PLC ζ sequence, protein levels, and distribution in human sperm do not correlate with semen characteristics and fertilization rates after ICSI

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Abstract

Purpose Sperm-borne PLC ζ protein induces Ca²⁺ oscillations in the oocyte and is believed to play a major role during oocyte activation. However, its implication in fertilization failure following ICSI is still debated. We analyzed PLC ζ gene sequence, protein expression level, and localization in both patients with previous failed fertilization by ICSI and sperm donors with proven fertility in order to assess the association of PLC ζ with both sperm characteristics and ability to fertilize. **Methods** Semen from 15 patients and 13 sperm donors with proven fertility was included in the study. Analysis of the PLC ζ gene sequence, protein expression through Western blot, and protein localization by immunofluorescence were performed. **Results** Two patients with total fertilization failure presented mutations in heterozygosis in the PLC ζ gene. Comparison with donor sample sequences displayed comparable SNP allele frequency. Distribution pattern of PLC ζ did not vary significantly between donor and patient samples. Levels of PLC ζ protein in sperm cells showed an interindividual variability both in patient and donor samples. Several SNPs previously reported in infertile patients were also present in fertile men.

Capsule PLC ζ seems to be a necessary but not sufficient factor in determining the molecular pathway involved in oocyte activation.

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Conclusion Failed fertilization occurs even when levels and distribution of PLC ζ protein are within normal range. PLC ζ seems to be a necessary but not sufficient factor in determining the molecular pathway involved in oocyte activation.

Keywords Sperm biomarker · PLC ζ · Fertilization failure · Oocyte activation · ICSI

Introduction

The fusion of the sperm membrane with the oocyte during fertilization triggers a molecular cascade that activates the oocyte, resumes meiosis, and initiates embryonic development [1]. Oocyte activation is induced by serial oscillations in the concentration of free cytosolic Ca²⁺, mainly released from the endoplasmic reticulum [2]. In mammals, these oscillations are triggered by a sperm-borne soluble factor that activates the phosphoinositide pathway, hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP₂) into diacyl-glycerol (DAG) and inositol 1,4,5-triphosphate (IP₃), which in turn elicits a transient Ca²⁺ release by binding to its receptor on the endoplasmic reticulum [3–6]. The sperm-specific phospholipase C zeta 1 (PLC ζ) has been identified as an oocyte activating factor in several species [7–11]. Several lines of evidence support the role of PLC ζ in inducing Ca²⁺ oscillation in oocytes; micro-injection of either PLC ζ complementary RNA (cRNA) or recombinant protein into bovine and murine oocytes induces Ca²⁺ oscillations and oocyte activation [8, 12–16]. Moreover, injection of human PLC ζ cRNA and recombinant PLC ζ protein into human oocytes triggers calcium oscillations and development to the blastocyst stage [9, 17]. On the other hand, injection of domain-deletion PLC ζ constructs leads to the inability of the protein to generate Ca²⁺ oscillations, while reduction of PLC ζ expression by RNA interference during

spermatogenesis hinders Ca^{2+} oscillations and subsequent oocyte activation [18, 19]. Furthermore, human sperm devoid of PLC ζ fail to induce Ca^{2+} oscillations [20], and two point mutations, H233L and H398P, have been characterized to affect PLC ζ functionality. Located at exons 6 and 11, respectively, these mutations disturb important functional domains (X and Y) and impair oocyte activation [16, 21–23].

Intracytoplasmic sperm injection (ICSI) is a widely used assisted reproduction technology (ART) to achieve fertilization by inserting the spermatozoon into the oocyte cytoplasm. Although ICSI bypasses all the steps of gamete interaction and fusion, total fertilization failure (FF) occurs in 1–3 % of all ICSI cycles [24, 25]. Oocyte activation failure is considered the main underlying cause of FF [25, 26]. Thus, to overcome FF, artificial oocyte activation (AOA) has been proposed, whereby the oocyte is placed in contact with an activation stimulus such as calcium ionophore at the time of fertilization [27].

The molecular pathogenesis underlying FF remains unclear. It has been proposed that patients who exhibit FF may have abnormal levels of PLC ζ and, therefore, may be unable to trigger oocyte activation [16, 20, 21, 28, 29]. A confounding factor in the definition of the PLC ζ role in FF is the use in most reported cases of the couple's own oocytes, rather than a donor of proven fertility, with the associated risk of an oocyte-borne alteration affecting the results.

To establish if FF in ICSI can be associated with aberrant levels and/or localization patterns of PLC ζ , we analyzed the PLC ζ gene and protein products in 15 FF cases and compared them with donors of proven fertility.

Materials and methods

Ethical considerations

Permission to conduct this study was obtained from the Ethical Committee for Clinical Research. Before inclusion in the study, all men were given an information sheet explaining the purpose of the test and the aim of the investigation; the patients could discuss their participation with a physician unrelated to the study, and written consent to participate was obtained from all patients.

Study population

The study period was June 2014 to May 2015. Inclusion criteria for the analysis of PLC ζ were low fertilization (≤ 20 %) to complete FF after at least one cycle of ICSI, independently of sperm parameters. The control group was composed by sperm donors of proven fertility.

All semen samples were analyzed according to World Health Organization (WHO) recommendations. Sperm count,

motility, and morphology were assessed by Sperm Class Analyzer[®] (Microptic), and samples were frozen according to the manufacturer's instructions (CryoProtecII, Nidacon) for future use.

The stimulation protocol for oocyte donors was a short antagonist protocol and GnRH agonist trigger in all cycles. Female patient stimulation was either long agonist or antagonist protocol depending on medical history, clinical characteristics, and ovarian reserve.

Genomic analysis of PLC ζ

Sperm was washed and at least 3×10^6 spermatozoa were centrifuged at 15,000g for 2 min at room temperature. Genomic DNA (gDNA) was isolated with QIAamp DNA Blood Mini Kit (QIAGEN, Germany) following the manufacturer's instructions. Amplicons of PLC ζ were amplified by polymerase chain reaction (PCR) with Phusion High-Fidelity DNA polymerase (NEB, USA) using previously described primer pairs [20, 21, 30].

PCR fragments were purified with a gel extraction kit (QIAGEN), and the sequence was 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 PLC ζ locus (*Homo sapiens* 12 BAC RP11-361114; Roswell Park Cancer Institute Human BAC Library) complete sequence.

Expression and localization of PLC ζ

PLC ζ protein expression and localization were analyzed following previously described protocols [31]. At least 5×10^6 spermatozoa were centrifuged at 15,000g for 2 min, and the cell pellet was resuspended in 100 μl of complete Laemmli sample buffer at 98 °C and lysed by 3 cycles of freezing/boiling (−20 °C, 98 °C). Cell lysates corresponding to 500,000 sperm cells were run on a 10 % sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE); separated proteins were transferred onto PVDF membranes (Millipore, USA).

For immunoblotting, blots were blocked in 5 % milk powder in PBST and incubated with 10 $\mu\text{g/ml}$ of an anti-human-PLC ζ antibody (pab0367-P, Covalab, France; batches of antibody 12E1, 14F2, and K1F1) in blocking buffer at 4 °C overnight, followed by several washes and incubation in secondary antibody (NA934, Amersham, USA) diluted 1:10,000. After development, blots were sequentially incubated with an anti- α -tubulin antibody (T6199, Sigma, USA) as a loading control. Each patient sample was run at least twice, and each donor sample was run at least three times.

Immunofluorescence was carried out as reported previously [31]. Briefly, at least 10^6 spermatozoa were fixed in PBS

containing 4 % paraformaldehyde (PFA) (Sigma, USA), centrifuged at 800g for 10 min, and washed in PBS. Samples were then permeabilized in 0.5 % Triton X-100 in PBS, blocked in 3 % BSA in PBS, and incubated overnight at 4 °C with 25 µg/ml of anti-human-PLCζ antibody. Samples were subsequently incubated in 5 µg/ml of secondary antibody (Alexa Fluor 568 F [ab']₂ fragment of goat anti-rabbit IgG; Invitrogen, UK). Slides were counterstained with 20 µg/ml FITC-peanut agglutinin (PNA) (Sigma, USA) and 2 µg/ml Hoechst-33342 (Sigma, USA) for 15 min at 37 °C in the dark. The localization of PLCζ, PNA, and the sperm nucleus was analyzed by confocal microscopy (LSM780 Zeiss, Germany). At least 25 optical fields were analyzed; if cell count was lower than 100 cells, up to 40 optical fields were observed. Acrosome status was classified as intact, reacted, or unlabeled according to PNA staining (Fig. 1a). The localization patterns of PLCζ expression were based on the previous work of Kashir and colleagues [32] with minor changes. Briefly, we assigned localization pattern into three categories: acrosomal (acrosomal alone, acrosomal + equatorial, acrosomal + postacrosomal, and all three together), equatorial (equatorial alone and equatorial + postacrosomal), and postacrosomal (Fig. 1b).

Statistical analysis

One-way analysis of variance (ANOVA) was performed to compare the distribution of PLCζ in acrosome intact cells from patients and donors. Level of significance was $p \leq 0.01$. Pearson correlation coefficient was calculated to measure correlation between the distribution patterns of PLCζ and the different sperm parameters (motility, concentration).

Results

Fifteen patients consented to participate in the study after a cycle with low fertilization or complete fertilization failure, of which six underwent ICSI with their partner oocytes and nine with donor oocytes (Table 1). All oocyte donors and three of the six female partners had proven fertility.

Semen parameters and ICSI cycles

Patients' semen parameters ranged from normozoospermia to oligoasthenoteratospermia (Supplementary Table 1). Regardless of the semen diagnosis, all patients had at least one ICSI cycle with low (≤ 20 %) to complete FF (Table 1). Most fertilized oocytes from low fertilization cycles reached D2/D3 of development and were suitable for transfer. Sperm donor samples with high fertilization (≥ 60 %) were analyzed as controls (Supplementary Table 2).

PLCζ gene sequence

The PLCζ gene is localized on chromosome 12 reverse strand (12p12.3) and consists of 15 exons. Two patients with total fertilization failure presented mutations in heterozygosis in the PLCζ gene. One patient (P13) displayed the H233L mutation

Fig. 1 **a** Acrosomal status of sperm cells according to PNA labeling and classified as A' intact acrosome, A'' reacted acrosome, or A''' unlabeled acrosome. **b** Localization pattern of PLCζ in the sperm head. B' PLCζ expressed in the acrosomal region of the cell. Acrosomal expression of PLCζ is sometimes accompanied by equatorial and/or postacrosomal expression of PLCζ. Asterisk marks weak staining at the equatorial region. B'' White arrow marks equatorial staining of PLCζ. B''' Postacrosomal only expression of PLCζ. Cells counterstained with Hoechst (nuclei) and PNA (acrosome). Scale bar = 5 µm

Table 1 Summary of oocyte source, female fertility, outcome of FF ICSI cycles, and outcome of cycle undergone after a first FF cycle

Patient	Oocyte source	Female fertility state	Fertilization FF cycle	Embryos D2/D3 FF	Cycle post-FF	Fertilization cycle post-FF	Embryos D2/D3 cycle post-FF
P1	Donor	Proven	0/6	NA	AOA	7/8	7
P2	Donor	Proven	1/5	0	Change male gamete	5/5	4
P3	Donor	Proven	0/5	NA	Change male gamete	5/6	4
P4	Partner	Proven	0/4	NA	Change male gamete	6/8	6
P5	Partner	Unproven	0/9	NA	No cycle	NA	NA
P6	Donor	Proven	1/7	1	No cycle	NA	NA
P7	Donor	Proven	1/7	1	No cycle	NA	NA
P8	Partner	Unproven	2/13	0	Change female gamete	3/5	3
P9	Partner	Unproven	0/2	NA	No cycle	NA	NA
P10	Partner	Proven	0/2	NA	AOA	1/2	1
P11	Donor	Proven	0/7	NA	AOA	6/8	5
P12	Donor	Proven	2/11	2	Same gametes	7/8	6
P13	Partner	Proven	0/2	NA	No cycle	NA	NA
P14	Donor	Proven	1/6	1	No cycle	NA	NA
P15	Donor	Proven	0/7	NA	AOA	9/12	9

NA not applicable

(exon 6) in heterozygosis. Furthermore, a novel point mutation, R197H, located in exon 6 in the X catalytic domain was found in heterozygosis in patient P15. In donor control samples, the regions where SNPs were found in patients were sequenced, i.e., exons 1, 2, 3, 5, 8, 9, 11, 12, and 13 and their neighboring intronic regions, identifying the presence of several SNPs (Fig. 2, Table 2). Presence of the allelic variants for most of the SNPs sequenced was similar both in donors and patients. All donors were homozygous for the alternative variant *rs5796766*, while several patients displayed the reference allele either in heterozygosis (3/15) or homozygosis (2/15). On the other hand, all donors were homozygous for the reference variant of the novel SNP located in intron 3 previously reported in a FF patient [20]. In this case, only one patient (P15) displayed the alternative allele in heterozygosis. Furthermore, all donors showed the homozygous variant of the reference allele for SNP *rs10505830*, located in exon 13, while one patient (P15) was heterozygous for the SNP. This SNP, which was also previously described in a FF patient [20], codes for a serine-to-leucine missense mutation with unknown effect on gene functionality to date.

PLCζ protein distribution

Distribution of the PLCζ protein in the sperm head was examined in all cells as well as in different subpopulations according to their acrosomal status, which were divided into intact acrosome, reacted acrosome, and unlabeled acrosome cells (Fig. 1, Supplementary Tables 3 and 4). When considering the total cell population, PLCζ postacrosomal only staining prevailed slightly over the other localization patterns and

showed higher expression proportion when comparing donors with patients (Fig. 3, Supplementary Table 5). In intact acrosome cells, PLCζ localized to the acrosomal region in the majority of cells and to a lesser extent in the equatorial or postacrosomal only regions. The proportion of each pattern between patient and donor samples was not significantly different. On the other hand, in cells displaying a reacted acrosome or with no acrosomal staining, PLCζ protein was mainly observed in the postacrosomal only region. In these subpopulations as well, no significant difference was found between donors and patients.

No difference was found when comparing fertile donors with patients who were normozoospermic. However, significantly higher PLCζ acrosomal staining was observed when comparing donors and non-normozoospermic ($p=0.003$). Also, there was significant variation in PLCζ acrosomal localization among patients when comparing normozoospermic samples with non-normozoospermic ones ($p=0.008$).

Finally, correlations between sperm characteristics such as concentration and motility with PLCζ distribution patterns were calculated for the different patterns (Supplementary Table 6). Both in donors and patients, neither motility nor concentration was correlated with PLCζ distribution patterns.

PLCζ protein levels

PLCζ protein levels were analyzed by Western blot and compared to tubulin levels as normalizer. Donor and patient samples displayed a wide range of PLCζ expression (Fig. 4, Table 3, and Supplementary Fig. 1). No analysis of protein levels was performed on P12–P15 as they were poor quantity

Fig. 2 Overview of the human genomic locus containing PLC ζ gene on chromosome 12 (12p12.3) in reverse strand, and schematics of PLC ζ gene and the span of single-nucleotide polymorphisms (SNPs) (*circles*) and point mutations (*asterisk*) found in patient (*P*) and donor (*D*) sperm samples. Location of allelic variants for 13 different haplotype-tagging SNPs was indicated. After comparison with a reference sequence (*Homo sapiens* 12 BAC RP11-361114; Roswell Park Cancer Institute Human BAC Library), *white circles* represent presence of reference alleles in homozygosis, *gray circles* represent presence of reference and alternative allele in heterozygosis, and *black circles* represent presence of alternative alleles in homozygosis. In addition, point mutations in heterozygosis were indicated (*, c.1457C>T, p.S500L; **, c.854C>T, p.R197H; ***, c.962G>A, p.H233L)

samples. Of note, samples from donors 4 and 7 (D4 and D7) showed a particularly low amount of PLC ζ ; we have tracked the reproductive performance of these two donors in the clinic, and they had a fertilization rate of 76 and 68 %, respectively, with a pregnancy rate of 46 and 45 %, respectively. These values were similar to those of donors with higher PLC ζ signal.

Discussion

We show that failed fertilization can occur across a wide range of PLC ζ protein expression levels and distribution, comparable to fertile donors, and not directly related to sperm characteristics. As opposed to most reports of PLC ζ in FF, which examine patients with poor sperm parameters in autologous ICSI cycles [20, 21, 32, 33], we considered patients with both

normal and abnormal semen analysis and included oocyte donation cycles with donors of proven fertility, minimizing the bias of female factors that are critical in FF [34, 35].

PLC ζ sequence and fertilization ability

Since alterations in PLC ζ protein levels and functionality might be related to mutations of the PLC ζ gene [20, 21], we analyzed the gene sequence focusing on particular exons and the intronic regions surrounding them. We found two mutations in patient samples, one of them, H233L, previously described in a FF patient with globozoospermia and familial history [23]. It is worth noting that, as suggested by the authors, infertile patients heterozygote for only one loss-of-function mutation might display subfertility rather than infertility. Also located in exon 6, we describe for the first time an arginine to histidine substitution (R197H) found in

Table 2 Summary of position and annotation of SNPs found in the *PLCζ* locus in donor and patient samples. Distribution of SNP alleles is reported as homozygous or heterozygous either for the reference (REF) or alternative (ALT) allele

Position	SNP annotation	Donors (<i>n</i> = 13)			Patients (<i>n</i> = 15)		
		REF/REF	REF/ALT	ALT/ALT	REF/REF	REF/ALT	ALT/ALT
5' UTR	rs1075421; A/G	5	6	2	8	5	2
Intron 1	rs7133682; C/T	12	0	1	15	0	0
Intron 1	rs5796766; A/–	0	0	13	2	3	10
Intron 3	rs12300257; A/G	8	4	1	11	4	0
Intron 3	Novel SNP ^a C/T	13	0	0	14	1	0
Intron 5	rs10841078; C/G	9	0	4	7	2	6
Intron 8	rs11279217; –/TCCTCCTCC	1	0	12	1	0	14
Intron 12	Novel SNP ^b A/G	12	1	0	15	0	0
Intron 12	rs12826151; A/C	9	0	4	5	2	8
Intron 12	rs73060535; G/T	12	0	1	15	0	0
Intron 12	rs11044253; C/T	11	0	2	12	1	2
Exon 13	S500L; rs10505830; C/T	13	0	0	14	1	0
Intron 13	rs2306798; A/G	9	3	1	11	3	1

^a Novel SNP genome coordinates 12:18736192, GATCCTATCCTATCCGT [20]

^b Novel SNP genome coordinates 12:18694791, AGTACCTGAAAGATTAA [20]

heterozygosis in another patient. This novel mutation is located in the X catalytic domain of the protein; however, only functional analysis of the mutation will help elucidate its real effect on protein function. In addition to these mutations, we found several polymorphisms that have been previously reported in the *PLCζ* locus [20]. We report for the first time a series of polymorphisms present not only in patients but also in donors, thus potentially excluding their impact on protein function. It is important to mention that the frequency ratio of reference and alternative alleles for the different SNPs remained similar between patient and donor sequences. *rs5796766* displayed a different ratio of homozygous/heterozygous variants in donor samples compared to patients; *rs5796766* is located in intron 1 of *PLCζ*, on the reverse strand and in *lncCAPZA3-1.1* on the forward strand. The role of this long noncoding RNA is unknown, although it is located at 5' UTR of *CAPZA3* (capping protein (actin filament) muscle Z-line, alpha 3), which is localized in the sperm acrosome and plays a role in maintaining polymerized actin during spermatogenesis [36]. Although disruptions in the *CAPZA3* protein have been clearly linked to male infertility in mice [37], where the *report32* mutated mice, identified in the course of an ENU mutagenesis screen, present disruption of F-actin, the role of *lncCAPZA3-1.1* and its SNP variants has not been evaluated yet in male infertility and deserves further exploration.

Both samples from low fertilization cycles (<20 %) and TFF cycles displayed the presence of the same SNPs. Exceptions were the three point mutations found (S500L, R197H, H233L) which were only found in FF cycles.

However, without a deeper functional analysis, no conclusions can be drawn regarding the presence of these mutations and the fertilization ability of the samples.

PLCζ protein localization and fertilization ability

Analysis of PLCζ localization by immunofluorescence has shown a variable pattern of distribution among individuals. PLCζ expression was reported to be predominantly present in the equatorial segment of the sperm head, with subsidiary expression at the acrosome and postacrosomal regions in fewer cells [31]. Another study did not find any predominant pattern of localization in the control group but found a predominant pattern of postacrosomal localization in FF samples [32]. Moreover, a more recent report from the same group showed a correlation between PLCζ localization and low fertilization rate in ICSI cycles [33]. Our findings show that proportions of the different localization patterns vary depending on the acrosomal status of the cell. While previous studies have considered the expression pattern of PLCζ in all sperm cells, we took a different approach and we also considered different cell subpopulations according to their acrosomal status. In our study, we report a slightly predominant postacrosomal expression pattern when considering all cell populations, which was significantly different between donors and patients. Moreover, a higher proportion of PLCζ acrosomal staining in intact acrosome cells is in agreement with (Grasa et al. [31], who described an increase in postacrosomal region localization concomitant to acrosomal reaction, suggesting a dynamic pattern of expression of the protein during

Fig. 3 Distribution of PLC ζ staining expressed as percentage of cells in **a** all cells and **b** cells with intact acrosome for each localization pattern: acrosomal (A.1, B.1), equatorial (B.1, B.2), and postacrosomal (C.1, C.2) in donors (light gray squares) and patients (dark gray diamonds)

fertilization. Interestingly, the proportion of PLC ζ acrosomal expression varies significantly when comparing non-normospermic patients with donors and non-normospermic patients with normospermic ones, respectively. This might be due to the inclusion of four teratospermic patients among the non-normospermic pool that might account for the existence of defective acrosomes.

When we compared the cycles with fertilization below 20 % with TFF, even if postacrosomal expression was the predominant pattern of localization, both groups displayed a wide range of proportions (12 to 57 % approx. in both groups), suggesting that different patterns of protein distribution might not account for the difference between low fertilization and TFF. Furthermore, no correlation could be found between sperm characteristics and PLC ζ distribution in patient and

donor samples, suggesting that PLC ζ expression within the sperm cell might be independent of motility and concentration, although studies with a larger number of samples will be needed to clarify this point.

In addition to protein localization, we also sought to determine protein levels in patient as well as donor samples. Total PLC ζ levels in individual control samples measured by fluorescence intensity varied significantly; although FF patients exhibited significantly reduced total levels of PLC ζ compared with controls, all showed fluctuations, with some controls presenting levels similar to FF samples [32]. Accordingly, we show that individual donors display a wide range of PLC ζ expression levels. Besides, overall levels of total PLC ζ in patient samples also presented great variability.

Fig. 4 PLC ζ protein levels in donors and patient samples. Western blots showing PLC ζ and tubulin expression in total protein lysates of donor (**a**) and patient (**b**) samples. Blots were run in different days and successively hybridized for PLC ζ and tubulin. *Ast* asthenospermic, *OAT* oligoasthenoteratospermic, *OA* oligoasthenospermic, *T* teratospermic

Only patient samples with teratospermia, oligoasthenospermia, and oligoasthenoteratospermia showed considerable lower levels of protein expression. Interestingly, the ratio between tubulin and PLC ζ protein levels did not differ much from the range seen in normospermic sperm samples. As already postulated for immunofluorescence [32], quantitative PLC ζ measurement might not be clinically significant as indicator of fertilization ability.

Of 15 patients included in the study, 9 underwent a new ART cycle (Table 1), 3 of them opting for a double gamete donation; 4 couples underwent AOA while keeping the same female gamete, all restoring normal fertilization rates. Two couples rejected AOA (P8, P12) and the couple using the female partner's oocyte (P8) changed to a donor oocyte. Both cycles resulted in ≥ 50 % fertilization rate.

In summary, we have shown that failed fertilization after ICSI often occurs in the presence of normal levels of PLC ζ protein and normal expression pattern, not directly related to sperm characteristics, although PLC ζ alterations could be suspected in cases of FF clearly due to male factor. Although two mutations that might affect the catalytic domain of the protein have been found in two patients, molecular analysis of the gene sequence of donor and patient samples has ruled out the role of several SNPs previously reported as associated to FF. Even if the inclusion of donor oocyte cycles strengthens our conclusions, larger populations might be needed to draw stronger conclusions. Therefore, the application of PLC ζ as a clinical diagnostic/prognostic biomarker for oocyte activation capability and as a putative therapeutic agent to overcome FF still has its limitations. We do not imply, of course, that PLC ζ does not have a central role as sperm-borne activation factor; for instance, looking specifically at globozoospermic patients, Escoffier and colleagues described PLC ζ absence in nine couples where globozoospermic sperm presented homozygous deletion of DPY19L2 [38]. These

patients had very low to absent fertilization. Moreover, Kuentz and colleagues showed that in a series of 66 cycles, in case of mutations of DPY19L2, and in the absence of AOA, the fertilization rate remains very low. The fertilization rate was restored completely through AOA [39]. Although the function of DPY19L2 is unknown, the absence of the protein produces a destabilization of the link between the acrosome and the nuclear envelope, indicating that the subcellular location of PLC ζ in human sperm is consistent with its role as the sperm-borne activating factor.

What we do question, however, is the utility of an assay of PLC ζ in clinical cases of low and failed fertilization given its wide range of expression and distribution across fertilizing samples. Finally, our results suggest that PLC ζ functions as an absolutely necessary sperm-borne activation factor [40] but that other yet unknown components in the complex activation pathway, especially in the oocytes, make PLC ζ expression not sufficient to guarantee correct oocyte activation.

Table 3 Relative expression of PLC ζ /tubulin in patients and donors

Patients	Ratio	Donors	Ratio
P1	1.123	D1	1.113
P2	0.902	D2	1.788
P3	2.776	D3	0.380
P4	0.515	D4	0.038
P5	0.827	D5	0.657
P6	0.346	D6	0.223
P7	0.397	D7	0.174
P8	0.494	D8	0.877
P9	0.237	D9	0.332
P10	0.639	D10	0.706
P11	2.393	D11	0.381
		D12	0.651

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Compliance with ethical standards

Competing interests The authors declare that they have no conflict of interest.

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