

Title

Total levels, localization patterns and proportions of sperm exhibiting phospholipase C Zeta (PLCζ) are significantly correlated with fertilization rates following intracytoplasmic sperm injection

Running title

PLCζ and fertilization rates in ICSI

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29 **Conflict of interest**

30 None of the authors have anything to disclose

Capsule

Total levels, localization patterns and proportions of sperm exhibiting phospholipase C Zeta are significantly correlated with fertilization rates following ICSI, but not with fertilization rates following IVF.

Abstract

OBJECTIVE: To study the relationship of total levels, localization patterns and proportions of sperm exhibiting phospholipase C zeta with fertilization rates following *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI).

DESIGN: Laboratory study. Controls versus patients following IVF (n = 27) or ICSI (n = 17) treatment.

SETTING: University Fertility Centers

PATIENT(S): A total of 44 semen samples subjected to either IVF or ICSI treatment. Oocyte collection, ICSI or IVF, determination of sperm concentration and motility and immunocytochemical analyses of PLC ζ .

INTERVENTION(S): None

MAIN OUTCOME MEASURE(S): Percentages of sperm exhibiting PLC ζ

RESULTS: Significant positive correlation between ICSI fertilization rates and total levels, localization patterns and the proportion (percentage) of sperm exhibiting PLC ζ . Total levels, localization patterns, and the proportion of sperm exhibiting PLC ζ are correlated with fertilization rates for ICSI, but not for IVF.

CONCLUSION(S): Evaluating total levels, localization patterns and proportions of PLC ζ may represent a useful diagnostic tool for clinical purposes in males for which IVF is unadvised or has previously failed. This clinical study also further supports the fundamental role of PLC ζ in the oocyte activation process.

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Key words: *In Vitro* Fertilization, IVF; Intracytoplasmic Sperm Injection, ICSI; total
fertilization failure, TFF; Phospholipase C Zeta, PLCζ; Oocyte activation

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Introduction

Fertility disorders affect 25% of couples worldwide and the increasing prevalence of infertility has led to the rapid growth and development of assisted reproductive technology (ART) (1). Indeed, ART treatments such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are now successful techniques to combat infertility, such that 2% of live births per year in the UK now result from the use of ART (2).

While IVF is a suitable treatment for many infertile couples, problems can still occur and lead to total fertilization failure (TFF) in 4-7% of cases (3). In this scenario, ICSI, which involves the direct injection of a single sperm into the oocyte (4), is considered as an alternative but effective technique with a mean fertilization rate of approximately 80% (5, 6). However, while 53.1% of ART cycles in the UK now involve ICSI (2), pregnancy and delivery rates resulting from this technique are no higher than 33% according to the European Society of Human Reproduction (ESHRE) (7). ICSI fertilization failure can be associated with several factors including technical issues such as incorrect injection and sperm expulsion during injection (8), sperm deficiencies such as globozoospermia or poor sperm nuclear chromatin condensation (9), or defects of the oocyte spindle (10). However, oocyte activation deficiency (OAD) is considered to be the principal factor underlying fertilization failure following ICSI (5, 6, 11-13).

Upon ovulation, oocytes remain arrested in metaphase-II (MII) (14) and are only alleviated from this state when they are fertilized. This process, known as 'oocyte activation' (15), is characterized, amongst other changes, by a specific pattern of periodic intracellular calcium (Ca^{2+}) oscillations that manifest in the ooplasm (16) following fusion of the oocyte with the fertilizing sperm. It is well established that these Ca^{2+} oscillations are triggered by a sperm-specific protein, phospholipase C zeta (PLC ζ) and lead the fertilized oocyte to initiate the

early phases of embryogenesis (13, 17, 18). The fundamental role of PLC ζ in this crucial process has been confirmed by several experiments in which the injection of recombinant PLC ζ , or cRNAs encoding for different mammalian PLC ζ isoforms, into mouse and human oocytes subsequently initiates their successful activation (19-22).

However, while the role of PLC ζ in oocyte activation is clearly evident in animal models, there have been far fewer studies investigating specific clinical links between male infertility and PLC ζ deficiency, especially with respect to infertile men involved in cases of ICSI fertilization failure (6, 23-26). As the use of ICSI continues to expand globally, it is highly prudent to develop methods to predict its likely success, particularly since ICSI requires heavy investment, both financially and emotionally. Sperm from infertile men presenting OAD have been reported to exhibit reduced or abolished forms of PLC ζ (23, 24). In addition, while Kashir et al. (25) showed that total levels of PLC ζ varied within a relatively small number of control and patient samples, and that the proportion of sperm exhibiting PLC ζ in a specific sample may represent a more reliable marker than total levels *per se*, a reliable study of PLC ζ as a potential clinical biomarker to predict the outcome of either IVF or ICSI has yet to be conducted.

While mounting biochemical and clinical evidence supports the vital role of PLC ζ in initiating the activation of oocytes at fertilization (18, 27, 28), important questions still remain, particularly relating to the localization profile of PLC ζ protein in the sperm head. Indeed, although previous studies have consistently reported equatorial and post-acrosomal are the most prominent PLC ζ localizations in control human sperm (23, 25, 26, 29-31), their actual impact upon fertilization rates has yet to be addressed clinically. Therefore, the present study sought to determine whether fertilization rates arising from IVF or ICSI treatment can be

110 specifically correlated to the total level of PLC ζ in human sperm, the actual proportion of
111 sperm exhibiting PLC ζ , and/or the discrete localization profile of this protein within the
112 sperm head.

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Materials and methods

Ethics and patients

All males involved in this study provided informed written consent and were recruited from the Oxford Fertility Unit (OFU; Oxford, UK) or the Assisted Conception Unit (ACU; Ninewells Hospital, Dundee, UK). In addition, this research study was approved by the National Research Ethics Service (South Central Oxford Committee C; Reference number: 10/H0606/65) and East of Scotland Research Ethics Service (EoSRES) (REC 1; Reference number: 13/ES/0091).

Samples were provided following at least three days of abstinence, and were further separated into two groups depending upon whether their clinical application was for IVF or ICSI. This separation was based upon sperm quality and past medical history. In the case of sperm quality, IVF was performed when all starting parameters (concentration, motility, morphology and volume) were at least, or close to, the World Health Organization (WHO) 2010 values, and when following DGW, these samples presented at least 70% progressive motile sperm, 0.3 mL resuspension volume, and a sperm count higher than 10 million per mL. ICSI was conducted when these thresholds were not reached. With regard to past medical history, ICSI was performed when very few oocytes (1-3) were collected, in the cases of fertility preservation, when previous IVF cycles presented less than 50% of fertilization success, when all previous fertilized oocytes presented one or three pronuclei, or when advised by consultants.

In total, 44 samples from 44 different men were used: 27 undergoing IVF cycles and 17 undergoing ICSI cycles. The age of the men ranged from 23 - 54 years (mean \pm standard error of the mean, SEM: 37.5 ± 0.8 years). Controls included those males with no history of

infertility or oocyte activation deficiency, males whose sperm were able to fertilize an oocyte following ART, and males that had fathered children via natural conception. Selection criteria for patients were based on sperm parameter analyses, such as progressive motility following density gradient washing (DGW), concentration (sperm·mL⁻¹), and morphological abnormalities (e.g. sperm presenting pin-shaped or round heads); fertilization rates; and history of IVF/ICSI failure or recurrent ICSI failure.

All sperm parameters were evaluated after DGW, according to World Health Organization guidelines (32).

Preparation of sperm samples

Upon reception of samples in the laboratory, semen volume, pH and viscosity, and sperm motility and concentration were evaluated, as described below. Thereafter, semen samples were subjected to density gradient washing (DGW) through PureSperm™ (PureSperm™ 40/80, Nidacon International AB, Gothenburg, Sweden), as described previously (30). Briefly, 2 mL of 80% PureSperm medium was layered onto the bottom of a 15 mL tube, followed by an overlay of 2 mL 40% PureSperm medium. Next, 1.5 mL of liquefied semen sample was layered on top of 40% PureSperm medium. Samples were then centrifuged at 300×g for 20 min at room temperature. After centrifugation, the supernatant was discarded, leaving approximately 0.5 mL of the pellet at the bottom of tube. This pellet was transferred to a clean tube containing 5 mL PureSperm™ Wash medium (Nidacon International AB) and the mixture was centrifuged for 10 min at 500×g. Aliquots intended for insemination were re-suspended in 1 mL Sydney IVF fertilization medium (Cook Medical Inc., Bloomington, IN, USA).

In those aliquots intended for PLCζ analysis, the remaining pellet was re-suspended with 200 μL of Phosphate Buffered Saline (PBS, Invitrogen Life Technologies; Carlsbad, CA, USA). After another centrifugation step at 800×g for 3 min, the supernatant was discarded without disturbing the pellet. The sperm pellet was then fixed with 100 μL 4% paraformaldehyde (PFA, Sigma-Aldrich; Gillingham, Dorset, UK) for 10 minutes. Following a final centrifugation and removal of PFA, the sperm pellet was re-suspended and stored in 200 μL PBS to await immunofluorescent analysis.

Evaluation of sperm concentration and motility

Sperm concentration and motility were evaluated before and after DGW. In both cases, sperm concentration was evaluated after diluting samples with sterile water (1/20) using a Neubauer chamber (Paul Marienfeld GmbH & Co. KG; Lauda-Königshofen, Germany). Sperm motility was evaluated in two independent replicates, and in each case this analysis was undertaken on the basis of the percentage of sperm exhibiting progressive sperm motility according to WHO guidelines (32). Motile count represented the number of sperm that exhibited progressive motility.

PLC ζ antibody purification and specificity testing

Anti-human PLC ζ was produced by Cova-Lab (UK) against two immunogenic peptides identified in the human PLC ζ amino acid sequence (C-RESKSYFNPSNIKE-coNH₂ and C-ETHERKGS DKRGDN; Accession Number: AF532185). Prior to use, the neat antibody was purified using the two original immunogenic peptides and a SulfoLink Kit (Pierce Biotechnology, Rockford, USA). Specificity of this antibody was determined previously by competitive pre-incubation studies with excessive peptide Grasa et al. (29). This particular antibody has been utilised in a number of publications arising from this laboratory, yielding consistent staining patterns each time (25, 29, 30, 33).

Immunofluorescent detection of PLC ζ and quantitative analyses

Samples were immunostained following the protocol described by Grasa et al. (29). Briefly, 100 μ L of each sperm sample were first processed by DGW and then added onto slides pre-coated with 0.01% Poly-L-Lysine and permeabilized overnight with 0.5% Triton X-100 in PBS (Sigma Aldrich, UK). After one hour of incubation with 3% bovine serum albumin (BSA, Sigma-Aldrich), slides were incubated at 4°C overnight with 100 μ L of 25 μ g·mL⁻¹

anti-PLC ζ antibody (Cova-Lab, UK) diluted in 0.05% BSA. Samples were then washed thrice with PBS and incubated at room temperature for 1 hour with 5 $\mu\text{g}\cdot\text{mL}^{-1}$ of a secondary goat anti-rabbit antibody conjugated with Alexa Fluor 488 (Invitrogen, Paisley, UK). Slides were subsequently washed thrice with PBS, mounted with 3 μL Vectashield H-1200 mounting medium containing 4'-6'-Diamidini-2-phenylindole (DAPI) (Vector, UK), and finally covered with 20mm x 20mm glass cover slips.

Samples were observed under a fluorescence microscope (Eclipse 80i, Nikon UK Ltd., Kingston upon Thames, UK) at 40 \times magnification using a fluorescein isothiocyanate filter (FITC; Excitation filter: 465-495 nm; Dichroic Mirror: 505 nm; Barrier Filter: 515-550 nm) specific for green PLC ζ fluorescence (excitation wavelength: 488 nm). Images were acquired with a Nikon DS-Ri1 camera (Nikon UK) at an exposure time of 400 ms and 100 sperm were counted per sample. Camera settings, such as resolution (1280 \times 1024), quality (16-bit) and color at high contrast, were standardized to ensure illumination intensity was the same for every image captured. Bright field images were used to select sperm heads for analysis. Only sperm where the head was still attached to the tail were chosen, ensuring that such sperm did not overlap with others. Sperm heads were outlined using the region of interest manager (ROI) tool from ImageJ software (Version 1.46a; National Institutes of Health, Bethesda, MD, USA). For each sample, several fields were captured and mean PLC ζ levels (arbitrary units) were quantified using Image J software. Simultaneously, the total proportion (percentage) of sperm exhibiting PLC ζ , as well as the localization profile of PLC ζ in each sperm, were evaluated following the methodology described by Kashir et al. (25). Each analyzed sperm was classified into one of the following eight categories in terms of PLC ζ localization: acrosomal (A); post-acrosomal (PA); equatorial (E); acrosomal and post-acrosomal (A+PA); acrosomal and equatorial (A+E); post-acrosomal and equatorial (PA+E);

acrosomal, post-acrosomal and equatorial (A+PA+E); or 'none' indicating a total absence of PLC ζ .

In Vitro Fertilization and Intracytoplasmic Sperm Injection

Sperm used for IVF and ICSI treatment were previously washed and re-suspended in fertilization medium as described below. Oocytes were collected from women aged from 24 to 44 years. Women were first down-regulated using a GnRH long agonist and were given 400 μ g Nafarelin (Synrel; Pfizer, Milton Keynes, UK) intra-nasally twice a day from the mid-luteal phase (day 21) of the menstrual cycle. After three weeks of Nafarelin administration, women were tested for pituitary suppression, which was confirmed when serum oestradiol levels were lower than 150 pmol·L⁻¹. Women were subsequently given between 125 and 375 IU recombinant FSH (Gonal-F; Merck-Serono, Feltham, UK) subcutaneously, depending upon the age of the patient, previous ovarian response to gonadotrophins and basal serum FSH levels. Subsequently, ultrasound was performed, and follicular response recorded, from day 8 of gonadotrophin stimulation. When a minimum of three leading follicles of at least 18 mm in diameter were observed by ultrasonography, 6500 IU hCG (Ovitrelle; Serono Pharmaceuticals Ltd., Feltham, UK) were administered subcutaneously to trigger oocyte retrieval. Collection of oocytes was performed from 35.5 to 37 h later and the oocytes were subsequently left in Sydney IVF fertilization medium (Cook Medical Inc.) or Sydney IVF cleavage medium (Cook Medical Inc.) depending on whether they were destined for subsequent IVF or ICSI.

For IVF treatment, a total of 2×10^5 sperm·mL⁻¹ in Sydney IVF Fertilization Medium (Cook Medical Inc.) were mixed with 2-4 collected oocytes ($n=218$; Supplementary Table 1) in a well (final volume: 0.5 mL), using Nunc™ 4-Well Dishes for IVF (Thermo Fisher Scientific;

Waltham, MA, USA). Oocytes were covered with mineral oil (Sydney IVF culture medium; Cook Medical Inc.) and plates were then incubated at 37°C, 5% CO₂ and 6% O₂, and 89% N₂ for 16-19h in a MINC™ incubator (Cook Medical Inc.). After evaluating fertilization rates as formation of pronuclei in fertilized oocytes, fertilization medium was replaced by Sydney IVF Cleavage medium (Cook Medical Inc.). A total of 201 oocytes were used (Supplementary Table 1) and fertilization outcomes for controls and patients were 73.91 ± 1.43 and 8.75 ± 1.93 , respectively (Supplementary Table 2).

In the case of ICSI, oocytes ($n=127$; Supplementary Table 1) were stripped from cumulus cells within 2 – 2.5 hours of collection. Prior to intracytoplasmic injection, sperm were left in 10% polyvinylpyrrolidone (PVP; Cook Medical Inc.) for between 5 and 30 min. Afterwards, they were injected individually into each oocyte within 4 h of oocyte collection with a micromanipulator (Narishige NT-88-V3; Nikon UK) and under an inverted phase-contrast microscope (Nikon Eclipse Ti-S; Nikon UK). Three oocytes per well, in individual drops of 15 µL each, were left in IVF Sydney Cleavage medium. Oocytes were covered with mineral oil (Sydney IVF culture medium; Cook Medical Inc.) and incubated at 37°C, 5% CO₂ and 6% O₂, and 89% N₂ in a MINC™ incubator. Oocytes presenting two pronuclei 16h-25h post-insemination were considered successfully fertilized. Oocytes not presenting two pronuclei 25h post-insemination were considered unsuccessfully fertilized. Fertilization outcomes for controls and patients were 80.44 ± 3.15 and 9.88 ± 1.76 , respectively (Supplementary Table 2).

Statistical analyses

Data were analyzed using a statistical package (IBM® SPSS® 21.0 for Windows; IBM corp., Chicago, Illinois, USA) and plots were made using Origin Pro 8.0 software (OriginLab Corp.,

Northampton, Massachusetts, USA). All data were first analyzed using the Shapiro-Wilk test to check data for normality and the Levene test was used to assess homogeneity of variances (homocedasticity). When required, data were transformed using arcsine root transformation ($\arcsin \sqrt{x}$). If, even transformed, data did not match parametric assumptions, alternative non-parametric tests were used. For fertilization rates (FR), data were weighted by the number of MII-oocytes retrieved per patient and transformed using logit transformation ($\text{logit} = \ln(\text{FR}/1 - \text{FR})$). Levels of PLC ζ , total proportions (in %) of sperm exhibiting PLC ζ , and the eight PLC ζ localization patterns were compared between controls and patients by *t*-test for independent samples or Mann-Whitney test. In addition, relationships of total levels of PLC ζ , total proportions of sperm exhibiting PLC ζ , and the eight PLC ζ localization patterns with progressive sperm motility, motile count and fertilization rates were evaluated by Pearson or Spearman correlation. Data are shown as mean \pm standard error of the mean (SEM), and level of significance was $P \leq 0.05$.

Results

Differences in the total number of sperm exhibiting PLC ζ in controls and patients subjected to both IVF and ICSI treatment

Figure 1 shows the percentage (mean \pm SEM) of sperm exhibiting PLC ζ in controls and patients undergoing IVF and ICSI cycles. While no significant differences ($P > 0.05$) were observed between controls and patients subjected to IVF treatment, patients intended for ICSI demonstrated significantly ($P < 0.05$) lower percentages of sperm exhibiting PLC ζ as compared to controls.

Differences in the total levels of PLCζ between controls and patients subjected to both IVF and ICSI treatment

Total levels of PLCζ in sperm from controls and patients in either IVF or ICSI treatment are shown in Figure 2 (mean ± SEM). For both IVF and ICSI, total levels of PLCζ were higher in controls than in patients. However, this increase was only statistically significant ($P<0.05$) in the case of ICSI patients.

Differences in PLCζ localization patterns in sperm from controls and patients subjected to IVF and ICSI treatment

Localization patterns of PLCζ in controls and patients were compared in males undergoing either IVF or ICSI treatment. In the case of IVF (Table 1, mean ± SEM), no significant differences between controls and patients were seen in any of the eight PLCζ localization patterns evaluated.

ICSI patients presented significantly ($P<0.05$) lower percentages of sperm exhibiting post-acrosomal and a combined pattern of PLCζ in equatorial, acrosomal and post-acrosomal regions (Table 1). Conversely, the proportion (%) of sperm that were devoid of PLCζ was significantly ($P<0.05$) higher in ICSI patients than in their control counterparts.

Correlations of conventional sperm parameters with total levels of PLCζ and the proportion of sperm exhibiting PLCζ

Total levels of PLCζ were found to be significantly ($P<0.05$) correlated with % progressive motile sperm and with sperm motility count. In addition, percentages of sperm exhibiting a post-acrosomal PLCζ-pattern were significantly ($P<0.05$) correlated with % progressive motile sperm (Supplementary Table 3).

Correlations between PLCζ and fertilization rates following ICSI and IVF procedures

The correlation between fertilization rates, the proportion of sperm exhibiting PLCζ, and the total levels of PLCζ was also examined (Table 2). While IVF fertilization rates were not seen to be significantly ($P>0.05$) correlated with any of the PLCζ parameters evaluated, the total levels of PLCζ, % total sperm exhibiting PLCζ, % sperm exhibiting PLCζ in post-acrosomal region, % sperm exhibiting PLCζ in equatorial region, % sperm exhibiting PLCζ in acrosomal and post-acrosomal regions, % sperm exhibiting PLCζ in acrosomal, post-acrosomal and equatorial regions demonstrated significant positive correlation ($P<0.05$) with fertilization rates following ICSI. Concomitantly, the proportion (%) of sperm that were devoid of PLCζ was negatively ($P<0.05$) correlated with fertilization rates following ICSI.

Discussion

Our results have shown for the first time that total levels, localization patterns and the proportion (%) of sperm exhibiting PLCζ are significantly correlated with fertilization rates following ICSI but not IVF treatment. This result is of paramount importance as it strongly indicates sperm PLCζ as being a useful prognostic and diagnostic marker for ICSI outcome. It is well understood that human infertility can be attributed to both male and female factors and that ICSI is predominantly implemented to treat male factor infertility (2). Although fertilization rates following ICSI routinely exceed 80%, 3-5% of ICSI cases still encounter fertilization failure (34, 35). In addition, while conventional semen analysis provides a general assessment with regard to sperm quality, an excessively large number of infertile patients tend to present a normal spermiogram (36-39). Therefore, it is fundamental to develop a novel or additional biological tool in order to predict the fertilizing ability of a given sperm sample, as this study sought.

Since the discovery of the sperm-soluble oocyte activation factor, PLC ζ (20), a strong body of both biochemical and clinical evidence has steadily accumulated in support of the fundamental role of PLC ζ as a universal trigger for oocyte activation in mammalian (19, 20, 24, 40, 41) and non-mammalian species (42-45). In spite of this, recent studies suggest that a post-acrosomal sperm protein found in the perinuclear theca, and known as PAWP, elicits calcium oscillations similar to those seen during fertilization in mammalian (46, 47) and non-mammalian oocytes (48). Furthermore, a significant correlation between PAWP levels in human sperm and fertilization rates following ICSI has been reported (49). These recent findings from Aarabi et al. (47, 49) have led to significant debate with regard to understanding the essential role of these two proteins (i.e. PLC ζ and PAWP) as oocyte activation factors (50-52). However, the true extent of the functional role of PAWP, and indeed its precise relevance in the oocyte activation process, should for now be treated with caution as the results of a recent study conflicted directly with those of Aarabi et al. (47) by failing to demonstrate calcium oscillations in mouse oocytes in response to the injection of recombinant PAWP (28). Our present study did not evaluate PAWP in our clinical patients, but our current dataset clearly demonstrates a strong clinical link between the total levels of PLC ζ in human sperm and fertilization rates following ICSI, thus strongly indicating PLC ζ as a biomarker for ICSI outcome.

From our data, the total levels and proportions of sperm exhibiting PLC ζ are significantly correlated with fertility outcomes following ICSI, but not IVF treatment. A previous study from this laboratory (25) showed that a high variation within control and patient groups existed, and suggested that this could limit the use of PLC ζ as a biomarker to predict fertility outcomes. While in the present study a high variation within control and patient groups was also observed, the correlations of total levels, localization patterns and proportions of sperm

exhibiting PLC ζ with fertility outcomes were found when ICSI and IVF were evaluated separately. Moreover, localization patterns of PLC ζ also demonstrated significant correlation with fertilization rates following ICSI. Seven localization patterns of PLC ζ in human sperm were identified by Kashir et al. (25); acrosomal (A), equatorial (E), post-acrosomal (P), A+P, A+E, P+E and all three patterns together (A+E+P). In the present study, four out of the seven localization patterns identified: percentages of sperm exhibiting PLC ζ (P) region, (E) region, (A+P) region and in (A+E+P) regions, were shown to be significantly correlated with fertilization rates following ICSI treatment. While our data suggest that a significant correlation is only observed in fertilization rates following ICSI, this does not preclude the critical role of PLC ζ in activating the oocyte during IVF treatment. This may further suggest that deficiency of PLC ζ may not be the major cause of IVF failure, but may also be associated with other factors such as the interaction of capacitated sperm with the oocyte zona pellucida and the acrosome reaction, since these events are bypassed during ICSI (53).

Apart from highlighting the potential value of PLC ζ as a fertility marker for individual sperm and ICSI outcome, the use of a significant number of clinical samples ($n=44$) in the present work provides significant support to the fundamental role of PLC ζ in determining oocyte activation and fertilization rates. However, approximately 40% of ICSI failure cases are attributed to oocyte activation deficiency (13, 18, 54). Thus, ICSI failure is not restricted to only sperm factor defects (13, 55), but may also include potential oocyte factors such as nuclear and cytoplasmic maturation (35). This might explain why correlation coefficients between PLC ζ and fertility outcomes in ICSI were statistically significant, but were never higher than 0.6. This, apart from indicating inter-individual differences, implies that other factors independent from PLC ζ levels, proportions, and localization pattern (e.g. idiopathic oocyte problems) may also contribute to ICSI failure.

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2 399 Finally, the differential localization patterns of PLC ζ across different mammalian species and
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4 400 their functional roles during oocyte activation and fertilization rates still remain unclear
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7 401 (reviewed in (27)). However, it is well understood that PLC ζ should reside at a readily
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9 402 accessible region within the sperm head, i.e. in an equatorial or post-acrosomal location (31),
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11 403 that would permit the rapid dispersion of this protein into the oocyte following gamete fusion
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13 404 (56). In this context, the significant correlation between ICSI fertilization outcomes and
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15 405 equatorial and post-acrosomal patterns observed in the present study confirms, for the first
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17 406 time using a significant number of clinical samples, the critical role of equatorial and post-
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19 407 acrosomal localizations of PLC ζ for oocyte activation.
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26 409 In conclusion, our findings demonstrate, for the first time, that evaluating total levels,
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28 410 localization patterns and proportions of sperm exhibiting PLC ζ may be used as a valuable
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30 411 prognostic marker for ICSI fertilization rates in couples for which IVF is unadvised or has
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32 412 failed previously. This provides important new data to assist with the translation of PLC ζ to
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34 413 the clinic, and extends our previous work conducted with a smaller sample size that did not
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36 414 allow us to distinguish between ART (25). While our data, compiled from a large clinical
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38 415 dataset, provide significant support for the fundamental role of PLC ζ in the oocyte activation
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40 416 process, they also show that causes other than PLC ζ , for example those related to molecular
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42 417 machinery inside the oocyte, may also underlie ICSI failure. Therefore, further research
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44 418 aiming to elucidate these other potential causes is warranted.
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Author's contribution

S.Y. and S.N.A. conducted the immunostaining experiments, acquired data and critically revised the manuscript. M.Y. analyzed and interpreted the data, and wrote the manuscript. C.J. contributed to experimental design and critically revised the manuscript. J.K. analyzed the stained samples and revised the manuscript. G.M. and S.J.M.D.S. recruited the patients and provided clinical data. E.McV. and C.L.B. provided clinical expertise/data and critically revised the manuscript. K.C. conceived and designed the experiment, contributed to data interpretation, and revised the manuscript. All authors read and approved the final version of this manuscript.

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

References

1. Mascarenhas MN, Flaxman SR, Boerma T, Vanderpoel S, Stevens GA National, regional, and global trends in infertility prevalence since 1990: a systematic analysis of 277 health surveys. *PLoS Med* 2012; 9: e1001356.
2. HFEA. Latest UK IVF figures—2011 and 2012. HFEA 2012, <http://www.hfea.gov.uk/docs/FertilityTreatment2012TrendsFigures.PDF>. [date accessed: 11th October 2014].
3. Bhattacharya S, Maheshwari A, Mollison J. Factors associated with failed treatment: an analysis of 121,744 women embarking on their first IVF cycles. *PLoS One* 2013; 8: e82249. doi: 10.1371/journal.pone.0082249.
4. Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 1992; 340: 17–18.
5. Vanden Meerschaut F, Leybaert L, Nikiforaki D, Qian C, Heindryckx B, De Sutter P. Diagnostic and prognostic value of calcium oscillatory pattern analysis for patients with ICSI fertilization failure. *Hum Reprod* 2013; 28: 87-98.
6. Vanden Meerschaut F, Nikiforaki D, Heindryckx B, De Sutter P. Assisted oocyte activation following ICSI fertilization failure. *Reprod Biomed Online* 2014; 28: 560-571.
7. Kupka MS, Ferraretti AP, de Mouzon J, Erb K, D'Hooghe T, Castilla JA, Calhaz-Jorge C, De Geyter C, Goossens V; European IVF-monitoring (EIM); Consortium, for the European Society of Human Reproduction and Embryology (ESHRE). Assisted reproductive technology in Europe, 2010: results generated from European registers by ESHRE. *Hum Reprod* 2014; 29: 2099-2113
8. Yanagida K, Fujikura Y, Katayose H. The present status of artificial oocyte activation in assisted reproductive technology. *Reprod Med Biol.* 2008; 7: 133-142.

9. Dam AH, Feenstra I, Westphal JR, Ramos L, van Golde RJ, Kremer JA. Globozoospermia revisited. *Hum Reprod Update* 2007; 13: 63–75.
10. Swain JE, Pool TB. ART failure: oocyte contributions to unsuccessful fertilization. *Hum Reprod Update* 2008; 14: 431-446.
11. Flaherty SP, Payne D, Matthews CD. Fertilization failures and abnormal fertilization after intracytoplasmic sperm injection. *Hum Reprod* 1998; 13 Suppl 1: 155–164.
12. Rawe VY, Olmedo SB, Nodar FN, Doncel GD, Acosta AA, Vitullo AD. Cytoskeletal organization defects and abortive activation in human oocytes after IVF and ICSI failure. *Mol Hum Reprod* 2000; 6: 510-516.
13. Kashir J, Heindryckx B, Jones C, De Sutter P, Parrington J, Coward K. Oocyte activation, phospholipase C zeta and human infertility. *Hum Reprod Update* 2010; 16: 690-703.
14. Jones KT, Carroll J, Merriman JA, Whittingham DG, Kono T. Repetitive sperm-induced Ca^{2+} transients in mouse oocytes are cell cycle dependent. *Development* 1995; 121: 3259-3266.
15. Horner VL, Wolfner MF. Transitioning from egg to embryo: Triggers and mechanisms of egg activation. *Dev Dyn* 2008; 237: 527–544.
16. Kashir J, Deguchi R, Jones C, Coward K, Stricker SA. Comparative biology of sperm factors and fertilization-induced calcium signals across the animal kingdom. *Mol Reprod Dev* 2013; 80: 787-815.
17. Swann K, Saunders CM, Rogers NT, Lai FA. PLCzeta(zeta): a sperm protein that triggers Ca^{2+} oscillations and egg activation in mammals. *Semin Cell Dev Biol* 2006; 17: 264-273.
18. Amdani SN, Jones C, Coward K. Phospholipase C zeta ($\text{PLC}\zeta$): oocyte activation and clinical links to male factor infertility. *Adv Biol Regul* 2013; 53: 292-308.

19. Cox L, Larman MG, Saunders CM, Hashimoto K, Swann K, Lai FA. Sperm phospholipase C ζ from humans and cynomolgus monkeys triggers Ca²⁺ oscillations, activation and development of mouse oocytes. *Reproduction* 2002; 124: 611–623.
20. Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, Swann K, Lai FA. PLC ζ : a sperm-specific trigger of Ca²⁺ oscillations in eggs and embryo development. *Development* 2002; 129: 3533–3544.
21. Yoon SY, Eum JH, Lee JE, Lee HC, Kim YS, Han JE, Won HJ, Park SH, Shim SH, Lee WS, et al. (2012). Recombinant human phospholipase C zeta 1 induces intracellular calcium oscillations and oocyte activation in mouse and human oocytes. *Hum Reprod* 2012; 27: 1768-1780.
22. Nomikos M, Yu Y, Elgmati K, Theodoridou M, Campbell K, Vassilakopoulou V, Zikos C, Livaniou E, Amso N, Nounesis G, Swann K, Lai FA. Phospholipase C ζ rescues failed oocyte activation in a prototype of male factor infertility. *Fertil Steril* 2013; 99: 76-85.
23. Yoon SY, Jellerette T, Salicioni AM, Lee HC, Yoo MS, Coward K, Parrington J, Grow D, Cibelli JB, Visconti PE, et al. Human sperm devoid of PLC, zeta 1 fail to induce Ca²⁺ release and are unable to initiate the first step of embryo development. *J Clin Invest* 2008; 118: 3671-3681.
25. Kashir J, Jones C, Mounce G, Ramadan WM, Lemmon B, Heindryckx B, de Sutter P, Parrington J, Turner K, Child T, et al. Variance in total levels of phospholipase C zeta (PLC- ζ) in human sperm may limit the applicability of quantitative immunofluorescent analysis as a diagnostic indicator of oocyte activation capability. *Fertil Steril* 2013; 99: 107-117.
26. Lee HC, Army M, Grow D, Dumesic D, Fissore RA, Jellerette-Nolan T. Protein phospholipase C Zeta1 expression in patients with failed ICSI but with normal sperm parameters. *J Assist Reprod Genet* 2014; 31: 749-756.

27. Kashir J, Nomikos M, Lai FA, Swann K. Sperm-induced Ca^{2+} release during egg activation in mammals. *Biochem Biophys Res Commun* 2014; 450: 1204-1211.
28. Nomikos M, Sanders JR, Theodoridou M, Kashir J, Matthews E, Nounesis G, Lai FA, Swann K. Sperm-specific post-acrosomal WW-domain binding protein (PAWP) does not cause Ca^{2+} release in mouse oocytes. *Mol Hum Reprod* 2014; 20: 938-947.
29. Grasa P, Coward K, Young C, Parrington J. The pattern of localization of the putative oocyte activation factor, phospholipase C ζ , in uncapacitated, capacitated, and ionophore-treated human spermatozoa. *Hum Reprod* 2008; 23: 2513-2522.
30. Kashir J, Heynen A, Jones C, Durrans C, Craig J, Gadea J, Turner K, Parrington J, Coward K. Effects of cryopreservation and density-gradient washing on phospholipase C ζ concentrations in human spermatozoa. *Reprod Biomed Online* 2011; 23: 263-267.
31. Escoffier J, Yassine S, Lee HC, Martinez G, Delaroche J, Coutton C, Karaouzène T, Zouari R, Metzler-Guillemain C, Pernet-Gallay K, Hennebicq S, Ray PF, Fissore R, Arnoult C. Subcellular localization of phospholipase C ζ in human sperm and its absence in DPY19L2-deficient sperm are consistent with its role in oocyte activation. *Mol Hum Reprod* 2015; 21: 157-168.
32. WHO. World Health Organization Laboratory Manual for the Examination and Processing of Human Semen. Geneva: World Health Organization, 2010.
33. Kashir J, Sermondade N, Sifer C, Oo SL, Jones C, Mounce G, Turner K, Child T, McVeigh E, Coward K. Motile sperm organelle morphology evaluation-selected globozoospermic human sperm with an acrosomal bud exhibits novel patterns and higher levels of phospholipase C ζ . *Hum Reprod* 2012; 27: 3150-3160.
34. Palermo GD, Neri QV, Takeuchi T, Rosenwaks Z. ICSI: where we have been and where we are going. *Semin Reprod Med* 2009; 27: 191-201.

35. Neri QV, Lee B, Rosenwaks Z, Machaca K, Palermo GD. Understanding fertilization through intracytoplasmic sperm injection (ICSI). *Cell Calcium* 2014; 55: 24-37.
36. Guzick DS, Overstreet JW, Factor-Litvak P, Brazil CK, Nakajima ST, Coutifaris C, Carson SA, Cisneros P, Steinkampf MP, Hill JA, Xu D, Vogel DL, National Cooperative Reproductive Medicine Network. Sperm morphology, motility, and concentration in fertile and infertile men. *N Engl J Med* 2001; 345: 1388-1393.
37. Keel BA. Within- and between-subject variation in semen parameters in infertile men and normal semen donors. *Fertil Steril* 2006; 85: 128-134.
38. Samplaski MK, Agarwal A, Sharma R, Sabanegh E. New generation of diagnostic tests for infertility: review of specialized semen tests. *Int J Urol* 2010; 17: 839-847.
39. Barratt CL, Mansell S, Beaton C, Tardif S, Oxenham SK. Diagnostic tools in male infertility-the question of sperm dysfunction. *Asian J Androl* 2011; 13: 53-58.
40. Kashir J, Konstantinidis M, Jones C, Heindryckx B, De Sutter P, Parrington J, Wells D, Coward K. Characterization of two heterozygous mutations of the oocyte activation factor phospholipase C zeta (PLC ζ) from an infertile man by use of minisequencing of individual sperm and expression in somatic cells. *Fertil Steril* 2012; 98: 423-431
41. Kashir J, Konstantinidis M, Jones C, Lemmon B, Lee HC, Hamer R, Heindryckx B, Deane CM, De Sutter P, Fissore RA, Parrington J, Wells D, Coward K. A maternally inherited autosomal point mutation in human phospholipase C zeta (PLC ζ) leads to male infertility. *Hum Reprod* 2012; 27: 222-231.
42. Coward K, Ponting CP, Chang H-Y, Hibbitt O, Savolainen P, Jones KT, Parrington J. Phospholipase C ζ , the trigger of egg activation in mammals, is present in a non-mammalian species. *Reproduction* 2005; 130: 157-163.

43. Coward K, Ponting CP, Zhang N, Young C, Huang CJ, Chou CM, Kashir J, Fissore RA, Parrington J. Identification and functional analysis of an ovarian form of the egg activation factor phospholipase C zeta (PLC ζ) in pufferfish. *Mol Reprod Dev* 2011; 78: 48–56.
44. Ito M, Shikano T, Oda S, Horiguchi T, Tanimoto S, Awaji T, Mitani H, Miyazaki S. Difference in Ca²⁺ oscillation-inducing activity and nuclear translocation ability of PLCZ1, an egg-activating sperm factor candidate, between mouse, rat, human, and medaka fish. *Biol Reprod* 2008; 78: 1081-1090.
45. Mizushima S, Takagi S, Ono T, Atsumi Y, Tsukada A, Saito N, Shimada K. Phospholipase Czeta mRNA expression and its potency during spermatogenesis for activation of quail oocyte as a sperm factor. *Mol Reprod Dev* 2009; 76: 1200-1207.
46. Wu ATH, Sutovsky P, Manandhar G, Xu W, Katayama M, Day BN, Park KW, Yi YJ, Xi YW, Prather RS, Oko R. PAWP, a Sperm-specific WW domain-binding protein, promotes meiotic resumption and pronuclear development during fertilization. *J Biol Chem* 2007; 282: 12164-12175.
47. Aarabi M, Balakier H, Bashar S, Moskovtsev SI, Sutovsky P, Librach CL, Oko R. Sperm-derived WW domain-binding protein, PAWP, elicits calcium oscillations and oocyte activation in humans and mice. *FASEB J* 2014; 28: 4434-4440.
48. Aarabi M, Qin Z, Xu W, Mewburn J, Oko R. Sperm-borne protein, PAWP, initiates zygotic development in *Xenopus laevis* by eliciting intracellular calcium release. *Mol Reprod Dev* 2010; 77: 249–256.
49. Aarabi M, Balakier H, Bashar S, Moskovtsev SI, Sutovsky P, Librach CL, Oko R. Sperm content of postacrosomal WW binding protein is related to fertilization outcomes in patients undergoing assisted reproductive technology. *Fertil Steril* 2014; 102: 440-447.
50. Aarabi M, Sutovsky P, Oko R. Re: Is PAWP the 'real' sperm factor? *Asian J Androl* 2015; doi: 10.4103/1008-682X.145071.

51. Nomikos M, Swann K, Lai FA. Is PAWP the "real" sperm factor? Asian J Androl 2015; doi:10.4103/1008-682X.142145.
52. Vadnais ML, Gerton LG. From PAWP to "Pop": opening up new pathways to fatherhood. Asian J Androl 2015; doi: 10.4103/1008-682X.142140.
53. Beck-Fruchter R, Lavee M, Weiss A, Geslevich Y, Shalev E. Rescue intracytoplasmic sperm injection: a systematic review. Fertil Steril 2014; 101: 690-698.
54. Mahutte NG, Arici A. Failed fertilization: is it predictable? Curr Opin Obstet Gynecol 2003; 15: 211-218.
55. Ramadan WM, Kashir J, Jones C, Coward K. Oocyte activation and phospholipase C zeta (PLC ζ): diagnostic and therapeutic implications for assisted reproductive technology. Cell Commun Signal 2012; 10: 12.
56. Nomikos M, Swann K, Lai FA. Starting a new life: sperm PLC-zeta mobilizes the Ca²⁺ signal that induces egg activation and embryo development: an essential phospholipase C with implications for male infertility. Bioessays 2012; 34: 126-134.

Figure legends

Figure 1

The proportion (%) of sperm exhibiting PLC ζ (mean \pm SEM) in controls and patients following IVF and ICSI treatment. Annotation with an asterisk (*) denotes significant ($P<0.05$) differences in patients with regard to controls

Figure 2

Mean fluorescent intensity (arbitrary units) of PLC ζ levels per sperm (mean \pm SEM) in controls and patients following IVF and ICSI treatment. Annotation with an asterisk (*) denotes significant ($P<0.05$) differences in patients with regard to controls

Supplementary Figure 1

Representative image of a human sperm showing equatorial and post-acrosomal PLC ζ immunostaining

Figure1

[Click here to download high resolution image](#)

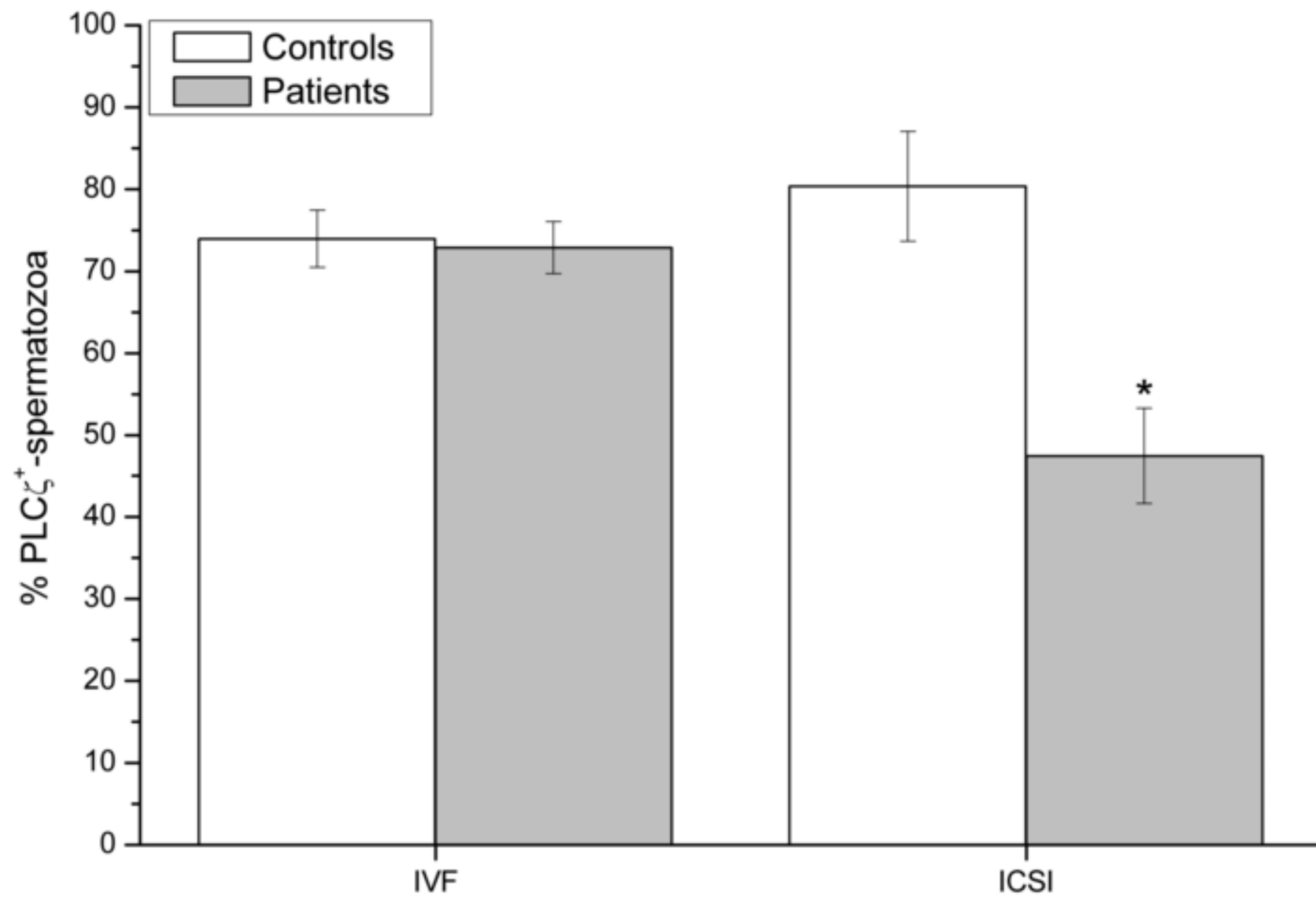
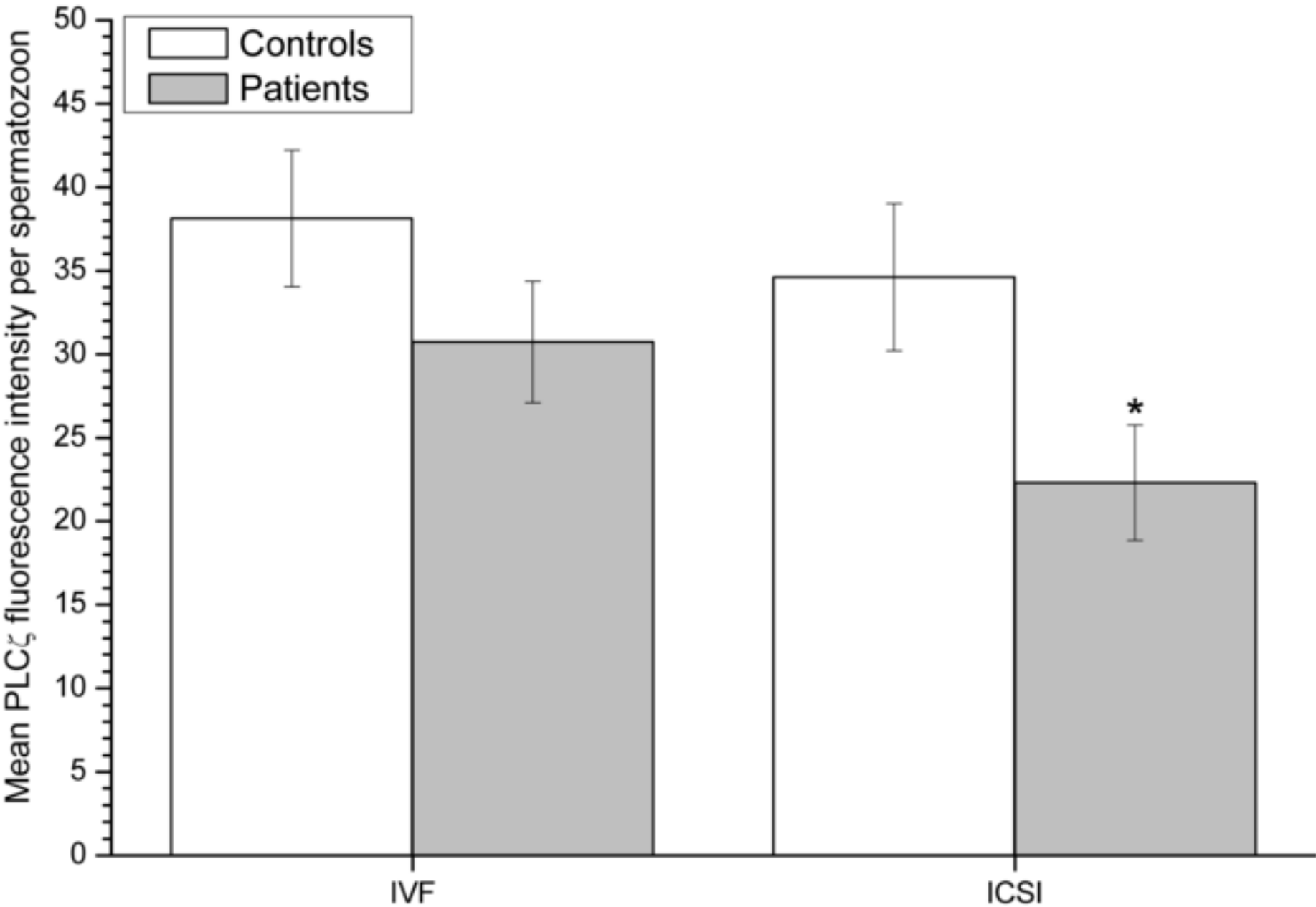


Figure2
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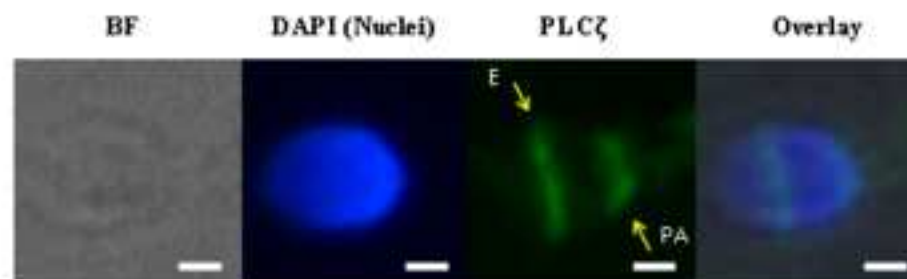


Table 1 Proportions (%) of sperm exhibiting PLC ζ (mean \pm SEM) in controls and patients subjected to IVF or ICSI. Annotation with an asterisk (*) denotes significant ($P<0.05$) differences between controls and patients. Localization of PLC ζ :

A=Acrosomal. PA=Post-acrosomal. E=Equatorial. None=Absence of PLC ζ

Localization of PLC ζ	IVF		ICSI	
	Controls	Patients	Controls	Patients
A	1.73 \pm 0.55	2.35 \pm 1.18	3.20 \pm 1.82	2.99 \pm 1.19
PA	12.21 \pm 3.26	10.12 \pm 3.04	11.49 \pm 3.38	2.77 \pm 2.01*
E	22.94 \pm 4.71	21.50 \pm 4.19	11.58 \pm 3.20	6.82 \pm 2.80
A + PA	1.48 \pm 0.43	1.23 \pm 0.58	2.93 \pm 1.44	1.98 \pm 1.61
A + E	1.25 \pm 0.45	2.12 \pm 0.83	0.50 \pm 0.34	0.96 \pm 0.19
PA + E	19.95 \pm 3.88	28.20 \pm 5.32	26.12 \pm 4.19	24.58 \pm 4.06
A + PA+ E	11.81 \pm 3.40	10.05 \pm 3.37	30.32 \pm 4.52	15.18 \pm 3.63*
None	28.63 \pm 4.55	24.43 \pm 4.90	13.86 \pm 3.35	44.72 \pm 5.71*

Table 2 Correlations of total levels and localization PLC ζ patterns and fertilization rates following IVF and ICSI treatment. Significant correlations are expressed with asterisks as follows: * $P < 0.05$, ** $P < 0.01$. Localisation of PLC ζ : A=Acrosomal. PA=Postacrosomal. E=Equatorial. None=Absence of PLC ζ .

	Fertilization rates	
	<i>IVF</i>	<i>ICSI</i>
Total levels of PLC ζ (fluorescence intensity)	0.15	0.35*
Total spermatozoa exhibiting PLC ζ (%)	-0.05	0.34*
Localization patterns (%)		
A	0.08	0.03
PA	-0.08	0.59**
E	-0.12	0.32*
A + PA	-0.10	0.39*
A + E	0.05	0.19
PA + E	-0.20	0.17
A + PA + E	0.06	0.47**
None	0.01	-0.30*

Supplementary Table 1 Numbers of controls, patients and oocytes retrieved during the course of this study.

	IVF		ICSI	
	Number of males	Number of oocytes retrieved	Number of males	Number of oocytes retrieved
Controls	17	138	6	46
Patients	10	80	11	81
Total	27	218	17	127

Supplementary Table 2 Fertilization outcomes for controls and patients following IVF and ICSI procedures

	IVF	ICSI
Controls	73.91 ± 1.43	80.44 ± 3.15
Patients	8.75 ± 1.93	9.88 ± 1.76

Supplementary Table 3 Pearson correlation between total levels and localization patterns of PLCζ and % progressive motile spermatozoa, motility count and age. (*) denotes a significant ($P<0.05$) Pearson correlation. Localization of PLCζ: A = Acrosomal, PA = Post-acrosomal, E = Equatorial, None = Absence of PLCζ.

	% Progressive motile spermatozoa	Sperm motility count
Total levels of PLCζ (fluorescence intensity)	0.28*	0.28*
Total spermatozoa exhibiting PLCζ (%)	0.19	0.18
Localization patterns (%)		
A	0.21	0.07
PA	0.30*	0.22
E	0.06	0.11
A + PA	0.18	-0.03
A + E	0.07	0.05
PA + E	0.12	0.15
A + PA + E	0.08	0.19
None	-0.08	-0.08

Title

Total levels, localization patterns and proportions of sperm exhibiting phospholipase C Zeta (PLC ζ) are significantly correlated with fertilization rates following intracytoplasmic sperm injection

Running title

PLC ζ and fertilization rates in ICSI

Authors

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29 **Conflict of interest**

30 None of the authors have anything to disclose

Capsule

Total levels, localization patterns and proportions of sperm exhibiting phospholipase C Zeta are significantly correlated with fertilization rates following ICSI, but not with fertilization rates following IVF.

Abstract

OBJECTIVE: To study the relationship of total levels, localization patterns and proportions of sperm exhibiting phospholipase C zeta with fertilization rates following *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI).

DESIGN: Laboratory study. Controls versus patients following IVF (n = 27) or ICSI (n = 17) treatment.

SETTING: University Fertility Centers

PATIENT(S): A total of 44 semen samples subjected to either IVF or ICSI treatment. Oocyte collection, ICSI or IVF, determination of sperm concentration and motility and immunocytochemical analyses of PLC ζ .

INTERVENTION(S): None

MAIN OUTCOME MEASURE(S): Percentages of sperm exhibiting PLC ζ

RESULTS: Significant positive correlation between ICSI fertilization rates and total levels, localization patterns and the proportion (percentage) of sperm exhibiting PLC ζ . Total levels, localization patterns, and the proportion of sperm exhibiting PLC ζ are correlated with fertilization rates for ICSI, but not for IVF.

CONCLUSION(S): Evaluating total levels, localization patterns and proportions of PLC ζ may represent a useful diagnostic tool for clinical purposes in males for which IVF is unadvised or has previously failed. This clinical study also further supports the fundamental role of PLC ζ in the oocyte activation process.

56

57 **Key words:** *In Vitro* Fertilization, IVF; Intracytoplasmic Sperm Injection, ICSI; total
58 fertilization failure, TFF; Phospholipase C Zeta, PLC ζ ; Oocyte activation

59

Introduction

Fertility disorders affect 25% of couples worldwide and the increasing prevalence of infertility has led to the rapid growth and development of assisted reproductive technology (ART) (1). Indeed, ART treatments such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are now successful techniques to combat infertility, such that 2% of live births per year in the UK now result from the use of ART (2).

While IVF is a suitable treatment for many infertile couples, problems can still occur and lead to total fertilization failure (TFF) in 4-7% of cases (3). In this scenario, ICSI, which involves the direct injection of a single sperm into the oocyte (4), is considered as an alternative but effective technique with a mean fertilization rate of approximately 80% (5, 6). However, while 53.1% of ART cycles in the UK now involve ICSI (2), pregnancy and delivery rates resulting from this technique are no higher than 33% according to the European Society of Human Reproduction (ESHRE) (7). ICSI fertilization failure can be associated with several factors including technical issues such as incorrect injection and sperm expulsion during injection (8), sperm deficiencies such as globozoospermia or poor sperm nuclear chromatin condensation (9), or defects of the oocyte spindle (10). However, oocyte activation deficiency (OAD) is considered to be the principal factor underlying fertilization failure following ICSI (5, 6, 11-13).

Upon ovulation, oocytes remain arrested in metaphase-II (MII) (14) and are only alleviated from this state when they are fertilized. This process, known as 'oocyte activation' (15), is characterized, amongst other changes, by a specific pattern of periodic intracellular calcium (Ca^{2+}) oscillations that manifest in the ooplasm (16) following fusion of the oocyte with the fertilizing sperm. It is well established that these Ca^{2+} oscillations are triggered by a sperm-specific protein, phospholipase C zeta ($\text{PLC}\zeta$) and lead the fertilized oocyte to initiate the

early phases of embryogenesis (13, 17, 18). The fundamental role of PLC ζ in this crucial process has been confirmed by several experiments in which the injection of recombinant PLC ζ , or cRNAs encoding for different mammalian PLC ζ isoforms, into mouse and human oocytes subsequently initiates their successful activation (19-22).

However, while the role of PLC ζ in oocyte activation is clearly evident in animal models, there have been far fewer studies investigating specific clinical links between male infertility and PLC ζ deficiency, especially with respect to infertile men involved in cases of ICSI fertilization failure (6, 23-26). As the use of ICSI continues to expand globally, it is highly prudent to develop methods to predict its likely success, particularly since ICSI requires heavy investment, both financially and emotionally. Sperm from infertile men presenting OAD have been reported to exhibit reduced or abolished forms of PLC ζ (23, 24). In addition, while Kashir et al. (25) showed that total levels of PLC ζ varied within a relatively small number of control and patient samples, and that the proportion of sperm exhibiting PLC ζ in a specific sample may represent a more reliable marker than total levels *per se*, a reliable study of PLC ζ as a potential clinical biomarker to predict the outcome of either IVF or ICSI has yet to be conducted.

While mounting biochemical and clinical evidence supports the vital role of PLC ζ in initiating the activation of oocytes at fertilization (18, 27, 28), important questions still remain, particularly relating to the localization profile of PLC ζ protein in the sperm head. Indeed, although previous studies have consistently reported equatorial and post-acrosomal are the most prominent PLC ζ localizations in control human sperm (23, 25, 26, 29-31), their actual impact upon fertilization rates has yet to be addressed clinically. Therefore, the present study sought to determine whether fertilization rates arising from IVF or ICSI treatment can be

110 specifically correlated to the total level of PLC ζ in human sperm, the actual proportion of
111 sperm exhibiting PLC ζ , and/or the discrete localization profile of this protein within the
112 sperm head.

113

Materials and methods

Ethics and patients

All males involved in this study provided informed written consent and were recruited from the Oxford Fertility Unit (OFU; Oxford, UK) or the Assisted Conception Unit (ACU; Ninewells Hospital, Dundee, UK). In addition, this research study was approved by the National Research Ethics Service (South Central Oxford Committee C; Reference number: 10/H0606/65) and East of Scotland Research Ethics Service (EoSRES) (REC 1; Reference number: 13/ES/0091).

Samples were provided following at least three days of abstinence, and were further separated into two groups depending upon whether their clinical application was for IVF or ICSI. This separation was based upon sperm quality and past medical history. In the case of sperm quality, IVF was performed when all starting parameters (concentration, motility, morphology and volume) were at least, or close to, the World Health Organization (WHO) 2010 values, and when following DGW, these samples presented at least 70% progressive motile sperm, 0.3 mL resuspension volume, and a sperm count higher than 10 million per mL. ICSI was conducted when these thresholds were not reached. With regard to past medical history, ICSI was performed when very few oocytes (1-3) were collected, in the cases of fertility preservation, when previous IVF cycles presented less than 50% of fertilization success, when all previous fertilized oocytes presented one or three pronuclei, or when advised by consultants.

In total, 44 samples from 44 different men were used: 27 undergoing IVF cycles and 17 undergoing ICSI cycles. The age of the men ranged from 23 - 54 years (mean \pm standard error of the mean, SEM: 37.5 ± 0.8 years). Controls included those males with no history of

139 infertility or oocyte activation deficiency, males whose sperm were able to fertilize an oocyte
140 following ART, and males that had fathered children via natural conception. Selection criteria
141 for patients were based on sperm parameter analyses, such as progressive motility following
142 density gradient washing (DGW), concentration (sperm·mL⁻¹), and morphological
143 abnormalities (e.g. sperm presenting pin-shaped or round heads); fertilization rates; and
144 history of IVF/ICSI failure or recurrent ICSI failure.

145

146 All sperm parameters were evaluated after DGW, according to World Health Organization
147 guidelines (32).

148

Preparation of sperm samples

Upon reception of samples in the laboratory, semen volume, pH and viscosity, and sperm motility and concentration were evaluated, as described below. Thereafter, semen samples were subjected to density gradient washing (DGW) through PureSperm™ (PureSperm™ 40/80, Nidacon International AB, Gothenburg, Sweden), as described previously (30). Briefly, 2 mL of 80% PureSperm medium was layered onto the bottom of a 15 mL tube, followed by an overlay of 2 mL 40% PureSperm medium. Next, 1.5 mL of liquefied semen sample was layered on top of 40% PureSperm medium. Samples were then centrifuged at 300×g for 20 min at room temperature. After centrifugation, the supernatant was discarded, leaving approximately 0.5 mL of the pellet at the bottom of tube. This pellet was transferred to a clean tube containing 5 mL PureSperm™ Wash medium (Nidacon International AB) and the mixture was centrifuged for 10 min at 500×g. Aliquots intended for insemination were re-suspended in 1 mL Sydney IVF fertilization medium (Cook Medical Inc., Bloomington, IN, USA).

In those aliquots intended for PLCζ analysis, the remaining pellet was re-suspended with 200 μL of Phosphate Buffered Saline (PBS, Invitrogen Life Technologies; Carlsbad, CA, USA). After another centrifugation step at 800×g for 3 min, the supernatant was discarded without disturbing the pellet. The sperm pellet was then fixed with 100 μL 4% paraformaldehyde (PFA, Sigma-Aldrich; Gillingham, Dorset, UK) for 10 minutes. Following a final centrifugation and removal of PFA, the sperm pellet was re-suspended and stored in 200 μL PBS to await immunofluorescent analysis.

Evaluation of sperm concentration and motility

Sperm concentration and motility were evaluated before and after DGW. In both cases, sperm concentration was evaluated after diluting samples with sterile water (1/20) using a Neubauer chamber (Paul Marienfeld GmbH & Co. KG; Lauda-Königshofen, Germany). Sperm motility was evaluated in two independent replicates, and in each case this analysis was undertaken on the basis of the percentage of sperm exhibiting progressive sperm motility according to WHO guidelines (32). Motile count represented the number of sperm that exhibited progressive motility.

PLCζ antibody purification and specificity testing

Anti-human PLCζ was produced by Cova-Lab (UK) against two immunogenic peptides identified in the human PLCζ amino acid sequence (C-RESKSYFNPSNIKE-coNH₂ and C-ETHERKGSDKRGDN; Accession Number: AF532185). Prior to use, the neat antibody was purified using the two original immunogenic peptides and a SulfoLink Kit (Pierce Biotechnology, Rockford, USA). Specificity of this antibody was determined previously by competitive pre-incubation studies with excessive peptide Grasa et al. (29). This particular antibody has been utilised in a number of publications arising from this laboratory, yielding consistent staining patterns each time (25, 29, 30, 33).

Immunofluorescent detection of PLCζ and quantitative analyses

Samples were immunostained following the protocol described by Grasa et al. (29). Briefly, 100 µL of each sperm sample were first processed by DGW and then added onto slides pre-coated with 0.01% Poly-L-Lysine and permeabilized overnight with 0.5% Triton X-100 in PBS (Sigma Aldrich, UK). After one hour of incubation with 3% bovine serum albumin (BSA, Sigma-Aldrich), slides were incubated at 4°C overnight with 100 µL of 25 µg·mL⁻¹

anti-PLC ζ antibody (Cova-Lab, UK) diluted in 0.05% BSA. Samples were then washed thrice with PBS and incubated at room temperature for 1 hour with 5 $\mu\text{g}\cdot\text{mL}^{-1}$ of a secondary goat anti-rabbit antibody conjugated with Alexa Fluor 488 (Invitrogen, Paisley, UK). Slides were subsequently washed thrice with PBS, mounted with 3 μL Vectashield H-1200 mounting medium containing 4'-6'-Diamidini-2-phenylindole (DAPI) (Vector, UK), and finally covered with 20mm x 20mm glass cover slips.

Samples were observed under a fluorescence microscope (Eclipse 80i, Nikon UK Ltd., Kingston upon Thames, UK) at 40 \times magnification using a fluorescein isothiocyanate filter (FITC; Excitation filter: 465-495 nm; Dichroic Mirror: 505 nm; Barrier Filter: 515-550 nm) specific for green PLC ζ fluorescence (excitation wavelength: 488 nm). Images were acquired with a Nikon DS-Ri1 camera (Nikon UK) at an exposure time of 400 ms and 100 sperm were counted per sample. Camera settings, such as resolution (1280 \times 1024), quality (16-bit) and color at high contrast, were standardized to ensure illumination intensity was the same for every image captured. Bright field images were used to select sperm heads for analysis. Only sperm where the head was still attached to the tail were chosen, ensuring that such sperm did not overlap with others. Sperm heads were outlined using the region of interest manager (ROI) tool from ImageJ software (Version 1.46a; National Institutes of Health, Bethesda, MD, USA). For each sample, several fields were captured and mean PLC ζ levels (arbitrary units) were quantified using Image J software. Simultaneously, the total proportion (percentage) of sperm exhibiting PLC ζ , as well as the localization profile of PLC ζ in each sperm, were evaluated following the methodology described by Kashir et al. (25). Each analyzed sperm was classified into one of the following eight categories in terms of PLC ζ localization: acrosomal (A); post-acrosomal (PA); equatorial (E); acrosomal and post-acrosomal (A+PA); acrosomal and equatorial (A+E); post-acrosomal and equatorial (PA+E);

acrosomal, post-acrosomal and equatorial (A+PA+E); or ‘none’ indicating a total absence of PLC ζ .

In Vitro Fertilization and Intracytoplasmic Sperm Injection

Sperm used for IVF and ICSI treatment were previously washed and re-suspended in fertilization medium as described below. Oocytes were collected from women aged from 24 to 44 years. Women were first down-regulated using a GnRH long agonist and were given 400 μ g Nafarelin (Synrel; Pfizer, Milton Keynes, UK) intra-nasally twice a day from the mid-luteal phase (day 21) of the menstrual cycle. After three weeks of Nafarelin administration, women were tested for pituitary suppression, which was confirmed when serum oestradiol levels were lower than 150 pmol·L⁻¹. Women were subsequently given between 125 and 375 IU recombinant FSH (Gonal-F; Merck-Serono, Feltham, UK) subcutaneously, depending upon the age of the patient, previous ovarian response to gonadotrophins and basal serum FSH levels. Subsequently, ultrasound was performed, and follicular response recorded, from day 8 of gonadotrophin stimulation. When a minimum of three leading follicles of at least 18 mm in diameter were observed by ultrasonography, 6500 IU hCG (Ovitrelle; Serono Pharmaceuticals Ltd., Feltham, UK) were administered subcutaneously to trigger oocyte retrieval. Collection of oocytes was performed from 35.5 to 37 h later and the oocytes were subsequently left in Sydney IVF fertilization medium (Cook Medical Inc.) or Sydney IVF cleavage medium (Cook Medical Inc.) depending on whether they were destined for subsequent IVF or ICSI.

For IVF treatment, a total of 2×10^5 sperm·mL⁻¹ in Sydney IVF Fertilization Medium (Cook Medical Inc.) were mixed with 2-4 collected oocytes ($n=218$; Supplementary Table 1) in a well (final volume: 0.5 mL), using Nunc™ 4-Well Dishes for IVF (Thermo Fisher Scientific;

Waltham, MA, USA). Oocytes were covered with mineral oil (Sydney IVF culture medium; Cook Medical Inc.) and plates were then incubated at 37°C, 5% CO₂ and 6% O₂, and 89% N₂ for 16-19h in a MINC™ incubator (Cook Medical Inc.). After evaluating fertilization rates as formation of pronuclei in fertilized oocytes, fertilization medium was replaced by Sydney IVF Cleavage medium (Cook Medical Inc.). A total of 201 oocytes were used (Supplementary Table 1) and fertilization outcomes for controls and patients were 73.91 ± 1.43 and 8.75 ± 1.93 , respectively (Supplementary Table 2).

In the case of ICSI, oocytes ($n=127$; Supplementary Table 1) were stripped from cumulus cells within 2 – 2.5 hours of collection. Prior to intracytoplasmic injection, sperm were left in 10% polyvinylpyrrolidone (PVP; Cook Medical Inc.) for between 5 and 30 min. Afterwards, they were injected individually into each oocyte within 4 h of oocyte collection with a micromanipulator (Narishige NT-88-V3; Nikon UK) and under an inverted phase-contrast microscope (Nikon Eclipse Ti-S; Nikon UK). Three oocytes per well, in individual drops of 15 µL each, were left in IVF Sydney Cleavage medium. Oocytes were covered with mineral oil (Sydney IVF culture medium; Cook Medical Inc.) and incubated at 37°C, 5% CO₂ and 6% O₂, and 89% N₂ in a MINC™ incubator. Oocytes presenting two pronuclei 16h-25h post-insemination were considered successfully fertilized. Oocytes not presenting two pronuclei 25h post-insemination were considered unsuccessfully fertilized. Fertilization outcomes for controls and patients were 80.44 ± 3.15 and 9.88 ± 1.76 , respectively (Supplementary Table 2).

Statistical analyses

Data were analyzed using a statistical package (IBM® SPSS® 21.0 for Windows; IBM corp., Chicago, Illinois, USA) and plots were made using Origin Pro 8.0 software (OriginLab Corp.,

Northampton, Massachusetts, USA). All data were first analyzed using the Shapiro-Wilk test to check data for normality and the Levene test was used to assess homogeneity of variances (homocedasticity). When required, data were transformed using arcsine root transformation ($\arcsin \sqrt{x}$). If, even transformed, data did not match parametric assumptions, alternative non-parametric tests were used. For fertilization rates (FR), data were weighted by the number of MII-oocytes retrieved per patient and transformed using logit transformation ($\text{logit} = \ln(\text{FR}/1 - \text{FR})$).

Levels of PLC ζ , total proportions (in %) of sperm exhibiting PLC ζ , and the eight PLC ζ localization patterns were compared between controls and patients by *t*-test for independent samples or Mann-Whitney test. In addition, relationships of total levels of PLC ζ , total proportions of sperm exhibiting PLC ζ , and the eight PLC ζ localization patterns with progressive sperm motility, motile count and fertilization rates were evaluated by Pearson or Spearman correlation. Data are shown as mean \pm standard error of the mean (SEM), and level of significance was $P \leq 0.05$.

Results

Differences in the total number of sperm exhibiting PLC ζ in controls and patients subjected to both IVF and ICSI treatment

Figure 1 shows the percentage (mean \pm SEM) of sperm exhibiting PLC ζ in controls and patients undergoing IVF and ICSI cycles. While no significant differences ($P > 0.05$) were observed between controls and patients subjected to IVF treatment, patients intended for ICSI demonstrated significantly ($P < 0.05$) lower percentages of sperm exhibiting PLC ζ as compared to controls.

Differences in the total levels of PLCζ between controls and patients subjected to both IVF and ICSI treatment

Total levels of PLCζ in sperm from controls and patients in either IVF or ICSI treatment are shown in Figure 2 (mean ± SEM). For both IVF and ICSI, total levels of PLCζ were higher in controls than in patients. However, this increase was only statistically significant ($P<0.05$) in the case of ICSI patients.

Differences in PLCζ localization patterns in sperm from controls and patients subjected to IVF and ICSI treatment

Localization patterns of PLCζ in controls and patients were compared in males undergoing either IVF or ICSI treatment. In the case of IVF (Table 1, mean ± SEM), no significant differences between controls and patients were seen in any of the eight PLCζ localization patterns evaluated.

ICSI patients presented significantly ($P<0.05$) lower percentages of sperm exhibiting post-acrosomal and a combined pattern of PLCζ in equatorial, acrosomal and post-acrosomal regions (Table 1). Conversely, the proportion (%) of sperm that were devoid of PLCζ was significantly ($P<0.05$) higher in ICSI patients than in their control counterparts.

Correlations of conventional sperm parameters with total levels of PLCζ and the proportion of sperm exhibiting PLCζ

Total levels of PLCζ were found to be significantly ($P<0.05$) correlated with % progressive motile sperm and with sperm motility count. In addition, percentages of sperm exhibiting a post-acrosomal PLCζ-pattern were significantly ($P<0.05$) correlated with % progressive motile sperm ([Supplementary Table 3](#)).

Correlations between PLCζ and fertilization rates following ICSI and IVF procedures

The correlation between fertilization rates, the proportion of sperm exhibiting PLCζ, and the total levels of PLCζ was also examined (Table 2). While IVF fertilization rates were not seen to be significantly ($P>0.05$) correlated with any of the PLCζ parameters evaluated, the total levels of PLCζ, % total sperm exhibiting PLCζ, % sperm exhibiting PLCζ in post-acrosomal region, % sperm exhibiting PLCζ in equatorial region, % sperm exhibiting PLCζ in acrosomal and post-acrosomal regions, % sperm exhibiting PLCζ in acrosomal, post-acrosomal and equatorial regions demonstrated significant positive correlation ($P<0.05$) with fertilization rates following ICSI. Concomitantly, the proportion (%) of sperm that were devoid of PLCζ was negatively ($P<0.05$) correlated with fertilization rates following ICSI.

Discussion

Our results have shown for the first time that total levels, localization patterns and the proportion (%) of sperm exhibiting PLCζ are significantly correlated with fertilization rates following ICSI but not IVF treatment. This result is of paramount importance as it strongly indicates sperm PLCζ as being a useful prognostic and diagnostic marker for ICSI outcome. It is well understood that human infertility can be attributed to both male and female factors and that ICSI is predominantly implemented to treat male factor infertility (2). Although fertilization rates following ICSI routinely exceed 80%, 3-5% of ICSI cases still encounter fertilization failure (34, 35). In addition, while conventional semen analysis provides a general assessment with regard to sperm quality, an excessively large number of infertile patients tend to present a normal spermiogram (36-39). Therefore, it is fundamental to develop a novel or additional biological tool in order to predict the fertilizing ability of a given sperm sample, as this study sought.

Since the discovery of the sperm-soluble oocyte activation factor, PLC ζ (20), a strong body of both biochemical and clinical evidence has steadily accumulated in support of the fundamental role of PLC ζ as a universal trigger for oocyte activation in mammalian (19, 20, 24, 40, 41) and non-mammalian species (42-45). In spite of this, recent studies suggest that a post-acrosomal sperm protein found in the perinuclear theca, and known as PAWP, elicits calcium oscillations similar to those seen during fertilization in mammalian (46, 47) and non-mammalian oocytes (48). Furthermore, a significant correlation between PAWP levels in human sperm and fertilization rates following ICSI has been reported (49). These recent findings from Aarabi et al. (47, 49) have led to significant debate with regard to understanding the essential role of these two proteins (i.e. PLC ζ and PAWP) as oocyte activation factors (50-52). However, the true extent of the functional role of PAWP, and indeed its precise relevance in the oocyte activation process, should for now be treated with caution as the results of a recent study conflicted directly with those of Aarabi et al. (47) by failing to demonstrate calcium oscillations in mouse oocytes in response to the injection of recombinant PAWP (28). Our present study did not evaluate PAWP in our clinical patients, but our current dataset clearly demonstrates a strong clinical link between the total levels of PLC ζ in human sperm and fertilization rates following ICSI, thus strongly indicating PLC ζ as a biomarker for ICSI outcome.

From our data, the total levels and proportions of sperm exhibiting PLC ζ are significantly correlated with fertility outcomes following ICSI, but not IVF treatment. A previous study from this laboratory (25) showed that a high variation within control and patient groups existed, and suggested that this could limit the use of PLC ζ as a biomarker to predict fertility outcomes. While in the present study a high variation within control and patient groups was also observed, the correlations of total levels, localization patterns and proportions of sperm

exhibiting PLC ζ with fertility outcomes were found when ICSI and IVF were evaluated separately. Moreover, localization patterns of PLC ζ also demonstrated significant correlation with fertilization rates following ICSI. Seven localization patterns of PLC ζ in human sperm were identified by Kashir et al. (25); acrosomal (A), equatorial (E), post-acrosomal (P), A+P, A+E, P+E and all three patterns together (A+E+P). In the present study, four out of the seven localization patterns identified: percentages of sperm exhibiting PLC ζ (P) region, (E) region, (A+P) region and in (A+E+P) regions, were shown to be significantly correlated with fertilization rates following ICSI treatment. While our data suggest that a significant correlation is only observed in fertilization rates following ICSI, this does not preclude the critical role of PLC ζ in activating the oocyte during IVF treatment. This may further suggest that deficiency of PLC ζ may not be the major cause of IVF failure, but may also be associated with other factors such as the interaction of capacitated sperm with the oocyte zona pellucida and the acrosome reaction, since these events are bypassed during ICSI (53).

Apart from highlighting the potential value of PLC ζ as a fertility marker for individual sperm and ICSI outcome, the use of a significant number of clinical samples ($n=44$) in the present work provides significant support to the fundamental role of PLC ζ in determining oocyte activation and fertilization rates. However, approximately 40% of ICSI failure cases are attributed to oocyte activation deficiency (13, 18, 54). Thus, ICSI failure is not restricted to only sperm factor defects (13, 55), but may also include potential oocyte factors such as nuclear and cytoplasmic maturation (35). This might explain why correlation coefficients between PLC ζ and fertility outcomes in ICSI were statistically significant, but were never higher than 0.6. This, apart from indicating inter-individual differences, implies that other factors independent from PLC ζ levels, proportions, and localization pattern (e.g. idiopathic oocyte problems) may also contribute to ICSI failure.

398

399 Finally, the differential localization patterns of PLC ζ across different mammalian species and
400 their functional roles during oocyte activation and fertilization rates still remain unclear
401 (reviewed in (27)). However, it is well understood that PLC ζ should reside at a readily
402 accessible region within the sperm head, i.e. in an equatorial or post-acrosomal location (31),
403 that would permit the rapid dispersion of this protein into the oocyte following gamete fusion
404 (56). In this context, the significant correlation between ICSI fertilization outcomes and
405 equatorial and post-acrosomal patterns observed in the present study confirms, for the first
406 time using a significant number of clinical samples, the critical role of equatorial and post-
407 acrosomal localizations of PLC ζ for oocyte activation.

408

409 In conclusion, our findings demonstrate, for the first time, that evaluating total levels,
410 localization patterns and proportions of sperm exhibiting PLC ζ may be used as a valuable
411 prognostic marker for ICSI fertilization rates in couples for which IVF is unadvised or has
412 failed previously. This provides important new data to assist with the translation of PLC ζ to
413 the clinic, and extends our previous work conducted with a smaller sample size that did not
414 allow us to distinguish between ART (25). While our data, compiled from a large clinical
415 dataset, provide significant support for the fundamental role of PLC ζ in the oocyte activation
416 process, they also show that causes other than PLC ζ , for example those related to molecular
417 machinery inside the oocyte, may also underlie ICSI failure. Therefore, further research
418 aiming to elucidate these other potential causes is warranted.

419

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Author's contribution

S.Y. and S.N.A. conducted the immunostaining experiments, acquired data and critically revised the manuscript. M.Y. analyzed and interpreted the data, and wrote the manuscript. C.J. contributed to experimental design and critically revised the manuscript. J.K. analyzed the stained samples and revised the manuscript. G.M. and S.J.M.D.S. recruited the patients and provided clinical data. E.McV. and C.L.B. provided clinical expertise/data and critically revised the manuscript. K.C. conceived and designed the experiment, contributed to data interpretation, and revised the manuscript. All authors read and approved the final version of this manuscript.

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

References

1. Mascarenhas MN, Flaxman SR, Boerma T, Vanderpoel S, Stevens GA National, regional, and global trends in infertility prevalence since 1990: a systematic analysis of 277 health surveys. *PLoS Med* 2012; 9: e1001356.
2. HFEA. Latest UK IVF figures–2011 and 2012. HFEA 2012, <http://www.hfea.gov.uk/docs/FertilityTreatment2012TrendsFigures.PDF>. [date accessed: 11th October 2014].
3. Bhattacharya S, Maheshwari A, Mollison J. Factors associated with failed treatment: an analysis of 121,744 women embarking on their first IVF cycles. *PLoS One* 2013; 8: e82249. doi: 10.1371/journal.pone.0082249.
4. Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 1992; 340: 17–18.
5. Vanden Meerschaut F, Leybaert L, Nikiforaki D, Qian C, Heindryckx B, De Sutter P. Diagnostic and prognostic value of calcium oscillatory pattern analysis for patients with ICSI fertilization failure. *Hum Reprod* 2013; 28: 87-98.
6. Vanden Meerschaut F, Nikiforaki D, Heindryckx B, De Sutter P. Assisted oocyte activation following ICSI fertilization failure. *Reprod Biomed Online* 2014; 28: 560-571.
7. Kupka MS, Ferraretti AP, de Mouzon J, Erb K, D'Hooghe T, Castilla JA, Calhaz-Jorge C, De Geyter C, Goossens V; European IVF-monitoring (EIM); Consortium, for the European Society of Human Reproduction and Embryology (ESHRE). Assisted reproductive technology in Europe, 2010: results generated from European registers by ESHRE. *Hum Reprod* 2014; 29: 2099-2113
8. Yanagida K, Fujikura Y, Katayose H. The present status of artificial oocyte activation in assisted reproductive technology. *Reprod Med Biol.* 2008; 7: 133-142.

- 467 9. Dam AH, Feenstra I, Westphal JR, Ramos L, van Golde RJ, Kremer JA. Globozoospermia
468 revisited. *Hum Reprod Update* 2007; 13: 63–75.
- 469 10. Swain JE, Pool TB. ART failure: oocyte contributions to unsuccessful fertilization. *Hum*
470 *Reprod Update* 2008; 14: 431-446.
- 471 11. Flaherty SP, Payne D, Matthews CD. Fertilization failures and abnormal fertilization after
472 intracytoplasmic sperm injection. *Hum Reprod* 1998; 13 Suppl 1: 155–164.
- 473 12. Rawe VY, Olmedo SB, Nodar FN, Doncel GD, Acosta AA, Vitullo AD. Cytoskeletal
474 organization defects and abortive activation in human oocytes after IVF and ICSI failure. *Mol*
475 *Hum Reprod* 2000; 6: 510-516.
- 476 13. Kashir J, Heindryckx B, Jones C, De Sutter P, Parrington J, Coward K. Oocyte activation,
477 phospholipase C zeta and human infertility. *Hum Reprod Update* 2010; 16: 690-703.
- 478 14. Jones KT, Carroll J, Merriman JA, Whittingham DG, Kono T. Repetitive sperm-induced
479 Ca^{2+} transients in mouse oocytes are cell cycle dependent. *Development* 1995; 121: 3259-
480 3266.
- 481 15. Horner VL, Wolfner MF. Transitioning from egg to embryo: Triggers and mechanisms of
482 egg activation. *Dev Dyn* 2008; 237: 527–544.
- 483 16. Kashir J, Deguchi R, Jones C, Coward K, Stricker SA. Comparative biology of sperm
484 factors and fertilization-induced calcium signals across the animal kingdom. *Mol Reprod Dev*
485 2013; 80: 787-815.
- 486 17. Swann K, Saunders CM, Rogers NT, Lai FA. PLCzeta(zeta): a sperm protein that triggers
487 Ca^{2+} oscillations and egg activation in mammals. *Semin Cell Dev Biol* 2006; 17: 264-273.
- 488 18. Amdani SN, Jones C, Coward K. Phospholipase C zeta (PLC ζ): oocyte activation and
489 clinical links to male factor infertility. *Adv Biol Regul* 2013; 53: 292-308.

490 19. Cox L, Larman MG, Saunders CM, Hashimoto K, Swann K, Lai FA. Sperm
 491 phospholipase C ζ from humans and cynomolgus monkeys triggers Ca²⁺ oscillations,
 492 activation and development of mouse oocytes. *Reproduction* 2002; 124: 611–623.

493 20. Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, Swann K, Lai
 494 FA. PLC ζ : a sperm-specific trigger of Ca²⁺ oscillations in eggs and embryo development.
 495 *Development* 2002; 129: 3533–3544.

496 21. Yoon SY, Eum JH, Lee JE, Lee HC, Kim YS, Han JE, Won HJ, Park SH, Shim SH, Lee
 497 WS, et al. (2012). Recombinant human phospholipase C zeta 1 induces intracellular calcium
 498 oscillations and oocyte activation in mouse and human oocytes. *Hum Reprod* 2012; 27: 1768-
 499 1780.

500 22. Nomikos M, Yu Y, Elgmati K, Theodoridou M, Campbell K, Vassilakopoulou V, Zikos
 501 C, Livaniou E, Amso N, Nounesis G, Swann K, Lai FA. Phospholipase C ζ rescues failed
 502 oocyte activation in a prototype of male factor infertility. *Fertil Steril* 2013; 99: 76-85.

503 23. Yoon SY, Jellerette T, Salicioni AM, Lee HC, Yoo MS, Coward K, Parrington J, Grow D,
 504 Cibelli JB, Visconti PE, et al. Human sperm devoid of PLC, zeta 1 fail to induce Ca²⁺ release
 505 and are unable to initiate the first step of embryo development. *J Clin Invest* 2008; 118: 3671-
 506 3681.

507 25. Kashir J, Jones C, Mounce G, Ramadan WM, Lemmon B, Heindryckx B, de Sutter P,
 508 Parrington J, Turner K, Child T, et al. Variance in total levels of phospholipase C zeta (PLC-
 509 ζ) in human sperm may limit the applicability of quantitative immunofluorescent analysis as a
 510 diagnostic indicator of oocyte activation capability. *Fertil Steril* 2013; 99: 107-117.

511 26. Lee HC, Army M, Grow D, Dumesic D, Fissore RA, Jellerette-Nolan T. Protein
 512 phospholipase C Zeta1 expression in patients with failed ICSI but with normal sperm
 513 parameters. *J Assist Reprod Genet* 2014; 31: 749-756.

514 27. Kashir J, Nomikos M, Lai FA, Swann K. Sperm-induced Ca^{2+} release during egg
515 activation in mammals. *Biochem Biophys Res Commun* 2014; 450: 1204-1211.

516 28. Nomikos M, Sanders JR, Theodoridou M, Kashir J, Matthews E, Nounesis G, Lai FA,
517 Swann K. Sperm-specific post-acrosomal WW-domain binding protein (PAWP) does not
518 cause Ca^{2+} release in mouse oocytes. *Mol Hum Reprod* 2014; 20: 938-947.

519 29. Grasa P, Coward K, Young C, Parrington J. The pattern of localization of the putative
520 oocyte activation factor, phospholipase C ζ , in uncapacitated, capacitated, and ionophore-
521 treated human spermatozoa. *Hum Reprod* 2008; 23: 2513-2522.

522 30. Kashir J, Heynen A, Jones C, Durrans C, Craig J, Gadea J, Turner K, Parrington J,
523 Coward K. Effects of cryopreservation and density-gradient washing on phospholipase C ζ
524 concentrations in human spermatozoa. *Reprod Biomed Online* 2011; 23: 263-267.

525 31. Escoffier J, Yassine S, Lee HC, Martinez G, Delaroche J, Coutton C, Karaouzène T,
526 Zouari R, Metzler-Guillemain C, Pernet-Gallay K, Hennebicq S, Ray PF, Fissore R, Arnoult
527 C. Subcellular localization of phospholipase C ζ in human sperm and its absence in DPY19L2-
528 deficient sperm are consistent with its role in oocyte activation. *Mol Hum Reprod* 2015; 21:
529 157-168.

530 32. WHO. World Health Organization Laboratory Manual for the Examination and
531 Processing of Human Semen. Geneva: World Health Organization, 2010.

532 33. Kashir J, Sermondade N, Sifer C, Oo SL, Jones C, Mounce G, Turner K, Child T,
533 McVeigh E, Coward K. Motile sperm organelle morphology evaluation-selected
534 globozoospermic human sperm with an acrosomal bud exhibits novel patterns and higher
535 levels of phospholipase C ζ . *Hum Reprod* 2012; 27: 3150-3160.

536 34. Palermo GD, Neri QV, Takeuchi T, Rosenwaks Z. ICSI: where we have been and where
537 we are going. *Semin Reprod Med* 2009; 27: 191-201.

538 35. Neri QV, Lee B, Rosenwaks Z, Machaca K, Palermo GD. Understanding fertilization
539 through intracytoplasmic sperm injection (ICSI). *Cell Calcium* 2014; 55: 24-37.

540 36. Guzick DS, Overstreet JW, Factor-Litvak P, Brazil CK, Nakajima ST, Coutifaris C,
541 Carson SA, Cisneros P, Steinkampf MP, Hill JA, Xu D, Vogel DL, National Cooperative
542 Reproductive Medicine Network. Sperm morphology, motility, and concentration in fertile
543 and infertile men. *N Engl J Med* 2001; 345: 1388-1393.

544 37. Keel BA. Within- and between-subject variation in semen parameters in infertile men and
545 normal semen donors. *Fertil Steril* 2006; 85: 128-134.

546 38. Samplaski MK, Agarwal A, Sharma R, Sabanegh E. New generation of diagnostic tests
547 for infertility: review of specialized semen tests. *Int J Urol* 2010; 17: 839-847.

548 39. Barratt CL, Mansell S, Beaton C, Tardif S, Oxenham SK. Diagnostic tools in male
549 infertility-the question of sperm dysfunction. *Asian J Androl* 2011; 13: 53-58.

550 40. Kashir J, Konstantinidis M, Jones C, Heindryckx B, De Sutter P, Parrington J, Wells D,
551 Coward K. Characterization of two heterozygous mutations of the oocyte activation factor
552 phospholipase C zeta (PLC ζ) from an infertile man by use of minisequencing of individual
553 sperm and expression in somatic cells. *Fertil Steril* 2012; 98: 423-431

554 41. Kashir J, Konstantinidis M, Jones C, Lemmon B, Lee HC, Hamer R, Heindryckx B,
555 Deane CM, De Sutter P, Fissore RA, Parrington J, Wells D, Coward K. A maternally
556 inherited autosomal point mutation in human phospholipase C zeta (PLC ζ) leads to male
557 infertility. *Hum Reprod* 2012; 27: 222-231.

558 42. Coward K, Ponting CP, Chang H-Y, Hibbitt O, Savolainen P, Jones KT, Parrington J.
559 Phospholipase C ζ , the trigger of egg activation in mammals, is present in a non-mammalian
560 species. *Reproduction* 2005; 130: 157–163.

561 43. Coward K, Ponting CP, Zhang N, Young C, Huang CJ, Chou CM, Kashir J, Fissore RA,
562 Parrington J. Identification and functional analysis of an ovarian form of the egg activation
563 factor phospholipase C zeta (PLC ζ) in pufferfish. *Mol Reprod Dev* 2011; 78: 48–56.

564 44. Ito M, Shikano T, Oda S, Horiguchi T, Tanimoto S, Awaji T, Mitani H, Miyazaki S.
565 Difference in Ca²⁺ oscillation-inducing activity and nuclear translocation ability of PLCZ1,
566 an egg-activating sperm factor candidate, between mouse, rat, human, and medaka fish. *Biol*
567 *Reprod* 2008; 78: 1081-1090.

568 45. Mizushima S, Takagi S, Ono T, Atsumi Y, Tsukada A, Saito N, Shimada K.
569 Phospholipase Czeta mRNA expression and its potency during spermatogenesis for activation
570 of quail oocyte as a sperm factor. *Mol Reprod Dev* 2009; 76: 1200-1207.

571 46. Wu ATH, Sutovsky P, Manandhar G, Xu W, Katayama M, Day BN, Park KW, Yi YJ, Xi
572 YW, Prather RS, Oko R. PAWP, a Sperm-specific WW domain-binding protein, promotes
573 meiotic resumption and pronuclear development during fertilization. *J Biol Chem* 2007; 282:
574 12164-12175.

575 47. Aarabi M, Balakier H, Bashar S, Moskovtsev SI, Sutovsky P, Librach CL, Oko R. Sperm-
576 derived WW domain-binding protein, PAWP, elicits calcium oscillations and oocyte
577 activation in humans and mice. *FASEB J* 2014; 28: 4434-4440.

578 48. Aarabi M, Qin Z, Xu W, Mewburn J, Oko R. Sperm-borne protein, PAWP, initiates
579 zygotic development in *Xenopus laevis* by eliciting intracellular calcium release. *Mol Reprod*
580 *Dev* 2010; 77: 249–256.

581 49. Aarabi M, Balakier H, Bashar S, Moskovtsev SI, Sutovsky P, Librach CL, Oko R. Sperm
582 content of postacrosomal WW binding protein is related to fertilization outcomes in patients
583 undergoing assisted reproductive technology. *Fertil Steril* 2014; 102: 440-447.

584 50. Aarabi M, Sutovsky P, Oko R. Re: Is PAWP the 'real' sperm factor? *Asian J Androl* 2015;
585 doi: 10.4103/1008-682X.145071.

586 51. Nomikos M, Swann K, Lai FA. Is PAWP the "real" sperm factor? Asian J Androl 2015;
587 doi:10.4103/1008-682X.142145.

588 52. Vadnais ML, Gerton LG. From PAWP to "Pop": opening up new pathways to fatherhood.
589 Asian J Androl 2015; doi: 10.4103/1008-682X.142140.

590 53. Beck-Fruchter R, Lavee M, Weiss A, Geslevich Y, Shalev E. Rescue intracytoplasmic
591 sperm injection: a systematic review. Fertil Steril 2014; 101: 690-698.

592 54. Mahutte NG, Arici A. Failed fertilization: is it predictable? Curr Opin Obstet Gynecol
593 2003; 15: 211-218.

594 55. Ramadan WM, Kashir J, Jones C, Coward K. Oocyte activation and phospholipase C zeta
595 (PLC ζ): diagnostic and therapeutic implications for assisted reproductive technology. Cell
596 Commun Signal 2012; 10: 12.

597 56. Nomikos M, Swann K, Lai FA. Starting a new life: sperm PLC-zeta mobilizes the Ca²⁺
598 signal that induces egg activation and embryo development: an essential phospholipase C
599 with implications for male infertility. Bioessays 2012; 34: 126-134.

600

601 **Figure legends**

602 **Figure 1**

603 The proportion (%) of sperm exhibiting PLC ζ (mean \pm SEM) in controls and patients
604 following IVF and ICSI treatment. Annotation with an asterisk (*) denotes significant
605 ($P<0.05$) differences in patients with regard to controls

606

607 **Figure 2**

608 Mean fluorescent intensity (arbitrary units) of PLC ζ levels per sperm (mean \pm SEM) in
609 controls and patients following IVF and ICSI treatment. Annotation with an asterisk (*)
610 denotes significant ($P<0.05$) differences in patients with regard to controls

611

612 **Supplementary Figure 1**

613 Representative image of a human sperm showing equatorial and post-acrosomal PLC ζ
614 immunostaining