article critically for important intellectual content. All authors read and approved the final version of the manuscript and agreed with the order of presentation of the authors.

## COMPETING INTERESTS

All authors declared no competing interests.

## ACKNOWLEDGMENTS

This work was supported by the National Science Centre Poland grant number: UMO-2016/23/D/NZ5/02604). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Supplementary information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

## REFERENCES

1 Inhorn MC, Patrizio P. Infertility around the globe: new thinking on gender, reproductive technologies and global movements in the 21st century. *Hum Reprod Update* 2015; 21: 411–26.
2 Bieniek JM, Lo KC. Recent advances in understanding and managing male infertility. *F1000Res* 2016; 5: 2756.
3 Bonache S, Mata A, Ramos MD, Bassas L, Larriba S. Sperm gene expression profile is related to pregnancy rate after insemination and is predictive of low fecundity in normozoospermic men. *Hum Reprod* 2012; 27: 1556–67.
4 Gianzo M, Muñoa-Hoyos I, Urizar-Arenaza I, Larreategui Z, Quintana F, *et al*. Angiotensin II type 2 receptor is expressed in human sperm cells and is involved in sperm motility. *Fertil Steril* 2016; 105: 608–16.
5 Carreau S, Bouraima-Lelong H, Delalande C. Estrogens: new players in spermatogenesis. *Reprod Biol* 2011; 11: 174–93.
6 Carreau S, Wolczynski S, Galeraud-Denis I. Aromatase, oestrogens and human male reproduction. *Philos Trans R Soc Lond B Biol Sci* 2010; 365: 1571–9.
7 Dostalova P, Zatecka E, Dvorakova-Hortova K. Of oestrogens and sperm: a review of the roles of oestrogens and oestrogen receptors in male reproduction. *Int J Mol Sci* 2017; 18: 904.
8 Carreau S, Bois C, Zanatta L, Silva FR, Bouraima-Lelong H, *et al*. Estrogen signaling in testicular cells. *Life Sci* 2011; 89: 584–7.
9 Walker WH. Testosterone signaling and the regulation of spermatogenesis. *Spermatogenesis* 2011; 1: 116–20.
10 Chimento A, Sirianni R, Casaburi I, Pezzi V. Role of estrogen receptors and g protein-coupled estrogen receptor in regulation of hypothalamus-pituitary-testis axis and spermatogenesis. *Front Endocrinol (Lausanne)* 2014; 5: 1.
11 Akingbemi BT. Estrogen regulation of testicular function. *Reprod Biol Endocrinol* 2005; 3: 51.
12 Carreau S, Hess RA. Oestrogens and spermatogenesis. *Philos Trans R Soc Lond B Biol Sci* 2010; 365: 1517–35.
13 Carreau S, Silandre D, Bourguiba S, Hamden K, Said L, *et al*. Estrogens and male reproduction: a new concept. *Braz J Med Biol Res* 2007; 40: 761–8.
14 Guido C, Perrotta I, Panza S, Middea E, Avena P, *et al*. Human sperm physiology:

estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) influence sperm metabolism and may be involved in the pathophysiology of varicocele-associated male infertility. *J Cell Physiol* 2011; 226: 3403–12.
15 Sebkova N, Cerna M, Ded L, Peknicova J, Dvorakova-Hortova K. The slower the better: how sperm capacitation and acrosome reaction is modified in the presence of estrogens. *Reproduction* 2012; 143: 297–307.
16 Ded L, Dostalova P, Dorosh A, Dvorakova-Hortova K, Peknicova J. Effect of estrogens on boar sperm capacitation *in vitro*. *Reprod Biol Endocrinol* 2010; 8: 87.
17 Mohamed ES, Park YJ, Song WH, Shin DH, You YA, *et al*. Xenoestrogenic compounds promote capacitation and an acrosome reaction in porcine sperm. *Theriogenology* 2011; 75: 1161–9.
18 Filannino A, Stout TA, Gadella BM, Sostaric E, Pizzi F, *et al*. Dose-response effects of estrogenic mycotoxins (zearalenone, alpha- and beta-zearalenol) on motility, hyperactivation and the acrosome reaction of stallion sperm. *Reprod Biol Endocrinol* 2011; 9: 134.
19 Rago V, Giordano F, Brunelli E, Zito D, Aquila S, *et al*. Identification of G protein-coupled estrogen receptor in human and pig spermatozoa. *J Anat* 2014; 224: 732–6.
20 Arkoun B, Gautier C, Delalande C, Barrier-Battut I, Guénon I, *et al*. Stallion spermatozoa: putative target of estrogens; presence of the estrogen receptors ESR1, ESR2 and identification of the estrogen-membrane receptor GPER. *Gen Comp Endocrinol* 2014; 200: 35–43.
21 Skibińska I, Kotwicka M, Jendraszak M, Jędrzejczak P. Influence of 17 β-estradiol, genistein and bisphenol A on spermatozoa mitochondrial function. *Acta Biochim Pol* 2011; 58: 69.
22 Skibińska I, Jendraszak M, Borysiak K, Jędrzejczak P, Kotwicka M. 17β-estradiol and xenoestrogens reveal synergistic effect on mitochondria of human sperm. *Ginekol Pol* 2016; 87: 360–6.
23 Kotwicka M, Skibinska I, Jendraszak M, Jedrzejczak P. 17β-estradiol modifies human spermatozoa mitochondrial function *in vitro*. *Reprod Biol Endocrinol* 2016; 14: 50.
24 Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* 2005; 307: 1625–30.
25 Revankar CM, Mitchell HD, Field AS, Burai R, Corona C, *et al*. Synthetic estrogen derivatives demonstrate the functionality of intracellular GPR30. *ACS Chem Biol* 2007; 2: 536–44.
26 Vadlamudi RK, Kumar R. Functional and biological properties of the nuclear receptor coregulator PELP1/MNAR. *Nucl Recept Signal* 2007; 5: e004.
27 Lonard DM, O'Malley BW. The expanding cosmos of nuclear receptor coactivators. *Cell* 2006; 125: 411–4.
28 Björnström L, Sjöberg M. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. *Mol Endocrinol* 2005; 19: 833–42.
29 Nagpal JK, Nair S, Chakravarty D, Rajhans R, Pothana S, *et al*. Growth factor regulation of estrogen receptor coregulator PELP1 functions via Protein Kinase A pathway. *Mol Cancer Res* 2008; 6: 851–61.
30 Gonugunta VK, Miao L, Sareddy GR, Ravindranathan P, Vadlamudi R, *et al*. The social network of PELP1 and its implications in breast and prostate cancers. *Endocr Relat Cancer* 2014; 21: T79–86.
31 Gur Y, Breitbart H. Mammalian sperm translate nuclear-encoded proteins by mitochondrial-type ribosomes. *Genes Dev* 2006; 20: 411–6.
32 World Health Organization. WHO laboratory manual for the examination and processing of human semen. 5th ed. Geneva: World Health Organization; 2010. p1–286.
33 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; 162: 156–9.
34 Skibinska I, Tomaszewski M, Andrusiewicz M, Urbaniak P, Czarnecka-Klos R, *et al*. Expression of estrogen receptor coactivator proline-, glutamic acid- and leucine-rich protein 1 within paraspinal muscles in adolescents with idiopathic scoliosis. *PLoS One* 2016; 11: e0152286.
35 Marchiani S, Tamburrino L, Ricci B, Nosi D, Cambi M, *et al*. SUMO1 in human sperm: new targets, role in motility and morphology and relationship with DNA damage. *Reproduction* 2014; 148: 453–67.
36 Marchiani S, Tamburrino L, Giuliano L, Nosi D, Sarli V, *et al*. Sumo1-ylation of human spermatozoa and its relationship with semen quality. *Int J Androl* 2011; 34: 581–93.
37 Raz L, Khan MM, Mahesh VB, Vadlamudi RK, Brann DW. Rapid estrogen signaling in the brain. *Neurosignals* 2008; 16: 140–53.
38 Brann DW, Zhang QG, Wang RM, Mahesh VB, Vadlamudi RK. PELP1 – a novel estrogen receptor-interacting protein. *Mol Cell Endocrinol* 2008; 290: 2–7.
39 Boonyaratanakornkit V. Scaffolding proteins mediating membrane-initiated extra-nuclear actions of estrogen receptor. *Steroids* 2011; 76: 877–84.
40 Wang G, Guo Y, Zhou T, Shi X, Yu J, *et al*. In-depth proteomic analysis of the human sperm reveals complex protein compositions. *J Proteomics* 2013; 79: 114–22.
41 Baldi E, Luconi M, Muratori M, Marchiani S, Tamburrino L, *et al*. Nongenomic activation of spermatozoa by steroid hormones: facts and fictions. *Mol Cell Endocrinol* 2009; 308: 39–46.
42 Girard BJ, Daniel AR, Lange CA, Ostrander JH. PELP1: a review of PELP1 interactions, signaling, and biology. *Mol Cell Endocrinol* 2014; 382: 642–51.
43 La Sala G, Farini D, De Felici M. Rapid estrogen signalling in mouse primordial germ cells. *Exp Cell Res* 2010; 316: 1716–27.
44 Li X, Li H, Jia L, Li X, Rahman N. Oestrogen action and male fertility: experimental and clinical findings. *Cell Mol Life Sci* 2015; 72: 3915–30.

**Supplementary Table 1: Demographic data and comparison of semen characteristics**

| | *Normal semen (n=17)* | *Abnormal semen (n=26)* |
|---|---|---|
| Age (year) | 31.9±6.5 | 33.6±6.7 |
| Sperm concentration ($\times 10^6$ ml$^{-1}$) | 87.6±18.7 | 18.3±21.3 |
| Total sperm number ($\times 10^6$) | 370.1±178.3 | 63.3±66.3 |
| Progressive motility (%) | 37.1±2.9 | 20.2±10.3 |
| Total motility (%) | 72.1±4.9 | 52.2±16.9 |
| Sperm morphology (%) | 31.0±8.2 | 2.2±0.7 |
| Viability (%) | 86.5±3.5 | 70.2±12.6 |

All the data are presented as mean±s.d. s.d.: standard deviation

**Supplementary Table 2: Statistical analysis results of proline-, glutamic acid-, and leucine-rich protein 1 mean fluorescence intensity values and semen parameters**

| *Parameter* | *Normal* | | *Abnormal* | | *P* |
|---|---|---|---|---|---|
| | *n* | *PELP1 expression (mean±s.d.)* | *n* | *PELP1 expression (mean±s.d.)* | |
| Sperm concentration ($\times 10^6$ ml$^{-1}$) | 25 | 44.12±10.31 | 18 | 67.32±23.31 | 0.0007[a] |
| Progressive motility (%) | 22 | 43.36±10.74 | 21 | 64.79±22.43 | 0.0005[a] |
| Total motility (%) | 37 | 51.20±17.18 | 6 | 70.03±31.52 | 0.2[b] |
| Total sperm number ($\times 10^6$) | 28 | 44.90±10.06 | 15 | 70.50±24.33 | 0.001[b] |
| Sperm morphology (%) | 17 | 42.78±11.77 | 26 | 61.05±21.70 | 0.001[b] |
| Viability (%) | 40 | 53.62±20.61 | 3 | 56.68±20.36 | 0.8[b] |

*n*: number of valid cases for parameter described in row; samples which did not meet the criteria for at least one standard WHO parameter were classified as abnormal. [a]Welch's *t*-test; [b]Mann–Whitney U-test. s.d.: standard deviation; PELP1: proline-, glutamic acid-, and leucine-rich protein 1; WHO: World Health Organization

**Supplementary Table 3: Correlation between semen parameters and proline-, glutamic acid-, and leucine-rich protein 1 expression**

| *Parameter* | *R* | *P** |
|---|---|---|
| Sperm concentration | –0.59 | <0.0001 |
| Progressive motility | –0.51 | <0.0005 |
| Total motility | –0.57 | 0.0001 |
| Total sperm number | –0.41 | 0.006 |
| Sperm morphology | –0.56 | <0.0001 |
| Viability | –0.43 | 0.004 |

*Significance level of *P* value. *R*: Spearman's rank correlation coefficient value; PELP1: proline-, glutamic acid-, and leucine-rich protein 1