**Phospholipase C zeta (PLCζ) and male infertility: clinical update and topical developments**

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**Abbreviations:**

AOA: Assisted oocyte activation; ICSI: Intracytoplasmic sperm injection; IVF: *In vitro* fertilisation; MOAT: mouse oocyte activation test; OAD: Oocyte activation deficiency; PAS-PT: Post-acrosomal sheath-perinuclear theca; SNP: single nucleotide polymorphism; SOAF: Sperm oocyte-activating factor; TFF: Total fertilisation failure

**Abstract**

The development of a mammalian embryo is initiated by a sequence of molecular events collectively referred to as ‘oocyte activation’ and regulated by the release of intracellular calcium in the ooplasm. Over the last decade, phospholipase C zeta (PLCζ), a sperm protein introduced into the oocyte upon gamete fusion, has gained almost universal acceptance as the protein factor responsible for initiating oocyte activation. A large body of consistent and reproducible evidence, from both biochemical and clinical settings, confers support for the role of PLCζ in this fundamental biological context, which has significant ramifications for the management of human male infertility. Oocyte activation deficiency (OAD) and total fertilisation failure (TFF) are known causes of infertility and have both been linked to abnormalities in the structure, expression, and localisation pattern of PLCζ in human sperm. Assisted oocyte activators (AOAs) represent the only therapeutic option available for OAD at present, although these agents have been the source of much debate recently, particularly with regard to their potential epigenetic effects upon the embryo. Consequently, there is much interest in the deployment of sensitive PLCζ assays as prognostic/diagnostic tests and human recombinant PLCζ protein as an alternative form of therapy. Although PLCζ deficiency has been directly linked to a cohort of infertile cases, we have yet to identify the specific causal mechanisms involved. While two genetic mutations have been identified which link defective PLCζ protein to an infertile phenotype, both were observed in the same patient, and have yet to be described in other patients. Consequently, some researchers are investigating the possibility that genetic variations in the form of single nucleotide polymorphisms (SNPs) could provide some explanation, especially since >6000 SNPs have been identified in the PLCζ gene. As yet, however, there is no consistent data to suggest that any of these SNPs influence the functional ability of PLCζ. Other laboratories appear to be focusing upon the PLCζ promoter, which is bi-directional and shared with the actin filament capping muscle Z-line alpha 3 gene (CAPZA3), or seeking to identify interacting proteins within the ooplasm. The aim of this review is to provide a synopsis of recent progress in the application of PLCζ in diagnostic and therapeutic medicine, to discuss our current understanding of how the functional

ability of PLCζ might be controlled, and thus how PLCζ deficiency might arise, and finally, to consider the potential implications of alternative sperm protein candidates, such as post-acrosomal WW-domain binding protein (PAWP), which has caused much debate and confusion in the field over the last few years.

**Keywords:** Phospholipase C zeta (PLCζ), calcium, sperm, oocyte activation, infertility, post-acrosomal WW-domain binding protein (PAWP), actin filament capping muscle Z-line alpha 3 gene (CAPZA3), bi-directional promoter, intron

## Introduction

Since its discovery in 2002 (Saunders et al., 2002), phospholipase C zeta (PLCζ) has continuously stood out from other sperm proteins that have claimed their possible role in oocyte activation. Owing to a myriad of biochemical and clinical evidence (**Table 1**), an association between PLCζ and male fertility has been increasingly recognised (reviewed by Amdani et al., 2013). PLCζ is a cytosolic sperm protein introduced into the oocyte following fertilisation, and induces oocyte activation via the release of intracellular calcium ions ($Ca^{2+}$) from endoplasmic reticulum stores (Saunders et al., 2007; Ducibella and Fissore, 2007; Swann and Yu, 2008; Kashir et al., 2013a). The $Ca^{2+}$ released, distinguished either as a series of oscillations (in mammals) or as a single transient (in non-mammals), regulates a sequence of molecular events that are collectively referred to as 'oocyte activation'. These events include the release of meiotic arrest, cortical granule exocytosis, maternal mRNA recruitment, pronuclear formation, polyspermy prevention, and the ultimate initiation of embryo development (**Figure 1**; Kline and Kline, 1992; Kashir et al., 2013a; Amdani et al., 2013; Yeste et al., 2015).

The unique biochemical structure of PLCζ makes it the smallest PLC isoform (~70 kDa in humans and ~74 kDa in mice), and its origin in sperm, along with its ability to induce $Ca^{2+}$ release in the oocyte, differentiates it from the other thirteen known PLC isozymes (Saunders et al., 2002; Gresset et al., 2012). PLCζ exhibits highest homology with phospholipase C delta 1 (PLCδ1), and a significant amount of research elucidating the regulation of PLCζ function in the oocyte has been based upon our structural knowledge of PLCδ1 (Saunders et al., 2002; Nomikos et al., 2013). Each characteristic PLC protein domain plays an important role in the release of $Ca^{2+}$ but in contrast to other PLC isoforms, PLCζ does not possess a pleckstrin homology (PH) domain localised at the N-terminus (Saunders et al., 2002; Nomikos et al., 2012). Indeed, the PH domain of PLCδ1 has been proven to mediate the hydrolysis of its substrate, phosphatidylinositol 4,5-biphosphate ($PIP_2$), in the plasma membrane to initiate cell signalling (Lomasney et al., 1996), and it was not clear until recently how PLCζ interacted with $PIP_2$ in order to exert function. In this regard, Nomikos et al. (2011a)

proposed that the XY-linker (the region between the catalytic X and Y domains) may regulate PLCζ function in the oocyte by acting as a site for $PIP_2$ interaction. In fact, unlike all other PLC isoforms, the PLCζ XY-linker exhibits positively-charged residues and is thus able to bind to negatively-charged $PIP_2$ via electrostatic interaction (Nomikos et al., 2011a). Removal of the XY-linker from PLCζ, however, does not completely terminate the release of $Ca^{2+}$ oscillations (Nomikos et al., 2011b), suggesting that an additional mechanism was involved. Nomikos et al. (2015b) subsequently demonstrated that the EF-hand domain of PLCζ also played a crucial role in binding $PIP_2$, as mutations within the EF-hand domain and XY-linker resulted in the complete termination of $Ca^{2+}$ oscillation-inducing ability.

The enzymatic activity of PLCζ is triggered upon entry into the oocyte via interaction with an as yet unidentified oocyte factor (or factors) (Swann and Lai, 2013). Additionally, early studies by Kurokawa et al. (2007) and Cooney et al. (2010) indicated the possible requirements for PLCζ to undergo proteolysis and post-translational modification, respectively, in order to establish enzymatic activity. The currently accepted mechanism for PLCζ signalling **(Figure 1)** is thought to begin with the diffusion of PLCζ from the fertilising sperm into the ooplasm. PLCζ stimulates the phosphoinositide signalling pathway by hydrolysing $PIP_2$-containing vesicles (Yu et al., 2012; Swann and Lai, 2013), generating inositol-1,4,5-triphosphate ($InsP_3$) and diacylglycerol (DAG). Then, $InsP_3$ binds to $InsP_3$ receptors ($InsP_3R$) localised on the surface of the endoplasmic reticulum causing the release of $Ca^{2+}$, while DAG activates the protein kinase C (PKC) pathway to translate $Ca^{2+}$ signals into cellular responses (Swann and Yu, 2008). One particular void to establish in our current understanding of the PLCζ signalling system is to interpret the critical communication between the sperm and oocyte following fertilisation. Indeed, PLCζ is widely believed to only achieve an active state upon entry into the oocyte and it remains very uncertain how the oocyte induces such activity (Kouchi et al., 2004; Nomikos et al., 2005). Furthermore, Swann and Lai, (2013) hypothesised the existence of specific receptors upon the surface of the $PIP_2$-containing vesicles within oocytes which might mediate the phosphoinositide signalling pathway. A current goal for researchers is to identify

this crucial oocyte component, as such receptors may also represent useful diagnostic or therapeutic targets.

Initial immunocytochemical localisation studies identified PLCζ in the acrosomal, equatorial or post-acrosomal regions of the human sperm head. However, in many cases, PLCζ has also been observed in a combination of locations, including acrosomal/equatorial, acrosomal/post-acrosomal, equatorial/post-acrosomal, or in all three regions (Grasa et al., 2008). Interestingly, some sperm also appear to be devoid of PLCζ altogether (Kashir et al., 2013b). The multiple localisation patterns suggest differential PLCζ function, not limited to oocyte activation, although this theory remains obscure and represents a very interesting challenge for future research. The localisation profile of PLCζ has met with some controversy recently as Aarabi et al. (2012) claimed that PLCζ was not a component of the post-acrosomal sheath-perinuclear theca (PAS-PT) and therefore, could not possibly represent a sperm oocyte-activating factor (SOAF) candidate. The perinuclear theca (PT) of the sperm head contains cytoskeletal proteins important for maintaining sperm head architecture and cytosolic proteins for fertilisation (Aul and Oko, 2002; Sutovsky et al., 2003), while the PAS-PT is a region in which the fertilising sperm interacts with the oocyte to transmit paternal DNA and the SOAF (Aul and Oko, 2002; Sutovsky et al., 2003). A study by Escoffier et al. (2015) later confirmed that the subcellular localisation of PLCζ was indeed at the PT of the equatorial and post-acrosomal regions of human sperm, thus contradicting the work of Aarabi et al. (2012). Contention regarding the localisation profile of PLCζ may therefore have been resolved but the precise expression profile during spermatogenesis remains debatable due to a lack of evidence describing PLCζ protein translation, particularly in humans. In mouse and pigs, mRNAs encoding for PLCζ have been found in round spermatids, and were suggested to undergo translation during the elongated spermatid stage, although no further evidence has been put forward on this topic (Yoneda et al., 2006).

Over recent years, the role of PLCζ has come under close scrutiny as a result of the emergence of other possible SOAF candidates, with post-acrosomal sheath WW domain-binding protein (PAWP) being the most prominent competitor (Reviewed by Amdani et al., 2015). Introduced in 2007 (Wu et

al., 2007), research has shown that PAWP exhibits the functional and localisation characteristics expected of a SOAF, although recent findings have provided compelling evidence to refute its functional role, at least in the mouse (Nomikos et al., 2014; Nomikos et al., 2015; Satouh et al., 2015). Most notably, the microinjection of a single sperm generated from a PAWP-null mouse was still able to initiate $Ca^{2+}$ oscillation-inducing activity similar to that seen during normal fertilisation. This proves, for the first time, that PAWP could not possibly be involved in activating oocytes via $Ca^{2+}$ release (Satouh et al., 2015). This breakthrough could potentially put an end to the ongoing conflict between groups working on their respective SOAF candidates and allow research attention to be fully focused upon the final translation of PLCζ to the clinic as a powerful diagnostic/therapeutic agent. The close association between PLCζ and male fertility has steadily become increasingly more evident since the first clinical study was published by Yoon et al. (2008). Accordingly, attention has since focused upon PLCζ as a new diagnostic/therapeutic for clinical application. However, there is still much to do, and many questions to be answered, in order to achieve this goal. The remainder of this synopsis first provides a historical overview of how PLCζ first became associated with male infertility, and secondly, provides a critical update in relation to how research in this discipline is beginning to change focus.

**PLCζ and its clinical association with male infertility: the formative years**

Male factor accounts for 25-30% of infertile cases and may arise as a result of abnormal semen and/or sperm qualities (Matzuk and Lamb, 2008; ESHRE, ART Fact Sheet, 2014) such as oligozoospermia, teratozoospermia, asthenozoospermia and azoospermia (Matzuk and Lamb, 2008). While specific causes for many of these conditions have been described in the literature, 10-25% of cases still remain unexplained (ESHRE, ART Fact Sheet, 2014). Assisted reproductive technology (ART) is continuously evolving to accommodate infertile patients with appropriate treatments and as a result, 1.5% of all births have been successful in the UK from these techniques; with ICSI (intracytoplasmic sperm injection) being the ultimate option for severe male infertile conditions (Neri

et al., 2014; HFEA, 2012). This procedure readily overcomes the limitations of other infertility treatments such as *in vitro* fertilisation (IVF), albeit having similar success rates (HFEA, 2014). However, despite its relative success, and labeling as the 'magic bullet' for oocyte activation deficiency cases, total fertilisation failure (TFF) occurs in 1-3% of ICSI cycles leading to much disappointment and confusion for these patients (Nasr-Esfahani et al., 2010; Vanden Meerschaut et al., 2014). The underlying cause is largely believed to be failure of the oocyte to activate, a condition known as oocyte activation deficiency (OAD) (Kashir et al., 2010), and over the years it has become very apparent that the deficiency of PLCζ is intimately related to OAD.

Yoon et al. (2008) identified sperm incapable of inducing $Ca^{2+}$ oscillations in oocytes. This population of sperm, obtained from patients who had experienced recurrent ICSI failure, was analysed as being PLCζ-deficient by immunohistochemistry. Activity was only re-established following the microinjection of sperm with PLCζ mRNA, thus indicating the need for PLCζ in order for oocytes to activate (Yoon et al., 2008). Moreover, sperm from patients diagnosed with partial or complete globozoospermia are unable to fertilise oocytes naturally as a result of reduced levels or the total absence of PLCζ (Dam et al., 2007; Kashir et al., 2010; Taylor et al., 2010). Globozoospermia is a rare male infertile condition caused by a defective *DPY19L2* gene in which sperm are round-headed and devoid of an acrosome (Chansel-Debordeaux at al., 2015; Escoffier et al., 2015). Following characterisation of the PLCζ localisation profile within the human sperm head (Grasa et al., 2008), evidence signifying the role of PLCζ as the SOAF (Saunders et al., 2002; Knott et al., 2005; Kashir et al., 2011; Nomikos et al., 2013), the findings of Yoon et al. (2008), and the association between PLCζ and globozoospermia (Dam et al., 2007; Kashir et al., 2010; Taylor et al., 2010; Escoffier et al., 2015), it became very apparent that PLCζ was intimately associated with the sperm's ability to activate the oocyte, and that PLCζ deficiency was likely to be the predominant causative factor underlying OAD.

Genetic anomalies detected in the gene encoding PLCζ have further emphasised the link between PLCζ and OAD, and screening of PLCζ exons has revealed two novel mutations, PLCζ$^{H398P}$

and PLCζ$^{H233L}$, in a single infertile non-globozoospermic patient. These variants generated an abnormal protein structure, which in turn, disrupted $Ca^{2+}$ oscillation-inducing activity and was therefore, the cause of the patient's infertile status (Heytens et al., 2009; Kashir et al., 2012). However, despite intense screening efforts by a variety of research groups, no other genetic abnormalities have been reported since. Consequently, PLCζ$^{H398P}$ and PLCζ$^{H233L}$ are most likely to represent rare mutations that occurred naturally, and unfortunately manifested in the same patient.

All previous genetic studies have focused purely upon the coding region of PLCζ (Yoon et al., 2008; Heytens et al., 2009; Kashir et al., 2012) and only recently has the promoter site been included. Indeed, Coward et al. (2011) discovered that PLCζ shares a bi-directional promoter with the actin filament capping muscle Z-line alpha 3 (CAPZA3), a protein that plays a key structural role for sperm during spermatogenesis **(Figure 2)**. Interestingly, PLCζ variants have already been reported in the bi-directional promoter of Chinese Holstein bulls. These genetic variants (-456G>A and +65T>C) were said to affect semen quality traits and, as a result, fertilisation outcome (Pan et al., 2013). However, no direct assay of PLCζ expression or activity was attempted in this particular study, and consequently it was impossible to elucidate how these particular variants influenced semen or fertilisation outcome. Nevertheless, this study has prompted genetic screening to include the PLCζ promoter region, and possibly, intronic regions to detect variants which may affect PLCζ expression, but which would not be detected with existing screening protocols.

The expression of PLCζ, however, has been proven to be more complicated as we might expect. We would anticipate, for example, that infertile patients would exhibit reduced total levels of PLCζ when compared to fertile patients. In fact, using quantitative immunofluorescent analysis in individual sperm, Kashir et al. (2013b) discovered a significant variation in the total levels of PLCζ, and identified predominant PLCζ localisation patterns from both fertile and infertile (OAD) men. Variations were heterogeneous and were observed between individuals and also between ejaculates (Kashir et al., 2013b) but the precise mechanisms underlying such variation remain unknown. Nonetheless, this has led to the need for a more robust PLCζ assay with which to diagnose PLCζ-

deficiency in human sperm, as assays determining the total levels of PLCζ, or identifying specific localisation patterns, may not be the most effective clinical diagnostic tool.

The current tools used to evaluate ICSI success in relation to sperm oocyte-activating capacity are via heterologous ICSI models, the mouse oocyte activation test (MOAT) and mouse oocyte calcium analysis (MOCA). These techniques are used most commonly for research purposes (Palermo et al., 1997; Gordo et al., 2000; Yoon et al., 2008;), but have also been introduced and used in a limited number of clinics (Heindryckx et al., 2005; Heindryckx et al., 2008; Vanden Meerschaut et al., 2012; Vanden Meerschaut et al., 2013; Durban et al., 2015). The MOAT categorises patients into three groups: group 1 (low MOAT activating capacity), group 2 (intermediate MOAT activating capacity) and group 3 (high MOAT activating capacity). For group 1 patients, sperm-related deficiency is the probable cause of previous fertilisation failures; for group 2 patients, the cause is inconclusive and can be due to both sperm and oocyte deficiencies; for group 3 patients, sperm-related deficiency is eliminated and other factors in the oocyte are likely causes (Vanden Meerschaut et al., 2013). It has been established that human PLCζ is more potent than mouse PLCζ, and as these models involve the injection of human sperm into mouse oocytes, analysis cannot strictly define the reasons for previous fertilisation failure and may only be beneficial in severe OAD cases presenting a lack of PLCζ (Swann et al., 2006; Vanden Meerschaut et al., 2013). Development of a homologous model would be a more effective and beneficial method as this would overcome the limitations of the heterologous model and thus, accurately deduce oocyte activation/calcium release ability and the cause of previous fertilisation failure. This would also enable more efficient diagnosis and the implementation of appropriate treatment management.

**Translating PLCζ to the clinic: topical updates and a time for change**

With increased awareness of PLCζ and its fundamental role in oocyte activation, from the clinical, scientific and public domains, the number of clinical cases identified which involve PLCζ-deficiency has continued to expand. Patient awareness of PLCζ and its role in OAD has had a

pronounced effect in promoting research in this area, notably by increased online discussion via fertility forums and blogs. Our major concern still involves the unknown factors underlying the expression of PLCζ. The variation in PLCζ expression in human sperm initially reported by Kashir et al. (2013b) has since been confirmed in a follow-up study by the same laboratory (Yelumalai et al., 2015). For the first time, Yelumalai et al. (2015) demonstrated that total levels and localisation patterns of sperm exhibiting PLCζ were significantly correlated to fertilisation rates following treatment by ICSI but not by IVF. This finding strongly suggests that PLCζ is a strong contender for a useful prognostic and diagnostic marker for ICSI cases. Moreover, this study further highlighted that the reasons for variation in PLCζ levels and localisation in human sperm, and the molecular mechanisms involved, are still unknown.

Abnormal PLCζ expression is particularly evident in globozoospermic sperm (Dam et al., 2007; Taylor et al., 2010), although the heterogeneity of PLCζ expression extends similarly to normozoospermic sperm. According to the World Health Organisation, normozoospermia refers to sperm with normal parameters (concentration, percentage of progressive motility and morphology) that are equal to, or above, lower reference limits (WHO, 2010). A number of recent cases have reported that normal-appearing sperm from patients with a history of repeated low fertilisation, or TFF, can be associated with reduced levels or abnormal localisation patterns of PLCζ (Lee et al., 2014; Chithiwala et al., 2015; Durban et al., 2015). The underlying mechanism for observed variation in PLCζ expression, and for why some males suffer from PLCζ deficiency while others do not, remains entirely speculative at present, but represents a major problem in translating PLCζ diagnosis/therapy into the clinic. Consequently, the next logical steps might be to investigate the non-coding regions of DNA that may be involved in the regulatory activity of PLCζ, particularly the bi-directional promoter shared with CAPZA3, and potentially intronic DNA. It is logical to hypothesise that any problems within the promoter or intronic regions could exert influence over PLCζ expression and thus, affect the status of fertility. Due to increasing evidence correlating PLCζ and its fundamental role in infertility, it is becoming essential that the regulatory mechanisms involved with

PLCζ expression are elucidated so as to shed light on the causes of deficiency, therefore allowing diagnosis and treatment to be carried out accordingly.

Following the discovery of a small number of PLCζ genetic variants that have affected fertilisation outcome (Heytens et al., 2009; Kashir et al., 2012; Pan et al., 2013), clinical cases involving single nucleotide polymorphisms (SNPs) within PLCζ have been gaining increasing attention over the past two years. Schrimpf et al. (2014) identified three intronic SNPs within the PLCζ of Hanoverian stallions that significantly affected equine fertility. This study suggested that these SNPs disrupted the transcriptional mechanism of PLCζ, which may have caused the abnormal regulation of PLCζ expression, although this was not proven experimentally. Although Pan et al. (2013) and Schrimpf et al. (2014) investigated PLCζ SNPs in the bovine and equine model, respectively, only a small case report by Durban et al. (2015) has been reported for human PLCζ. Three intronic PLCζ SNPs were identified in a normozoospermic patient with recurrent complete fertilisation failure (Durban et al., 2015). It was proposed that these SNPs, one of which had been previously reported by Yoon et al. (2008), and two novel SNPs which had neither been reported nor associated with a disease previously, may have resulted in the observed reduction in PLCζ expression in the patient under investigation. The precise reasons underlying abnormal PLCζ expression in this patient remain unknown but the authors hypothesised that altered transcription, translation, or turnover, were possible mechanisms. Our own unpublished research, using the 'Single Nucleotide Polymorphism database (dbSNP)', resulted in the detection of 6224 SNPs within the human PLCζ gene. While the common conception is that SNPs rarely cause a disorder with a pathological consequence, it has been reported that SNPs may be associated with a particular phenotypic diversity. Consequently in the case of PLCζ, it could be plausible that certain SNPs might influence gene expression, as implied in the limited number of studies thus far.

Both Schrimpf et al. (2014) and Durban et al. (2015) detected relevant SNPs that appeared to alter the expression of PLCζ; all of these SNPs occurred within intronic DNA. For many years, introns were perceived as representing "junk DNA" which served no purpose in the eukaryotic

genome (Chow et al., 1977; Berk et al., 1977). However, over recent times, a number of studies have concluded that introns appear to be necessary prerequisites to achieve normal protein expression. Introns in eukaryotic organisms are now thought to be multi-functional and heavily involved in all steps of mRNA processing (initiation, termination, and the regulation of transcription, genome organisation and alternative splicing) (Reviewed by Chorev and Carmel, 2012). Several studies, in both humans and plants, have shown that intron-bearing genes have increased protein expression in comparison to intronless genes, as intron-bearing genes produce more mRNA; the mechanism involved in this phenomenon, however has yet to be determined (Gruss et al., 1979; Buchman and Berg, 1988; Le Hir et al., 2003; Akua et al., 2010). Therefore, it could be hypothesised that genetic variants (whether SNP or mutation in origin) located within intron-bearing genes might exert effects upon mRNA production and therefore, influence expression of the final protein. The human *PLCζ* gene consists of 15 exons with introns spanning the intervening sequence (Yoon et al., 2008). It therefore appears worthwhile to investigate the PLCζ introns in order to assess whether they are susceptible to SNPs with the ability to influence protein expression. This could be a plausible explanation to account for the significant variation of PLCζ expression seen between fertile and infertile patients. Moreover, it was estimated that over 50% of human genetic disorders are the result of an interrupted splicing pattern, thus emphasising the importance of introns and their function at the genomic level (Lopez-Bigas et al., 2005; Wang and Cooper, 2007).

It is widely believed that factors such as DNA fragmentation, oxidative stress, or PLCζ expression levels are potential biomarkers for sperm quality and thus, fertility status. Although research on these factors have been performed separately, a recent study by Park et al. (2015) linked all three factors together and concluded that there was a negative correlation between PLCζ immunoreactivity and 8-hydroxy-2’-deoxyguanosine (8-OH-dG), a biomarker for oxidative stress). These authors suggested that this data could provide a potential prognostic biomarker with which to assess sperm quality for ART success. However, there were a number of limitations to this study including low sample size, unknown fertility status of the subjects, and even though the authors stress

the association between the three factors, there was no specific correlation between DNA fragmentation and PLCζ levels.

While the main remit of this review was to discuss OAD in relation to PLCζ-related deficiencies, it is also important to consider the specific role of interacting factors within the oocyte, particularly in OAD cases where sperm-related deficiencies have been eliminated. Although specific oocyte factors which activate PLCζ enzymatic activity or factors which bind with PLCζ for $PIP_2$ hydrolysis have yet to be identified, abnormalities within the molecular machinery of the oocyte following fertilisation may also have an impact on fertilisation failure and infertility. The specific frequency and amplitude of $Ca^{2+}$ release are crucial for successful oocyte activation, and the conditions or availability of protein factors involved in these mechanisms should also be investigated for unrelated sperm-deficiency OAD cases. Such protein factors may include $PIP_2$, $InsP_3R$, DAG, PKC and store-operated calcium-entry systems (e.g., STIMI, ORAII and SERCA). Utilising these protein factors for future diagnostic or prognostic assays would signify an additional crucial step in diagnosing OAD and also represent novel therapeutic targets (Reviewed by Yeste et al., 2015).

**The urgency for an endogenous approach for OAD therapy**

The only available treatment for OAD at present is the use of artificial oocyte activators (AOAs), particularly the use of $Ca^{2+}$ ionophores (Nasr-Esfahani et al., 2010; Amdani et al., 2013). Although proven to be successful, such agents may potentially exert cytotoxic, mutagenic, and epigenetic effects upon oocytes and embryos. This is due to the manner in which such agents release $Ca^{2+}$ in the oocyte following ICSI; ionophores produce a single $Ca^{2+}$ transient instead of a series of natural oscillations (Santella and Dale, 2015). Given that the pattern of $Ca^{2+}$ oscillations is vital in regulating oocyte activation, the use of AOAs may represent a possible threat to successful embryogenesis (Nasr-Esfahani et al., 2010; Amdani et al., 2013). Although supported by the HFEA (Human Fertilisation and Embryology Authority, 2015), the regulatory body responsible for ART within the UK, and the increased awareness of the public in the United Kingdom with regard to the

use of such agents, there is widespread caution in the use of AOAs (Santella and Dale, 2015; van Blerkom et al., 2015). As a result, it has been specified that AOAs should only be used if justified by additional data, such as proven PLCζ deficiency (van Blerkom et al., 2015). It is therefore of great importance for researchers to produce an alternative, safer and more endogenous therapy for OAD, namely in the form of a human recombinant PLCζ protein (hrPLCζ) (For review, see Amdani et al., 2013). In addition to its potential therapeutic use, the successful production of this protein may lead to other benefits, including the generation of a PLCζ monoclonal antibody, and as the foundation for protein crystallisation studies.

Generation of a monoclonal antibody could provide a powerful new diagnostic/prognostic assay, and could create a more accurate foundation for the analysis of PLCζ localisation in human sperm (Grasa et al., 2008; Kashir et al., 2013b; Yelumalai et al., 2015). Thus far, current studies have consistently utilised antibodies, which are polyclonal in nature. Although the use of such antibodies has been supported by appropriate peptide-blocking assays, we cannot ignore the fact that the specificity of current antibodies could have an impact upon experimental outcomes (Nomikos et al., 2013; Amdani et al., 2013; Kashir et al., 2013b; Yelumalai et al., 2015; Amdani et al., 2015). At present, the three-dimensional structure of PLCζ protein remains unknown, although computer modeling suggests it shares strong homology to PLCδ1. Consequently, a predicted three-dimensional structure for PLCζ protein has been generated, derived upon the established structure of PLCδ1 (Heytens et al., 2009). Although this has been useful in understanding the functional effects of PLCζ$^{H398P}$ and PLCζ$^{H233L}$ mutations upon PLCζ structure and function (Heytens et al., 2009; Kashir et al., 2012), it remains a major requirement to generate a definitive structure. This can be achieved via X-ray crystallography and will help us to comprehend the structural attributes of PLCζ which are presently either unavailable or remain questionable; the precise shape and domain structure, the conformational changes PLCζ goes through during oocyte activation and interaction with other macromolecules in the oocyte, to evaluate post-translation modifications, and for the development of

structural-based drugs to regulate PLCζ activity and function (Smyth and Martin, 2000; Acharya and Lloyd, 2005).

A prerequisite to the development of a monoclonal antibody, or protein crystallisation studies, is the large-scale purification of PLCζ recombinant protein. The production of hrPLCζ has, however, represented a major challenge for researchers working in the field, with successful purification of a functionally active protein representing the biggest obstacle (Yoon et al., 2012). Initial attempts by Cooney et al. (2010) resulted in an inactive hrPLCζ. Work by Kashir et al. (2011) successfully reported the expression of an active hrPLCζ but this was present in mammalian cell lysates, and not in a purified form. The first purified hrPLCζ was produced in a bacterial cell line by Yoon et al. (2012). However, in addition to a clearly abnormal $Ca^{2+}$ release profile when injected into human eggs, excessively high concentrations of hrPLCζ were required to induce $Ca^{2+}$ oscillations with this particular hrPLCζ, thus representing a major concern from a clinical point of view. More recently, Nomikos et al. (2013) successfully generated a purified and active hrPLCζ, using the Nus-A fusion recombinant protein system. This protein induced $Ca^{2+}$ oscillations similar to those observed during normal fertilisation and rescued the activation ability of mouse oocytes that had been previously injected with cRNA synthesised to PLCζ$^{H398P}$ and PLCζ$^{H233L}$, providing further proof of concept for PLCζ as a powerful therapeutic agent (Nomikos et al., 2013). Concerns however remain with respect to the relative size of the tag used in this study (60 kDa) and its requirements for activity and stability. A purification tag of this size may affect potential use of the protein therapeutically and therefore, more work is warranted to ensure that activity and stability are sustained in the absence of the tag. In a more recent study, Sanusi et al. (2015) were able to rescue failed oocyte activation following ICSI in a mouse model using a hrPLCζ, thus demonstrating that hrPLCζ can be used safely for ICSI failure cases in which the injected sperm may already contain endogenous $Ca^{2+}$ releasing activity. Nomikos et al. (2014) further utilised their hrPLCζ to justify that PLCζ induces oocyte activation when injected into mouse oocytes while recombinant PAWP protein (recPAWP) did not. While this data directly refuted the latest findings from Aarabi et al. (2014) who claimed that recPAWP induced $Ca^{2+}$

oscillations upon injection in both mouse and human oocytes, it should be noted that differences in expression host systems, reagents, protein purification tags, or even experimental conditions, could have resulted in the very different outcomes reported by Nomikos et al. (2014) and Aarabi et al. (2014). The hrPLCζ from Nomikos et al. (2013) remains the most promising option for future applications although no further advancements in terms of clinical therapy or diagnostics have been reported as yet.

**Conclusion**

While substantial progress has been made in the study of PLCζ both from a scientific and clinical point of view, there are still many outstanding concerns and priorities. Firstly, there is a clear need to fully comprehend the precise mechanism underlying PLCζ function in the phosphoinositide signalling pathway and its proposed interactions with an as yet unidentified oocyte-borne factor, or factors, to achieve this action. Secondly, to investigate non-coding regions of PLCζ as these may help us determine how the expression of this fundamental protein is regulated. Screening of these regions in a wider population of OAD patients may help us to detect genetic variants which are able to influence PLCζ expression, or even localisation. However, the limitations imposed by the current methodologies used for genetic screening may prompt us to integrate new technologies including next generation sequencing (NGS) and dedicated promoter studies. NGS represents a more sensitive and less time-consuming method for screening PLCζ genetic variants, and carefully designed promoter studies may help us to understand the impact of variants occurring within this critical regulatory region upon PLCζ expression. The most outstanding priority is to synthesise a pure hrPLCζ with which to generate a monoclonal antibody and protein crystals, as these will benefit both scientific and clinical settings.

Recent conflict regarding the identification of the appropriate SOAF candidate between PLCζ and PAWP understandably raised concerns over the eligibility of PLCζ as the sole therapeutic target for development. A PAWP knockout model was recently created and successfully proved that PAWP

is not involved in $Ca^{2+}$ release during oocyte activation (Satouh et al., 2015). This has created renewed emphasis on the need to create a similar model for PLCζ. Creating a PLCζ knockout model has, however, proved to be a difficult task; an early attempt resulted in mice exhibiting a failure to fully complete spermatogenesis (Ito et al., 2010), suggesting perhaps that the bi-directional promoter shared by the *PLCζ* and *CAPZA3* genes had been compromised. This suggests that future studies should exert extreme caution but also stresses the urgent need for such work. Unraveling this series of outstanding concerns is imperative in giving the growing body of OAD patients around the world an endogenous alternative to current AOA treatments, which are increasingly facing skepticism and concern from the scientific community while also encouraging reproductive tourism to the limited number of clinics offering such therapy.

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**Figure Legends**

**Figure 1.** The signalling mechanism of PLCζ leading to oocyte activation. Following gamete fusion, PLCζ is introduced into the ooplasm and interacts with an as yet unknown oocyte factor (or factors) located on the surface of vesicles containing $PIP_2$, leading to the hydrolysis of $PIP_2$ into $InsP_3$ and DAG. $InsP_3$ subsequently binds to $InsP_3Rs$ on the endoplasmic reticulum causing the release of calcium ($Ca^{2+}$). $Ca^{2+}$ activates CaMKII and in turn inhibits CSF. This inhibition liberates APC and subsequently degrades the levels of Cyclin BI from the MPF complex, composed of CDK1 and Cyclin B1. Reduction of Cyclin B1 inactivates MPF causing the release of the oocyte from meiotic arrest. Simultaneously, the $Ca^{2+}$ released also inactivates MAPK allowing formation of the pronucleus. DAG activates the PKC pathway and translates $Ca^{2+}$ signals into cellular responses such as the release of cortical granules. APC: Anaphase-promoting complex/cyclosome; CaM/CaMKII: Calcium/Calmodulin-dependent protein kinase II; CDK1: Cyclin-dependent kinase I; CSF: Cytostatic factor; DAG: Diacylglycerol; $InsP_3$: Inositol-1,4,5-triphosphate; $InsP_3R$: $InsP_3$ receptor; MAPK: Mitogen-activated protein kinase; MPF: Maturation promoting factor; $PIP_2$: Phosphatidylinositol 4,5-biphosphate; PKC: Protein Kinase C. Reproduced from Yeste et al. (2015) with permission.

**Figure 2.** Genetic linkage of chromosome 12 in humans. **(A)** PLCζ is located back-to-back with CAPZA3. **(B)** PLCζ shares a bi-directional promoter with CAPZA3, and each gene is transcribed in opposite directions to carry out their functions, oocyte activation and sperm structure during spermatogenesis, respectively. Gene abbreviations: PIK3C2G, phosphoinositide-3-kinase, class 2, gamma polypeptide; PLCζ, phospholipase C, ζ; CAPZA3, actin filament capping muscle Z-line alpha 3; PEPP2, phosphatidylinositol 3-phosphate-binding PH domain protein 2; AEBP2, Adipocyte-enhancer (AE) binding protein 2. Reproduced and modified from Coward et al. (2011) with permission.

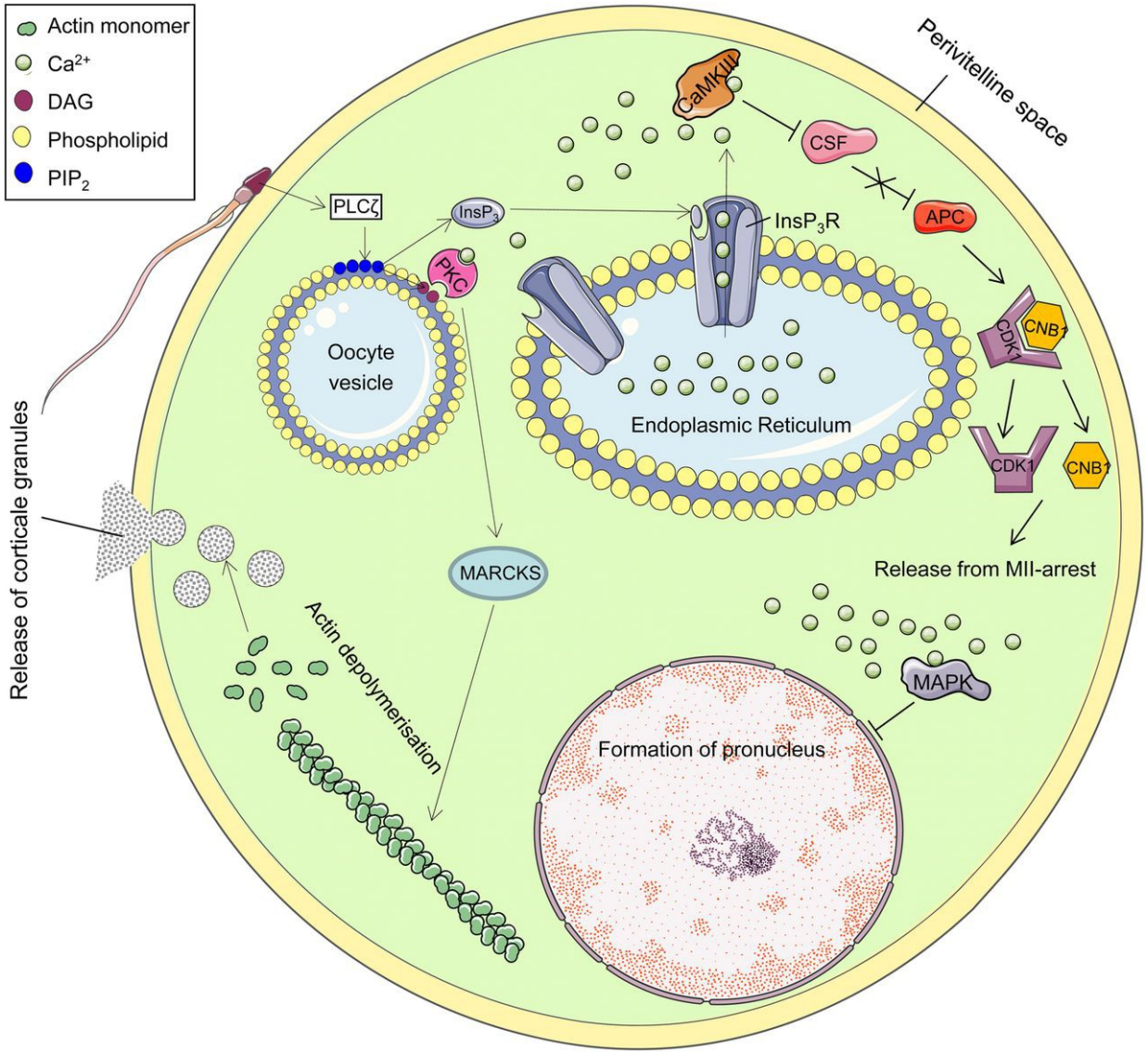
## Tables

**Table 1.** Key features of PLCζ with respect to its candidature as a SOAF. Table reproduced and modified from Amdani et al. (2015).

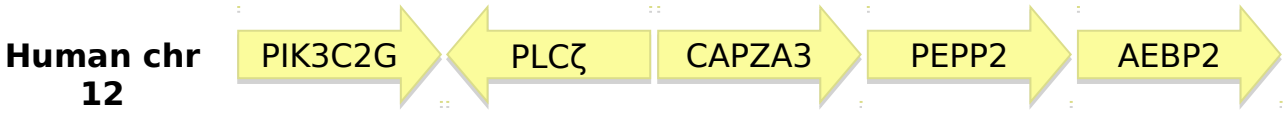
| Features | PLCζ | Study (Year) |
|---|---|---|
| Expression Profile | PLCζ mRNA is first expressed in round spermatids and likely to be translated in elongating spermatids. PLCζ is detectable in all subsequent stages, both in the testes and epididymis. | Saunders et al. (2002); Yoneda et al. (2006); Young et al. (2009). |
| Localisation Patterns | PLCζ localisation patterns have been detected in the acrosomal, equatorial, post-acrosomal and/or the PAS-PT region of the sperm head. | Fujimoto et al. (2004); Swann et al. (2006); Yoon and Fissore (2007); Grasa et al. (2008); Escoffier et al. (2015). |
| Structure | PLCζ is the smallest PLC isoform consisting of a C2 domain, four tandem EF-hand domains, and X and Y catalytic domains. Each domain plays a crucial role in PLCζ function. The XY-linker is located between the X and Y domains. | Saunders et al. (2002, 2007); Swann et al. (2006); Nomikos et al. (2011a, 2011b, 2015b). |
| Calcium Signalling Mechanism | PLCζ hydrolyses $PIP_2$ upon introduction into the oocyte generating $InsP_3$. $InsP_3$ binds to $InsP_3R$ causing the release of $Ca^{2+}$ from the ER (supported by a large body of experimental evidence). | Swann et al. (2006); Nomikos et al. (2011a); Swann and Lai (2013). |
| Oocyte Activation | MII-arrested mouse oocytes microinjected with PLCζ cRNA induced a normal $Ca^{2+}$ release profile and encountered normal development to the blastocyst stage. Sperm extracts devoid of PLCζ failed to induce $Ca^{2+}$ activity. Microinjection of human recombinant PLCζ protein (hrPLCζ) in either lysate or purified form induced normal $Ca^{2+}$ oscillations in mouse oocytes. | Saunders et al. (2002); Kashir et al. (2011); Nomikos et al. (2013); Nomikos et al. (2014); Sanusi et al. (2015). |
| Links to Infertility | Sperm with reduced levels, total absence or abnormal localisation patterns of PLCζ failed to evoke $Ca^{2+}$ oscillations. Activity can be restored following the microinjection of sperm coincident with PLCζ mRNA. Two novel mutations in PLCζ, H398P and H233L, were discovered in an infertile non-globozoospermic patient and produced an abnormal $Ca^{2+}$ release profile. Two genetic variants from bull PLCζ affected semen quality traits. SNPs located within the intronic regions of PLCζ (equine and human) were proposed to be the cause of reduced PLCζ expression. Globozoospermic sperm unable to induce oocyte activation demonstrated reduced or absent PLCζ. Normozoospermic patients with a history of low fertilisation failure or TFF showed reduced PLCζ levels and/or abnormal localisation patterns. | Yoon et al. (2008); Heytens et al. (2009); Kashir et al. (2012); Pan et al. (2013); Lee et al. (2014); Schrimpf et al. (2014); Chansel-Debordeaux et al. (2015); Chithiwala et al. (2015); Durban et al. (2015); Escoffier et al. (2015). |

[a] $Ca^{2+}$, calcium; ER, endoplasmic reticulum; ICSI, intracytoplasmic sperm injection; $InsP_3$, inositol-1,4,5-triphosphate; $InsP_3R$, $InsP_3$ receptor; PAS-PT, post-acrosomal sheath-perinuclear theca; $PIP_2$, phosphatidylinositol 4,5-biphosphate; PLCζ, phospholipase C zeta; SNP, single nucleotide polymorphism; TFF, total fertilisation failure

# Figure 1


Actin monomer
Ca2+
DAG
Phospholipid
PIP2
Perivitelline space
CaMKII
CSF
APC
PLCζ
InsP3
InsP3R
PKC
Oocyte vesicle
Endoplasmic Reticulum
CDK1
CNB1
CDK1
CNB1
Release from MII-arrest
MARCKS
Actin depolymerisation
MAPK
Formation of pronucleus
Release of corticale granules

## Figure 2 (A)




## Figure 2 (B)