F & S 2nd revision

The use of phospholipase C zeta (PLCζ) analysis to identify candidates for artificial oocyte activation (AOA): a case series of clinical pregnancies and a proposed algorithm for patient management

Running title: PLCζ identifies candidates for AOA (30/40 letters)

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Capsule (20/30)

Immunofluorescence analysis of PLCζ in sperm of men with previous fertilization failure can guide clinicians to identify candidates for ICSI/AOA

Abstract

Objective: To investigate the applicability of PLCζ analysis in assisting the clinical decision-making process when considering artificial oocyte activation (AOA) for infertile males in assisted reproductive technology (ART).

Design: To screen 46 males (43 infertile/13 fertile) using our PLCζ assay.

Setting: Fertility unit/university laboratory.

Patients: Infertile males with (1) abnormal sperm morphology, (2) total fertilization failure, low fertilization rate (<50%), or repeated fertilization failure in ART.

Intervention(s): We analysed PLCζ levels in sperm from fertile and infertile males. Eligible patients subsequently underwent ICSI/AOA with calcimycin (GM508).

Main Outcome Measure(s): PLCζ localization, level and the proportion of sperm expressing PLCζ. Thresholds of PLCζ-deficiency, fertilization rates, pregnancy rates and live birth rates of AOA and non-AOA cycles.

Results: Compared with 13 controls, 34 of the 43 infertile males had significantly lower levels of PLCζ and/or a significantly lower proportion of sperm exhibiting PLCζ. Of these 34 patients, 15 showed a significant PLCζ reduction in both parameters, which we termed 'PLCζ deficiency'. Five PLCζ-deficient patients opted for AOA; all 5 achieved fertilization and 4 achieved clinical pregnancies and live births. Fertilization rate improved significantly from 18.6% (intracytoplasmic sperm injection, ICSI) to 56.8% (ICSI/AOA) ($p<0.001$). The clinical pregnancy rate and live birth rate with AOA were both 40% per initiated cycle. Youden index analysis revealed that the cut-offs below which infertile males were likely to benefit from AOA were 71% for the proportion of sperm expressing PLCζ and 15.57 arbitrary units for mean PLCζ level.

Conclusion: PLCζ analysis is a useful diagnostic tool to determine patient eligibility for subsequent AOA treatment.

Key words

artificial oocyte activation, calcium ionophore, oocyte activation deficiency, phospholipase C zeta and PLCζ deficiency

Introduction

In recent decades, assisted reproductive technology (ART) has significantly improved pregnancy outcomes for infertile couples. Conventional *in-vitro* fertilization (IVF) has been mainly used in cases of unexplained and female factor infertility, while intracytoplasmic sperm injection (ICSI) specifically addresses severe male factor. In addition, ICSI is an effective option for couples who experience recurrent fertilization failure with conventional IVF, although its use varies according to different national guidelines and local protocols. Worldwide, ICSI was used in 66% of non-donor IVF cycles between 2008 and 2010 (1), while in the UK it accounted for only 36% of cycles in 2016 (2). According to figures released by the HFEA in 2014, male factor is associated with 10% of failed IVF cases and 50.4% of failed ICSI cases (2). Despite ensuring the direct injection of an individual sperm into one oocyte, total fertilization failure (TFF), where all mature oocytes fail to fertilize, remains a problem and occurs in 1-3% of ICSI cycles (3-5). In addition, the chance of recurrent fertilization failure can be as high as 30% (6). The principal cause of TFF is oocyte activation failure, known as oocyte activation deficiency (OAD) (7).

Oocyte activation is a spatial-temporal process that includes a series of fundamental embryonic events such as meiosis resumption, pronuclei development, polyspermy blockage, maternal messenger RNA degradation, protein synthesis and cytoskeleton rearrangement (8). These phenomena take place shortly after gamete fusion, and mark the transition of a mature oocyte into an embryo (7). The main biochemical process behind mammalian oocyte activation involves a series of oscillations in calcium ions (Ca^{2+}) released from the intracellular stores of the endoplasmic reticulum (9). Calcium oscillation has been observed in mouse oocytes injected with fertile human sperm (10). Furthermore, sperm from patients with failed ICSI has been shown to trigger abnormal Ca^{2+} oscillatory patterns, or no oscillations in mouse oocytes,

suggesting a diagnostic and prognostic use to the analysis of calcium responses in patients with TFF (11).

OAD can result from female- or male-related factors. The main reason leading to OAD is a deficiency in phospholipase C zeta (PLCζ), a sperm-specific factor (12). Other causes contributing to OAD include oocyte arrest in the mitotic metaphase, nuclear deficiency, and abnormal formations of polar body and spindle structures (13-15). There is now substantial scientific and clinical evidence to support the role of PLCζ in triggering Ca^{2+} release and the initiation of oocyte activation. PLCζ is a testis-specific protein located in the sperm head of both mammalian and non-mammalian species (16, 17). The microinjection of sperm extracts containing PLCζ into mouse oocytes elicits fertilization-like Ca^{2+}oscillations and oocyte activation (12, 18). Injecting PLCζ cRNA (19) and recombinant PLCζ (20) into mouse oocytes has been shown to effectively induce the release of Ca^{2+}, illustrating the trigger effect of PLCζ upon the initial stages of embryogenesis.

The critical role of PLCζ in oocyte activation has been further substantiated by evidence that an abnormally low amount of PLCζ, or a reduction in its activity, leads to OAD (21, 22). The injection of PLCζ-devoid sperm from patients with a history of ICSI failure into mouse oocytes has been shown to result in a lack of Ca^{2+} oscillations and oocyte activation failure (21). Moreover, the relative immunofluorescent intensity of PLCζ in infertile patients diagnosed with OAD has been found to be significantly lower than in fertile controls, and different localization patterns of PLCζ in sperm have also been associated with OAD (23). In addition, PLCζ has been linked with semen parameters (24), sperm morphology (25) and male age (26).

In human sperm, PLCζ is predominantly expressed in the equatorial region of sperm heads (27), or distributed between the equatorial and acrosomal/post-acrosomal regions (23). The spatial pattern of PLCζ expression within spermatozoa is thought to be important for

oocyte activation, although the dominant localization is the subject of some debate (28). Indeed, the evidence on how PLCζ levels and localization patterns correlate to fertilization rates in ICSI is conflicting (28-30). Collectively, previous data have suggested the use of PLCζ as a diagnostic marker for predicting OAD (29-31).

Currently, the only viable clinical option for OAD rescue is artificial oocyte activation (AOA). AOA involves the use of various mechanical, physical or chemical stimuli to artificially reproduce the release of Ca^{2+} (3), and it has been successful in triggering oocyte activation in human (32, 33). However, AOA may induce a monotonic rise of Ca^{2+} in oocytes, instead of Ca^{2+} oscillations (34, 35), and it may also lead to aneuploidy during mammalian embryogenesis (36). Therefore, the Human Fertilisation and Embryology Authority (HFEA) recommends that treatment with AOA should be limited to infertile patients with clear evidence of oocyte activation deficiency, for example, PLCζ deficiency (37, 38). Nevertheless, the diagnostic applicability of AOA in clinical practice remains unclear and varies between clinics. Most IVF clinics carry out AOA mainly based on patient history of TFF, recurrent fertilization failure, low fertilization rate in ICSI, abnormal sperm morphology, or suspected OAD (39-42). Furthermore, the cut-off for low fertilization rate of couples in previous IVF/ICSI treatments often varies between 0 – 50% of total fertilized oocytes (32, 43, 44).

The present study aimed to use our in-house PLCζ assay to investigate PLCζ protein expression in patients with a history of fertilization failure following IVF/ICSI, and to identify patient eligibility criteria for AOA treatment. For the first time, we present the diagnostic applicability of our in-house assay in identifying patients eligible for AOA, as demonstrated by the four successful ICSI-AOA cases in men with clear evidence of PLCζ deficiency.

Materials and methods

Patients

Written informed consent was obtained from all participants. Forty-three infertile male patients and 13 fertile controls were recruited from Oxford Fertility, UK, between October 2016 and April 2019. Eligibility criteria for case study subjects included males with at least one of the following features: (1) abnormal sperm head morphology (i.e., either grossly abnormal, 100% globozoospermic, or pin-shape/ enlarged/diminished head), (2) a history of total fertilization failure, low fertilization rate (<50%), or recurrent fertilization failure in previous IVF/ICSI cycles. Participants' fertilization history was recorded either by our fertility centre, or other IVF centres in the UK previously involved in their care. Exclusion criteria included (1) severe oligozoospermia where the number of sperm identified on the staining glass slide (required for subsequent immunofluorescence staining) were fewer than 100 and hence insufficient for testing with our PLCζ assay, and (2) sperm that was surgically retrieved. Control subjects included: (1) men who had previously fathered a child via natural conception within 10 years prior to the time of consent, or (2) men who had normal parameters in semen analysis according to World Health Organization (WHO) guidelines (45), and whose sperm had fertilized one or more oocytes in previous IVF cycles.

According to PLCζ expression in sperm, infertile patients were divided into three groups: (1) PLCζ-deficient group, including patients who showed significantly lower mean PLCζ levels in sperm, and significantly lower proportion of sperm exhibiting PLCζ, when compared to those of fertile controls; (2) PLCζ-reduced group, including patients who showed either significantly lower mean PLCζ levels in sperm, or a significantly lower proportion of sperm exhibiting PLCζ, when compared to those of fertile controls; and (3) PLCζ-normal group, including patients who showed no significant difference in the aforementioned two parameters when compared to fertile controls. Patients from the PLCζ-deficient group were offered clinical advice on AOA treatment.

Sperm preparation

All semen samples were collected after 2-7 days of abstinence, as recommended by the WHO in 2010 (46). Ejaculated semen from all 56 males (43 infertile and 13 fertile) was subjected to density gradient washing (DGW) described elsewhere (26). After DGW, the sperm was fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich, UK) for 10 mins. Fixed sperm were resuspended in PBS and stored at 4°C before staining.

Antibody purification

A rabbit polyclonal antibody was synthesized by Cova-lab (Villeurbanne, France), with peptides derived from two human PLCζ amino acid sequences (C-ETHERKGSDKRGDN, and C-RESKSYFNPSNIKE-coNH_2; Accession Number: AF532185). The specific antibody was purified using immunogenic peptides binding to an agarose column according to the manufacturer's instructions (SulfoLink Kit, Pierce Biotechnology, USA) (47). Antibody specificity was determined by preincubation with excessive PLCζ peptides (27).

Immunofluorescence staining and quantitative analyses

Sperm concentration was determined using a Nikon microscope before staining. The PLCζ assay requires three days for immunostaining and was performed as previously described with modifications (27). Briefly, 50 µL of fixed sperm were loaded onto hydrophobic molds coated with 0.01% Poly-L-Lysine solution (Sigma-Aldrich, UK) and left to settle for 30 mins. Sperm were then permeabilized with 0.5% Triton X-100 in PBS overnight at 4 ℃. Slides were subsequently incubated with 3% BSA (Sigma, UK) in PBS at room temperature (RT), followed by probing with 25 µg/mL rabbit anti-human PLCζ antibody in 0.05% BSA-PBS and overnight incubation at 4 ℃. Slides were then incubated with 5 µg/mL of AlexaFluor-488-conjugated secondary goat anti-rabbit antibody (Thermo Fisher Scientific, UK) for 1 h at RT. Washing

with PBS was performed three times in between all the aforementioned steps. Slides were then mounted with Vectashield H-1200 medium containing 4'-6'-diamidini-2-phenylindole (Vector, UK) and observed at x40 magnification using a fluorescence microscope equipped with a fluorescence and an isothiocyanate filter (Eclipse 80i, Nikon, UK). A Nikon camera (Nikon UK, Ltd) was used to photograph the slides. Sperm were viewed in a bright-field channel and PLCζ fluorescence was observed using the FITC channel at 400 ms exposure time. Multiple fields of view were captured. Light settings of the microscope and camera remained the same for each experiment.

The in-house PLCζ assay requires at least 100 cells on the staining glass slides for sample analysis (i.e., at least 100 sperm heads can be circled for the assay to work). It is sometimes difficult to predict whether a sperm sample will meet the minimum number of 100 cells before running the PLCζ analysis, as sperm loss is inevitable during the immunofluorescence staining. Therefore, in cases of severe low sperm count, clinicians advised patients that the laboratory would make every endeavour to successfully analyse samples. In such circumstances, we used all sperm after DGW for the assay, hoping that at least 100 cells would remain on the slides after staining was completed. After capturing multiple fields of view, images were analysed using Image J (National Institute of Health, USA). Sperm heads were first circled in brightfield, and only those with attached tails were selected for analysis. The fluorescence intensity of PLCζ in sperm heads was quantified with Image J, using the 'region of interest (ROI)' tool, and normalized to fluorescence background. The mean level of ROI was then calculated (termed "relative fluorescence intensity") and compared between individuals. The proportion of patient sperm exhibiting PLCζ and the localization patterns of PLCζ were also assessed. Analysed sperm were categorized into one of eight patterns of recognised PLCζ localization (23). Quality control of the in-house assay was carried out by ensuring that different researchers obtained the same analysis results using sperm

from the same male. PLCζ analysis was also performed on different days by the same researcher, in order to ensure a consistent performance. Furthermore, we performed the in-house PLCζ assay following an established protocol, which has been verified in published literature (23, 25-27, 29, 48).

Ovarian stimulation, ICSI-AOA, embryo culture and transfer

The protocol used for ovarian stimulation has been described elsewhere (41). Women received either a long stimulation protocol using a GnRH agonist (Buserelin, Sanofi-Aventis, Germany), or a short protocol with a GnRH antagonist (Cetrorelix, Merck Serono, Germany). Recombinant FSH (rFSH) was used in both protocols (FSH, Merck-Serono). Ultrasound was subsequently performed to observe follicular response. When the leading follicle reached ≧18 mm diameter, ovulation induction was performed by using 250 μg of β-hCG (Merck Serono, Germany). Oocyte collection was carried out 36 hrs later, followed by IVF or ICSI as previously described (29).

Following ICSI, oocyte activation was performed as described elsewhere (29). Five female patients whose partners showed PLCζ deficiency underwent ICSI-AOA using protocols with additional GM508 (Cult-Active, Gynemed, Germany) treatment. Briefly, GM508 incorporating calcimycin was first equilibrated at 37.5 ℃ with 5% CO_2 for 4 hrs before use. Immediately after ICSI, oocytes were exposed to 200 μL GM508 for 15 mins, followed by three thorough washes in 500 μL culture medium. Injected oocytes were then transferred to 30 μL sequential culture media and were cultured until a cleavage check was performed (16-20 hrs after ICSI). Embryo quality was checked and scored 43-35, 67-69, and 91-93 hrs post-ICSI (i.e., Days 2, 3 and 4), following ESHRE guidelines (49). Top- and medium-quality Day 5 blastocysts were then transferred, and remaining blastocysts were cryopreserved for future use.

Fertilization rate was calculated by dividing the number of oocytes forming pronuclei by the number of oocytes injected. Pregnancy was defined as positive serum β-hCG levels at 14 days after embryo transfer. Clinical pregnancy was defined as the presence of a fetal heartbeat on ultrasound ~4 weeks after embryo transfer. Pregnancy rate and clinical pregnancy rate were calculated per initiated cycle. Live birth rate was defined as the number of deliveries that resulted in at least one live born baby expressed per initiated cycle.

Analysis

The mean levels of PLCζ in sperm from each patient were quantified as 'PLCζ immunofluorescence intensity' and compared to those of a matched fertile control of similar age and similar sperm parameters according to WHO criteria. Fertile controls could be used in more than one PLCζ analysis. Parametric assumption was tested with Prism 5.0 (Graph-Pad, CA, USA), and normality of the data was confirmed by the D'Agostino & Pearson omnibus normality test. Statistical PLCζ analysis was performed using the Mann-Whitney test or Student's *t*-test (fluorescence PLCζ levels), and Bonferroni's multiple comparisons by one-way ANOVA (localization of PLCζ). The proportion (%) of sperm exhibiting PLCζ first underwent arcsine transformation, in order to stabilize variances, and was then analysed by Student's *t*-test. Medcalc software (Version 18.6) was used to determine a receiver operating characteristic curve (ROC), area under the ROC curve (AUC), and Youden's index analysis, to indicate the cut-off values for PLCζ analysis. Data were expressed as mean ± S.E.M unless otherwise stated, and considered statistically significant where $p<0.05$, $p<0.01$ or $p<0.001$.

Ethical approval

This research study was approved by the National Research Ethics Service (Reference number: 10/H0606/65).

Results

Patients characteristics

No significant age difference was identified between patients (35.7±4.3 years, mean ± SD) and controls (38.0±5.0 years, mean ± SD) (p=0.1105).

PLCζ protein levels and localization pattern analysis in infertile males

In total, 34 of 43 patients had significantly lower levels of PLCζ in sperm ($p<0.05$), and/or significantly lower proportions of sperm exhibiting PLCζ ($p<0.05$) than fertile controls. Among these 34 patients, 15 showed significant PLCζ reduction in both parameters; we termed this condition 'PLCζ deficiency' (patients 1-15, Supplementary table 1 and 2).

Figure 1 is representative of our analysis of a PLCζ-deficient sample; in this case, patient 3 (Figure 1 A). Patient 3 showed a significantly lower mean PLCζ level than a fertile control respectively ($p<0.0001$, Figure 1 B) and a significantly lower proportion of sperm exhibiting PLCζ compared to a respective fertile control (p=0.0032, Figure 1 C). One-way ANOVA and Bonferroni's post-test revealed that PLCζ was predominantly located in the equatorial region of patient and control sperm ($p<0.0001$, Figure 1 D).

We also compared PLCζ levels between the group of 43 infertile patients and the 13 fertile controls. The mean total protein level of PLCζ in sperm from the infertile group was significantly lower ($p<0.001$) than that of the fertile control group (relative fluorescence: 16.41±0.96 and 22.85±0.90 [arbitrary units, a.u], respectively, Supplementary figure 1 A). Furthermore, the mean proportion of sperm expressing PLCζ in the infertile group was significantly lower ($p<0.001$) than that of the control group (relative fluorescence 78.80±2.82 and 89.99±1.06 [a.u], respectively, Supplementary figure 1 B). PLCζ parameters of all 43 patients are shown in Supplementary table 1.

Outcomes following ICSI-AOA

Following our PLCζ analysis, 15 PLCζ-deficient patients were offered AOA treatment, of which 5 opted to undergo ICSI/AOA (patients 3-7, Supplementary table 2). All 5 couples achieved fertilized oocytes. The fertilization rate of PLCζ-deficient patients was significantly improved from 18.6% in ICSI cycles to 56.8% in ICSI-AOA cycles (Table 1). The overall pregnancy rate in ICSI-AOA was 60.0% per started cycle, with a clinical pregnancy rate of 40.0%.

The clinical outcomes of the 43 infertile males are summarized in Supplementary table 1, and the 5 patients who received ICSI/AOA will be discussed on a case-by-case basis. Of these 5 ICSI/AOA patients, 4 had live births (patients 3, 5, 6, and 7), resulting in a live birth rate of 40.0 % per initiated cycle. Although patient 4 did not achieve clinical pregnancy after ICSI/AOA, the couple experienced an early pregnancy loss (i.e., positive β-hCG but no ultrasonic evidence) and had 2 good-quality blastocysts frozen.

Case 1

Patient 3 presented at the age of 34 years with a normal semen analysis; sperm concentration 15 million/ml, sperm motility A+B 46%, and normal morphology (6%). His female partner was 32 years old and had previously conceived with a different partner. The couple had experienced failure of one previous IVF cycle (0 of 5 fertilized oocytes). Following a diagnosis of male PLCζ deficiency (PLCζ relative fluorescence: 11.62±0.26 *vs*. fertile control 28.44±0.53 [a.u], 70.23% of sperm containing PLCζ *vs*. 95.64% in fertile control), the female underwent a long stimulation protocol and 13 oocytes were collected. ICSI was subsequently carried out on 8 mature oocytes with supplementary calcimycin (GM508) treatment. Three of the 8 oocytes were successfully fertilized and developed into blastocysts; 2 good quality

blastocysts were transferred resulting in a successful singleton pregnancy. The remaining blastocyst was cryopreserved. This represents the first pregnancy in Oxford following treatment with ICSI-AOA, with a healthy male baby born in September 2018 (birth weight: 4.3 kg).

Case 2

Patient 5 presented at the age of 36 years, with a history of fertilization failure in 1 previous IVF cycle (0 of 12 fertilized oocytes). Our analysis indicated that his sperm was deficient in PLCζ (relative fluorescence: 15.57±0.19 *vs.* fertile control 23.40±0.22 [a.u], 71.00% of sperm containing PLCζ *vs.* 94.00% in fertile control). In the subsequent cycle with GM508, sperm concentration was low (12 million/ml), but the other semen parameters were normal (sperm motility A+B 54% and 6% normal morphology). Fifteen of 20 oocytes were fertilized. Of these, 2 embryos were cryopreserved, and 2 embryos were transferred, resulting in the birth of a live male baby in December 2018 (birth weight: 4.4 kg).

Case 3

Patient 6 (27 years old) had two previous failed IVF/ICSI cycles before undergoing ICSI-AOA (4 of 8 fertilized oocytes in IVF, and 2 of 9 in ICSI). On each occasion, two embryos were transferred but did not result in a pregnancy. In-house analysis revealed PLCζ deficiency (relative fluorescence: 15.53±0.26 *vs.* fertile control 27.85±0.52 [a.u], 45.00% of sperm containing PLCζ *vs.* 90.00% in fertile control). At the time of receiving GM508 treatment, patient 6 showed normal sperm concentration (52 million/ml) and sperm motility (A+B 58%), but abnormal sperm morphology (3%). Following ICSI/AOA, 8 of 8 oocytes retrieved were successfully fertilized. Of these, 2 were transferred and 2 were cryopreserved. No pregnancy

was observed at the first transfer attempt, however the subsequent transfer of 2 frozen embryos resulted in a singleton live birth in May 2019.

Case 4

Patient 7 was 36 years old at the time of treatment. Our group has previously identified a point mutation in exon 6 of the PLCζ gene in patients 7's brother. Patient 7 exhibited PLCζ deficiency (relative fluorescence: 12.12±0.54 *vs.* fertile control 16.51±0.25 [a.u], 70.53% of sperm containing PLCζ *vs.* 97.00% in fertile control) and had no fertilization in a previous IVF cycle (0 of 9 fertilized oocytes). In the subsequent cycle using GM508, he exhibited normal semen parameters (sperm concentration 88 million/ml, sperm total motility A+B 55% and 6% normal morphology), and 8 of 12 oocytes fertilized; one good-quality blastocyst was transferred and 2 were frozen. However, no pregnancy was observed. Subsequent frozen embryo transfers resulted in no pregnancy and a missed miscarriage at 6 weeks. Following a second attempt with AOA, 8 of 11 oocytes were fertilized and two blastocysts were transferred, resulting in a live female infant (birth weight: 4.2 kg).

Diagnostic accuracy of PLCζ analysis to predict AOA applicability in infertile male patients

The diagnostic accuracy of PLCζ analysis to determine eligibility for AOA was evaluated. Cut-off values were determined using the Youden index and justified AUC (Supplementary figure 2), based on the PLCζ data of all 56 males (43 infertile and 13 fertile males). The Youden index calculation indicated that the cut-offs associated with OAD were 15.57 a.u for the mean PLCζ level (AUC: 0.722, $P<0.01$), and 71% for the proportion of sperm containing PLCζ (AUC: 0.977, $p<0.001$). We then plotted a graph of mean PLCζ level in sperm (M, X-axis) against the proportion of sperm containing PLCζ (P, Y-axis), using data from

individual infertile males (Figure 2) and fertile controls (Supplementary figure 3). Cut-off points divided these males into four categories; I. patients with M < 15.57 a.u, but P > 71%; II. patients with M>15.57 a.u and P> 71%; III. M≤15.57 a.u and P≤71% (PLCζ deficiency); IV, M>15.57 a.u, but P<71%.

Strikingly, 13 of the 15 infertile males with PLCζ deficiency in our cohort were in category III (Figure 2). Moreover, all 4 cases of successful ICSI-AOA pregnancy were in this category (Figure 2). Patients in categories I and IV were those who had either form of defective PLCζ (low mean PLCζ levels or low percentage of sperm containing PLCζ, respectively) but not overall PLCζ deficiency (i.e. reduced mean PLCζ per sample and reduced percentage of sperm containing PLCζ). Further follow-up revealed that patients 18 and 23, with category I PLCζ reduction, had had previous live births with their respective current partners without AOA (Figure 2 and Supplementary table 2). Furthermore, our analysis revealed that patient 4 (globozoospermia) with PLCζ deficiency was in category IV. The couple then received AOA treatment, which yielded 2 fertilized oocytes from 7, although no pregnancy was observed following transfer of the 2 embryos. Subsequent ICSI-AOA achieved 6 fertilized oocytes from 22. One embryo was transferred, and an early pregnancy loss was observed. Category II included patients who exhibited normal (or better) parameters of PLCζ quantification when compared to fertile controls (Figure 2). In line with this, patients 20, 27 and 36 from category II have experienced live births/on-going pregnancy without AOA (Supplementary table 2). However, patient 8 is in category III and PLCζ analysis identified that he may have PLCζ deficiency.

PLCζ categorization also showed that all fertile controls were classified into category III and one was assigned to category I (Supplementary figure 3).

Discussion

This study aimed to quantify PLCζ protein levels in the sperm of infertile males and to investigate the applicability of a PLCζ assay as a diagnostic tool in the assessment of patient eligibility for AOA. Our results comparing relative fluorescence PLCζ levels in the sperm of infertile and fertile males were in line with our previous findings (23, 25, 26, 29). Infertile patients had PLCζ deficiency in their sperm, indicating that any abnormalities in the PLCζ gene and/or protein are likely to play a key role in IVF/ICSI failure (50, 51). Indeed, the crucial role of PLCζ in oocyte activation is highlighted by evidence showing that PLCζ levels, localization patterns, and the proportion of human sperm exhibiting PLCζ significantly correlate with ICSI fertilization rates (29, 30). In addition, PLCζ has been associated with abnormal sperm morphology. Sperm from teratozoospermic males showed significantly reduced PLCζ levels compared to fertile males (52), and sperm from globozoospermic males exhibited significantly lower PLCζ mRNA and protein levels, and abnormal localization patterns compared to fertile males (22). Collectively, our results suggest that PLCζ is a promising biomarker for diagnosing OAD and predicting fertilization outcomes in ICSI.

AOA is a potential option for low fertilization or TFF and is the only option with which to rescue OAD (34, 41). Unsurprisingly, bias may exist when choosing AOA treatment for patients; indeed the effect of patient choice to undergo self-funded treatment and clinician bias cannot be excluded. Furthermore, AOA is not beneficial in all cases of suspected OAD (15, 53, 54), which suggests that a consensus guidance in clinical practice for the use of AOA is needed. In the absence of objective diagnostic criteria to support the use of AOA, our data demonstrate that PLCζ analysis has significant potential in accurately informing clinicians about patient eligibility to undergo AOA as well as treatment prognosis.

Previous attempts have been made to associate oocyte activation capacity and successful fertilization with PLCζ mRNA, RNA, or protein levels in human sperm (30, 55, 56). The immunochemistry method used in our PLCζ assay was the same to those previously

published by our research group (23, 25, 26, 29). Here we report, for the first time, that categorization of infertile males by mean PLCζ level in sperm and proportion of sperm containing PLCζ can help with the clinical decision-making process in AOA treatment. The success of 4 ICSI-AOA cases in achieving clinical pregnancy (patient 3, 5, 6 and 7) indicates that quantitative PLCζ analysis is able to elucidate, at least partially, the mechanisms underlying fertilization failure in such patients, and supports the use of AOA according to our suggested algorithm (Figure 3).

Furthermore, we also calculated the appropriate cut-off thresholds of PLCζ characteristics, in order to examine the accuracy of the PLCζ assay. Accuracy analysis attested to the reliability of our PLCζ results (Figure 2), further indicating that these thresholds have the potential to serve as reference standards for evaluating PLCζ deficiency. Fertile males exhibit variable levels of PLCζ in sperm (23), and for this reason fertile controls recruited in the current study were first tested to ensure they exhibited similar parameters of PLCζ to previous controls.

As discussed, 5 patients (patients 18, 20, 23, 27, and 36) achieved live births following subsequent IVF/ICSI treatment without AOA (Table 1 and Supplementary table 2). Individual PLCζ analysis revealed that these patients either had normal PLCζ protein levels or reduced PLCζ (i.e., categories I and II, Figure 2), which further supports the use of our suggested algorithm in the selection of AOA-eligible patients (Figure 3). It is worth mentioning that patient 7 (from category IV, Figure 2) had AOA treatment following the diagnosis of PLCζ deficiency. As a result, the couple experienced an early pregnancy loss. This suggests that PLCζ analysis may be helpful with predicting the chances of fertilization success, and that combining PLCζ analysis results and the PLCζ categorization may better predict clinical pregnancy success.

Interestingly, two of ICSI-AOA patients had normal semen parameters upon undergoing ICSI-AOA, suggesting that PLCζ analysis may complement routine semen analysis and target AOA-eligible patients with normozoospermia, not only teratozoospermia (52). The present study did not correlate semen parameters (Supplementary table 3) with PLCζ expression, as previous evidence has shown that PLCζ characteristics significantly correlate with semen quality (sperm concentration, motility and morphology). Mean PLCζ levels in sperm correlated significantly with sperm motility (29), and the proportion of sperm exhibiting PLCζ was significantly correlated with the percentage of progressive sperm, sperm concentration and morphology (24, 29). However, Ferrer-Vaquer *et al.* suggested that PLCζ localizations did not correlate with sperm motility and concentrations (28). Furthermore, routine semen analysis does not always correlate with the functional quality of sperm and cannot be used to predict pregnancy outcomes (57). Our data support the use of immunofluorescent staining of PLCζ as an adjuvant to routine basic semen analysis in accurately evaluating AOA applicability.

Importantly, we expect that AOA success, as well as the accuracy of our in-house quantitative assay, should be more significantly related to the chances of fertilization success, rather than to clinical pregnancy outcomes. Indeed, AOA aims to rectify oocyte activation problems, while pregnancy success remains largely dependent upon other factors such as abnormal maternal hormone levels, poor endometrium receptivity and embryo aneuploidy (58). In addition, data relating to IVF/ICSI outcomes among infertile patients with normal PLCζ (category II, Figure 2) were not available, precluding matched comparisons between participants, specifically with regards to clinical pregnancy and live birth rates. Some of the infertile patients in our cohort with normal PLCζ experienced IVF failure, reiterating that OAD is not exclusively the result of PLCζ abnormalities and likely relates to additional unknown male and female factors (7).

The PLCζ assay and PLCζ categorization revealed different results for one patient; patient 8 was classified in category III, whereas the PLCζ assay revealed that he exhibited PLCζ deficiency. Clinical ICSI/AOA outcomes of the patient would have provided useful information with which to examine the disparity between the two assays and the accuracy of the two. However, the patient has not yet undergone ICSI/AOA. Interestingly, patient 8 exhibited round sperm with small acrosomes, and studies have shown that globozoospermic males can benefit from AOA treatment (59).

Undergoing ICSI treatment after fertilization failure in IVF is a standard clinical approach in ART. In this study, we also analysed PLCζ levels in males who had IVF failure to investigate the extent of the PLCζ analysis in predicting AOA outcomes. Patients 3 and 5 experienced total fertilization failure in their previous IVF cycles, and PLCζ analysis showed they both had PLCζ deficiency. Subsequent ICSI with AOA resulted in live births in both participants. More evidence could be acquired if further studies involve split cycles in which oocytes are equally distributed to ICSI and ICSI/AOA treatment. Fertilization rates from each method can be then correlated with PLCζ expression in sperm and provide evidence as to whether PLCζ analysis is efficient in predicting AOA outcomes.

The functional release of Ca^{2+} at the time of oocyte activation requires specific periodic frequency, appropriate amplitude and an adequate number of oscillations (60). Accordingly, insufficient Ca^{2+} oscillation ability in a human oocyte leads to a failure to activate (11). Admittedly, the use of AOA generates some concerns. One is that it may lead to either insufficient or excessive oscillations in the oocyte by altering physiological Ca^{2+} oscillatory patterns (35, 61, 62). In turn, this may impair embryogenesis in both the pre- and post-implantation stages (63). Another concern is that AOA protocols vary between IVF units, with significant differences in reagents (ionomycin, $SrCl_2$, or calcimycin combined with $SrCl_2$), incubation times and the use of supplements. Furthermore, responses using the same protocol

may be discrepant due to individual factors (43). There is a paucity of data to support the universal recommendation of AOA in clinical practice, and the HFEA states that randomized controlled trials and follow-up studies are required (37).

Calcium ionophores remain, thus far, the most commonly used reagents for OAD rescue in a clinical setting. One study showed that GM508 is efficient in promoting zygote development to the cleavage stage (64), and in improving implantation, fertilization and pregnancy rates (32, 41). Furthermore, safety data from AOA-born children have been investigated (65, 66). These studies specifically focused on congenital abnormalities, birth weight and neurodevelopmental outcomes of AOA-born children, and the overall results have been reassuring so far. Long-term follow-up data would be paramount in informing clinicians about the immediate and delayed risks associated with calcium ionophores. A recombinant form of human PLCζ protein (rhPLCζ) has been proposed as a suitable candidate given its ability to trigger Ca^{2+} oscillation in a mouse model (67). Producing a pure, functional rhPLCζ molecule using mammalian cells, better mimicking its endogenous equivalent, has thus far proven a challenge that our group endeavours to solve.

Fertilization is a complex process dependent on factors not exclusive to PLCζ deficiency. Other male factors that may also lead to fertilization failure include sperm structural and morphological defects, sperm DNA damage, or sperm flagellar abnormalities. Sperm DNA damage has been associated with fertilization failure in IVF/ICSI cycles, and is commonly tackled by different techniques: alkaline and neutral Comet assay, Sperm Chromatin Dispersion Test, TUNEL assay and Sperm Chromatin Structure Assay (68). In addition, cation channel of sperm (CatSper) in the sperm tail is important in the sperm's ability to penetrate the oocyte (69), while the K^+ ion channels-associated factor, IKSper, is expressed in the sperm flagellum and plays a key role in the acrosome reaction (70). Other forms of dysfunction of these factors can also lead to OAD, such as inadequate oocyte maturation, low oocyte numbers,

or even technical problems encountered during ART (15, 71). As mentioned earlier, there are several other methods that have been experimentally applied to evaluate sperm ability for oocyte activation, such as the heterologous injection of human sperm into mouse oocytes (mouse oocyte activation test, MOAT) (72), or the measurement of calcium levels in human oocytes upon sperm injection (11). Sperm DNA integrity, in particular, has been associated with successful pregnancy in IVF/ICSI, although its applicability as a diagnostic tool requires further research (73, 74).

In the absence of a gold standard test to diagnose OAD and due to the small number of AOA cases in this study, we are unable to fully validate the sensitivity, specificity and overall accuracy of PLCζ analysis as a diagnostic tool. However, PLCζ categorization of fertile controls at least elucidates, to some extent, that our PLCζ assay is able to accurately identify PLCζ deficient patients. In the present study, only PLCζ-deficient patients were offered AOA treatment, as the HFEA requires that administration of AOA treatment should be justified by a known factor contributing to fertilization failure (38). Patients who are non-deficient or found to have partially abnormal PLCζ are usually not eligible for AOA treatment. However, they can undergo AOA if repeated (total) fertilization failure has been an issue in previous ICSI cycles, or as advised by clinicians and embryologists on a suspicion of activation problems suggested, for example, by late cleavage of embryos. Unfortunately, some patients came from other fertility units, and it is thus difficult to obtain exact details of their previous treatment cycles. With regard to the PLCζ non-deficient patients from our fertility centre, none of them underwent AOA. Further studies, in larger numbers of patients undergoing ICSI/AOA, are needed.

Another limitation of this study is that our assay requires fixed sperm and is thus not necessarily reflective of the PLCζ levels and activity within live sperm used in IVF/ICSI. Further research is now required to investigate whether patients with normal PLCζ parameters

would benefit from receiving AOA. In addition, in the present study, we recruited fertile controls who had proven fertility in the past ten years. Although our group has previously demonstrated that male age does not affect PLCζ expression (26), other factors may contribute to subfertility. Future studies should recruit fertile controls who have conceived as recently as possible.

In conclusion, this study demonstrated the efficacy and accuracy of our in-house assay to identify PLCζ abnormalities and inform clinical decisions in the treatment of couples with male-factor infertility. Our findings provide further evidence of the association between abnormalities in PLCζ and OAD and support the use of a PLCζ assay to identify patients who would benefit from AOA while undergoing ICSI. Crucially, we show the success rate of AOA treatment in this cohort, as measured by a fertilization rate of 56.8% and a pregnancy rate of 60.0% per initiated cycle. We propose an algorithm for the diagnosis and management of patients in whom AOA is likely to be beneficial, allowing for a targeted and individualized approach. Further research should focus on short- and long-term complications of AOA and the development of an effective form of rhPLCζ in order to best mimic its endogenous counterpart and increase therapeutic success.

Author's roles

CJ/TC/KT/KC participated in study design, XM/CJ/GM/CR/TC participated in study execution, XM/CJ/KC participated in data analysis, XM/PM worked on manuscript drafting, PM/CJ/CR/GM/KT/TC/KC revised the manuscript, XM/CJ/PM/KC/TC finished critical discussion.

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Trial registration number

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Conflict of interest

No financial competing interests, personal competing interests, professional competing interests to declare.

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Figure legends

Figure 1 Example of our in-house PLCζ assay of a PLCζ-deficient patient (patient 3). **(A):** Representative immunofluorescence images of a sperm from patient 3 and a fertile control exhibiting PLCζ at different localizations. Images was captured under (a) Bright-field, (b) DAPI (4',6-diamidino-2-phenylindole), (c) FITC-PLCζ, and were merged as (d) Overlay. First and second lanes represent PLCζ localizations in the fertile sperm, and the last lane is the immunofluorescence staining using infertile patient 3 sperm. Yellow arrows indicate presence of PLCζ in the equatorial region of sperm head. White arrow indicates PLCζ acrosomal localization, and asterisk indicates PLCζ post-acrosomal localization. PLCζ was barely can be found in patient 3 sperm. Images were captured at 40x magnification, FITC filter (wavelength 488nm) and at 400ms exposure. Scale bar = 5μm. **(B):** Total relative fluorescence of sperm exhibiting PLCζ in patient 3 and a fertile control. **(C):** Proportion of sperm exhibiting PLCζ in patient 3 and a fertile control. (B and C): Data are expressed as mean ± SEM. *** denotes statistically significant differences at $p<0.001$ and ** denotes statistically significant differences at $p<0.01$, as demonstrated by the Mann Whitney test. **(D):** Proportion of sperm exhibiting PLCζ in different localization patterns in patient 3. *** denotes statistically significant differences as demonstrated by a one-way ANOVA test ($p<0.001$). Data are expressed as mean percentages.

Figure 2 Categorization of infertile males by mean PLCζ level in sperm (X-axis) against proportion of sperm containing PLCζ (Y-axis). The vertical dashed line represents the cut-off (15.57 a.u) of mean PLCζ level (M) per patient's sperm, while the horizontal dashed line represents the cut-off (71%) of the proportion of sperm containing PLCζ (P) per patient's sperm. "I, II, III, IV" represent the four categories associated with PLCζ parameters. I. patients with $M < 15.57$ a.u, but $P > 71\%$; II. patients with $M>15.57$ a.u and $P> 71\%$; III. $M\leq15.57$ a.u and

P≤71% (PLCζ deficiency, and likely favors AOA); IV, M>15.57 a.u, but P<71%. Patients with PLCζ deficiency are labelled in red; the red triangle represents patients who have had clinical pregnancies/live-birth with AOA, red dots represent those did not receive AOA, and the red star represents those received AOA and experienced chemical pregnancy. Green cubes represent patients who have had live-births or on-going pregnancy without AOA, and black dots depict patients with unknown outcomes of subsequent IVF/ICSI treatment.

Figure 3

The suggested clinical algorithm for assessing the eligibility of patient for AOA. The laboratory-based PLCζ analysis is an individual targeted approach, which can save effort, time and cost for both IVF clinics and patients.

Supplementary figure 1 Comparison of the quantitative immunofluorescence analysis of PLCζ between infertile (black) and fertile (grey) males. **(A):** Comparison between the mean total PLCζ level in the infertile and fertile groups. **(B):** Comparison between the mean proportion of sperm exhibiting PLCζ in infertile and fertile patients**.** "n" represents the total number of PLCζ analyses. Thirteen fertile male samples were repeatedly used as controls in a number of 43 fluorescence analysis. Data are expressed as mean ± SEM, *** denotes a significant difference at p<0.001 demonstrated by the Mann Whitney test (A), and the Student's *t*-test (B).

Supplementary figure 2 Cut-offs of (A) mean PLCζ level and (B) proportion of sperm containing PLCζ to identify infertile males for AOA treatment. Cut-offs were calculated

according to receiver operating characteristic curve (ROC), and the area under the ROC curve (AUC). Cut-offs were chosen when the corresponding sensitivity and specificity both reached at the best values, as calculated using Youden's index. The value of AUC was 0.722 for the mean PLCζ level ($P<0.01$) and 0.977 for the proportion of sperm containing PLCζ ($p<0.001$), thus verifying the accuracy of the cut-offs.

Supplementary figure 3

Categorization of fertile males by mean PLCζ level in sperm (X-axis) against proportion of sperm containing PLCζ (Y-axis). The vertical dashed line represents the cut-off (15.57 a.u) of mean PLCζ level (M) per patient's sperm, while the horizontal dashed line represents the cut-off (71%) of the proportion of sperm containing PLCζ (P) per patient's sperm. Fertile controls were classified to category II ($M>15.57$ a.u and $P> 71\%$) and I ($M < 15.57$ a.u, but $P > 71\%$).

Table 1 ICSI and ICSI-AOA outcomes in PLCζ deficiency group, and ICSI outcomes in PLCζ-reduced and PLCζ normal groups.

	PLCζ deficiency group		PLCζ-reduced group	PLCζ normal group
	Previous ICSI cycle	ICSI/AOA cycle	Previous ICSI cycle	Previous ICSI cycle
Paternal age (M ± SD)	34.7 ± 4.3		36.9 ± 4.1	35.3 ± 3.8
Maternal age (M ± SD)	32.8 ± 5.3		35.0 ± 4.2	33.3 ± 3.6
Patient numbers (n)	9	5	12	6
ICSI cycles (n)	13	10	14	6
ICSI fertilization rate % (2pn/n)	18.6 (21/113)	56.8 (50/88)***	34.3 (36/105)	20.0 (17/85)
Pregnancy rate (%)	7.7 (1/13)	60.0 (6/10)	28.6 (4/14)	33.3 (2/6)
Clinical pregnancy rate (%)	0 (0/13)	40.0 (4/10)	28.6 (4/14)	16.7 (1/6)
Live-birth rate (%)	0	40.0 (4/10)	28.6 (4/14)	16.7 (1/6)

PLCζ deficient group includes patients who showed (1) significantly lower mean PLCζ levels in sperm, and (2) significantly lower proportion of sperm exhibiting PLCζ, when compared those to fertile controls. PLCζ-reduced group includes patients who showed (1) significantly lower mean PLCζ levels in sperm, or (2) significantly lower proportion of sperm exhibiting PLCζ, when compared those to fertile controls. PLCζ normal group includes patient who showed no significant difference in aforementioned two parameters when compared to fertile controls. Patient age were expressed as Mean ± Standard Division (M ± SD); n represents the number of cycles; 2pn/n represents numbers of cells with pronuclei out of injected oocytes. *** denotes a significant difference at $p<0.001$ between ICSI/AOA and ICSI cycles in PLCζ deficiency group. Pregnancy: positive serum β-hCG levels at 14 days after embryo transfer. Clinical pregnancy: the presence of a fetal heartbeat on ultrasound ~4 weeks after embryo transfer. Pregnancy rate and clinical pregnancy rate were determined per initiated cycle. Live birth rate was defined as the number of deliveries that resulted in at least one live born baby expressed per initiated cycle.

Supplementary table 1 PLCζ parameters in 43 infertile males

Patient No.	PLCζ Deficiency?	Mean PLCζ level a.u (95% CI)	Proportion of PLCζ in sperm a.u (95% CI)
1	Yes	6.54 (5.55-7.53)	38.00 (30.53-45.47)
2	Yes	8.58 (7.56-9.59)	29.83 (19.74-39.92)
3	Yes	11.63 (9.74-13.51)	70.23 (56.33-84.13)
4	Yes	21.50 (19.73-23.26)	47.00 (35.79-58.21)
5	Yes	15.57 (14.18-16.96)	71.00 (62.44-79.56)
6	Yes	15.53 (13.67-17.39)	45.00 (36.57-53.43)
7	Yes	12.12 (8.23-16.02)	70.54 (60.57-80.51)
8	Yes	20.11 (17.63-22.58)	79.68 (70.45-88.92)
9	Yes	13.41 (11.6-15.22)	47.62 (34.88-60.36)
10	Yes	14.01 (12.59-15.43)	70.87 (60.02-81.72)
11	Yes	14.02 (10.18-17.85)	46.70 (22.28-71.13)
12	Yes	12.28 (11.06-13.50)	61.85 (48.66-75.04)
13	Yes	12.33 (10.80-13.86)	48.84 (38.55-59.14)
14	Yes	10.00 (9.154-10.85)	39.05 (29.05-49.04)
15	Yes	12.17 (9.43-14.91)	57.44 (44.15-70.72)
16	Reduced PLCζ level	9.70 (7.47-11.92)	90.13 (84.19-96.06)
17	Reduced PLCζ level	13.59 (12.42-14.76)	80.40 (73.30-87.50)
18	Reduced PLCζ level	9.53 (7.63-11.42)	80.48 (69.90-91.70)
19	Reduced PLCζ level	20.90 (18.07-23.74)	95.27 (88.87-101.70)
20	Reduced PLCζ level	20.11 (16.24-23.97)	98.18 (94.07-102.30)
21	Reduced PLCζ level	13.30 (11.12-15.47)	89.00 (80.99-97.01)
22	Reduced PLCζ level	11.12 (9.62-12.61)	87.45 (79.42-95.49)
23	Reduced PLCζ level	8.065 (5.57-10.56)	83.36 (73.09-93.63)
24	Reduced PLCζ level	13.58 (11.06-16.09)	82.17 (69.20-95.13)
25	Reduced PLCζ level	19.20 (17.94-20.45)	88.97 (80.93-97.02)
26	Reduced PLCζ level	16.62 (14.43-18.81)	88.06 (82.01-94.12)
27	Reduced PLCζ level	19.72 (15.68-23.77)	77.00 (59.79-94.21)
28	Reduced PLCζ level	18.47 (15.88-21.06)	88.18 (80.89-95.47)
29	Reduced PLCζ level	15.67 (13.58-17.77)	95.17 (91.47-98.88)
30	Reduced PLCζ level	12.64 (11.12-14.17)	99 (96.74-101.3)
31	Reduced PLCζ level	18.73 (16.61-20.85)	92.89 (87.89-97.90)
32	Reduced PLCζ level	18.04 (16.44-19.63)	87.18 (78.30-96.60)
33	Reduced PLCζ level	12.14 (10.87-13.41)	74.43 (63.60-85.26)
34	Reduced PLCζ proportion	24.92 (20.41-29.43)	77.21 (65.25-89.16)
35	No	15.13 (11.59-18.68)	74.59 (61.64-87.53)
36	No	21.04 (18.76-23.33)	85.92 (75.62-96.22)

37	No	21.13 (17.18-25.08)	92.61 (83.83-101.40)
38	No	25.36 (21.31-29.41)	98.26 (95.63-100.90)
39	No	16.01 (12.36-19.66)	92.75 (83.73-101.80)
40	No	38.71 (35.33-42.09)	99 (96.74-101.30)
41	No	35.67 (30.55-40.80)	78.07 (67.55-88.59)
42	No	18.96 (16.42-21.50)	82.02 (68.39-95.65)
43	No	19.56 (17.47-21.64)	86.96 (79.33-94.59)

a.u: arbitrary units; 95% CI: 95% confidence interval.

Supplementary table 2 Patient information and IVF/ICSI outcomes (n=43).

Patient No.	Male age at date of sample	Female age at date of sample	IVF/ICSI history			AOA treatment			Pregnancy outcome
			Cycles (n)	Fertilization rate % (2pn/n)	ET (n)	AOA used?	Fertilization rate % (2pn/n)	ET (n)	
1	38	36	1	16.7 (2/12) (ICSI)	2		No		Early miscarriage
2	37	35	2	0.0 (0/6) (IVF)			No		N.A
				0.0 (0/4) (ICSI)			No		N.A
3	34	32	1	0.0 (0/5) (IVF)		Yes,	37.5 (3/8) (ICSI)	2	AOA live-birth, singleton
4	25	21	1	20.8 (5/24) (ICSI)	1	Yes,	Cycle 1, 37.5 (2/7) (ICSI)	2	Negative
							Cycle 2, 27.3 (6/22) (ICSI)	1	Early miscarriage
5	36	38	1	0.0 (0/12) (IVF)		Yes,	75.0 (15/20) (ICSI)	2	AOA-live birth, singleton
6	27	28	2	50.0 (4/8) (IVF)	2	Yes,	100.0 (8/8) (ICSI)	2	Negative
				22.2 (2/9) (ICSI)	2			2 (FET)	AOA-live birth, singleton
7	36	35	1	0.0 (0/9) (IVF)		Yes,	Cycle 1, 66.7 (8/12) (ICSI)	1	Negative
								1 (FET)	Early miscarriage
								1(FET)	Negative
							Cycle 2, 72.7 (8/11) (ICSI)	2	AOA-live birth, singleton

8	32	25	0	Round heads and small acrosomes		No	
9	34	34	1	0 (0/2) (IVF)		No	
10	36	40	1	0 (0/9) (ICSI)		No	
11	42	34	1	0 (0/5) (IVF)		No	
12	42	35	1	0 (0/10) (ICSI) Severe oligoasthenozoospermia		No	
13	34	38	2	14.3 (1/7) (ICSI)	N.A	No	
14	32	29	1	20 (1/5) (ICSI)	1	No	Negative
15	32	N.A	5	30.3 (10/33) (ICSI x 4 times) Frozen cycle	1x twice ET 1	No	Negative Negative
16	43	32	1	0.0(0/16) (IVF)		No	N.A
17	32	37	1	0.0 (0/12) (IVF)		No	N.A
18	36	35	2	0.0 (0/4) (ICSI) 25.0 (2/8) (ICSI)	 2	No No	N.A Live birth
19	33	39	1	2.6 (1/38) (IVF)	1	No	Negative
20	38	35	2	0.0 (0/12) (IVF)		No	N.A

				80.0 (4/5) (ICSI)	1	No	Live birth
21	43	41	1	0.0 (0/2) (ICSI)		No	N.A
22	33	36	2	10.0 (1/10) (IVF)	1	No	Negative
				45.5 (5/11) (ICSI)	2	No	Negative
23	32	30	2	0.0 (0/7) (IVF)		No	N.A
				88.9 (8/9) (ICSI)	1	No	Live birth
24	37	31	1	0.0 (0/17) (IVF)		No	N.A
25	43	42	1	0.0 (0/8) (ICSI)		No	N.A
26	32	27	1	0.0 (0/16) (IVF)		No	N.A
27	43	29	2	9.1 (1/11) (IVF)	1	No	Negative
				83.3 (10/12) (ICSI)	2	No	Live birth
28	41	39	1	15.4 (2/13) (IVF)	N.A	No	
29	38	36	2	0 (0/4) (IVF))		No	
				0 (0/5) (ICSI)			
30	34	35	1	0 (0/5) (ICSI)		No	
31	38	38	3	0 (0/14) (IVF)		No	

				27.8 (5/18) (ICSI twice)	1		Negative
32	36	33	2	14.3 (2/14) (ICSI)	1x twice ET	No	Negative
33	37	N.A	0	Family history, bother of patient 12		No	
34	32	N.A	2	0.0 (0/6) (IVF)		No	N.A
				0.0 (0/4) (ICSI)		No	N.A
35	37	N.A	4	12.5 (4/32) (ICSI)	1	No	Negative
36[#]	33	30	1	66.6 (8/12) (ICSI)	1	No	Live birth (at week 36-40)
37	44	41	1	0.0 (0/14) (IVF)		No	N.A
38	33	33	2	25.0 (1/4) (IVF)	1	No	N.A
				0.0 (0/7) (ICSI)		No	N.A
39	31	31	2	15.4 (2/13) (IVF)	2	No	Negative
				21.4 (3/14) (ICSI)	1	No	Early miscarriage
40	33	30	1	0.0 (0/3) (IVF)		No	N.A
41	36	35	1	0 (0/11) (ICSI)		No	N.A
42	35	33		0 (0/5) (IVF)		No	
43	36	34	1	22.2 (2/9) (ICSI)	1	No	Negative

IVF/ICSI history may be displayed as mixed cycles performed by different IVF clinics. Abbreviations: N. A: not applicable; IVF: *in-vitro* fertilization; ICSI: intracytoplasmic sperm injection; ET: embryo transfer; FET: frozen embryo transfer. Early miscarriage: human chorionic gonadotropin was detected after embryo transfer; however, ultrasound showed no fetal heart at the week 6. #: Patient 36 was recruited because his bother (Patient 7) had total fertilization failure.

Supplementary table 3 Semen analysis of patient from Oxford Fertility (n=25).

Patient No.	Count (million/ml)	Progressive (%)	Non-progressive (%)	Immotile (%)	Morphology (%)
1	4	30	3	67	2
3	15	46	3	51	6
4	160	32	8	60	0
5	12	54	14	32	6
6	52	58	1	43	3
7	88	55	4	41	6
16	55	60	3	37	6
18	14	32	25	43	2
19	212	48	7	45	5
20	85	52	7	41	7
22	118	55	5	40	4
23	106	42	16	42	4
24	58	45	12	43	4
25	12	56	17	27	6
26	65	55	10	35	3
27	47	54	8	38	2
28	102	50	10	40	4
29	120	68	4	28	5
30	115	77	7	16	4
31	51	66	0	34	4
36	16	48	3	49	3
37	163	68	3	29	6
39	139	46	8	46	5
40	58	46	12	42	4
43	33	58	9	33	2

Figure(s)

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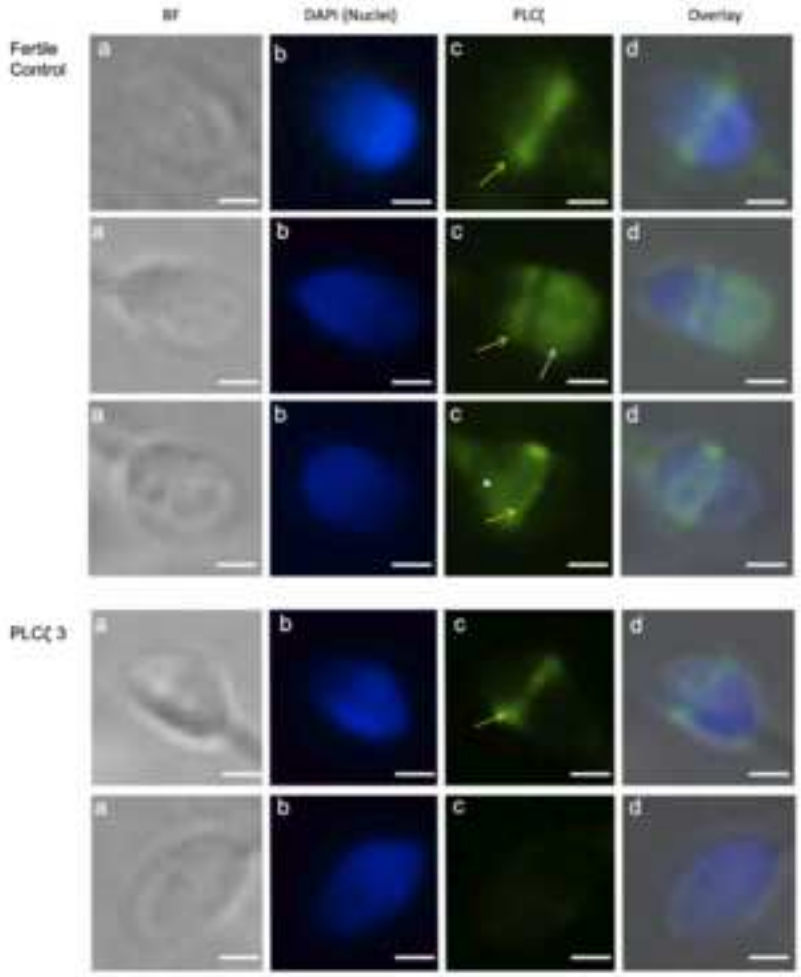
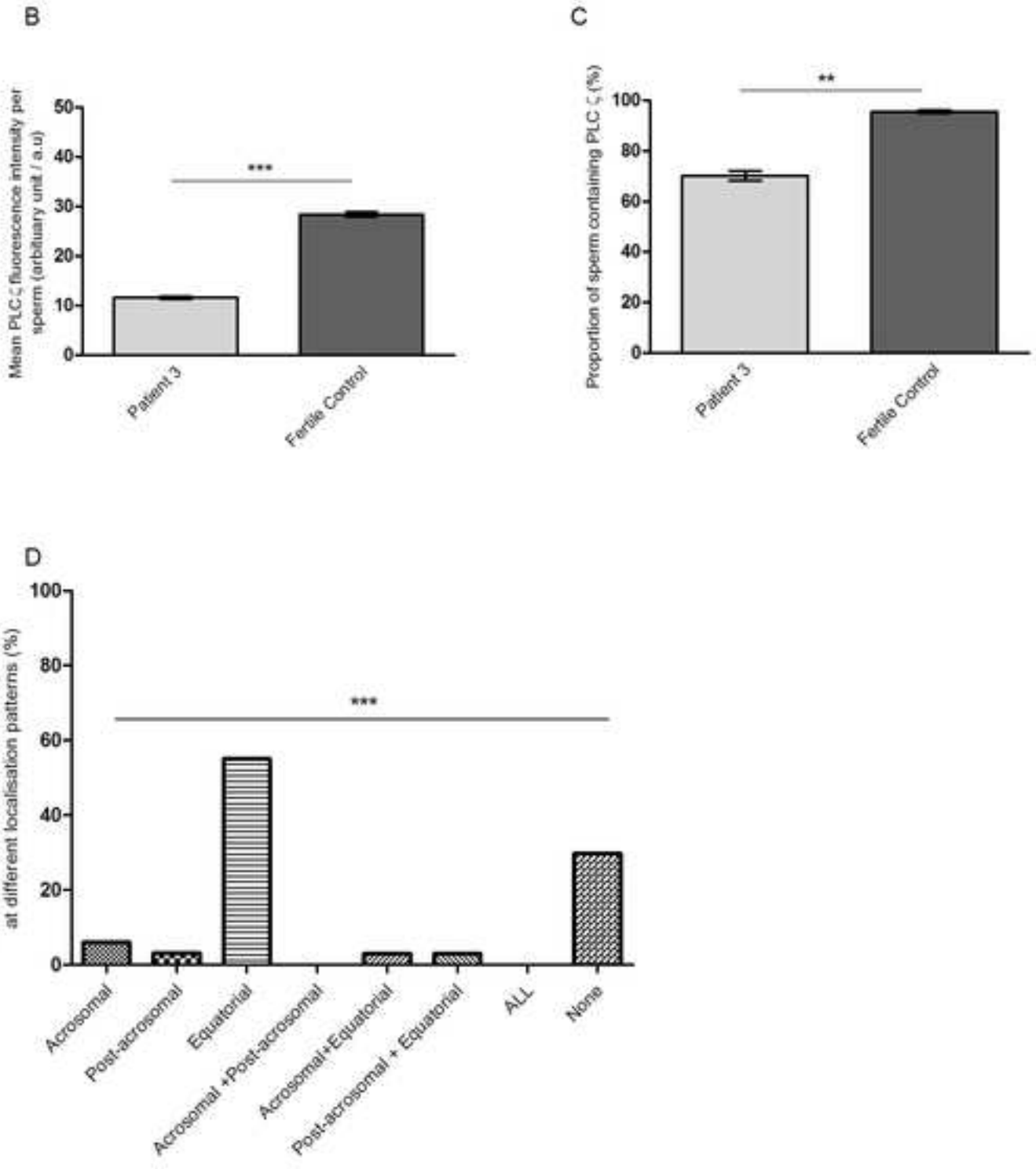
Figure 1 A





Figure(s)

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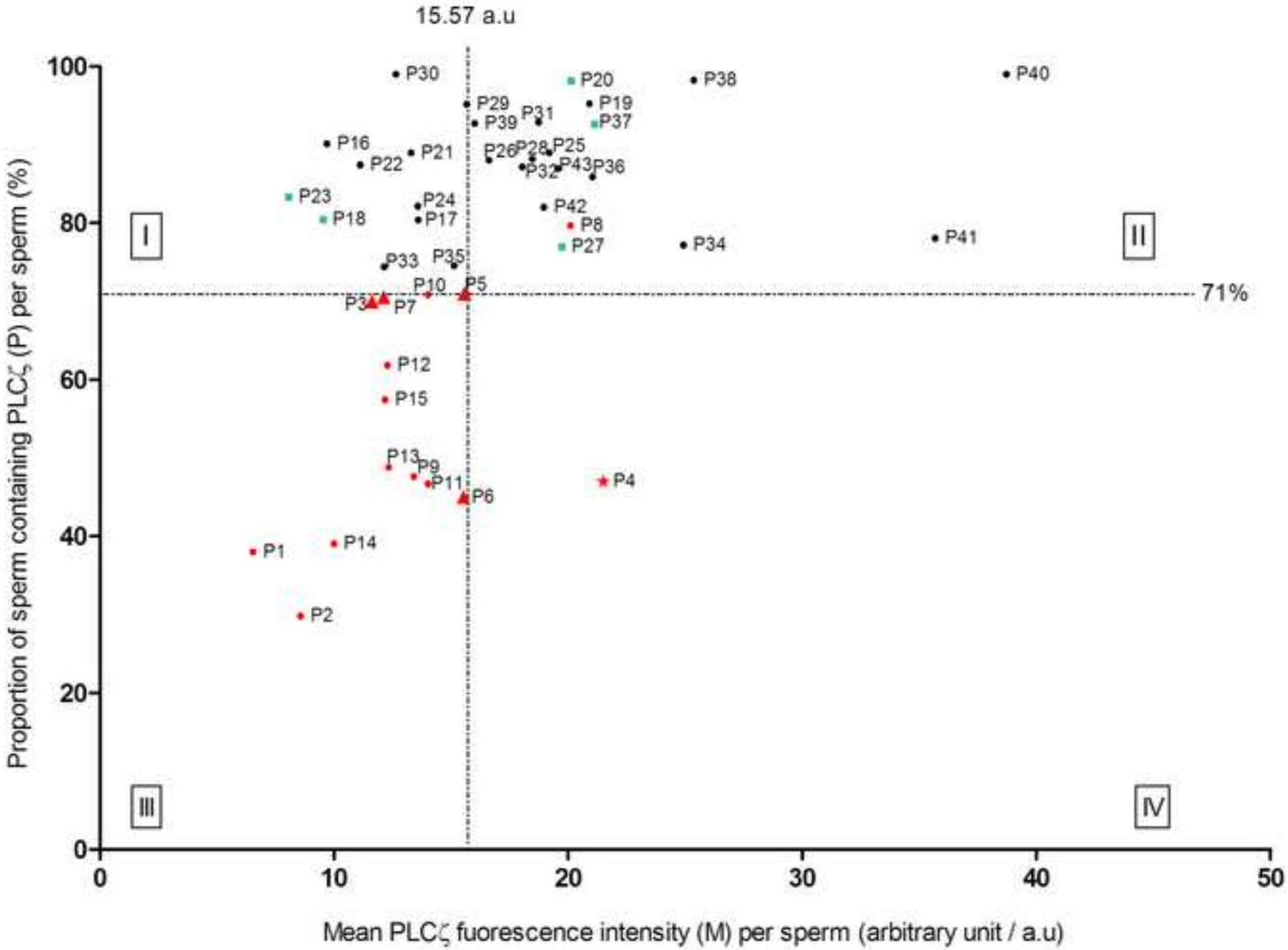
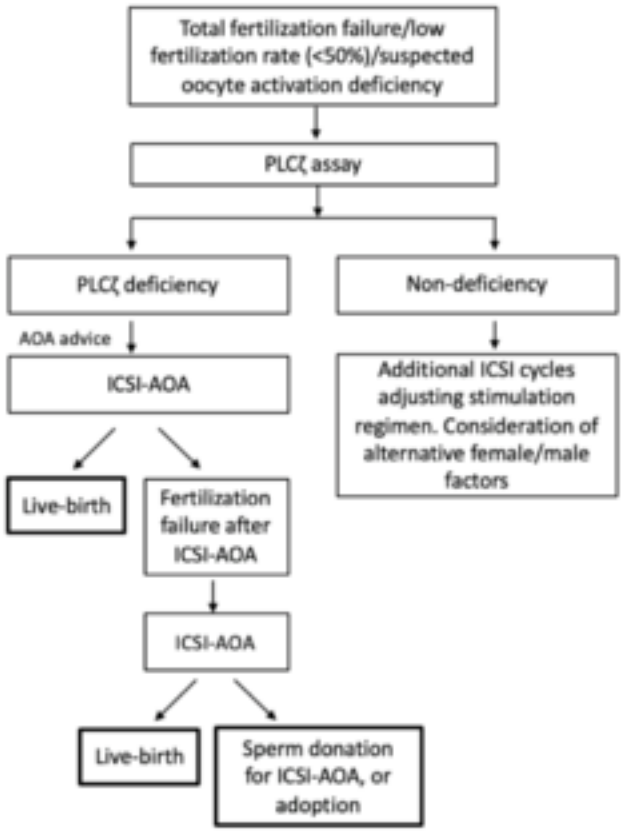
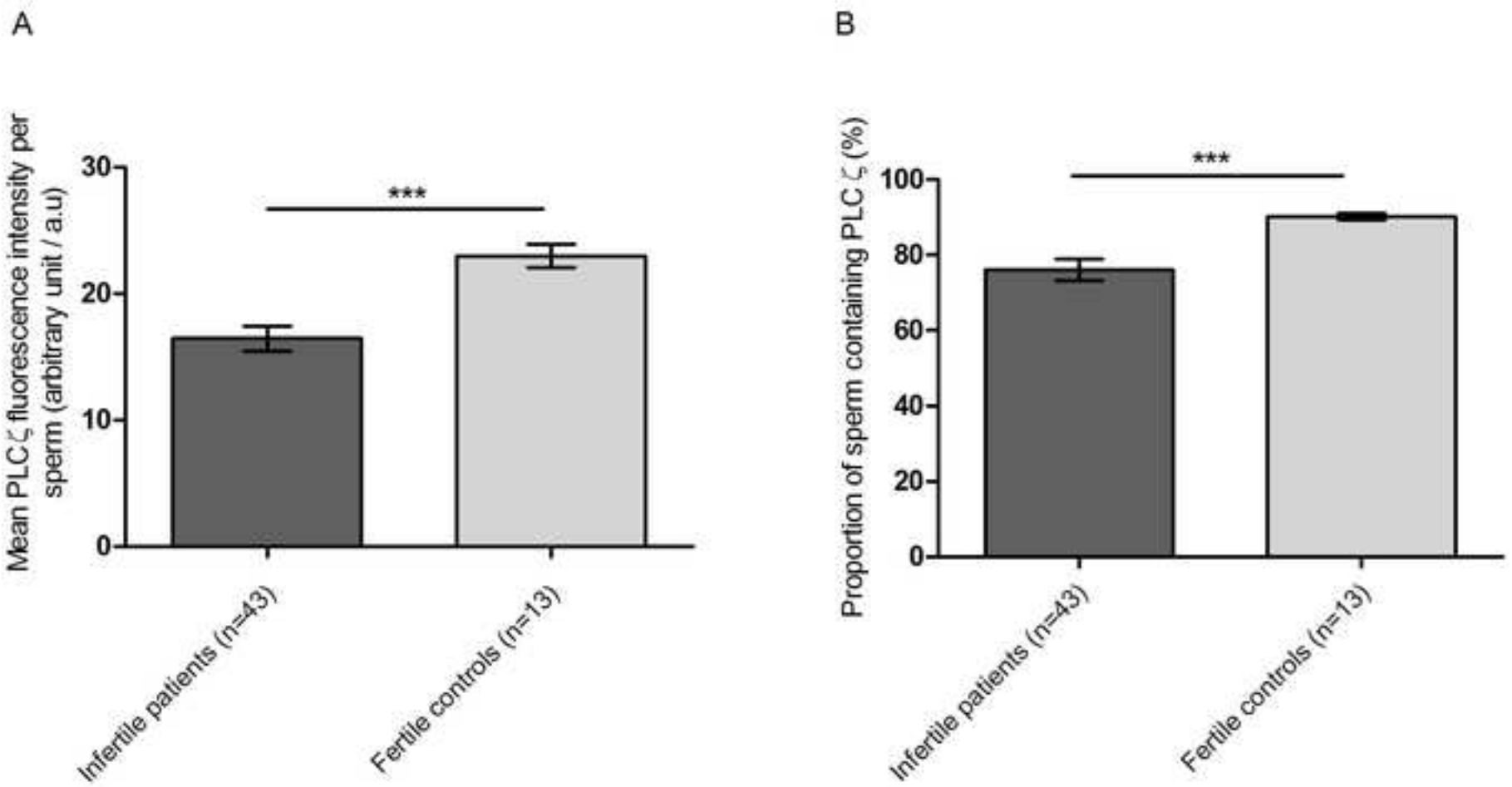


Figure 3





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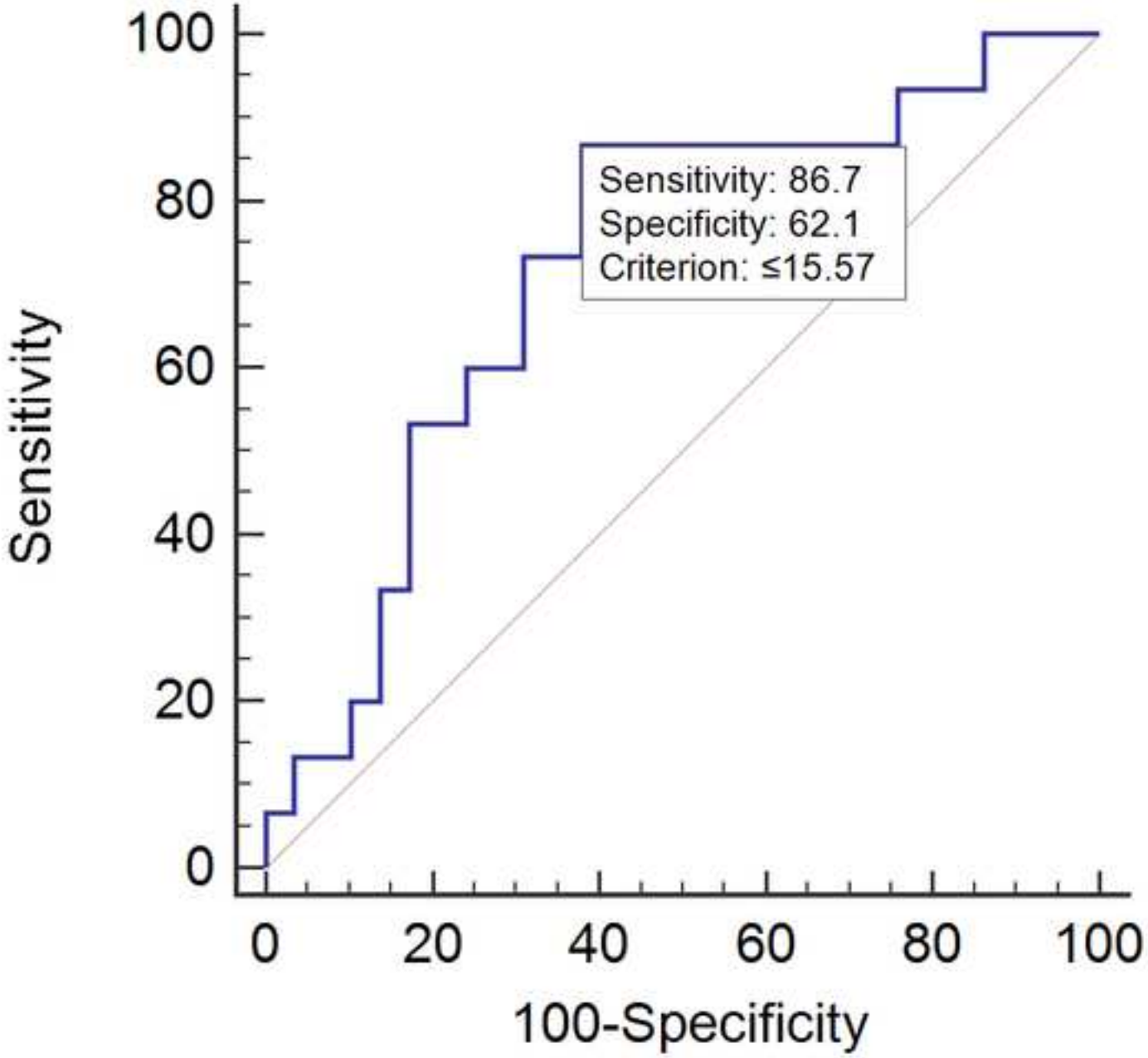

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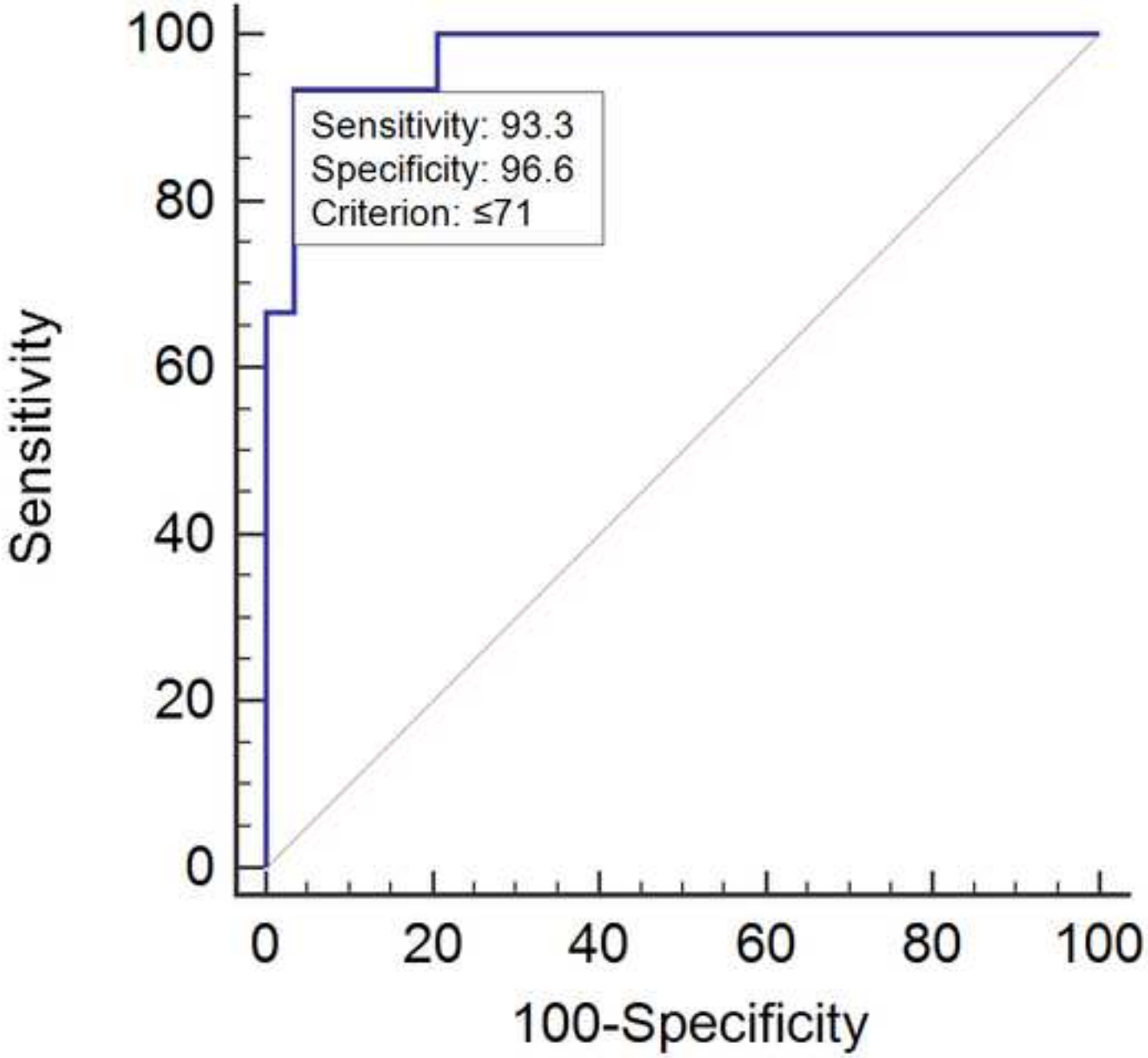

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