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GAMETE BIOLOGY

WBP2NL/PAWP mRNA and protein expression in sperm cells are not related to semen parameters, fertilization rate, or reproductive outcome

T. Freour[1,2,3,4] · M. Barragan[1] · A. Ferrer-Vaquer[1] · A. Rodríguez[1] · Rita Vassena[1]

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Abstract
Purpose WBP2NL/PAWP, a protein found in the post-acrosomal region of mammalian spermatozoa, has been proposed as a sperm-borne oocyte-activating factor (SOAF) contributing to Ca^{2+} release within the oocyte and subsequent fertilization and embryo development. However, its relevance as either a diagnostic or a prognostic marker of fertilization failure has been questioned in the recent literature. We analyzed WBP2NL/PAWP gene and protein expression level and localization in patients without previous intracytoplasmic sperm injection (ICSI) cycles in order to assess its association with both sperm characteristics and ability to fertilize.
Methods Raw frozen-thawed semen samples from 33 couples referred for oocyte donation were included in the study during 2015. Relative protein expression versus α-tubulin (western blot, WB), proportion of post-acrosomal WBP2NL/PAWP-positive spermatozoa over the total number of sperm cells (immunofluorescence), and WBP2NL/PAWP gene expression (RT-qPCR) were analyzed and correlated with semen analysis parameters (number, motility, and morphology) and with reproductive outcomes.
Results WBP2NL/PAWP protein was expressed in all samples with high variability: relative protein expression (1.77 ± 0.8, range [0.4–3.7]), proportion of positive cells (49.6% ± 16.1, range [22–89]), and relative gene expression (7.3 ± 8.2). No significant correlation ($R^2 < 0.1$) was found between gene and protein expression, neither between WBP2NL/PAWP gene or protein expression, and fertilization rate or other reproductive outcomes (i.e., pregnancy). In contrast, we found significant correlation between sperm morphology and WBP2NL/PAWP semiquantitative analysis in WB ($r = -0.42$, $p < 0.05$) and for sperm motility and WBP2NL/PAWP expression in IF ($r = 0.52$, $p < 0.05$).
Conclusion Taken into account that WBP2NL/PAWP gene and protein levels and distribution did not correlate with fertilization rates, this study questions the interest of WBP2NL/PAWP protein and gene expression analysis in sperm cells as a prognostic factor for the outcome of ICSI cycles. Larger studies focusing on WBP2NL/PAWP protein and gene expression are needed in order to evaluate the role of WBP2NL/PAWP as a prognostic factor for ART.

Keywords Sperm · WBP2NL/PAWP · Fertilization · ICSI · Oocyte donation · Gene expression

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✉ Rita Vassena
rvassena@eugin.es

[1] Clínica Eugin, Traversera de les Corts, 322 - 08029 Barcelona, Spain

[2] Service de médecine de la reproduction, CHU de Nantes, 44093 Nantes, France

[3] Inserm UMR1064 – ITUN, 44000 Nantes, France

[4] Faculté de médecine, Université de Nantes, 44000 Nantes, France

Introduction

The advent of intracytoplasmic sperm injection (ICSI) in the 1990s has revolutionized the treatment of human infertility by assisted reproductive technology (ART). Although ICSI was initially developed to treat severe male infertility, its use has increased rapidly and has been extended to other indications without a clear biological rationale [1]. Despite the artificial bypass of gamete interaction, fertilization and embryo development still

do not reach 100% in ICSI cycles, and approximately two thirds of all ICSI cycles fail to produce a viable pregnancy [2]. Successful fertilization in mammals depends on the intracellular release of Ca^{++} elicited by one or several sperm-borne oocyte-activating factors (SOAFs), whose identity and mechanisms of action are still highly debated [3]. A quantitative and/or qualitative defect in one or several SOAF(s) should thus result in fertilization failure (FF) or affect embryonic development, regardless of the parameters of conventional semen analysis. Phospholipase C-zeta (PLCζ) has been reported to be the most critical SOAF candidate, although whether PLCζ is the only SOAF is still debated [4–6].

More recently, post-acrosomal WW domain-binding protein (PAWP), also known as WW domain-binding protein 2 N-terminal Like (WBP2NL), a protein located in the post-acrosomal sheath region of the perinuclear theca, has been proposed as a putative SOAF [3, 7], although its capability to initiate oocyte activation and fertilization has been questioned [8–11].

Despite extensive debate on the topic, only a handful of clinical studies have reported so far on the predictive value of SOAF candidates for successful fertilization in ART cycles. For example, the clinical interest of PLCζ as a prognostic factor of fertilization in ICSI cycles has not been confirmed owing to the large variance of its total levels and localization pattern in sperm cells [5]. Conversely, WBP2NL/PAWP has been reported to be significantly associated with fertilization success in animal models [12] and humans [13]. However, one of the two single available studies conducted in patients lacked critical information on male and female demographic, biological, and clinical characteristics, preventing from drawing firm conclusions on the potential relationship between the expression of a single protein, i.e., WBP2NL/PAWP, and ICSI cycle outcome. The oocyte donation model, where oocytes are donated by young fertile women, mitigates the bias associated with testing a heterogeneous infertile population and provides a fair basis for studies on male parameters and ART outcome.

Although spermatozoa are generally considered both transcriptionally and translationally quiescent, they do retain messenger RNAs (mRNAs), useful both for the spermatozoon itself and for the zygote after fertilization [14–16], with potential for being used in the assessment of male fertility [17]. Only one very recent study reported WBP2NL/PAWP mRNA expression in sperm cells in humans, but was conducted in globozoospermic patients, with potential technical issues concerning normalization and RNA integrity testing [18].

We conducted a prospective cohort study in which we analyze WBP2NL/PAWP mRNA and protein expression in couples referred for oocyte donation cycles to evaluate its relationship with sperm parameters and its possible use as a prognostic factor of both fertilization rates and clinical outcomes.

Materials and methods

Study population, ethical approval, and laboratory procedures

This study has been performed per Good Clinical Practice (CPMP/ICH/ 135/95) and per the ethical principles stated in the Declaration of Helsinki 1964, as revised in 2013. The protocol was approved by the local Ethical Committee for Clinical Research. Written informed consent was obtained from all patients.

The study cohort consisted of 33 couples referred for a cycle with donated oocytes. Semen samples were obtained by masturbation after 2–5 days of abstinence. After 30 min of liquefaction at room temperature, sperm samples were analyzed with a computer-assisted semen analyzer (Sperm Class analyzer, SCA Human Edition; Microptic S.L., Barcelona, Spain) and classified following WHO guidelines [19]. The ejaculate was then cryopreserved in straws with Sperm Cryoprotect II (Nidacon, Molndal, Sweden) and stored in liquid nitrogen up to sample preparation. After 10 min thawing at room temperature, spermatozoa were washed in PBS and split into three tubes for cell fixation, protein extraction, and immediate RNA extraction, respectively. No further sperm selection was performed for this study.

Twenty-three of the 33 couples decided to go forward with the oocyte reception cycle. In agreement with the legal requirement, all oocyte donors were between 18 and 35 years of age, with a BMI <30, had normal karyotype, and no evidence of systemic or reproductive conditions. All donors were negative for HIV, sexually transmitted diseases, and hepatitis C and B. Further, 23 of 25 donors were of proven fertility, having either had a child themselves or having provided a successful pregnancy in a cycle with their oocytes. Finally, as per clinic protocol, all donors underwent the same stimulation protocol, consisting of a GnRH antagonist protocol with GnRH agonist trigger in all cases, and underwent ovum pick-up strictly 36 h after triggering.

Briefly, all mature oocytes obtained after denudation in hyaluronidase (HYASE-10X®, Vitrolife, Göteborg) were inseminated by ICSI, following standard protocols [20, 21]. After insemination, oocytes were cultured independently in 25 μl drops of G1® medium (Vitrolife, Göteborg) covered with mineral oil (OVOIL®, Vitrolife, Göteborg); fertilization was assessed 16–19 h post ICSI by visualization of two pronuclei and two polar bodies. Embryos were transferred to the uterus of the recipient on days (D) 2 to 5 of embryo culture,

depending on medical indication and patient preference. At day 3 of embryo culture, G1 medium was replaced by G2 (G-2®, Vitrolife, Göteborg). All embryos were morphologically scored and ranked using a scale 1 to 10 of combined embryo score [22], considering number and symmetry of blastomeres, and percentage of embryo fragmentation, where 10 corresponds to a perfect score and 2 points are deducted for cell number irregularities (e.g., any number of cells different from 4 to 6 for D2 and 6–10 for D3) and 2 points for cell asymmetries (e.g., four cells of uneven size or three cells of even size); 1 more point can be subtracted for any percentage of fragmentation higher than 5%. The highest scoring embryos were selected for transfer, which was carried out with a hyaluronan-rich transfer medium (UTM®, Origio, Denmark). Biochemical pregnancy was evaluated by β-hCG concentration in blood 14 days after embryo transfer. Implantation failure was defined by negative β-hCG test in blood 14 days after embryo transfer. Clinical pregnancy was determined by ultrasound examination showing a gestational sac with heartbeat at 6–7 weeks of amenorrhea.

Western blotting

Protein expression of WBP2NL/PAWP was analyzed as follows: at least 3×10^6 spermatozoa were centrifuged at 15,000*g* for 2 min, washed in PBS, before resuspension in 50 to 100 μl of Laemmli buffer and lysis by 3 cycles of freezing/boiling (−20 °C, 98 °C). In each case, sperm extract corresponding to 500,000 cells was applied on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were then transferred on PVDF membranes (Millipore, USA). Blots were blocked in 5% non-fat milk powder in Tris-buffered saline-Tween 0.1% (TBS-T) and probed with 1:1000 dilution of a rabbit anti-human WBP2NL/PAWP polyclonal antibody (ab170115, Abcam, USA) in blocking buffer at 4 °C overnight, following by incubation in secondary HRP-conjugated anti-rabbit antibody (NA934, Amersham, USA) diluted 1:10,000. A mouse anti-human-tubulin antibody (T6199, Sigma, USA) was then used on the same membranes to normalize the signal (dilution 1:2000). The immunoreactivity was detected with the use of chemiluminescent substrate (Luminata Classico, Millipore) and exposure to X-ray films. Due to the impossibility to use a blocking peptide, validation of the polyclonal antibody used (anti-human-WBP2NL/PAWP) was performed comparing spermatozoon extracts with OV-90 cell extract, showing bands with apparent MW of 35 kDa (upper band), 31 kDa (mid-band), and 28 kDa (lower band), which should correspond to WBP2NL/PAWP isoform 1 (theoretical MW = 31.9 kDa), WBP2 (theoretical MW = 28.1 kDa; 49% identity with WBP2NL/PAWP N-terminus), and WBP2NL/PAWP isoform 2 (theoretical MW = 23.7 kDa), respectively. In agreement, relative semi-quantification of WBP2NL/PAWP expression was performed by a comparative densitometry analysis using upper and lower bands shown in WB with ImageJ software.

Immunofluorescence staining

For immunofluorescence (IF), at least 10^6 spermatozoa were washed and fixed in PBS containing 4% paraformaldehyde (PFA, Sigma, Saint Louis, MO, USA) for 20 min at room temperature. Fixed sperm cells were loaded on poly-lysine-coated slides, permeabilized in 0.5% Triton X-100 in PBS for 15 min, blocked in 3% BSA for 1 h in PBS, and incubated overnight at 4 °C with 500 μg/ml of anti-human-WBP2NL/PAWP antibody in 0.05% BSA/PBS (dilution 1:200) (ab170115, Abcam, USA). Samples were then incubated in 5 μg/ml of secondary antibody (Alexa Fluor 568 F [Ab']2 fragment goat anti-rabbit IgG; Invitrogen, Paisley, UK). Slides were counterstained with 20 μg/ml FITC-PNA and 2 μg/ml Hoechst-33342 (Sigma) for 15 min at 37 °C in the dark. The presence and localization of WBP2NL/PAWP in sperm cells was determined by laser scanning confocal microscopy (Sp5, Leica, Heidelberg, Germany) at ×63 magnification. Images were analyzed with ImageJ software and the proportion of cells showing WBP2NL/PAWP staining (acrosomal or post-acrosomal) was calculated both in all sperm cells with stained nucleus and in cells with acrosome status visible (either intact or reacted).

RNA isolation and real-time quantitative RT-qPCR

When at least 10 million sperm cells were available after protein extraction and cell fixation, total RNA was extracted with phenol-free RNeasy mini kit (Qiagen, Hilden, Germany) followed by DNase treatment I (DNA-Free; Ambion, Austin, TX, USA) according to manufacturer's instructions. cDNA synthesis was performed as previously described [23]. SsoAdvanced™ Universal SYBR® Green Supermix (BioRad, Hercules, CA, USA) was used for qPCR according to manufacturer's instructions. All qPCR reactions were performed in triplicate, including no template and minus reverse transcription controls, as described before [23]. WBP2NL/PAWP gene expression was analyzed with primers designed on Primerdesign website (Table 1). The specificity of qPCR results was confirmed by agarose gel electrophoresis for single band of expected size of amplicon. Additionally, PCR fragments were purified with Gel Extraction Kit (QIAGEN) and the sequence determined by BigDye Terminator v3.1 at Sanger ABI 3730xl (GATC Biotechnologies AG, Germany) and analyzed with Chromas Software (Technelysium Ltd., Australia). BLAST analysis was performed against the published sequence of the genomic *WBP2NL/PAWP* locus complete sequence.

Table 1 Primer sequences used for quantitative PCR

Gene	Accession no.	Gene ontology (GO)	Primer (5′-3′)	Amplicon size (bp)
PAWP	NM_152613		F: CTCACTTCATACCGGGTGATTTT R: TTCGTCATCAGATCAAATGGCAT	89
RPLP1	BC003369	GO:0000184	F: CACGACGATGAGGTGACAGT R: CAGATGAGGCTCCCAATGTT	134
RPLP2	BC007573	GO:0000184	F: GAAGATCTTGGACAGCGTGG R: ACCAGCAGGTACACTGGCA	133
RPL13A	NM_012423	GO:0000184	F: CCTGGAGGAGAAGAGGAAAGAGA R: TTGAGGACCTCTGTGTATTTGTCAA	125

RPLP1, RPLP2, and RPL13A were chosen as reference genes (RGs) according to the type of sample and extraction method [23] (Table 1). Results were normalized applying geNorm algorithm [24].

Statistical analysis

WBP2NL/PAWP protein and gene expression was compared between patients with normal and patients with abnormal sperm analysis parameters, and between patients with successful clinical outcome (pregnancy) and those failing in achieving pregnancy. The correlation between each sperm parameter and WBP2NL/PAWP protein or gene expression was also calculated. The correlation of WBP2NL/PAWP protein and gene expression with fertilization rate and average embryo quality score was also studied. Finally, WBP2NL/PAWP protein and gene expression was compared between patients with successful oocyte donation cycle and those failing in achieving pregnancy. Medcalc software version 15.11.4 was used for statistical analysis. p value <0.05 was considered statistically significant.

Results

A total of 33 patients were included in the study, and 23 of them performed oocyte reception cycle with their partner at the time of the analysis. Demographic data, semen parameters, and reproductive outcome are reported in Table 2 and S1.

WBP2NL/PAWP protein was expressed in all samples with a high variability (mean relative expression in WB 1.77 ± 0.8 arbitrary units (AU), range [0.4–3.7] (Figure S1). The average WBP2NL/PAWP-positive sperm cells by IF was 49.6% ± 16.1 (range [22–89]); WBP2NL/PAWP acrosomal staining was found in 22.1% ± 11.6 [2–55] of all sperm cells, and post-acrosomal staining was found in 27.5% ± 15.1 [9–64] of sperm cells (Fig. 1). Some cells presented unspecific tail staining. The analysis of WBP2NL/PAWP expression by IF was significantly different between sperm cells with intact acrosome and those with reacted acrosome, with a significant decline in acrosome staining and a significant increase in post-acrosomal staining in acrosome-reacted cells compared to acrosome intact cells (5.1% ± 8.2 vs 50.5% ± 20 and 48.7% ± 22.3 vs 20.4% ± 15.7, respectively, $p < 0.05$). No significant correlation was found between WBP2NL/PAWP WB expression levels and the proportion of WBP2NL/PAWP-positive cells ($R^2 = 0.04$, $p > 0.05$).

RNA extraction could be performed in 20 patients. WBP2NL/PAWP mRNA was expressed in all tested patients with high variability (Figure S2). Mean mRNA expression after normalization with geNorm algorithm was 7.3 ± 8.2 AU, range [0.2–13.7]; mRNA expression levels were not significantly correlated with either WBP2NL/PAWP WB protein level ($r = -0.08$, $p > 0.05$) or proportion of WBP2NL/PAWP-positive cells in IF ($R^2 = 0.05$, $p > 0.05$).

Mean WBP2NL/PAWP protein and mRNA expression was comparable between normozoospermic patients ($n = 26$) and non-normozoospermic patients ($n = 7$) (mean relative expression in WB for isoform 1, apparent MW = 35 kDa, 1.77 ± 0.7 AU vs 1.86 ± 0.8 AU, respectively, $p > 0.05$; mean proportion of WBP2NL/PAWP-positive cells in IF 49.8% ± 16.7 vs 53.7% ± 14.8, respectively, mean gene expression 8.4 ± 8.3 AU vs 3.8 ± 4, respectively, $p > 0.05$) (Table S2). However, when WBP2NL/PAWP isoform 2, apparent MW = 28 kDa was analyzed, statistically significant differences were observed (mean relative expression in WB 0.07 ± 0.1 AU vs 0.23 ± 0.2 AU, respectively; $p < 0.05$) (Figure S4A). No significant correlation was found between WBP2NL/PAWP mRNA or protein expression and sperm parameters, i.e., sperm concentration, total motility (progressive + non-progressive sperm cells), or morphology, except for sperm morphology and WBP2NL/PAWP (isoform 1, apparent MW = 35 kDa) semiquantitative analysis in WB ($r = -0.42$, $p < 0.05$) and for sperm motility and WBP2NL/PAWP expression in IF ($r = 0.52$, $p < 0.05$) (Figure S3 and Figure S4B). No significant correlation was found with fertilization rate (Fig. 2) or embryo quality score (Fig. 3) ($R^2 = 0.03$, $p > 0.05$). In addition, polynomial regression curves were applied and no significance was found for any of the coefficients. The same results were also observed when normozoospermic and non-normozoospermic patients were evaluated separately for WBP2NL/PAWP semiquantitative analysis in WB

Table 2 Demographic characteristics, semen parameters, and reproductive outcomes of patients included in the study. Values are presented as mean ± standard deviation [range]

Age (years)		41 ± 5 [33–58]
Body mass index (kg/m^2)		25.6 ± 3.3
Semen parameters	Volume of ejaculate (ml)	4.8 ± 2.0 [2–10]
	Sperm concentration (10^6/ml)	75.5 ± 46.6 [6–184]
	Total sperm cell number in the ejaculate (10^6)	337.9 ± 248.7 [50–1145]
	Progressive motility (%)	51.0 ± 18.2 [18–88]
	Non-progressive motility (%)	3.2 ± 2.2 [0.5–10]
	Immotile cells (%)	45.8 ± 18.3 [8–80]
	Round cells (10^6/ml)	0.1 ± 0.1 [0–0.3]
	Normal forms (%)	8.1 ± 4.3 [2–20]
Fertilization rate (%)		69.7 ± 20.7 [38–100]
Average embryo quality score (AU)		6.9 ± 1.2

(ANOVA, $p = 0.957$), but differences in distribution for mRNA expression (ANOVA, $p < 0.009$). WBP2NL/PAWP mRNA expression remained not significantly different when stratified according to pregnancy results (ANOVA, $p = 0.212$) while there is a difference in the value distribution (ANOVA, $p = 0.0032$) when protein expression was analyzed (Fig. 4).

Discussion

We report here for the first time the analysis of WBP2NL/PAWP gene and protein expression in sperm cells in a cohort of patients referred for oocyte donation cycles. We did not find any correlation between WBP2NL/PAWP expression and conventional semen parameters, or between WBP2NL/PAWP expression and oocyte donation cycle outcome.

To our knowledge, WBP2NL/PAWP protein analysis and its correlation with IVF outcome have only been reported twice [13, 18]. In the first study, the authors concluded that a significant correlation existed between sperm WBP2NL/PAWP protein levels and both fertilization rates and normal embryonic development after ICSI [13]. In the second recent study evaluating WBP2NL/PAWP, as well as two other SOAF candidates, i.e., PLCζ and truncated KIT, only globozoospermic patients were included, providing results that might not be applicable to the general male population [18]. We also chose to detect WBP2NL/PAWP protein by focusing on semiquantitative analysis methods, i.e., western

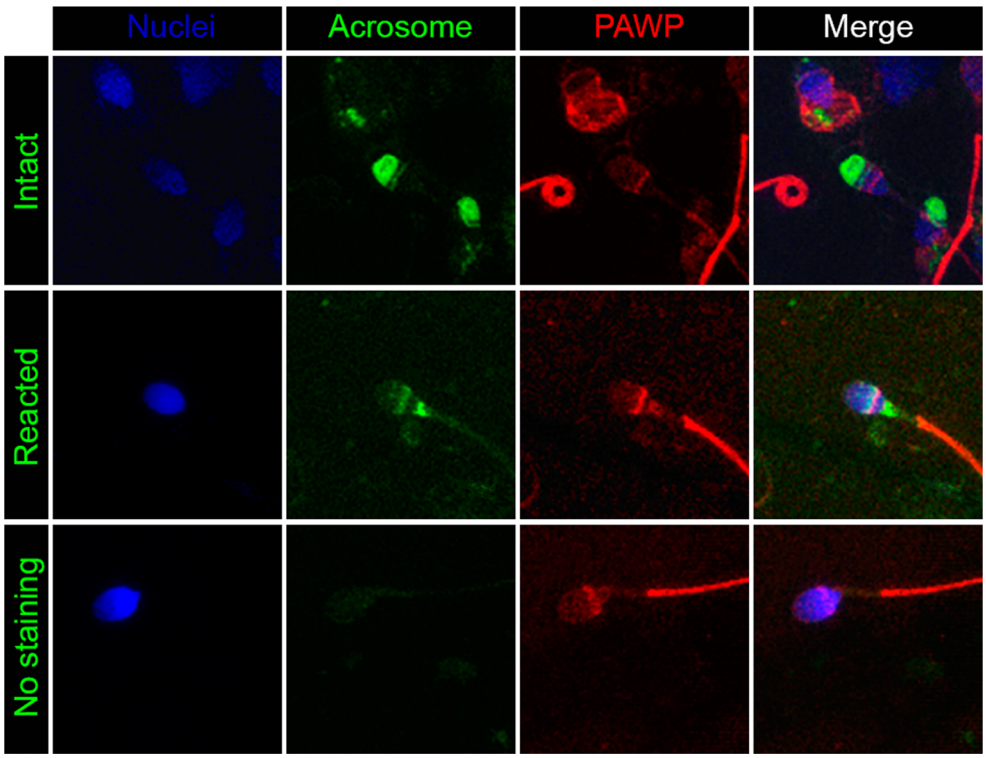

Fig. 1 Acrosomal status of sperm cells according to PNA labeling and classified as intact acrosome, reacted acrosome, or unlabeled acrosome, and localization pattern of WBP2NL/PAWP in the sperm head. Cells counterstained with Hoechst (nuclei) and PNA (acrosome)

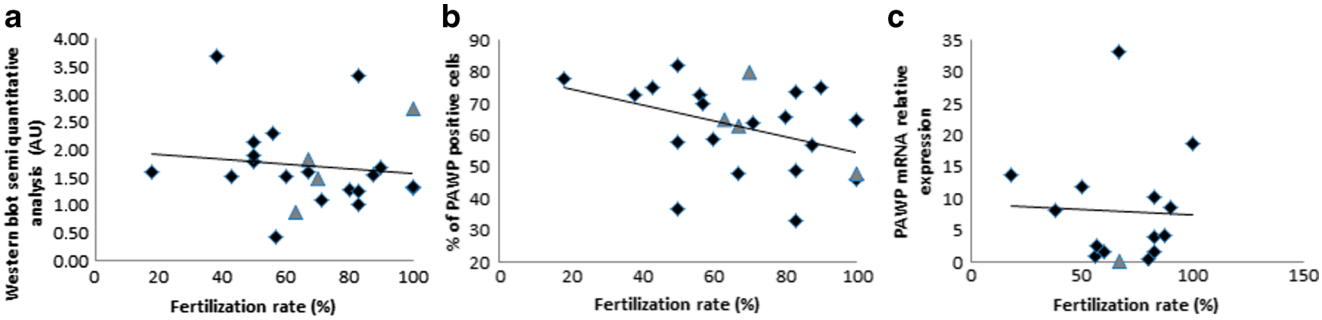

Fig. 2 Correlation of WBP2NL/PAWP protein expression measured with immunofluorescence (R^2 = 0.05) (**a**) or western blot (R^2 = 0.03) (**b**), and WBP2NL/PAWP gene expression (R^2 = 0.05) (**c**) with fertilization rate in oocyte donation cycle. Individual values are presented as *black diamond dots* and *gray triangle dots* for patients with normozoospermia and abnormal sperm analysis, respectively

blot and immunofluorescence. We do acknowledge that these methods suffer from some limitations, mainly related to the performance and specificity of the antibody used, and that results should be interpreted in the light of such limitations. As reported in Aarabi et al. [13], we found a high inter-patient variability in the expression of WBP2NL/PAWP. However, WBP2NL/PAWP expression was observed in all patients, whereas this was apparently not the case in Aarabi's study [13], suggesting perhaps the existence of a specific subgroup of patients not included in our study. We also found a significantly lower WBP2NL/PAWP acrosomal expression as well as an increase in the proportion of WBP2NL/PAWP post-acrosomal staining in acrosome-reacted sperm cells compared to those with intact acrosome. This could suggest a post-acrosomal delocalization occurring during acrosome reaction. However, this preliminary result should be interpreted and should be confirmed further on more samples in specifically designed studies, based on more sensitive technologies, such as electronic microscopy.

Whenever possible, we further added a WBP2NL/PAWP mRNA analysis in sperm cells. One issue when performing mRNA analysis is related to the choice of relevant reference genes and normalization algorithm. In this respect, we referred to our previously published work and chose the three most relevant reference genes in frozen sperm cells after phenol-free RNA extraction instead of the most widely used housekeeping genes such as GAPDH who were shown to perform poorly in these cells [23]. We observed that WBP2NL/PAWP gene expression in sperm cells was highly variable in patients. Furthermore, we also found that WBP2NL/PAWP gene and protein expression were not correlated. This should be expected, as sperm cells are terminally differentiated, with little if any active transcription, although de novo translation has been shown to occur at low level [15]. It can be assumed that the majority of stored mRNAs in sperm cells are produced during earlier stages of spermatogenesis and will either remain present until fertilization if they have a critical role or be progressively degraded [25]. In this view, the sperm cell's mRNA content might not be stable, but changing over time. It is currently impossible to know how long a sperm cell has been matured in the epididymis to interpret its dynamic mRNA content. Therefore, the heterogeneity of sperm cells' population, along with the impossibility to anticipate mRNA half-life in sperm cells, makes any attempt to correlate mRNA expression with protein expression and further biological processes imprecise at the very least. Moreover, whether WBP2NL/PAWP mRNA expression in sperm cells can be related to a biological process after fertilization remains to be studied. Only one very recent study also reported WBP2NL/PAWP gene analysis in human sperm cells [18]. The authors concluded that its gene expression was lower in globozoospermic patients than in fertile controls,

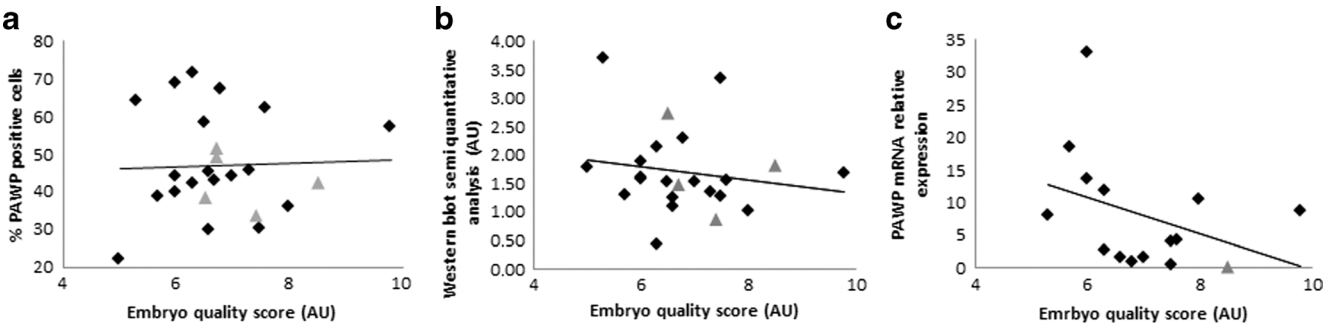

Fig. 3 Correlation of WBP2NL/PAWP protein expression measured with immunofluorescence (**a**) or western blot (**b**), and WBP2NL/PAWP gene expression (**c**) with average embryo quality score in oocyte donation cycle. Individual values are presented as *diamond dots* and *triangle dots* for patients with normozoospermia and abnormal sperm analysis, respectively

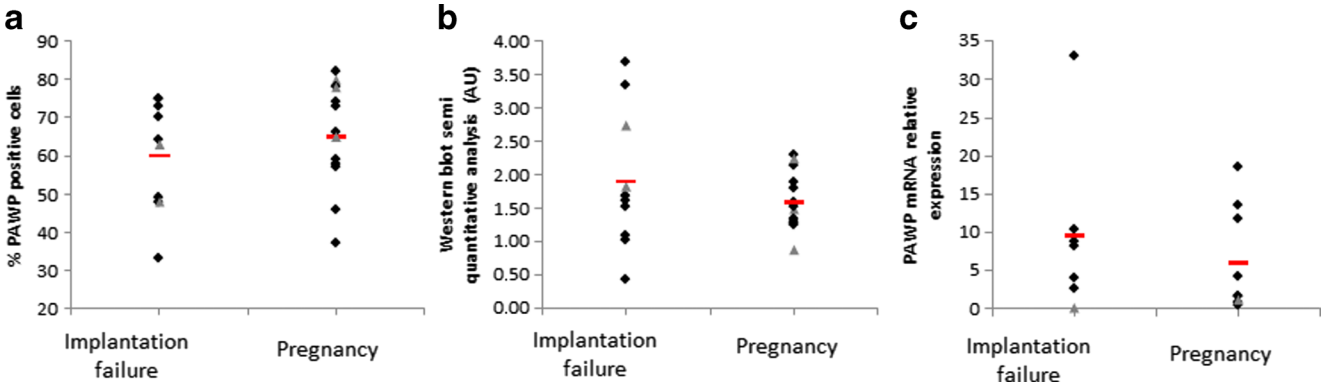

Fig. 4 Comparison of the respective proportion of WBP2NL/PAWP positive cells (**a**), WBP2NL/PAWP protein expression in WB (**b**), and WBP2NL/PAWP gene expression (**c**) in implantation failure and pregnancy groups. Results are presented as individual values (*black diamond* and *gray triangle* for patients with normozoospermia and abnormal sperm analysis, respectively) and mean (*red line*). All comparisons were not statistically significant ($p > 0.05$)

although this interesting study could suffer from a few technical biases such as the normalization method used based on GAPDH, a transcript which lacks stability in sperm cells [23]. Moreover, the specificity of PCR amplification did not seem to have been checked by sequencing.

Despite the use of oocyte donation cycles which mitigate the female factor bias, and the fact that, indeed, 23 out of 25 donors had proven fertility, correlations between the expression of a unique sperm protein in a heterogeneous population of cells and ICSI outcome after injection of just one cell should be interpreted carefully. The difficulty of translating aggregate results in heterogeneous cellular populations to one cell performance highlights the need for accurate non-invasive methods to assess sperm quality.

WBP2NL/PAWP is known to be progressively assembled during spermatogenesis [26], but whether this protein is involved in sperm maturation and has a structural role remains unknown. Although sperm parameters appear to remain unchanged in PAWP-KO mice [10], a report in human suggested a correlation between WBP2NL/PAWP expression and sperm morphology, although no precise data was given [13]. We did not find any correlation between the sperm parameters analyzed and WBP2NL/PAWP mRNA or protein expression, which was comparable in patients with normal and abnormal sperm parameters.

Recently, a study conducted on WBP2NL/PAWP null mice demonstrated that depletion of WBP2NL/PAWP did not alter Ca^{2+} oscillations or subsequent embryo development after ICSI, suggesting that WBP2NL/PAWP is not critical for fertilization in mouse [10]. Although this study provides strong arguments against the role of WBP2NL/PAWP as the unique SOAF, the KO construct model allowed for the expression of part of WBP2NL/PAWP, although the suspected active domain was removed. As the mechanism of action of WBP2NL/PAWP has not been deciphered yet, one might be cautious in considering this a definitive demonstration of the absence of role for WBP2NL/PAWP in fertilization.

We do recognize some limitation of the current study, specifically those related to the technical limitations given by the use of immune fluorescence over, for instance, cytometry; furthermore, the use of whole ejaculate rather than a sample following sperm selection might make the results of our study harder to correlate with reproductive outcomes. Although our study was not designed to determine whether WBP2NL/PAWP is a SOAF or not, our results question the possibility of using WBP2NL/PAWP protein and gene expression in sperm cells as a prognostic factor of fertilization in ICSI cycle. Larger studies focusing on WBP2NL/PAWP protein and gene expression are needed in order to evaluate the role of WBP2NL/PAWP as a prognostic factor for ART.

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Compliance with ethical standards

Informed consent Informed consent was obtained from all the couples involved in the study.

Ethical approval All procedures performed were in accordance with the ethical standards of the institutional research committees and with the 1964 Helsinki declaration and its subsequent amendments. This study has been performed per Good Clinical Practice (CPMP/ICH/ 135/95). The protocol was approved by the local Ethical Committee for Clinical Research.

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

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