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Acknowledgments

Firstly, I would like to extend my utmost gratitude to my supervisor, Dr. Kevin Coward. I have been through my journey in Oxford with him since I started my MSc in 2012, and his belief in me has not faltered once. While I was an MSc student, I saw the hardships his DPhil students went through to create a better version of themselves and along with that, I also saw how supportive he was during their troughs and from this I knew I was in very good hands. There are not enough words to express how truly grateful I am for having Kevin give me this opportunity to do a DPhil under his supervision. Secondly, I am truly indebted to my laboratory manager, Mrs. Celine Jones. Like Kevin, she has also been there from the start and I am thankful, as she has always made sure my research ran smoothly and being in the laboratory with her has been nothing less than infinite delight. I would also like to thank previous and current members of the Coward group (Dr. Marc Yeste, Dr. Rebecca Dragovic, Dr. Patricia Grasa-Molina, Mrs. Laura Rose). They have all created a friendly atmosphere in the work place, and I am very thankful for their countless advice and enthusiasm. The previous and current DPhil students in the Coward group (Natalia, Suseela, Lien, Eisa, and Xin), who have made this DPhil just that much exciting and with some I know I have created everlasting friendships with.

Without my funding bodies, my DPhil would not have been possible so I would like to express my gratitude to Universiti Brunei Darussalam and the Ministry of Education (Brunei Darussalam) for their generosity.

The scientific collaborations I have made for this thesis helped facilitate my research and I am eternally grateful to each and every one of the individuals involved. For Chapter two: Miss Katharina Spath (NGS), Dr. Tomas Malinauskas (predicted three-dimensional models), Miss Minerva Ferrer-Buitrago (PLCζ cRNA microinjections), Mrs. Tracey Griffiths (single sperm isolation), Miss Dhruti Babariya (mini-sequencing), and Professor Dagan Wells, who is also my secondary supervisor, for his advice on genetics and allowing me to use his laboratory facilities. For Chapter three: Dr. Donatien Chedem Fotso (NGS analysis), and previous MSc and FHS students (Miss Felicitas Azpiroz, Miss Sanya Arora, Miss Alice Macneill, and Mr Oliver Lloyd-Parry) for their assistance on the dbSNP analysis. For Chapter four: Miss Goli Ardestani and Professor Rafael Fissore (PLCζ recombinant protein microinjections). Additionally, Professor Elspeth Garman, Dr. Karl Morten and Dr. Andrew Greenfield for their sincere advice and constructive criticisms during my transfer and confirmation of status.

The many friends I have made in Oxford who made my living experience here, both during my MSc and DPhil years, extremely beautiful. A special mention goes to Sylvia, Ragya, Nada, Chidambra, Victoria, Yiota, Tomi, Feli, Amal, Faez, Zeynep, Araz, and Helena. To my Oxford family, Dhruti and Katharina, we have gone through our most ups and downs together, yet, we continue to remind each other every time that everything will fall into place and for now it has. I have so much love for the both of you. To Cheryl, my best friend who has been with me every step of the way during my undergraduate and graduate years, I cannot thank you enough. I would like to extend my gratitude and love to my Godparents in London for their unwavering support. I would like to thank my siblings for being the best brother and sister anyone could ever ask for.

Lastly, of course, to my parents who have given me this wonderful life I live. To my father, who has taught me appreciation in the hardest way possible. To my mother, who has provided me unconditional love, and for being the most honest and strongest person I know.
**Glossary of abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AOA(s)</td>
<td>Artificial oocyte activators</td>
</tr>
<tr>
<td>ART</td>
<td>Assisted reproductive technology</td>
</tr>
<tr>
<td>AUM</td>
<td>Antigen unmasking/retrieval</td>
</tr>
<tr>
<td>C2</td>
<td>PKC-homology type II</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium ions</td>
</tr>
<tr>
<td>CAPZA3</td>
<td>Actin filament capping muscle Z-line alpha 3</td>
</tr>
<tr>
<td>cRNA</td>
<td>Complementary ribonucleic acid</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DGW</td>
<td>Density gradient washing</td>
</tr>
<tr>
<td>ECD</td>
<td>Enrichment control DNA</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EF</td>
<td>Elongation-factor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotropin hormone</td>
</tr>
<tr>
<td>HEK293T</td>
<td>Human embryonic kidney cells expressing a mutant SV40 large T-antigen</td>
</tr>
<tr>
<td>HFEA</td>
<td>Human fertilisation and embryology authority</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>hPLCζ</td>
<td>human PLCζ</td>
</tr>
<tr>
<td>hrPLCζ</td>
<td>human recombinant PLCζ</td>
</tr>
<tr>
<td>ICSI</td>
<td>Intracytoplasmic sperm injection</td>
</tr>
<tr>
<td>IGV</td>
<td>Integrated genome viewer</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol-1,4,5-triphosphate</td>
</tr>
<tr>
<td>IP₃Rs</td>
<td>IP₃ receptors</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>IVF</td>
<td>In vitro fertilisation</td>
</tr>
<tr>
<td>KSOM</td>
<td>Potassium simplex optimised medium</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth medium</td>
</tr>
<tr>
<td>MOAT</td>
<td>Mouse oocyte activation test</td>
</tr>
<tr>
<td>MOCA</td>
<td>Mouse oocyte calcium analysis</td>
</tr>
<tr>
<td>mPLCζ</td>
<td>mouse PLCζ</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NGS</td>
<td>Next generation sequencing</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localisation signal</td>
</tr>
<tr>
<td>OA</td>
<td>Oocyte activation</td>
</tr>
<tr>
<td>OAD</td>
<td>Oocyte activation deficiency</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAS-PT</td>
<td>Post-acrosomal sheath-perinuclear theca</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphoinositide</td>
</tr>
<tr>
<td>PIK3C2G</td>
<td>Phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing gamma polypeptide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4,5-biphosphate</td>
</tr>
<tr>
<td>PLC</td>
<td>PI-specific phospholipase C</td>
</tr>
<tr>
<td>PLCβ</td>
<td>Phospholipase C beta</td>
</tr>
<tr>
<td>PLCδ</td>
<td>Phospholipase C delta</td>
</tr>
<tr>
<td>PLCζ</td>
<td>Phospholipase C zeta</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>PN</td>
<td>Pronuclear</td>
</tr>
<tr>
<td>PT</td>
<td>Perinuclear theca</td>
</tr>
<tr>
<td>PTMs</td>
<td>Post-translation modifications</td>
</tr>
<tr>
<td>RE</td>
<td>Restriction enzyme</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SOAF</td>
<td>Sperm oocyte-activating factor</td>
</tr>
<tr>
<td>sPLCζ</td>
<td>simian PLCζ</td>
</tr>
<tr>
<td>TTF</td>
<td>Total fertilisation failure</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
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Abstract

Oocyte activation deficiency (OAD) is an infertile condition observed in patients who have experienced recurrent total fertilisation failure (TFF) following intracytoplasmic sperm injection treatment. This condition was considered to be an idiopathic factor for a long time but strong clinical evidence now suggests that dysfunctional forms of phospholipase C zeta (PLCζ) may be predominant causative factors for OAD. Genetic contribution has played a role in patients suspected of having OAD, as four PLCζ exonic mutations have been discovered and characterised as being the cause of infertility. In this study, a novel nonsense mutation, PLCζK322Stop, was identified in the PLCζ XY-linker region of Patient LR. This variant results in the truncation of approximately half of PLCζ, therefore was non-functional when activity was tested. Patient LR, which also exhibited a previously reported mutation, PLCζH233L, may suggest that the patient is sub-fertile, as opposed to being infertile, as initially expected. Although research has purely focused upon the coding regions of PLCζ, it was obvious that our knowledge of PLCζ regulatory elements remain very limited. Next generation sequencing (NGS) was therefore employed to detect variants in the non-coding regions of PLCζ, promoter and introns, which may have resulted in the observed phenotypic diversity of PLCζ expression in fertile and infertile patients. As a result of mapping failure, an alternative approach was considered to identify variants within human PLCζ, and this involved using the single nucleotide polymorphism (SNP) database. Over 2500 SNPs were localised in the intronic regions of PLCζ and thus, it could be speculated that these variants may help elucidate the wide variation of PLCζ expression reported. Additionally, two particular patients with TFF (79 and 107) were investigated in this study to identify an association with PLCζ and their infertile state. For Patient 79, multiple PLCζ immunofluorescence analysis was performed and a significant improvement in PLCζ expression was observed one year after his first investigation. This may have been the result of an external factor, which influenced protein expression. As for Patient 107, a novel substitution mutation, PLCζV193E, was identified and was predicted to affect PLCζ stability and folding. There is global interest to create a safer and alternative OAD therapy, namely a human recombinant PLCζ protein (hrPLCζ). The first method, using a bacterial cell line resulted in successful purification and identification but the product proved to be inactive following mouse oocyte microinjection. The second method involved production of a mammalian-expressed hrPLCζ, which was successfully purified and identified but due to time restrictions, could not be tested for functionality. Concurrently, the findings in this thesis have reinforced the association between PLCζ and OAD, and provided improved options for the diagnosis and treatment of OAD.

Keywords: Oocyte activation deficiency (OAD), phospholipase C zeta (PLCζ), variants, next generation sequencing (NGS), promoter, introns, and human recombinant PLCζ protein (hrPLCζ).
Chapter 1: An insight into the sperm oocyte-activating factor, phospholipase C zeta (PLCζ)
1.1 Oocyte activation

Oocyte activation (OA) is a series of fundamental processes that are crucial for embryo development which takes place following fertilisation (Kline and Kline, 1992; Stricker, 1999). OA is implicitly regulated by the release of intracellular calcium ($\text{Ca}^{2+}$) within the oocyte, characterised as a single transient in non-mammals or a series of oscillations in mammals (Swann, 1990; Miyazaki, 2006; Whitaker, 2006; Figure 1). Earlier studies reported that this crucial change in $\text{Ca}^{2+}$ levels occurs as a result of the phosphoinositide (PI) signalling pathway, in which the hydrolysis of a substrate, phosphatidylinositol 4,5-biphosphate (PIP$_2$), causes the production of inositol-1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG). The binding of IP$_3$ to IP$_3$ receptors (IP$_3$Rs), localised on the surface of the endoplasmic reticulum (ER) membrane, liberates stored $\text{Ca}^{2+}$ into the ooplasm (Swann, 1990; Miyazaki et al., 1993).

![Figure 1](image.png)

**Figure 1.** Calcium release following fertilisation in the oocyte of non-mammalian and mammalian species. (A) Strongylocentrotus nudus, (B) Xenopus laevis, (C) Homo sapiens and (D) Mus musculus. Reproduced from Miyazaki (2006) and Nomikos et al. (2013).

The release of $\text{Ca}^{2+}$ is a prerequisite to many essential processes including cortical granule exocytosis, release of meiotic arrest, regulation of gene expression, pronuclear (PN) formation, recruitment of maternal mRNA, and ultimately the initiation of
embryogenesis (reviewed by Amdani et al., 2013). The signalling mechanism by which this specialised Ca\textsuperscript{2+} signal induces this response has yet to be fully explained, but our current understanding is depicted in Figure 2. Briefly, the release of Ca\textsuperscript{2+} following fertilisation causes the release of cortical granules in the oocyte via exocytosis to prevent polyspermy, and the activation and inhibition of protein kinases, CaM/CaMKII (Calcium/Calmodulin-dependent protein kinase) and MAPK (Mitogen-activated protein kinase). This, in turn, relieves the oocyte from meiotic arrest and induces PN formation, respectively.

**Figure 2.** Signalling mechanism of oocyte activation following fertilisation. Ca\textsuperscript{2+} oscillations triggered by the sperm following fertilisation mediate cortical granule exocytosis, and inhibit MAPK activity to allow PN formation. Additionally, the Ca\textsuperscript{2+} oscillations activate CaMKII to inhibit CSF. This inhibition liberates APC, which subsequently reduces the levels of Cyclin B1 in the MPF complex comprised of Cdk1 and Cyclin B1. Cyclin B1 reduction inactivates MPF, which in turn relieves meiotic arrest. CaM/CaMKII: Calcium/Calmodulin-dependent protein kinase II; CSF: Cytostatic factor; APC: Anaphase-promoting complex/cyclosome; MPF: Maturation promoting factor; Cdk1: Cyclin-dependent kinase 1; MAPK: Mitogen-activated protein kinase. Modified from Yeste et al. (2016).
The mechanisms and consequences of Ca\textsuperscript{2+} oscillations are crucial for all of the major events that occur during OA and subsequent embryogenesis (Swann and Yu, 2008; Ramadan \textit{et al}., 2012). Oocytes are distinctively sensitive to the specific frequency and amplitude of the oscillations whereby each Ca\textsuperscript{2+} spike progressively stimulates the OA process (Swann and Yu, 2008). The initial increase in Ca\textsuperscript{2+} release regulates both the early and late events of OA, and modulates peri-implantation effects upon gene expression and development to term (Ducibella \textit{et al}., 2006). Following an initial single transient, which tends to be of longer duration and higher amplitude, the frequency and amplitude of the remaining Ca\textsuperscript{2+} oscillations influences events such as the activity of cell cycle protein kinases, cortical granule exocytosis and embryonic development (Ducibella \textit{et al}., 2002; Miyazaki and Ito, 2006).

Previous hypotheses suggested that the Ca\textsuperscript{2+} released via the PI signalling pathway is induced by fusion of the sperm head to the oocyte membrane upon fertilisation (Swann, 1990). This was evident from experiments in which the direct microinjection of a spermatozoon, or soluble sperm extracts, or mRNA derived specifically from spermatogenic cells, induced Ca\textsuperscript{2+} oscillations which were indistinguishable to that of normal fertilisation (Swann \textit{et al}., 1989; Swann, 1990; Parrington \textit{et al}., 1996). The general consensus of opinion, and the advent of intracellular calcium imaging technology, eventually identified a sperm protein responsible for this phenomenon (Swann, 1990; Parrington, 2001). As OA was revealed to occur via the PI signalling pathway due to the production of IP\textsubscript{3}, it was evident that a PI-specific phospholipase C (PLC) might be involved (Swann, 1990; Miyazaki \textit{et al}., 1993), and additionally, that the sperm extracts used to study the release of Ca\textsuperscript{2+} in the ooplasm displayed a high level of PLC enzyme activity (Jones \textit{et al}., 1998). Consequently, subsequent research focused upon the specific identity of the PLC protein.
1.2 Phospholipase C zeta (PLCζ): A biochemical view

1.2.1 Discovery

The identity of the precise sperm factor had been the source of much contention but a landmark study reported by Saunders et al. (2002) introduced, for the first time, phospholipase C zeta (PLCζ) into the PLC class of enzymes. PLCζ was discovered using a mouse expressed sequence tag database, and this revealed single-pass reads of short fragments identified as novel PLC sequences - all derived from the testis. Immunoblot analysis and molecular cloning ultimately deduced a 74kDa protein, which the authors named PLCζ (Saunders et al., 2002). In contrast to the other twelve PLC isozymes (Table 1), PLCζ is sperm-specific, thus making it a dominant candidate for establishing OA.

Table 1. Key features of the twelve PLC isozymes.

<table>
<thead>
<tr>
<th>PLC</th>
<th>Expression</th>
<th>Primary function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ (δ1, δ3 and δ4)</td>
<td>Ubiquitous</td>
<td>Tumour suppressor, fertilisation, trophoblast and placental development</td>
<td>Fukami et al. (2004) Nakamura et al. (2005) Fu et al. (2007)</td>
</tr>
<tr>
<td>β (β1, β2, β3 and β4)</td>
<td>Brain, hematopoietic cells and eyes</td>
<td>Cell differentiation</td>
<td>Gresset et al. (2012) Cocco et al. (2016)</td>
</tr>
<tr>
<td>γ (γ1 and γ2)</td>
<td>Ubiquitous</td>
<td>Actin reorganisation and cell migration</td>
<td>Gresset et al. (2012)</td>
</tr>
<tr>
<td>ε</td>
<td>Heart, liver and lung</td>
<td>Cell survival, actin organisation and T-cell activation</td>
<td>Gresset et al. (2012)</td>
</tr>
<tr>
<td>ζ</td>
<td>Testis</td>
<td>Oocyte activation</td>
<td>Saunders et al. (2002)</td>
</tr>
<tr>
<td>η (η1 and η2)</td>
<td>Brain</td>
<td>Formation and maintenance of neuronal network</td>
<td>Cockcroft (2006)</td>
</tr>
</tbody>
</table>
Saunders et al. (2002) successfully demonstrated the ability of PLCζ to generate Ca^{2+} oscillations by microinjecting various forms of the protein, and concluded that the microinjection of PLCζ complementary RNA (cRNA) into metaphase II-arrested mouse oocytes could evoke Ca^{2+} oscillations similar to those triggered during normal fertilisation (Saunders et al., 2002). Furthermore, the immunodepletion of PLCζ from sperm extracts eliminated Ca^{2+} release, proving that the ability of a sperm to induce OA relied solely upon the presence of PLCζ (Saunders et al., 2002). Subsequent supplementary work from Cox et al. (2002) identified human PLCζ (hPLCζ) and simian PLCζ (sPLCζ), each with a molecular mass of 70kDa and 74kDa, respectively. Genomic organisation localised the hPLCζ gene in chromosome 12, where it comprises 15 exons (Cox et al., 2002). As originally observed for mouse PLCζ (mPLCζ; Saunders et al., 2002), immunoblot analysis confirmed the expression of hPLCζ in both the testis and spermatozoa. Additionally, microinjecting cRNA for hPLCζ into mouse oocytes induced Ca^{2+} oscillations, which triggered embryo development to the blastocyst stage. The same was observed during the microinjection of cRNA derived from sPLCζ into mouse oocytes. However, Cox et al. (2002) deduced that hPLCζ exhibits a higher potency than its simian counterpart as hPLCζ was able to evoke fertilisation-like Ca^{2+} oscillations at lower cRNA concentrations. Further confirmation that PLCζ was a fundamental requirement for physiological OA arose from the use of RNA interference technology. This created partially PLCζ-deficient sperm in transgenic mice and upon injection into mouse oocytes, Ca^{2+} oscillations were observed to terminate prematurely (Knott et al., 2005).

Cox et al. (2002) compared PLCζ sequences from human, simian and mouse, and concluded that primates shared a higher percentage sequence identity than between mPLCζ and hPLCζ. However, its cost and difficulty to rear in the laboratory made the simian an unfavourable model organism and thus, the mouse has become the premier mammalian model system (Perlman, 2016). Due to its high homology with hPLCζ (mPLCζ sequence
identity: 71%), the mouse model has contributed greatly to our understanding of the protein (Cox et al., 2002). The important discovery of PLCζ, and its putative role, disclosed a plethora of research opportunities and therefore, determining the structure of PLCζ was crucial to elucidate the signalling mechanism involved and confirm its involvement in OA.

1.2.2 Structure

Saunders et al. (2002) characterised PLCζ as having a typical PLC domain structure: X and Y catalytic domains – which constitute the active site in all other PLC isoforms, XY-linker, a single PKC-homology type II (C2) domain at the C-terminus, and a four tandem elongation-factor (EF) hand domain at the N-terminus (Figure 3).

**Figure 3.** Structural domains of the mammalian phospholipase C isozymes. All of these isozymes consist of a typical PLC domain structure: EF-hands, X-domain, Y-domain, C2 domain and XY-linker. PLCζ is the smallest mammalian PLC as it does not contain additional structural domains like that of the remaining PLCs. The difference in structure confers a specific function for each PLC isozyme as described in Table 1. PH: Pleckstrin homology domain; SH: Src homology domain; RasGEF: guanine-nucleotide-exchange factor for Ras; RA: Ras-binding domain. Reproduced and modified from Cockroft (2006).
To date, the other mammalian PLC isozymes differ according to their structure and regulatory mechanisms (Gresset et al., 2012; Figure 3). PLCζ is unique when compared to other PLC isoforms and this is because it lacks both the pleckstrin homology (PH) and Src homology domains, thus making it the smallest known mammalian PLC (Saunders et al., 2002; Cox et al., 2002). Each PLCζ domain plays a crucial role in OA but the scope of our knowledge towards these domains remains insufficient and therefore continues to be the source of much research.

1.2.2.1 *XY catalytic domains and the XY-linker region*

The enzymatic activity of PLCζ is provided by the XY catalytic domains, the most highly conserved region amongst all PLCs. Mutagenesis of key residues within the active site has been shown to result in the absence of PIP₂ hydrolysis *in vitro* and PLCζ-dependent Ca²⁺-oscillations in mouse oocytes (Saunders et al., 2002; Nomikos et al., 2011a). The region between the X and Y domains is known as the XY-linker and plays an established role as a key regulator of function in most PLCs. However, there are key structural and biochemical differences in the PLCζ XY-linker across different species, as well as other PLC isoforms, which imply differential physiological roles.

Studies by Yoda et al. (2004) and Saunders et al. (2007) identified a nuclear localisation signal (NLS) within the XY-linker region of mPLCζ, which was proposed to modulate enzymatic activity. During zygotic interphase, mPLCζ is translocated to the pronuclei via the NLS and associates with the nuclear transport receptor. This translocation prevents PLCζ from associating with its PIP₂ substrate and thus terminates Ca²⁺ oscillations. However, at the present time, it is unclear as to whether human PLCζ exhibits a similar NLS region (Ito et al., 2008; Swann and Lai, 2013).

Structural and biochemical studies have determined that in PLCδ, β, and γ isozymes, the XY-linker regulates potent auto-inhibition of enzyme function as a result of
their negatively-charged XY-linker region. Binding of the linker region to specific target molecules removes inhibition and initiates activity (Ellis et al., 1993; Fernald et al., 1994; Schnabel and Camps, 1998). Conversely, PLCζ exhibits positively-charged residues within its XY-linker region, which is also much longer than in other PLCs (Cox et al., 2002; Saunders et al., 2002). The putative role of the XY-linker is to mediate PLCζ enzymatic activity by targeting membrane-bound PIP2. The positively-charged XY-linker interacts with negatively-charged PIP2, with high affinity, via electrostatic interaction (Nomikos et al., 2011b). Therefore, the XY-linker plays an essential role in targeting PLCζ to membrane substrate PIP2 to initiate OA.

1.2.2.2 EF hand domains

A notable feature of PLCζ is its high sensitivity to Ca^{2+}, and experimental evidence indicates that PLCζ is 100-fold more sensitive to Ca^{2+} than PLCδ1 (Kouchi et al., 2004). This increased level of sensitivity is thought to arise as a result of the EF hand domains, which occur as pairs in order to enhance stability. The deletion of one or both EF hand domains completely terminates in vivo Ca^{2+} oscillations in mouse oocytes, although, interestingly, PIP2 hydrolysis in vitro is maintained (Kouchi et al., 2005; Nomikos et al., 2005). This continuity is due to the presence of the XY-linker and XY catalytic domains, segments known to be involved in targeting PIP2 and PIP2 hydrolysis, respectively. Further to this, the C2 domain may also be involved in targeting PIP2, thus implying the complex co-ordination of multiple segments within PLCζ to function in an appropriate manner (Swann et al., 2006).
1.2.2.3 C2 domain

The C2 domain is a conserved motif consisting of approximately 130 amino acids, and was estimated to be found in 300 known proteins in which its function was predominantly related to intracellular signaling and membrane trafficking (Nalefski and Falke, 1996; Lander et al., 2001). The C2 domain has been proposed to exert a key role in regulating PLCζ activity. Similar to the three-dimensional structure of PLCδ1 (PLCζ exhibits the highest homology with PLCδ (33%)), in which the XY-linker region of PLCδ1 is folded to generate contact between the EF-hand domains and C2 domain to form the catalytic core, PLCζ is predicted to undergo a specific functional conformation in order to exert functional activity. Although the three-dimensional structure of PLCζ has yet to be established, computer modeling predicts that the protein bends in response to association between the EF hands and C2 domain, thus exposing the NLS (Kuroda et al., 2006; Figure 4).

![Diagram of PLCζ structure](image)

Figure 4. Predicted three-dimensional structure of PLCζ and its conformational change, exposing the putative NLS region and binding to the nuclear transport receptor. Reproduced and modified from Kuroda et al. (2006).

An alternative role for the C2 domain includes facilitating PLCζ in targeting membrane-bound PIP2 and initiating OA (Swann et al., 2006). A study by Swann and Lai (2013) proposed that the C2 domain may also be involved in the binding of PLCζ to a
putative receptor expressed on the surface of PIP$_2$–containing vesicles within the ooplasm to facilitate PIP$_2$ hydrolysis in the absence of a PH domain (Swann and Lai, 2013; Figure 5). Therefore, it seems likely that both the C2 domain and the XY-linker region are involved in substrate binding, with the latter potentially playing a more prominent role. However, the lack of detailed kinetic analysis, and high-resolution protein structure, does not yet definitively designate these domains as being involved in substrate binding and more importantly, the biochemical activity of each domain has yet to be fully elucidated upon the introduction of PLCζ into the oocyte.

Figure 5. Putative role of the C2 domain. The C2 domain of PLCζ interacts with a receptor (red rectangle) localised on the surface of the intracellular small vesicles containing PIP$_2$. Additionally, the XY-linker has also been proposed to be involved in substrate binding. Reproduced and modified from Swann and Lai (2013).

1.2.3 Expression profile and localisation of PLCζ

Much controversy lies within the expression profile of PLCζ during spermatogenesis as there is insufficient data regarding its precise translation at present. However, it was reported that PLCζ mRNA in the mouse and boar was first expressed in round spermatids and possibly translated during the elongated spermatid stage (Saunders et al., 2002; Yoneda et al., 2006). However, Young et al. (2009) detected PLCζ mRNA in the earlier
stages of spermatogenesis in hamsters, notably spermatocyte cells. This earlier pattern of expression suggested that it might be species-specific. Collectively, however, it has been proposed that \( \text{PLC}_\zeta \) is detectable in all subsequent stages, both in the testes and the epididymis (Saunders et al., 2002; Yoneda et al., 2006; Young et al., 2009). To date, no follow-up study has been performed to confirm this hypothesis and more importantly, the precise expression profile of \( \text{PLC}_\zeta \) in the human testis remains unknown.

The localisation of \( \text{PLC}_\zeta \) in sperm is a seemingly more complex profile as different patterns have been identified in a number of species (Fujimoto et al., 2004; Yoon and Fissore, 2007; Young et al., 2009). Each pattern, although only speculative and has yet to be proven, corresponds to that of a specific function, not limited to oocyte activation. This theory remains obscure and therefore, represents a very interesting challenge for future research (Grasa et al., 2008; Kashir et al., 2010). Immunocytochemical localisation studies identified \( \text{PLC}_\zeta \) in the acrosomal, equatorial or post-acrosomal regions of the human sperm head (Grasa et al., 2008; Kashir et al., 2013; **Figure 6**). Additionally, \( \text{PLC}_\zeta \) 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; Escoffier et al., 2015). Further to this, for a sperm factor to be classified as a sperm oocyte-activating factor (SOAF), it must be a component of the post-acrosomal sheath-perinuclear theca (PAS-PT). 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 recent study by Escoffier et al. (2015) reported that a subcellular localisation of \( \text{PLC}_\zeta \) was indeed at the PT of the equatorial and post-acrosomal regions of human sperm (**Figure 7**).
**1.2.4 Function**

PLCζ remains in an inactive state within the sperm and enzymatic activity is only conferred when the protein is released into the oocyte. The exact mechanisms of PLCζ inactivity in the sperm remain unknown but it is strongly believed that factors within the oocyte are necessary for the activation of PLCζ (Swann *et al.*, 2006; Kashir *et al.*, 2010). Further to this, studies in both the porcine and mouse model have indicated the need for
PLCζ to undergo spontaneous proteolysis (Kurokawa et al., 2007). Using porcine sperm, the authors showed that sperm fractions (SF), cytosolic (SF\textsuperscript{C}) and high-pH soluble (SF\textsuperscript{pH}), both induced PLCζ-like activity and Ca\textsuperscript{2+}-oscillations when injected into mouse oocytes. SF\textsuperscript{C} contained PLCζ but SF\textsuperscript{pH} lacked it, and thus further investigation revealed that this fraction actually contained N- or C-terminal fragments of PLCζ, which may have been cleaved and then formed stable complexes capable of retaining PLCζ activity. To consolidate these findings, Kurokawa et al. (2007) co-expressed two complimentary mPLCζ cRNAs (residues 1-361 and 361-647), derived from the cleavage of the XY-linker region. Co-injection of these cRNAs induced Ca\textsuperscript{2+} oscillations in mouse oocytes, indistinguishable to those triggered by wild type (WT) mPLCζ cRNA. Therefore, PLCζ may initially interact with proteases present in the oocyte prior to achieving enzymatic activity but the process in humans has yet to be determined (Kurokawa et al., 2007).

In addition to proteolytic cleavage, other post-translation modifications (PTMs) in PLCζ are also thought to be a highly likely requirement in the bovine model for the establishment of enzymatic activity (Cooney et al., 2010). The authors observed that injecting a range of bovine PLCζ recombinant protein (1pg – 10pg) into either bovine or mouse oocytes failed to induce Ca\textsuperscript{2+} oscillation-inducing activity. The authors proposed that as the recombinant protein was made via an E. coli expression system, PTMs could not occur, and hence the lack of in vivo activity (Cooney et al., 2010). Since this study, advancements in protein purifications, and the production of recombinant PLCζ protein in other species have overcome the absence of in vivo activity (described further in Section 1.3.3), although there has been no follow-up study investigating the requirement for PTMs in mammalian PLCζ. Therefore, it is essential for future studies to consider both proteolysis and PTMs to understand fully the mechanisms regulating PLCζ activity and whether they occur in hPLCζ.
The amount of PLCζ in mouse sperm is estimated to be around 20-50fg and the level of PLCζ protein required to induce Ca^{2+} oscillations in a single mouse oocyte is within the range of 4-75fg (equivalent to 0.002-0.02mg/ml PLCζ cRNA) (Saunders et al., 2002; Nomikos et al., 2011b). This was established by the injection of either myc-tagged or luciferase-tagged PLCζ fusion proteins into mouse oocytes (Saunders et al., 2002; Nomikos et al., 2011b). It was then deduced that PLCζ is the most potent PLC isoform, as an increased concentration (~20-fold higher) of PLCδ1 protein is required to generate Ca^{2+} oscillations compared with PLCζ when injected into mouse oocytes (Kouchi et al., 2004), although this level of effectiveness from PLCζ remains unclear.

Upon fertilisation, it was initially proposed that PLCζ hydrolyses membrane-bound PIP_2 but supplementary work provided evidence for the colocalisation of PLCζ to PIP_2-containing vesicles in mouse oocytes (Kashir et al., 2011a; Yu et al., 2012). It was reported that following the injection of PLCζ, PIP_2 was reduced within these vesicles, thus providing clear evidence that these intracellular vesicles are a prominent source of substrate for hydrolysis (Yu et al., 2012). The mode of communication between PLCζ and such vesicles remain unknown, although it was proposed that a specific oocyte binding protein expressed by these vesicles may be responsible and could operate via a receptor-mediated pathway (Yu et al., 2012; Swann and Lai, 2013). A key goal for researchers at present is therefore to identify the receptor mechanism involved, and to relate the number and distribution of oocyte-borne PIP_2-containing vesicles to Ca^{2+} release ability. This is imperative as OAD is not limited to sperm-related deficiencies but may also be due to issues in their oocyte counterpart (Yeste et al., 2016). As reviewed by Yeste et al. (2016), there are many factors within the oocyte, which regulate the Ca^{2+} oscillation-inducing activity following PLCζ introduction, therefore it would be negligent to deny the role of the oocyte in OA.
1.2.4.1 Mechanism of action

The signaling mechanism of PLCζ upon fertilisation is described in Figure 8. PLCζ is introduced into the ooplasm and hydrolyses PIP2-containing vesicles, generating IP3 (Swann and Lai, 2013). IP3 diffuses across the ooplasm to the ER membrane where it binds to IP3Rs causing the release of Ca2+ via membrane channels (Nomikos et al., 2011a; Ramadan et al., 2012; Swann and Lai, 2013). DAG is produced in concert with IP3 and activates the protein kinase C pathway, which is believed to translate Ca2+ signals into cellular responses (Swann and Yu, 2008).

Figure 8. Mechanism action of PLCζ. Upon fertilisation, PLCζ is introduced into the ooplasm and hydrolyses PIP2, stored in intracellular vesicle compartments, and liberates IP3 and DAG. IP3 subsequently triggers the release of Ca2+ from the endoplasmic reticulum via IP3Rs, and concomitantly DAG activates the PKC pathway. PIP2: Phosphotidylinositol 4,5-biphosphate; IP3: Inositol triphosphate; DAG: Diacylglycerol; PKC: Protein kinase C. Modified and reproduced from Amdani et al. (2015a).

A multitude of studies have investigated the IP3-induced release of Ca2+ in oocytes but research involving DAG production following PIP2 hydrolysis remains limited, and Yu et al. (2008) proposed this occurs in concert with IP3 production. Another study reported
that over-expression of PLCζ in mouse oocytes, which in turn would result in increased DAG production, exhibited abnormal secondary Ca²⁺ oscillations (Swann and Yu, 2008). This was suggested to be due to PKC-mediated Ca²⁺ influx and the subsequent excess of Ca²⁺ stores as a result of DAG over-production. These secondary Ca²⁺ oscillations were proven to be undesirable to mouse oocytes, as all failed to reach development to the blastocyst stage following injection of increased PLCζ concentrations (Yu et al., 2008). Therefore, an optimal range of PLCζ introduced into the oocyte is vital during fertilisation, as this would retain the subsequent production of the secondary messengers to a physiological minimal level so as to not disrupt oocyte Ca²⁺ homeostasis (Swann and Yu, 2008; Yu et al., 2008; Fukami et al., 2010).

Along with these pertinent findings, recent studies have focused on the clinical aspects of PLCζ and its strong association with fertility. Clinical studies described in the following section indicate that OAD may no longer be an idiopathic factor in infertility and its development is strongly proposed to be due to deficiencies in PLCζ expression, structure, and thus function. As a result, more research is warranted for the potential diagnostic, prognostic and therapeutic applications of PLCζ for OAD.

1.3 Phospholipase C zeta (PLCζ): A clinical view

1.3.1 Male infertility

Male infertility has been reported in 25-30% of cases and may arise as a result of genetic factors or abnormal semen and/or sperm qualities such as oligozoospermia, teratozoospermia, asthenozoospermia and azoospermia (Matzuk and Lamb, 2008; European Society of Human Reproduction and Embryology (ESHRE), assisted reproductive technology (ART) Fact Sheet, 2014). Although the aetiology for many of these conditions has been described, 10-25% of cases are idiopathic (ESHRE, ART Fact Sheet, 2014). ART is revolutionising the field of reproductive medicine, and is
continuously striving to accommodate infertile patients with suitable treatments; with ICSI (intracytoplasmic sperm injection) being the primary option for severe male infertile conditions (Human Fertilisation and Embryology Authority (HFEA), 2012; Neri et al., 2014)). ICSI readily overcomes the limitations of other conventional treatments such as in vitro fertilisation (IVF; HFEA, 2014). Regardless of its relative success, total fertilisation failure (TFF) still occurs in 1-3% of ICSI cycles (Nasr-Esfahani et al., 2010; Vanden Meerschaut et al., 2014). The underlying cause is largely believed to be failure of the oocyte to activate, an infertile condition known as oocyte activation deficiency (OAD) (reviewed by Amdani et al., 2013; Amdani et al., 2015b), and over recent years this condition has been causally linked to abnormalities in the structure, expression, and localisation pattern of PLCζ in human sperm.

1.3.1.1 Initial associations between clinical infertility and PLCζ

Yoon et al. (2008) identified a population of sperm obtained from patients who had experienced recurrent ICSI failure, and showed that such sperm lacked the ability to induce Ca²⁺ oscillations in oocytes. Immunocytochemistry analysis showed that PLCζ in this sperm population was reduced, devoid, or abnormally localised. Ca²⁺ oscillation-inducing activity was only restored following the microinjection of sperm concomitant with mPLCζ mRNA into mouse oocytes, thus deducing the need for PLCζ in order for OA to occur (Yoon et al., 2008; Figure 9). This landmark paper thus provided the first evidence that PLCζ may represent a novel treatment for OAD.

Subsequent findings described the inability of human globozoospermic sperm to evoke long-term Ca²⁺ oscillations when injected into mouse oocytes, and additional immunocytochemical staining showed reduced levels or abnormal localisation patterns of PLCζ (Heytens et al., 2009). This was consistent with an initial review from Dam et al. (2007), which suggested that globozoospermic sperm showed reduced ability to activate
oocytes following ICSI. Globozoospermia is a rare male infertile condition in which sperm are round-headed and devoid of an acrosome, which is proposed to be due to a defective DPY19L2 gene (Chansel-Debordeaux et al., 2015; Escoffier et al., 2015). Previous reports have shown that 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 (Kashir et al., 2010; Taylor et al., 2010). Along with immunocytochemical staining, which was performed by Yoon et al. (2008), Heytens et al. (2009), and Taylor et al. (2010), additional investigations involved genetic screening of PLCζ in order to determine if genetic factors might play a role in the aberrant expression and/or localisation patterns observed from these infertile patients.

Figure 9. Injection of mPLCζ mRNA to rescue sperm from a patient unable to induce Ca²⁺ oscillations. (A) Injection of sperm from Patient W into mouse oocyte showed Ca²⁺ oscillations indistinguishable to normal fertilisation, (B) Sperm from Patient K lacked PLCζ, and injection of his sperm failed to trigger Ca²⁺ oscillations, (C) Introducing mPLCζ mRNA (0.1μg/μl) 2 hours following sperm injection from Patient K initiated Ca²⁺ oscillations, and rescued OA. F(340/380): Fluorescence ratio 340/380nm (a.u.). Reproduced from Yoon et al. (2008).

1.3.1.2 Genetic links between PLCζ and oocyte activation deficiency

This section is covered in greater detail in Chapter 2. In brief, sequential reports from Heytens et al. (2009) and Kashir et al. (2012a) genetically associated OAD with PLCζ-deficiency from the same non-globozoospermic infertile patient. Both authors discovered variants, described as substitution mutations, which may have been responsible for the patient’s infertile phenotype: Proline for Histidine at position 398 (PLCζH398P) and Leucine
for Histidine at position 233 (PLCζ^{H233L}), corresponding to the Y and X catalytic domains, respectively. Three-dimensional modeling of each mutant variant compromised the local protein folding of PLCζ. The injection of cRNA derived from PLCζ^{H398P} or PLCζ^{H233L} into mouse oocytes failed to evoke Ca^{2+} oscillations similar to that of normal fertilisation (Heytens et al., 2009; Kashir et al., 2012a). Subsequently, Nomikos et al. (2013) showed that the Ca^{2+} release profile caused by variants PLCζ^{H398P} and PLCζ^{H233L} could be rectified by the microinjection of a WT human recombinant PLCζ protein (hrPLCζ) into mouse oocytes previously injected with cRNA derived from the mutant variants. This is further proof of concept that PLCζ remains the strongest candidate for potential OAD therapy, particularly in patients with abnormal PLCζ features affecting their fertility status.

The discovery of these variants further emphasises the link between OAD and PLCζ-deficiency. It should be noted that these variants were detected following screening of only the PLCζ exonic regions and a small range of their neighbouring introns. Sequencing all PLCζ introns was not a viable option at the time due to the unavailability of technology and in addition to that, using Sanger sequencing to screen the intervening sequences would have been very costly. However, due to the rarity of these exonic mutations, there is now interest in expanding PLCζ screening into the non-coding regions (promoter and introns). It is logical to propose that abnormalities within the promoter region could affect the transcriptional regulatory mechanism of PLCζ, altering the expression of the protein. Interestingly, PLCζ shares a bi-directional promoter with the actin filament capping muscle Z-line alpha 3 (CAPZA3; Figure 10), a protein responsible for the architecture of the sperm head during spermatogenesis (Coward et al., 2011).
Figure 10. PLCζ shares a bi-directional promoter with CAPZA3. Each gene is transcribed in the opposite direction to carry out their respective functions, oocyte activation and sperm structure during spermatogenesis, respectively. Reproduced from Amdani et al. (2015b).

Further to this, while the introns were initially characterised as “junk DNA” due to its unknown function in the eukaryotic genome, it is widely established that introns may be essential for normal protein expression. Introns are suggested to be multi-functional, and may influence steps of mRNA processing (reviewed by Chorev and Carmel, 2012). The link between PLCζ and its role in infertility is increasingly becoming prominent. Consequently, it is crucial that we now expand our knowledge of the regulatory mechanisms involved with PLCζ expression so as to shed light on the causes of deficiency, therefore allowing diagnosis and appropriate treatment to be designed and carried out accordingly. This section will be further explained in Chapter 4.

Pan et al. (2013) were the first to report variants within the promoter region of PLCζ in Chinese Holstein bulls; these affected semen quality traits and thus, fertilisation outcome. The authors, however, failed to correlate their findings with specific PLCζ expression and activity. No follow-up studies have been performed since Pan et al. (2013) but this, however, should only encourage further research into exploring the non-coding regions, particularly in humans. It could be speculated that variants identified within these regions may influence protein expression, and two important reports by Grasa et al. (2008) and Kashir et al. (2013) have shown significant variability in PLCζ levels in fertile and infertile patients, which currently represents a major conundrum in the research. This will be described further in the following section and Chapter 3. Although no association has been established between the wide variation observed and the screening of non-coding
regions, it is still a necessary investigation to understand the regulatory mechanisms involved in \( \text{PLC}_\zeta \) expression, as our current knowledge is limited to the coding regions of the gene only.

1.3.2 Prognostic and diagnostic tools for patients suspected of having OAD

This section will be explored in greater detail in Chapter 3. The most taxing conundrum in \( \text{PLC}_\zeta \) research is the significant variability of \( \text{PLC}_\zeta \) expression reported in fertile and infertile patients (Grasa et al., 2008; Kashir et al., 2013). Immunofluorescent analysis in single sperm, from both fertile and infertile (OAD) men, showed a wide variation from two parameters of interest: total levels and localisation patterns of \( \text{PLC}_\zeta \) (Grasa et al., 2008; Kashir et al., 2013). In addition to fertile controls exhibiting similar levels of \( \text{PLC}_\zeta \) expression to infertile patients, each ejaculate from patients also showed a variation in expression (Kashir et al., 2013). Furthermore, only one fertile control exhibited a predominant localisation pattern for \( \text{PLC}_\zeta \), whereas widespread variation was observed in all other samples. The reason for this heterogeneity remains unanswered but Kashir et al. (2013) proposed that assaying total levels or determining specific localisation patterns might not illustrate the best clinical diagnostic tool. Therefore, there is a need to establish a more robust and reproducible \( \text{PLC}_\zeta \) assay for human sperm in order to diagnose \( \text{PLC}_\zeta \)-deficiency.

The current tools used for patients who have repeatedly failed ICSI treatment, and to distinguish sperm- from oocyte-related causes of this failure is the mouse oocyte activation test (MOAT) and mouse oocyte calcium analysis (MOCA) (Heindryckx et al., 2005; Grasa et al., 2008; Yoon et al., 2008; Heindryckx et al., 2008; Kashir et al., 2010; Vanden Meerschaut et al., 2012; Vanden Meerschaut et al., 2013). These tests involve the injection of a single sperm from the patient into a mouse oocyte (heterologous ICSI) and
while these tests are widely utilised for research purposes (Palermo *et al*., 1997; Gordo *et al*., 2000; Yoon *et al*., 2008), only a limited number of clinics have introduced them (Heindryckx *et al*., 2005; Heindryckx *et al*., 2008; Vanden Meerschaut *et al*., 2012; Vanden Meerschaut *et al*., 2013). MOAT categorises sperm-related, oocyte-related or inconclusive via the proportion of two-cell formation following injection (Vanden Meerschaut *et al*., 2013; **Table 2**).

**Table 2.** Patient categories and their cause of fertilisation failure according to the mouse oocyte activation test (MOAT). Reproduced from Vanden Meerschaut *et al*. (2013).

<table>
<thead>
<tr>
<th>Mouse oocyte activation test (%)</th>
<th>Group</th>
<th>Cause of failed fertilisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 20</td>
<td>1</td>
<td>Sperm-related</td>
</tr>
<tr>
<td>21-84</td>
<td>2</td>
<td>Inconclusive (sperm- or oocyte-related)</td>
</tr>
<tr>
<td>≥ 85</td>
<td>3</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

The MOCA is a more thorough diagnostic test as it defines sperm-oocyte activating capacity by measuring the amplitude and frequency of the Ca$^{2+}$ peaks produced in each mouse oocyte injected. Although these assays are currently the most reliable, it is undeniable that hPLCζ is more potent than mPLCζ, and thus the reasons for fertilisation failure cannot be strictly deduced from these tests (Nomikos *et al*., 2014; Amdani *et al*., 2015b). These tests may, however, be beneficial for extreme OAD cases in which patients lack PLCζ (Swann *et al*., 2006; Vanden Meerschaut *et al*., 2013).

An abstract published by Ferrer-Buitrago *et al*., (2015) introduced the human oocyte calcium analysis (HOCA) test, and this homologous model was shown to overcome the limitations of the current diagnostic tools as the authors were able to extrapolate the cause of previous fertilisation failure from subjects in MOAT group 3. While this
represented encouraging progress in our understanding of OAD and calcium dynamics upon fertilisation, a major concern is the need to produce a more robust PLCζ test to diagnose PLCζ-deficiency in human sperm, as the current immunofluorescent analysis described previously may not be the most effective tool.

At present, the antibody widely used for PLCζ immunofluorescent analysis is polyclonal in nature. Although this has been supported by appropriate peptide-blocking assays, there are some concerns over specificity and how this could have an influence upon experimental outcomes (Nomikos et al., 2013; Kashir et al., 2013). An alternative method to overcome the limitations that arise from the current immunofluorescent analysis is the generation of a monoclonal antibody. This could provide a more powerful and accurate diagnostic/prognostic assay, and widespread speculation of the precise localisation pattern of PLCζ in human sperm (Grasa et al., 2008; Kashir et al., 2013). However, creating a monoclonal antibody would require a pure hrPLCζ, a quest researchers have been trying to achieve for over two decades. The reasons for this delay are described in Section 1.3.3.

1.3.3 Current and potential therapy for OAD

OAD is characterised by the absence or abnormal release of Ca²⁺ oscillations upon fertilisation. Artificial agents, which are able to increase the level of Ca²⁺ within the ooplasm, can overcome this condition (Heindryckx et al., 2005). These agents are known as artificial oocyte activators (AOAs) and the most common forms are calcium ionophores (A23187 or ionomycin); these agents form the only current treatment option for OAD patients with the exception of donor sperm (Yanagida et al., 2008; Nasr-Esfahani et al., 2010). Only recently have AOAs, which are utilised concomitantly with ICSI, become accessible within clinics in the United Kingdom. The theoretical risks of using these agents are acknowledged and are normally only applied with appropriate indications such as globozoospermia or PLCζ-deficiency (Yoon et al., 2008; Heytens et al., 2009; Vanden
Meerschaut et al., 2014). Although this technique has accommodated patients with previous fertilisation failure, and has resulted in pregnancy, there are concerns surrounding the potential detrimental effects of these agents on embryo viability (Nasr-Esfahani et al., 2010). Calcium ionophores may exert cytotoxic and mutagenic effects upon both oocytes and embryos, and additionally they are known to induce a single transient, as opposed to the Ca$^{2+}$ oscillations observed during normal fertilisation. Given that the Ca$^{2+}$ release profile plays a critical role in regulating gene expression and coordinating the events of activation, the use of these agents may represent a possible threat to successful embryo development (Nasr-Esfahani et al., 2010).

It is therefore of great importance for researchers to develop an alternative, safer and more endogenous therapy for OAD, for example, in the form of a hrPLCζ (for review, see Amdani et al., 2013). In addition to its potential therapeutic use and its being more robust diagnostic/prognostic tool, the successful production of this protein may lead to other benefits, including the generation of a PLCζ monoclonal antibody, and the foundation for protein crystallisation studies.

### 1.3.3.1 Generation of a human recombinant PLCζ protein

The production of a hrPLCζ has represented a major challenge for researchers working in the field, with successful purification of a functionally-active protein representing the largest obstacle (Yoon et al., 2012). Its generation will not only benefit clinics but also research laboratories, such as in the development of a monoclonal antibody (described in Section 1.3.2) and high-resolution PLCζ protein three-dimensional structure production (explored further in Chapter 4). At present, only a predicted three-dimensional structure of PLCζ has been created via the established structure of PLCδ1, due to its strong homology to PLCζ (Heytens et al., 2009). This model has helped us to understand the functional effects of genetic variants discovered in PLCζ (Heytens et al., 2009; Kashir et
These findings are still based on a predicted model and therefore, the ability to generate a definitive structure will enable the effects of the variants on PLCζ structure and function to be examined in a precise manner.

PLCζ cRNA was the initial suggestion for OAD therapy, following its ability to induce Ca^{2+} oscillations when injected into aging human oocytes, which resulted in the generation of parthenogenetic blastocysts (Rogers et al., 2004; Yoon et al., 2008). However, cRNA is not clinically robust and is potentially harmful to embryo development, as it exerts uncontrollable transcriptional and endogenous transcriptase activity (Spadafora, 2004; Kashir et al., 2010). Focus was soon redirected to creating hrPLCζ, as a safer means of activating oocytes, since this would mimic the physiological conditions of native PLCζ in sperm.

Grasa and colleagues created the first bacterial-expressed hrPLCζ in 2008. The authors validated the identity of the protein as PLCζ using immunoblot analysis but failed to test its activity by microinjection into mouse oocytes (Grasa et al., 2008). Thereafter, Kashir et al. (2011a) produced the first hrPLCζ in the form of mammalian cell lysates, which was successful in inducing Ca^{2+} oscillations when microinjected into mouse oocytes. However, its use in the clinic was not feasible, as it could not be purified from the cell lysates in an active form. Yoon et al. (2012) also generated hrPLCζ using a bacterial cell line. However, there were concerns due to an abnormal Ca^{2+} release profile when introduced in human oocytes, and an excessively high concentration of hrPLCζ was necessary to induce the Ca^{2+} peaks, thus making it an undesirable candidate for future OAD treatment. Most recently, Nomikos et al. (2013) generated an active and purified hrPLCζ using the Nus-A fusion protein (60kDa). This protein induced Ca^{2+} oscillations similar to those observed during normal fertilisation and rescued the activation ability of mouse oocytes that had previously been injected with cRNA synthesised to PLCζ^{H398P} and PLCζ^{H233L}, thus providing further proof of concept for PLCζ as a powerful therapeutic
agent (Nomikos *et al.*, 2013). Although the Nus-A tag used facilitated the stability, solubility, and functionality of the protein, the authors failed to present any findings showing that its removal can still preserve the favorable features of the hrPLCζ and this may be seen as an obstacle for future clinical use.

While substantial progress has been made in the study of PLCζ both scientifically and clinically since its discovery, there are still many outstanding concerns and priorities. There is a clear need to determine how the expression and localisation of this fundamental protein are regulated, physiologically and genetically. Furthermore, it is evident that PLCζ exerts functionality by interacting with an as yet unidentified oocyte-borne factor, or factors, in order to bind with its substrate. It is equally important to expand the clinical screening of PLCζ in patients to help understand the extent of PLCζ deficiency in the wider population and how knowledge of this condition might influence diagnosis and treatment. From a clinical and research standpoint, the key priority is to synthesise a pure and functionally-active hrPLCζ protein, which does not require the use of excessive protein concentrations or the use of large protein fusion tags in order to preserve purity, solubility, and stability.
1.4.1 Aims of this thesis

1. To identify novel PLCζ variants from patients suspected of having OAD

To genetically screen two particular regions of PLCζ, promoter and XY-linker, from patients suspected of having OAD, and to identify and characterise possible mutations that may play a role in their infertile phenotype. This will be explored in Chapter 2.

2. To investigate the wide variation of PLCζ characteristics observed in fertile and infertile patients by exploring PLCζ regulatory elements

To explore the non-coding regions (promoter and introns) of human PLCζ, and the regulatory mechanisms involved in expression. This may support the reasons for the wide variation of PLCζ characteristics reported by Grasa et al. (2008) and Kashir et al. (2013). This will be explored in Chapter 3.

3. To produce an active human recombinant PLCζ protein

To create a hrPLCζ expressed from both a bacterial and mammalian cell line. Attempts made by Yoon et al. (2012) and Nomikos et al. (2013) using the bacterial cell system have resulted in unsuccessful purification, low yield and a hrPLCζ, which was not clinically viable. Kashir et al. (2011a) successfully produced hrPLCζ from a mammalian cell system but the resulting product prevents it from being used clinically. The successful production of hrPLCζ would significantly benefit research and clinical settings. This will be explored in Chapter 4.
Chapter 2: Identification of novel PLCζ variants from patients suspected of having OAD
2.1 Introduction

ICSI is the primary treatment for severe male infertile conditions and overcomes the limitations of other conventional treatments such as IVF (HFEA, 2012; HFEA, 2014; Neri et al., 2014). Regardless of its relative success, total fertilisation failure (TFF) still occurs in 1-3% of ICSI cycles (Nasr-Esfahani et al., 2010; Vanden Meerschaut et al., 2014). The underlying reason is largely believed to be failure of the oocyte to activate, a condition termed as OAD. Over recent years this condition has been causally linked to abnormalities in sperm or oocyte factors involved in OA. Strong clinical and scientific evidence has now suggested that the SOAF, PLCζ, may be responsible for OAD cases whereby sperm-related deficiency is the main cause (Kashir et al., 2010). OAD, defined as the absence or aberrant release of Ca\(^{2+}\) in the oocyte following fertilisation, disrupts the events of OA. OAD was initially categorised as an idiopathic factor and this may no longer be the case as its development is strongly proposed to be due to deficiencies in PLCζ expression, structure, and thus function (Kashir et al., 2010).

Yoon et al. (2008) were the first to report the clinical association between OAD and PLCζ-deficiency. These authors identified an abnormal population of sperm from patients who had experienced recurrent ICSI failure, and such sperm were observed to lack Ca\(^{2+}\) oscillation-inducing activity in oocytes (Yoon et al., 2008). Immunocytochemistry analysis showed that PLCζ in this sperm population was reduced, lacking, or abnormally localised. The ability to induce Ca\(^{2+}\) oscillations in oocytes was only restored following the microinjection of sperm with mPLCζ mRNA - defining the need for PLCζ to activate oocytes (Yoon et al., 2008). This landmark paper also briefly suggested a possible genetic contribution to the abnormal PLCζ features observed. The authors screened all of the PLCζ exonic regions (15 exons including at least 50bp of the introns spanning each exon) from a fertile control and three patients exhibiting abnormal PLCζ expression. This revealed 8 single nucleotide polymorphisms (SNPs), two of which were novel, one that resulted in a
missense mutation, and the remaining five were previously reported from a software the authors used to annotate the SNPs identified, CodonCode Aligner (Yoon et al., 2008). The missense mutation resulted from a change of a Serine to Leucine residue at position 500 of the amino acid sequence (S500L) and was homozygous to the respective patient. The authors did not demonstrate the impact of S500L on PLCζ function but the SNPs were not thought to be detrimental. Furthermore, Yoon et al. (2008) speculated that the abnormal localisation or absence of PLCζ might have been due to mutations localised within transcription factors that regulate PLCζ expression. Alternatively, PLCζ could have been degraded or discarded during the final stages of spermatogenesis. Although detailed characterisation was not accomplished on the variants detected, this study provided preliminary indication that genetic anomalies detected in PLCζ may be the cause of sperm-related OAD cases (Yoon et al., 2008).

Genetic anomalies identified in PLCζ appear to be very rare occurrences and prior to this thesis only two novel mutations had been discovered, both of which were from the same non-globozoospermic infertile patient (Heytens et al., 2009; Kashir et al., 2012a). The first mutant isoform was a substitution of a Proline to Histidine residue at position 398 (H398P) from the amino acid sequence, and occurred within a cleft of the catalytic Y domain (Heytens et al., 2009). Computer modeling revealed an altered secondary structure that lead to a disrupted PLCζ function (Figure 11). Ca²⁺ oscillation-inducing activity was abnormal as none of the mouse oocytes (n=10) injected with cRNA derived from PLCζ^{H398P} were able to produce >8 calcium spikes per hour (a characteristic of the normal Ca²⁺ release profile at fertilisation) (Heytens et al., 2009; Figure 12). Immunofluorescence analysis showed that localisation appeared to be absent in the equatorial region but there was some degree of positive staining in the post-acrosomal region of the patient’s sperm head (Heytens et al., 2009).
Figure 11. Schematic of the predicted three-dimensional model of PLCζ mutations discovered from a non-globozoospermic infertile patient. Top and below panel denotes PLCζ^{H398P} and PLCζ^{H233L}, respectively. The mutation disrupts the local protein folding of PLCζ (red circle) resulting in no side-chain-side-chain hydrogen bonds (PLCζ^{H398P}) or reduces the number of neighbouring amino acid contacts (PLCζ^{H233L}). Reproduced from Heytens et al. (2009) and Kashir et al. (2012a).

Figure 12. The Ca^{2+} release traces following the microinjection of cRNA corresponding to PLCζ^{WT} (left) and PLCζ^{H398P} (right) at a concentration of 0.1mg/μl. Reproduced from Heytens et al. (2009).
Subsequent detailed analysis of genomic DNA from the same patient identified a second variant, a substitution of Histidine to Leucine at position 233 (H233L) in the X catalytic domain (Kashir et al., 2012a). PLCζ_{H233L} was observed to be less detrimental than PLCζ_{H398P} as cRNA derived from PLCζ_{H233L} was able to evoke Ca^{2+} oscillations when injected in mouse oocytes, albeit in an abnormal manner, when compared to WT cRNA (Figure 13). Three-dimensional modeling of mutant variant PLCζ_{H233L} revealed a disrupted local protein fold, similar to that described in PLCζ_{H398P} (Kashir et al., 2012a; Figure 11).

![Figure 13](image.png)

**Figure 13.** The Ca^{2+} release profile following the microinjection of cRNA corresponding to PLCζ_{WT} (left) and PLCζ_{H233L} (right) at a concentration of 172ng/μl and 176ng/μl, respectively. Reproduced from Kashir et al. (2012a).

Both mutations are heterozygous, such that PLCζ_{H398P} and PLCζ_{H233L} were inherited from the father and mother, respectively, and demonstrate that the maternal germ line can convey male infertility (Kashir et al., 2012a; Kashir et al., 2012b). The mix of sperm populations observed from the patient, derived from the paternal and maternal alleles, was suggested to arise during the meiotic stages of spermatogenesis (Figure 14). A subsequent study by Nomikos et al. (2013) was able to show that the Ca^{2+} release profile caused by variants PLCζ_{H398P} and PLCζ_{H233L} could be rescued by the microinjection of a WT hrPLCζ into mouse oocytes previously injected with cRNA derived from the mutant.
variants. This classifies PLCζ as the strongest candidate for potential OAD therapy, particularly for patients with abnormal PLCζ features whose fertility status is affected.

As of yet, no other variants have been identified, thus signifying the rarity of mutations in PLCζ. It should, however, be noted that since the discovery of PLCζ, clinical research has since peaked, with particular interest surrounding its association with OAD.
Ethical constraints concerning the acquisition of patient samples, both buccal and sperm, has gradually been lifted as crucial studies have revealed the importance of \( \text{PLC}_\zeta \) in OA, and therefore, OAD. This has allowed for more \( \text{PLC}_\zeta \)-related genetic screening to be performed in patients whose cause of recurrent ICSI failures remain unknown. The Coward group has collaborated with Ninewells Hospital and Medical School (Dundee, Scotland), Oxford Fertility (Oxford, UK) and the Department of Reproductive Medicine, Ghent University (Belgium), to acquire both buccal and semen samples from patients suspected of having OAD for routine assays, including \( \text{PLC}_\zeta \) immunofluorescent analysis and \( \text{PLC}_\zeta \) exonic screening. These assays have been performed for 9 years and as the link between \( \text{PLC}_\zeta \)-deficiency and OAD has slowly become more prominent, patients are becoming more informed. This information has been spread from either the recommendations of other patients who have agreed to have these assays, or from Internet forums, and as a result there has been a recent influx of samples from collaborators. This development is a clear indication that \( \text{PLC}_\zeta \) has become more acceptable for research and thus, an important factor in the progression of reproductive medicine.

In this present study, genetic screening of patients suspected of having OAD was targeted to the promoter-exon 1 and XY-linker regions (Figure 15).

**Figure 15.** The exonic structure of human \( \text{PLC}_\zeta \) cDNA sequence and its corresponding functional domains. \( \text{PLC}_\zeta \) consists of 15 exons and shares a bi-directional promoter with \( \text{CAPZA3} \). The red arrows indicate the particular \( \text{PLC}_\zeta \) regions screened in this chapter. Exons sizes are not to scale.
These particular regions of interest (ROIs) were selected for several reasons. Firstly, no screening has ever been performed in the promoter region of human PLCζ and therefore, such studies may potentially explain their infertile phenotype. Secondly, the promoter region is a crucial element of the gene, which initiates transcription, and thus, anomalies identified within it may undermine PLCζ protein expression. As for the XY-linker, this region plays an important role in PLCζ function, particularly in initiating Ca²⁺ oscillation-inducing activity for OA by hydrolysing substrate PIP₂ (Saunders et al., 2002; Nomikos et al., 2011a). Therefore, variants detected in these regions may underlie the patients’ inability to activate the oocyte following fertilisation.
2.1.1 Aims

The aim of this chapter was to perform a similar clinical screening protocol to that carried out routinely in our laboratory. However, this was implemented such that only two particular ROIs were sequenced, as opposed to all of the exonic regions of PLCζ. This implementation was developed to introduce, for the first time, a genetic screen to identify anomalies in the promoter region of PLCζ. In addition to the promoter, the region between exons 8 to 10, corresponding to the XY-linker was also screened. Previous work involving the screening of this region has been very problematic and often sequencing has failed, and this may be due to the complex nature of the sequence, of which has yet to be determined. Therefore, new primers were synthesised and an optimal polymerase chain reaction (PCR) was utilised in order to improve the efficiency of screening this critical region. It was hoped that genetic anomalies detected within this fragment from infertile patients might help elucidate their phenotype.

Hypothesis: Genetic anomalies detected within PLCζ ROIs, promoter and XY-linker, may explain the compromised state of fertility from patients suspected of having OAD.
2.2 Materials and methods

In this study, the standard protocol used for routine clinical screening in the Coward laboratory was employed to detect genetic anomalies in the promoter-exon 1 and XY-linker regions of PLCζ, which might be present in the patients’ samples. Specific procedures were integrated into this protocol in the event that further validation and characterisation was necessary upon the detection of a variant (Figure 16).

Figure 16. Schematic flow diagram depicting the methods used to detect potential variants from patient genomic DNA. Following identification of a possible variant(s), additional protocols were performed for further validation and characterisation.
2.2.1 Sample acquisition and processing

The male subjects, fertile control and patient, used in this study agreed to participate and provided written consent. Buccal and semen samples were obtained with appropriate ethical permission from Ghent University (Belgium) and the Oxford Tropical Research Ethics Committee (OXTREC: 31/09), Oxford Fertility (Oxford, UK) and the National Research Ethics Service (NRES; South Central Oxford Committee C; Reference number: 10/H0606/65), and the Ninewells Hospital (Dundee, Scotland) East of Scotland Research Ethics Service (EoSRES; REC 1; Reference number: 13/ES/0091). The male subjects undergoing fertility treatment were aged over 18. The fertile controls were participants with proven fertility, normal semen parameters, and were undergoing treatment at Oxford Fertility. Patients receiving treatment from their respective clinics were characterised clinically as OAD cases. These markers included globozoospermia and/or a history of ICSI failure, specifically when eight or more oocytes from the same patient failed to fertilise following ICSI, or when an experienced clinical opinion indicated possible OA issues.

2.2.2 Mutation detection from patient samples

2.2.2.1 Genomic DNA extraction from buccal and semen samples

Buccal samples were obtained from each patient using either the Oragene DNA collection kit (DNA Genotek, USA) or FTA cards (Whatman, UK). The buccal cells present in the Oragene DNA collection kit were first incubated (50°C, 1h). The Oragene Purifier (Genotek, USA) was then added to the buccal cells which were further incubated on ice (10min). Following incubation, the sample was harvested (5min, 14,000g) and the pellet discarded. Ethanol (100%; Sigma-Aldrich, UK) was added to the supernatant and incubated (room temperature (RT), 10min)) to allow DNA precipitation, and then centrifuged (2min, 14,000g). Ethanol (75%) was used to resuspend the pellet which was
centrifuged (1 min, 14,000g). The supernatant was discarded and the pellet left to dry to ensure complete ethanol evaporation. The pellet was then resuspended in nuclease-free water (Ambion Life Technologies, UK) and DNA concentration measured (ng/μl) by recording the absorbance at a wavelength of 260 nm using a spectrophotometer. The DNA was diluted 1:50 with nuclease-free water (Ambion Life Technologies, UK), which was also used as a blank. The DNA and blank were transferred into plastic cuvettes (Eppendorf, UK) and absorbance (A_{260}) recorded prior to calculating the DNA concentration with the formula below.

\[
\text{Absorbance value (A}_{260}\text{) x Dilution factor (50) x Optical density of 1 unit of double-stranded DNA (50 ng/μl)}
\]

Where necessary, patient semen samples were subjected to density gradient washing (DGW) with PureSperm 40/80 (Nidacon, Sweden), using the manufacturer’s protocol. Genomic DNA (gDNA) extraction from the sperm sample was performed using the QIAamp DNA Mini kit (Qiagen, UK), following the manufacturer’s instructions.

2.2.2.2 Polymerase chain reaction (PCR) and amplicon analysis

PLCζ gDNA (from a buccal sample) was amplified using the High Fidelity PCR Master kit (Roche, Germany). Newly synthesised primers (Table 3) were designed for the promoter-exon 1 and XY-linker regions (Figure 17) using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). The physical constants of these primers were characterised using OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html), and SNPCheck (https://secure.ngrl.org.uk/SNPCheck/snpcheck.htm) was used to assess the presence of SNPs within the primers. PCR reactions were performed using a G-Storm Thermal Cycler (Model GS4, GSI, UK) with optimised cycle conditions. This involved initial steps such as heating the lid (110°C) and denaturation (94°C, 4 min), followed by 10 cycles of
denaturation, annealing, and elongation (94°C for 10s, 62°C for 70s, and 72°C for 2min, respectively). A second round of 20 cycles included further denaturation, annealing, and elongation (94°C for 15s, 62°C for 30s, and 72°C for 2min, respectively), followed by a final extension step (72°C, 7m) prior to storing the amplicons at 4°C to await further analysis.

Table 3. Screening of the promoter-exon 1 and XY-linker regions for the detection of variants in PLCζ.

<table>
<thead>
<tr>
<th>Region</th>
<th>Size (bp)</th>
<th>Primers (F: forward; R: reverse)</th>
<th>PCR amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter-exon 1</td>
<td>371</td>
<td>F: GCCACCTTTCCCATCAAGGTGT R: CAACACCATTGTGATTCCTGAAG</td>
<td>371</td>
</tr>
<tr>
<td>XY-linker (exon 8, 9, and 10)</td>
<td>1988</td>
<td>F: TCTCTCAAATTGTTTCCTTAGGCAC R: GCAGCTAACATAAGAAAAGGCG</td>
<td>1988</td>
</tr>
</tbody>
</table>

Figure 17. Screening of the PLCζ promoter-exon 1 and XY-linker regions. This was performed to detect variants which may have played a role in the compromised fertility of a patient suspected of having OAD.

The amplicons were detected and analysed using agarose gel electrophoresis. The concentration of agarose used for gels and the type of DNA ladder, Quick-Load DNA Ladder (1kbp; New England Biolabs, UK) or O’GeneRuler (50bp; Thermo Fisher Scientific, UK), were selected according to the size of the expected amplicon. Analytical agarose (Promega, UK) was dissolved in Tris-Acetate-EDTA buffer (1X; Thermo Fisher Scientific, UK) using a microwave oven. Ethidium bromide (0.625mg/ml; Thermo Fisher Scientific, UK) was added into the gel mixture, poured into a gel tank and allowed to set. Loading dye was added to the DNA samples and DNA ladder. The gel was then run at 120V (1hr) and visualised using the GelDOC-IT TS imaging system (Bio-Rad, UK).


2.2.2.3  **PCR purification and sequencing (Sanger and NGS)**

Amplicons that presented bands at the expected molecular size from gels (see Appendix Figure 62 and 63) were purified using the QIAquick PCR purification kit (Qiagen, UK), following the manufacturer’s protocol. Purified DNA was then sent for Sanger sequencing (Source Bioscience, Oxford, UK). The FASTA data obtained were then analysed for variance and similarity by aligning each sequence to its appropriate WT fragment using the ClustalW Multiple Sequence Alignment application (BioEdit Sequence Alignment Editor Software).

Following the identification of a possible novel variant located within the XY-linker region of an infertile patient (Patient LR) by Sanger sequencing, further PCR amplifications from the gDNA extracted (buccal and sperm cells) were performed by creating a shorter amplicon targeted at the region of the variant with primers (forward: 5’-GGAGGAGGAAGAGGATAAATTC-3’; reverse: 5’-ACAAAACACCACCTCACCTTC-3’) and PCR conditions described in Section 2.2.2.2. These amplicons were then subjected to NGS, which was performed by Miss Katharina Spath (from Professor Dagan Wells’ laboratory) using the Ion Personal Genome Machine (Thermo Fisher Scientific, UK).

2.2.2.4  **Three-dimensional modeling of the novel variant**

The presence of the novel variant in both the buccal and sperm sample of Patient LR was confirmed by NGS, and following this it was therefore necessary to determine the effect upon PLCζ structure. This analysis was kindly performed by Dr. Tomas Malinauskas (from the Division of Structural Biology, University of Oxford, Oxford, UK). A homology model of hPLCζ was constructed based on the crystal structure of human phospholipase C beta-2 (PLCβ2) and rat PLCδ1 using Modeller (https://salilab.org/modeller/). This was established from the amino acid sequence alignment generated using the HHpred
homology detection server (http://toolkit.tuebingen.mpg.de/hhpred). The structures were then analysed using PyMOL (http://pymol.org) and Photoshop.

### 2.2.3 Functionality of the novel variant

The following methods attempted to test the functionality of the novel variant identified in Patient LR to determine whether this was the reason for the patient’s infertile phenotype. This consisted of synthesising PLCζ cRNA encoding the WT and novel variant, and the microinjection of these cRNAs into mouse oocytes to analyse the resultant Ca^{2+} release profile.

#### 2.2.3.1 cRNA synthesis

PLCζ<sup>WT</sup> cRNA was generated using the molecular construct described in Kashir et al. (2011a). cRNA for the novel variant was synthesised using the Quikchange II site-directed mutagenesis kit (Agilent Technologies, USA). Following the manufacturer’s instructions, the novel variant was created using the aforementioned vector as a template and primers (forward: 5’-AGCGTGGAGACAAATCAAGACTAGGAAACAGGGGTAAAAAAG-3’; reverse: 5’-CTTTTTTACCCCTGTTTCCTAGTCTTGATTGTCTCCACGCT-3’). To ensure that the point mutation was correctly inserted, the molecular construct was submitted for Sanger sequencing and verified (as described in Section 2.2.2.3, and the sequencing result can be seen in the Appendix Figure 64). cRNA was then transcribed and purified using the mMessage Machine (T3) kit (Ambion Life Technologies, UK) and RNeasy MinElute Cleanup kit (Qiagen, UK), following the manufacturer’s protocol, respectively. cRNA was measured using the Qubit Fluorometer 3.0 (Thermo Fisher Scientific, UK) and aliquoted in single-use tubes and stored at -80°C.

The purity of the cRNA synthesised was evaluated using 1% agarose gel electrophoresis. Analytical agarose was dissolved in diethylpyrocarbonate (DEPC) treated
water (Ambion Life Technologies, UK) and dissolved using a microwave oven. Paraformaldehyde (4%; Sigma Aldrich, UK) and NorthernMax running buffer (10X; Thermo Fisher Scientific, UK) were added into the solution once cooled. The gel mixture was poured into a gel tank and immersed in RNA gel running buffer (NorthernMax running buffer, paraformaldehyde, and DEPC-treated water).

The cRNA samples were mixed with RNA gel loading dye (Thermo Fisher Scientific, UK). The ladder used was the RiboRuler high range RNA ladder (Thermo Fisher Scientific, UK). Both samples and ladder were incubated at 65°C for 10 minutes, and simultaneously the gel was ‘pre-run’ at 70V (10m). The samples and ladder were loaded into the gel, and run at 90V for 2 hours and 30 minutes. The gel was visualised using the GelDOC-IT TS imaging system.

2.2.3.2 Mouse oocyte collection, culture, cRNA microinjection, and Ca^{2+} imaging

Miss Minerva Ferrer-Buitrago from the Department of Reproductive Medicine, Ghent University Hospital, Belgium performed the following protocol. Female mice (B6D2F1, 7 to 10-weeks-old) were stimulated by the intraperitoneal administration of follicle-stimulating hormone (FSH; 10IU; Folligon Intervet, Boxmeer, The Netherlands) and ovulation was induced with human chorionic gonadotropin hormone (hCG; 10IU; Chorulon Intervet, Boxmeer, The Netherlands) 46 to 48 hours after FSH administration. Oocyte retrieval was performed 13 to 14 hours upon hCG injection. Protein-free potassium simplex optimised medium (KSOM) and HEPES-buffered KSOM (KSOM-HEPES) supplemented with bovine serum albumin (0.4%; MP Biochemicals, Asse-Relegem, Belgium) were used for culture and during manipulation, respectively.

Prior to cRNA injection, oocytes were loaded with a Ca^{2+} indicator by direct exposure to Fura-2-AM (7.5uM, 30min). Fluorescence variation was recorded using a radiometric method at 340/380nm ratio every 5s for duration of 2 hours. Ca^{2+}
measurements were performed 30 minutes following cRNA injection. Ca$^{2+}$ oscillation-inducing activity was assessed by the microinjection of each cRNA into the mouse oocytes. The concentration in the injection pipette was 0.1mg/ml for both WT and novel variant cRNA. The volume of cRNA injected into each oocyte was 7-12pl (approximately 1-3% of the total oocyte volume).

2.2.4 Immunofluorescence studies

Immunofluorescent analysis in patient and control fertile sperm was performed to analyse the effects of the novel variant on three parameters of interest: total levels of PLCζ, the proportion (%) of sperm exhibiting PLCζ and the localisation patterns of PLCζ. This protocol has been previously described by Grasa et al. (2008), Heytens et al. (2009), Kashir et al. (2013), and Nomikos et al. (2013). The DGW sperm sample was fixed with paraformaldehyde (4%, RT, 10min). Fixed samples were then resuspended in phosphate buffered saline (PBS; Oxoid, UK) and smeared onto 0.1% poly-L-lysine (Sigma Aldrich, UK) coated slides (30min). The slides were then permeabilised with 0.5% Triton-X-100-PBS (Sigma Aldrich, UK), and incubated (4°C, overnight). The following day, slides were washed with PBS and blocked with 3% bovine serum albumin-PBS (BSA-PBS; 1h; Sigma Aldrich, UK). The slides were then labeled with a polyclonal anti-PLCζ antibody (25μg/ml; Covalab, France) in 0.05% BSA-PBS and incubated (4°C, overnight). As described by Grasa et al. (2008), two immunogenic peptide sequences in human PLCζ (C-RESKSYFNPSNIKE-coNH$_2$; C-ETHERKGSDKRGDN-coNH$_2$) were injected into rabbits to allow production of the polyclonal antibody. Antibodies were purified using column chromatography, following the manufacturer’s protocol (Covalab, France). Antibody specificity was tested and confirmed by pre-incubation with an excess of immunogenic peptide, and cross-reactivity with other PLCs by Covalab (Grasa et al., 2008). The following day, the slides were washed with PBS and incubated with Alexa
Fluor 488 goat anti-rabbit IgG (5μg/ml; 1h; Life Technologies, UK). The slides were washed with PBS and mounted with Vectashield H-1200 mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) (Vector, UK), and a glass coverslip prior to analysis.

PLCζ immunofluorescence in each sample was identified with an 80i fluorescence microscope (Nikon Instruments, UK), using a fluorescein isothiocyanate (FITC, 488nm wavelength) filter for green fluorescence and DAPI fluorescence using a DAPI filter (exposure time: 400ms). Slides were magnified at 40X using the NIS elements viewer program (Nikon Instruments, UK) for imaging. Approximately 100 sperm were analysed from each sample and analysis consisted of several parameters: total levels of PLCζ, the proportion (%) of sperm exhibiting PLCζ and localisation patterns of PLCζ. Analysis was performed using Image J software (National Institute of Health, USA) and the ROI tool.

Statistical analysis by Prism 5.0 software (Graphpad, USA) was used to compare the parameters of interest between patient and control fertile sperm. Statistical difference was determined using the student’s t-test, and one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparisons post-hoc test.

2.2.5 Mutation detection in single sperm

To ensure that no other variants were present from Patient LR, complete exon screening (exons 2-7 and 11-15) was performed from the patient’s gDNA (buccal) using in-house primers (Table 4), which were previously designed by Dr. Kevin Coward using Mutation Discovery Software (www.mutationdiscovery.com). The exons were amplified and the resulting amplicons were analysed using the methods described in Section 2.2.2.2.

Sanger sequencing revealed an additional variant from Patient LR, and this was the same mutation reported in a previous patient (PLCζ^{H233L}; Kashir et al., 2012a). Therefore, direct mutation detection was performed to analyse the distribution of both variants from
single sperm, and to investigate its potential effect on the patient’s fertility state (as described by Kashir et al., 2012a).

### Table 4. PLCζ Exonic screening for the detection of additional variants in Patient LR.

<table>
<thead>
<tr>
<th>Region</th>
<th>Size (bp)</th>
<th>Primers (F: forward; R: reverse)</th>
<th>PCR amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 2</td>
<td>151</td>
<td>F: GTTTCTATGGTTAAGGAGTGGCTA R: GGACTTTAATTGCAACTCCCTCG</td>
<td>329</td>
</tr>
<tr>
<td>Exon 3</td>
<td>124</td>
<td>F: CAGTAAAATCAAATTTTCTTTAGGAAT R: CTAAACCTTTATGCAAATTACCTGAC</td>
<td>286</td>
</tr>
<tr>
<td>Exon 4</td>
<td>232</td>
<td>F: CGTCACTCCATGCAGGTTTGAT R: CGGATAAATTTTGATAACACACAAATCAT</td>
<td>509</td>
</tr>
<tr>
<td>Exon 5</td>
<td>202</td>
<td>F: GAGTAAAGTTTCAAGTGAGTGATG AG R: CCTACTCAAGGTAGACACTGCCA</td>
<td>361</td>
</tr>
<tr>
<td>Exon 6</td>
<td>145</td>
<td>F: TTAATGCAAAATTTCCTAGAGACTCAA R: TATCATCTAAAGGCTAAAGCA</td>
<td>421</td>
</tr>
<tr>
<td>Exon 7</td>
<td>150</td>
<td>F: AGGTACTAAACGTAGTAAAGT R: CACAGGCAATATAACAGTGACA</td>
<td>335</td>
</tr>
<tr>
<td>Exon 11</td>
<td>117</td>
<td>F: TTCTTTGAAATGATCAGTGATACATA R: TACTTCTGCAAACACTCAATATC</td>
<td>413</td>
</tr>
<tr>
<td>Exon 12</td>
<td>170</td>
<td>F: TCAGTAAGTGTTTGCTTCTT R: GAAATTGGAAGCAAATCAGTGGAAT</td>
<td>339</td>
</tr>
<tr>
<td>Exon 13</td>
<td>130</td>
<td>F: GCTGACTTAGAGTCCATCAACTT R: CAGTACATTTCTGTATACATC</td>
<td>458</td>
</tr>
<tr>
<td>Exon 14</td>
<td>150</td>
<td>F: AAAAGTTTGAGAGAAATAATGTAATCTAT R: CTTATGATAGGCTATTGTTATGT</td>
<td>330</td>
</tr>
<tr>
<td>Exon 15</td>
<td>143</td>
<td>F: TTCTCAGCAAGGCTATTGGAGAC R: GAATGGGGCTCAATTTCTAGTTT</td>
<td>290</td>
</tr>
</tbody>
</table>

### 2.2.5.1 Single sperm isolation

The sperm sample previously prepared by DGW in Section 2.2.2.1 was used for single sperm isolation. Tracey Griffiths (senior clinical embryologist from Oxford Fertility, UK) performed the single sperm isolation using a Nikon Eclipse Ti-S micromanipulator system (Nikon Instruments, UK). Each single sperm (n=38) was washed in non-stick washing buffer (NWB; Reprogenetics, UK) and transferred to an individual sterile 0.2ml microcentrifuge tube, and stored at -80°C to await analysis.
2.2.5.2 Multiplex and inner hemi-nested PCR

Prior to amplification, each single sperm was lysed using alkaline lysis buffer (PCR grade water; 200mM NaOH; 250mM DTT) and incubated using a G-storm Thermal Cycler (65°C, 20min). Following the manufacturer’s protocol, first round amplifications with the Multiplex PCR kit (Qiagen, UK) was used to obtain two fragments of the PLCζ gene containing PLCζ^{K322Stop} (forward primer: 5’-GAAGTGGCAGATGGAGAGGA-3’; reverse primer: 5’-ACAAAACACCACCTCA-3’) and PLCζ^{H233L} (forward primer: 5’-TTTTGCTAGTGCCCTTG-3’; reverse primer: 5’-AGGCTAAGCATTATAGGA-3’) variants. The PCR conditions involved a holding step (95°C, 15min) followed by 55 cycles of denaturation, annealing, and elongation (94°C for 30s, 58°C for 90s, and 72°C for 1min, respectively). A final extension step (72°C, 7m) was carried out prior to storing the amplicons at 4°C.

The resulting DNA amplicons (outer reactions) were then subjected to two inner hemi-nested PCR to amplify the PLCζ^{K322Stop} (forward primer: 5’-CTGTTTTAGGAGACAATCAAGAC-3’; reverse primer: 5’-ACAAAACACCACCTCA-3’) and PLCζ^{H233L} (forward primer: 5’-ATTGACTGCTGGGATGGA-3’; reverse primer: 5’-AGGCTAAGCATTATAGGA-3’) fragments (inner reactions), separately. The reagents used for the inner hemi-nested PCR consisted of HotMaster Taq DNA polymerase (0.6 units; VWR International, UK), HotMaster Taq buffer (1X; VWR International, UK), dNTPs (200mM each; Thermo Fisher Scientific, UK), each primer (0.8mM; Eurogentec, Belgium), outer reaction amplicon, and PCR-grade water (Qiagen, UK). The PCR conditions involved a holding step (96°C, 1min) followed by 40 cycles of denaturation, annealing, and elongation (94°C for 15s, 58°C for 15s, and 65°C for 45s, respectively). A final extension step (65°C, 2m) was carried out prior to storing the reactions at 4°C.
2.2.5.3  

Mini-sequencing

Mini-sequencing was performed with the help of Miss Dhruti Babariya (from Professor Dagan Wells’ laboratory). The inner reaction products from inner hemi-nested PCR were treated with EXOSAP-it (Reprogenetics, UK) to remove unincorporated primers and dNTPs. The products were then subjected to mini-sequencing using the SNaPshot Multiplex kit (Thermo Fisher Scientific, UK). The products derived from mini-sequencing were then analysed by capillary electrophoresis on a 3130 genetic analyser (Thermo Fisher Scientific, UK), and data analysed by GeneMapper v4.0 software (Thermo Fisher Scientific, UK).
2.3 Results

2.3.1 Identification of a novel variant in the PLCζ XY-linker region

The aim of this chapter was to specifically screen two regions of PLCζ, promoter-exon 1 and XY-linker, to identify genetic anomalies, which may play a role in the patients’ compromised state of fertility. In total, five patients, and three fertile controls, were subjected to screening (Table 5). Anomalies were identified by comparing the patient DNA alignment to that of the WT PLCζ sequence, and validated after two replicates.

Table 5. Summary of the sequencing results following screening of PLCζ promoter-exon 1 and XY-linker regions. ✓: Region normal compared to WT PLCζ sequence. ×: Insufficient genomic DNA or sequencing failure. *: Possible variant revealed.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Location</th>
<th>PLCζ region screened</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Promoter-exon 1</td>
</tr>
<tr>
<td>Control 1</td>
<td>Oxford</td>
<td>✓</td>
</tr>
<tr>
<td>Control 2</td>
<td>Oxford</td>
<td>✓</td>
</tr>
<tr>
<td>Control 3</td>
<td>Oxford</td>
<td>✓</td>
</tr>
<tr>
<td>OI</td>
<td>Belgium</td>
<td>✓</td>
</tr>
<tr>
<td>HB</td>
<td>Belgium</td>
<td>✓</td>
</tr>
<tr>
<td>KS</td>
<td>Belgium</td>
<td>×</td>
</tr>
<tr>
<td>BL</td>
<td>Belgium</td>
<td>×</td>
</tr>
<tr>
<td>LR</td>
<td>Belgium</td>
<td>✓</td>
</tr>
</tbody>
</table>

From Table 5, only Controls (1 and 3) and Patient OI revealed 100% similarity in the regions screened. Patient HB and Control 2 showed only a normal promoter-exon 1 segment, whereas screening of the XY-linker region were inconclusive as a result of insufficient genomic DNA. The sequencing for Patient KS and BL for both regions were inconclusive as a result of sequencing failure, and this persisted following multiple attempts. Interestingly, while Patient LR exhibited a normal promoter-exon 1 region,
screening of his XY-linker revealed a potential novel variant and this was confirmed following eight replicates.

Genetic screening of Patient LR’s XY-linker region revealed a base change from adenine (A) to thymine (T) at position 964 of the PLCζ open reading frame (ORF) sequence. This corresponded to the substitution of a lysine residue (K or Lys) at position 322 (K322Stop) creating a stop codon in the XY-linker region. Chromatograms produced from sequencing showed green and red peaks at this specific position, corresponding to bases A and T, respectively (Figure 18).

Next generation sequencing (NGS) confirmed the presence of the variant in both buccal and sperm samples from Patient LR, and identified a mixed population of normal (53%) and abnormal (47%) sperm in the patient’s sample. Further screening of Patient LR’s PLCζ exons were performed to verify that no other variants were present and interestingly, in addition to PLCζ<sup>K322Stop</sup>, PLCζ<sup>H233L</sup> which was previously reported by Kashir et al. (2012a), was also identified and confirmed in five replicates.

Our collaborator, Professor Björn Heindryckx at the Department of Reproductive Medicine, Ghent University Hospital, Belgium, provided clinical data and samples for Patient LR. Patient LR and his wife were being treated for infertility and their clinical
history stated that they had previously failed four successive ICSI cycles, and only in their fifth ICSI attempt were they able to achieve a successful pregnancy (see Table 6). MOAT was carried out to identify the possible cause of their previous fertilisation failures, and the test revealed that 5 out of 29 (19%) oocytes showed activation. It was then deduced that patient LR was a MOAT group 1 subject hence, sperm-related deficiency was the likely cause. The cause of his infertile phenotype may then be associated to PLCζ<sup>K322Stop</sup> and therefore, extensive investigation was carried out on this novel variant to analyse its potential effect on PLCζ structure, expression, and function.

**Table 6.** Summary of Patient LR’s ICSI cycles and partner’s pregnancy outcome.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Number of oocytes fertilised (method used)</th>
<th>Pregnancy outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0/12 (ICSI with vigorous aspiration)</td>
<td>No pregnancy</td>
</tr>
<tr>
<td>2</td>
<td>0/5 (ICSI)</td>
<td>No pregnancy</td>
</tr>
<tr>
<td></td>
<td>2/4 (ICSI with electrical pulses)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5/13 (ICSI with electrical pulses)</td>
<td>No pregnancy</td>
</tr>
<tr>
<td>4</td>
<td>0/7 (ICSI with calcium injection)</td>
<td>No pregnancy</td>
</tr>
<tr>
<td></td>
<td>2/4 (ICSI with electrical pulses)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>8/20 (ICSI with calcium and ionomycin)</td>
<td>Pregnant</td>
</tr>
</tbody>
</table>

Predicted three-dimensional models of PLCζ<sup>WT</sup> and PLCζ<sup>K322Stop</sup> were generated based on the crystal structures of human PLCβ2 and rat PLCδ1 to understand the potential effect of the variant on PLCζ structure and function (Figure 19). These structures revealed an evolutionary-conserved pocket, which accommodates substrate, PIP<sub>2</sub>, and Ca<sup>2+</sup> required for PLCζ catalysis within the triosephosphate isomerase (TIM) domain. The side chains of residues from both X and Y regions of the TIM domain form hydrophilic and van der Waals interactions with PIP<sub>2</sub>, and therefore the mutation would eliminate three crucial residues (Ser378, Arg405 and Tyr407) in PLCζ which corresponded to the PIP<sub>2</sub>-binding residues (Ser522, Arg549 and Tyr551) in PLCδ1. Essentially, this mutation would result in
the truncation of PLCζ by 47% in contrast to WT PLCζ and thus, would result in the major disruption of PLCζ structure and function.

Figure 19. Predicted three-dimensional model of PLCζ^WT (top) and PLCζ^K322Stop (bottom). PLCζ^K322Stop localised within the XY-linker truncates the expression of the protein to almost half of its wild type counterpart.
Immunofluorescent sperm analysis measuring the total levels of PLCζ from patient LR (8.1 arbitrary units; a.u) showed no significant difference ($P>0.05$) when compared to the fertile control (10.4 a.u; **Figure 20**). It appeared that total levels of PLCζ from the fertile control sperm were also low, and this phenomenon has been reported previously by Kashir *et al.* (2013). Patient LR (46.9%), however, showed a significantly ($P<0.05$) lower proportion (%) of sperm exhibiting PLCζ when compared to the fertile control (78.9%; **Figure 20**).

![Figure 20](image)

**Figure 20.** Immunofluorescent analysis of total levels (top panel) and proportion of sperm exhibiting PLCζ (bottom panel). The analysis compared sperm from patient LR and fertile control. Hash tag (#) denotes no significant difference ($P>0.05$), whereas asterisk (*) indicates a significant difference ($P<0.05$). The top panel represents mean±SEM, and the bottom panel represents the data as mean percentages.

The localisation profile of patient LR was shown to be statistically ($P<0.05$) significant, when compared to each different patterns (**Figure 21**). There was no visible
staining in the combined regions (acrosomal and post-acrosomal, acrosomal and equatorial, and post-acrosomal and equatorial) and all the patterns combined. There was a lower proportion of staining in the acrosomal (3.4%) and post-acrosomal regions (2.8%). 53.1% and 40.7% of the sperm head analysed lacked PLCζ and was localised within the equatorial region, respectively (Figure 21). Furthermore, PLCζ in the equatorial region was shown to appear as a round signal in the patient’s sperm, as compared to the whole equatorial segment seen in the fertile control (as pointed out by the yellow arrows at the bottom panel of Figure 21).

![Figure 21. Immunofluorescent analysis of patient LR’s sperm exhibiting PLCζ at different localisation patterns (top panel). The asterisk (*) indicates a significant difference (P<0.05). Representative image of (a) Bright-field, (b) DAPI, (c) PLCζ, and (d) Overlay showing sperm from patient LR and fertile control exhibiting PLCζ (bottom panel). The yellow arrows indicate the presence of PLCζ. Images were captured at 40X magnification, FITC filter (wavelength: 488nm), and exposure at 400ms. Scale bar: 5µm.](image-url)
As PLCζ\textsuperscript{K322Stop} truncated the expression of PLCζ to approximately half of its molecular weight, this may have impacted upon the interaction between the polyclonal antibody and peptide sequences during immunofluorescence. Figure 22 reveals that in the presence of PLCζ\textsuperscript{K322Stop}, only one of the two peptide sequence was present in the protein as the second is localised downstream of the mutation site. Therefore, the second peptide sequence was absent in 47% of Patient LR’s sperm population. This may have influenced the efficiency of the immunofluorescent analysis in this patient.

**Figure 22.** Regions of immunogenic peptide sequences in PLCζ protein. The yellow highlight denotes the immunogenic peptide sequences which were injected into rabbits for antibody production and the red highlight indicates the position of the novel variant in Patient LR.

As mentioned earlier in this section, the mutation previously reported by Kashir et al. (2012a), PLCζ\textsuperscript{H233L}, was also detected following PLCζ exonic screening in Patient LR.

The distribution of each variant in single sperm (n=38) was analysed using DNA mini-sequencing technology. Chromatogram peaks indicated the base present at the location of each variant (Figure 23). Two peaks, bases A (green) and T (red), were present in 34 out of 38 sperm (89.5%; Figure 23A). Sperm receive one single allele and therefore, the double peaks indicate the presence of multiple alleles. Four out of 38 sperm showed a single peak (Figure 23 B and C), thus a single allele. Table 7 confirmed each sperm possessed either PLCζ\textsuperscript{K322Stop} or PLCζ\textsuperscript{H233L}, but never both at the same time.
Figure 23. Chromatogram peaks of single sperm from Patient LR exhibiting PLCζ\textsuperscript{K322Stop} or PLCζ\textsuperscript{H233L}. (A) Two peaks (green and red) which correspond to bases A and T, respectively, were shown from single sperm (n=34). Four out of 38 exhibited a single peak of adenine (green; B) or thymine (red; C) for either PLCζ\textsuperscript{K322Stop} or PLCζ\textsuperscript{H233L}. The size standard is depicted as the orange peak.

Table 7. Direct mutation detection of PLCζ\textsuperscript{K322Stop} and PLCζ\textsuperscript{H233L} from four individual sperm of the patient. The plus (+) and minus (-) indicates the presence and absence of the variant.

<table>
<thead>
<tr>
<th>Sperm</th>
<th>PLCζ\textsuperscript{K322Stop}</th>
<th>PLCζ\textsuperscript{H233L}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
2.3.2 Functional effects of PLCζK322Stop upon oocyte activation

Prior to testing the functionality effects of PLCζK322Stop, the purity and integrity of the cRNA synthesised was assessed using agarose gel electrophoresis (Figure 24).

![Figure 24. Assessment of the purity and integrity of the cRNA synthesised. The clean single band produced on the agarose gel indicates un-degraded cRNA (PLCζWT and PLCζK322Stop), which could be utilised for downstream experiments such as the microinjection into mouse oocytes for functionality studies. The red box denotes cRNA that has degraded and therefore, production must be repeated.]

The microinjection of PLCζK322Stop cRNA demonstrated that none of the mouse oocytes (0/17) induced any Ca²⁺ oscillation-inducing activity (Figure 25). In contrast, 6 out of 9 (66%) mouse oocytes microinjected with PLCζWT cRNA exhibited strong characteristic oscillation patterns (Figure 26). The Ca²⁺ release pattern was indistinguishable to that of normal fertilisation (> 10 peaks during the 2 hour measurements).

![Figure 25. Ca²⁺ release profile following microinjection of cRNA corresponding to PLCζK322Stop at a concentration of 0.1mg/ml. Ca²⁺ oscillation-inducing activity was absent following microinjection.]
Further to this, a rescue experiment was carried out to determine whether PLCζ\textsuperscript{K322Stop} was linked to the patient’s OAD. A total of 17 mouse oocytes were microinjected with PLCζ\textsuperscript{K322Stop} cRNA; Ca\textsuperscript{2+} oscillations were absent in all cases. One hour following the administration of PLCζ\textsuperscript{K322Stop} cRNA, the oocytes were re-microinjected with PLCζ\textsuperscript{WT} cRNA. Only 8 out of the 17 original oocytes survived after the second injection. However, five out of the eight oocytes responded to PLCζ\textsuperscript{WT} cRNA, as demonstrated by the successful release of Ca\textsuperscript{2+}, characterised as oscillations, within a period of two hours (Figure 27).
2.4 Discussion

Yoon et al. (2008) were the first to report the association between dysfunctional forms of PLCζ and OAD, and we have since become more knowledgeable of this infertile condition. These authors selected patients with a history of previous fertilisation failures, and following a thorough investigation to identify their infertile status, their sperm population was shown to exhibit abnormal PLCζ features. Further to this, the sperm lacked the ability to induce oocyte activation, and the process was only restored following microinjection of mPLCζ mRNA. Yoon et al. (2008) identified eight single nucleotide polymorphisms (SNPs) following genetic screening of PLCζ from these patients but failed to deduce if such variants played a role in their infertile phenotype.

The first genetic link was observed in a non-globozoospermic infertile patient in which a heterozygous mutation, PLCζ<sup>H398P</sup>, disrupted the secondary structure of PLCζ leading to abnormal Ca<sup>2+</sup> oscillation-inducing activity (Heytens et al., 2009). A second heterozygous mutation, PLCζ<sup>H233L</sup>, was later detected from the same patient. PLCζ<sup>H233L</sup> was less detrimental than PLCζ<sup>H398P</sup> as it was still able to sustain at least some functional activity when PLCζ<sup>H233L</sup> cRNA was microinjected into mouse oocytes (Kashir et al., 2012a). Further investigation of these mutations revealed that PLCζ<sup>H398P</sup> and PLCζ<sup>H233L</sup> were inherited from the father and mother, respectively, and demonstrated that the maternal germ line can convey male infertility (Kashir et al., 2012a; Kashir et al., 2012b). Absent or abnormal Ca<sup>2+</sup> oscillation-inducing activity resulting from these variants was rectified by the microinjection of a WT hrPLCζ into mouse oocytes previously injected with cRNA derived from PLCζ<sup>H398P</sup> and PLCζ<sup>H233L</sup> (Nomikos et al., 2013). This signifies the potential application of PLCζ for OAD therapy, particularly for patients with abnormal forms of PLCζ, in which fertility status had been affected.

Since this thesis commenced, only two other novel variants have been discovered, from two additional patients suspected of having OAD; a missense homozygous mutation,
which converted Isoleucine into Phenylalanine at position 489 of the PLCζ amino acid sequence (PLCζ^{Ile489Phe}) and a point mutation in the X domain, which replaced Arginine with Histidine at position 197 (PLCζ^{R197H}) (Escoffier et al., 2016; Ferrer-Vaquer et al., 2016). **Figure 28** characterises the four mutations discovered in PLCζ, as of yet.

![Figure 28. Schematic representation of the mutations discovered within the exonic structure of human PLCζ. Four mutations have been reported in PLCζ and each had compromised the fertility state of the patient exhibiting the variant.](image)

PLCζ^{Ile489Phe} occurred in the C2 domain and was deleterious as it resulted in the total absence of the protein in sperm, abnormal Ca^{2+} release profile and early embryonic arrest (Escoffier et al., 2016). Interestingly, the screening investigation reported by Ferrer-Vaquer et al. (2016) also detected PLCζ^{H233L}, previously reported by Kashir et al. (2012a), from an infertile patient with TFF. It should however be noted that only two oocytes were injected (one fertilisation cycle) and this was insufficient to suggest that PLCζ^{H233L} may be responsible for the patients infertile condition. Furthermore, Kashir et al. (2012a) proposed that infertile patients who were heterozygous for only one loss-of-function mutation might be sub-fertile, so further investigation on this patient is necessary. Additionally, Ferrer-Vaquer et al. (2016) failed to analyse the structural and functional effects of PLCζ^{R197H}, and deduce whether the variant resulted in the patient’s infertile phenotype. Nevertheless,
the discovery of these mutations further emphasises the link between OAD and PLCζ-deficiency but also signify that exonic mutations are very rare, as these have only been reported in a total of three patients in the span of almost a decade.

In this thesis, initial genetic screening involved targeting promoter-exon 1 and XY-linker regions of five infertile patients. These patients had all experienced recurrent failed fertilisation and had sought help to identify the cause of their infertile condition, which was possibly OAD. These particular ROIs were selected for several reasons. Firstly, no screening has ever been performed in the promoter region of human PLCζ and therefore, this may help to elucidate their infertile phenotype. Secondly, the promoter region is a crucial element of the gene, which initiates transcription, and thus, anomalies identified within it are a consequence of the processes leading to PLCζ expression. As for the XY-linker, this region plays an important role in PLCζ function, particularly in initiating Ca²⁺ oscillation-inducing activity for OA by hydrolysing the substrate PIP₂ (Saunders et al., 2002; Nomikos et al., 2011a). Variants detected in this region may therefore underlie the patients' inability to activate the oocyte following fertilisation. However, Nomikos et al. (2011b) has shown that the deletion of the XY-linker does not completely terminate in vivo Ca²⁺ oscillation-inducing activity and a subsequent study demonstrated that, similarly to PLCδ1, the first PLCζ EF-hand domain also showed affinity for PIP₂ (Nomikos et al., 2015). It can therefore be assumed that as a result of the rich basic residues found within the XY-linker and the first EF-hand domain, both components play an essential role in targeting PLCζ to membrane substrate PIP₂ (Nomikos et al., 2015).

Previous work in the Coward laboratory has consistently failed to screen the segment between exons 8 to 10, which encodes the XY-linker region. However, newly synthesised primers and PCR conditions were utilised in this present study. As reported by Zhou et al. (2004), gDNA is filled with sequences containing homopolymeric tracts, or more generally simple repetitive sequences, and, analysis of the XY-linker has showed that
it possessed such regions. Homopolymeric tracts may result, although not always, in sequencing failure due to their ability either to cause enzyme slippage or even the formation of secondary structures during PCR (Personal communications with Mr. Kenny Wheeler, previous laboratory team leader at Source Bioscience Oxford). Therefore, it was necessary that extensive primer design and validation were performed to ensure optimal amplification.

Screening of five infertile patients (OI, HB, KS, BL, and LR) and 3 fertile controls had either failed or was successful. DNA degradation or insufficient amount of DNA was the reasons for the sequencing failure observed from patients HB (only XY-linker failed), KS, BL, and fertile control 2 (only XY-linker failed). Due to limited buccal samples from these patients, repeating the gDNA extraction for additional sequencing was not possible. As for patients (OI, HB, LR, and all fertile controls) who showed successful sequencing in both or either regions, their promoter-exon 1 and/or XY-linker segments lacked variants. These segments were therefore normal when compared to the WT sequences of the respective regions. It could then be deduced that other regions of the gene may be responsible for OAD.

Patient LR, however, exhibited a possible novel variant in his XY-linker region although his promoter-exon 1 sequence was normal. The variant was a change of amino acid, from a lysine residue to a stop codon (PLCζ<sup>K322Stop</sup>). This is known as a “nonsense mutation”, and prematurely terminates protein translation at the location of the variant (Turner and Choy, 2015). A predicted three-dimensional model of PLCζ<sup>K322Stop</sup> revealed a severely compromised protein, in which 286 amino acids (47%) were missing in contrast to PLCζ<sup>WT</sup>. The absence of amino acids resulted in the omission of the entire Y and C2 functional domains. Interestingly, Sanger sequencing revealed that in the nine replicates performed, and at the position of the variant, there was an alternate presence of either A or T bases. This may suggest that Patient LR has a mixed population of sperm. NGS
confirmed this population and showed that, 47% and 53% contained mutant and normal PLCζ DNA sequences, respectively, suggesting $\text{PLC}_{\zeta}^{K322\text{Stop}}$ to be heterozygous. Theoretically, it should have been an equal (50%) population of mutant and normal sperm but due to the multiple preparative procedures of NGS, the values observed in this present study were proposed to be an artifact (Professor Dagan Wells, Personal communication). Nevertheless, the values (47% and 53%) corresponded to the low Ca²⁺ oscillation-inducing activity of the patient, evident from his MOAT results. In the event that $\text{PLC}_{\zeta}^{K322\text{Stop}}$ was homozygous, his MOAT results would have been 0% but this was not the case. Presumably, when taking into consideration his mixed sperm population, his MOAT value should be comparable. Practically, this was not the case as only 26 sperm were injected, thus, only increasing the sperm number would increase the probability of reaching approximately 50% observed by NGS. Regardless of this mixed population, following the characterisation of $\text{PLC}_{\zeta}^{K322\text{Stop}}$, this is the first mutation ever reported which results in only half the expression of PLCζ.

As the variant results in the incomplete expression of PLCζ, it would be considered as non-functional. This was evident following the microinjection of $\text{PLC}_{\zeta}^{K322\text{Stop}}$ cRNA into mouse oocytes, which failed to induce any Ca²⁺ oscillation-inducing activity. However, the microinjection of $\text{PLC}_{\zeta}^{\text{WT}}$ into mouse oocytes resulted in Ca²⁺ release in a manner similar to that at fertilisation. Additional investigation demonstrated that $\text{PLC}_{\zeta}^{\text{WT}}$ could rescue failed Ca²⁺ oscillation-inducing activity in mouse oocytes, which was previously expressed with $\text{PLC}_{\zeta}^{K322\text{Stop}}$ cRNA. This further supports the notion that PLCζ can be used as a novel therapeutic agent for OAD, as previously described (Yoon et al., 2008; Nomikos et al., 2013; Sanusi et al., 2015). PLCζ therapy could then have been used, as a corrective treatment for Patient LR instead of AOAs, if a recombinant PLCζ had been available in the clinic.
This present study demonstrated the detrimental structural and functional effects of PLCζ^{K322Stop}, and immunofluorescent analysis of the patient’s sperm when compared to a fertile control male showed a statistically significant difference in the proportion of sperm exhibiting PLCζ and the localisation patterns of PLCζ. The proportion of sperm exhibiting PLCζ was reduced in Patient LR, when compared to fertile control sperm, and this correlated with the localisation pattern in which over half of the sperm analysed lacked PLCζ. In the remaining sperm from Patient LR, PLCζ was localised in the equatorial region, as expected as it is mainly localised in this segment of the sperm head (Escoffier et al., 2015). Additional expression was observed in the post-acrosomal and acrosomal regions, although this was very minimal. Although PLCζ was mainly localised in the equatorial region, its expression was punctate, appearing as a rounded signal, in comparison to the whole equatorial segment as shown in most fertile control sperm.

It should, however, be noted that the sperm sample from Patient LR was transported to our laboratory in cryopreserved form. Kashir et al. (2011b) reported that cryopreserved sperm from four out of seven fertile men showed a significant reduction in PLCζ immunofluorescence compared to fresh DGW sperm from the same subjects. The reason for this could be the generation of excessive reactive oxygen species during freeze-thaw, which may alter sperm membrane function and structure (Gadea et al., 2011), and result in the distortion of PLCζ localisation, or even possible leakage. This could have therefore influenced the reduction or abnormal PLCζ signal observed from Patient LR and thus, a fresh sperm sample would be required to provide accurate immunofluorescent signal for the patient. Furthermore, the antibody used during this study should be considered as a possible factor underlying the reduction observed in PLCζ expression. Two immunogenic peptide sequences were used to immunise rabbits for the production of the polyclonal antibody. Upon immunostaining, the antibody would bind to these sequences if present thus creating a signal and in the case of PLCζ, this is green fluorescence. It should
be noted that in sperm exhibiting \( \text{PLC}_\zeta^K322\text{Stop} \), the second peptide sequence would be absent. Whether this has a significant effect on staining has yet to be determined but it may be the case that the antibody may bind only to the sequence present thus, resulting in a reduction of \( \text{PLC}_\zeta \) signal. There was, however, no significant difference in the total relative fluorescence of sperm exhibiting \( \text{PLC}_\zeta \) when compared between Patient LR and the fertile control. This was not surprising as Grasa et al. (2008) and Kashir et al. (2013) reported significant variability of \( \text{PLC}_\zeta \) expression between fertile and infertile patients, particularly total levels and localisation patterns of \( \text{PLC}_\zeta \).

Interestingly, complete screening of Patient LR’s exons identified an additional variant, \( \text{PLC}_\zeta^{H233L} \), previously reported by Kashir et al. (2012a) and Ferrer-Vaquer et al. (2016). When \( \text{PLC}_\zeta^{H233L} \) was first discovered (Kashir et al., 2012a), it was already known from the work of Heytens et al. (2009) that this patient’s sperm also had an additional variant, \( \text{PLC}_\zeta^{H398P} \). Both of these mutations were reported to be heterozygous, and mutation detection showed that each individual sperm possessed either variant \( \text{PLC}_\zeta^{H233L} \) or \( \text{PLC}_\zeta^{H398} \) but never both. Furthermore, \( \text{PLC}_\zeta^{H398P} \) and \( \text{PLC}_\zeta^{H233L} \) were inherited from the father and mother, respectively (Kashir et al., 2012a; Kashir et al., 2012b). In the present study, it would be presumptuous to make this assumption without access to the DNA of the patient’s parents. It was therefore difficult to determine the precise genetic mode of inheritance without further ethical permission, which was not possible in the time constraints imposed by this thesis. However, as the nature of both variants was heterozygous, either parent could have passed on \( \text{PLC}_\zeta^{K322\text{Stop}} \) and \( \text{PLC}_\zeta^{H233L} \) but this is purely an assumption. Mutation detection in Patient LR’s single sperm (n=4) showed that the variants never appeared together, similar to the observations made by Kashir et al. (2012a). Due to the very low sample size, it could be proposed that the variants were present on separate chromosomes, and a stronger conclusion could be made if the procedure was repeated with more individual sperm than the four used. Provided that each
variant is in close genetic proximity, recombination translocating PLCζ^K322Stop and PLCζ^H233L onto a single chromosome is unlikely. Furthermore, single sperm isolation is regarded to be a highly sensitive procedure resulting in both a high failure and contamination rate (Caragine et al., 2009). Failure to amplify occurred in the present case, as initially 38 individual sperm were obtained by an experienced embryologist and only four gave a final outcome. The amplification protocol followed that of Kashir et al. (2012a), and the cycles used (first amplification: 55; second amplification: 40) may have been excessive for this study. The number of optimal total cycles is approximately 30-40, and increasing the number of cycles may lead to an increase in non-specific products (Cha and Thilly, 1993). This might account for the high contamination rate observed, as two peaks were consistently seen from the mini-sequencing analysis, except for the four single sperm. Sperm are haploid so only one allele (one peak) should have been observed in this analysis.

The data collected from this study suggest that Patient LR may be sub-fertile, as evident from his MOAT findings, and it was important to identify the cause so as to understand the effects PLCζ may have on OAD caused by sperm-related deficiencies. As in previous reports (Heytens et al., 2009; Kashir et al., 2012a; Escoffier et al., 2016; Ferrer-Vaquer et al., 2016), this study only involved investigating the exonic regions of PLCζ and as aforementioned, mutations are very rare in this segment of DNA. Furthermore, these studies are the first to examine the possible association between variants localised in the non-coding regions (promoter and introns) of PLCζ and the wide variation of PLCζ expression observed by Grasa et al. (2008) and Kashir et al. (2013). The further investigations in Chapter 3 may explore the cause of the significant variability of PLCζ expression in fertile and infertile patients. Furthermore, Patient LR received ICSI concomitantly with AOAs to achieve a successful pregnancy, and while this may be encouraging progress, there are concerns over the use of such agents. In particular, Patient
LR and his partner underwent five attempts of ART to achieve pregnancy using vigorous AOA-ICSI protocols and more research is therefore warranted to produce a safer and more physiological form of OAD therapy, namely in the form of hrPLCζ injection. If hrPLCζ had been accessible clinically, it could have rectified Patient LR’s infertile condition, resulting in a more favourable approach to pregnancy as opposed to the multiple ART cycles performed. The progress of hrPLCζ production will be explained in greater detail in Chapter 4.
2.5 Key findings

In this chapter, 5 infertile patients suspected of having OAD were screened from two particular regions of PLCζ, promoter-exon 1 and XY-linker. Screening confirmed a novel nonsense mutation from Patient LR, PLCζ^K322Stop, which was characterised to be detrimental as the variant results in the truncation of half the expression of PLCζ. Although this would result in the infertile state of Patient LR, as initially expected, the patient was also heterozygous for a mutation previously discovered, PLCζ^H233L (Kashir et al., 2012a). Thus, Patient LR could be categorised as sub-fertile but more research is required to confirm this. The discovery of PLCζ^K322Stop would make this the fifth novel mutation found within the exonic region of the gene, implicating its rarity, and consolidates the association between PLCζ and OAD.
2.6 Future work

- To repeat the mutation detection experiment. The four sperm, which showed that both variants were not present in the same sperm, were not sufficient to conclude the heterozygous nature of \( \text{PLC}_\zeta^{K322\text{Stop}} \) and \( \text{PLC}_\zeta^{H233L} \) in Patient LR. It is important that the high level of contamination is controlled prior to repeating the experiment. This would include optimising the PCR condition such that the cycles used are reduced so as to prevent the amplification of non-specific products, therefore excluding contaminants in the reaction.

- To validate Patient LR’s heterozygosity for \( \text{PLC}_\zeta^{K322\text{Stop}} \) and \( \text{PLC}_\zeta^{H233L} \). NGS should be repeated to determine if the patient’s sperm population consists of an equal distribution of the each variant, as the presence of the novel mutation only was determined. From this it would be possible to confirm that patient LR is sub-fertile, as opposed to being completely infertile as suggested initially.

- Although the mutation was discovered within the XY-linker segment of \( \text{PLC}_\zeta \), it is important that this region, and the promoter-exon 1 region, be incorporated into routine screening in the laboratory for patients suspected of having OAD. The promoter-exon 1 and XY-linker regions play a crucial role in \( \text{PLC}_\zeta \) expression and function, respectively, and thus variants identified within these fragments may play a role in patients suspected of having OAD, as was the case for Patient LR.
Chapter 3: Investigating the phenotypic diversity of PLCζ expression in infertile patients suspected of having OAD
3.1 Introduction

Chapter 2 of this thesis explored the genetic link between OAD and PLCζ-deficiency by describing the effect of a novel nonsense mutation, \( \text{PLC}_\zeta^\text{K322Stop} \), on the structural and functional features of PLCζ from an infertile patient. While this variant, and the four already published, serve to emphasise the genetic contribution of PLCζ-deficiency in males suspected of having OAD, their discovery was established by screening the coding regions of the gene (Heytens et al., 2009; Kashir et al., 2012a; Escoffier et al., 2016; Ferrer-Vaquer et al., 2016). This seemed to be a sensible approach since abnormalities detected within the exons would result in a dysfunctional or non-functional protein, as was the case for variants \( \text{PLC}_\zeta^\text{H398P}, \text{PLC}_\zeta^\text{Ile489Phe}, \) and \( \text{PLC}_\zeta^\text{K322Stop} \).

Interestingly, only Pan et al. (2013) have investigated the promoter region of PLCζ (in Chinese Holstein bulls) and identified variants that affected semen quality traits and thus, fertilisation outcome. Although they did not correlate their findings with downstream events such as PLCζ expression or \( \text{Ca}^{2+} \) inducing ability, it was still of great interest that a non-coding region was finally being explored. It is also worth noting that PLCζ shares a bi-directional promoter with the actin filament capping muscle Z-line alpha 3 (CAPZA3), a protein responsible for the architecture of the sperm head during spermatogenesis (Coward et al., 2011; Figure 29). Bidirectional promoters are present in 11% of the human genome, and interestingly, a substantial number of gene pairs sharing a bidirectional promoter display parallel expression (Trinklein et al., 2004; Wakano et al., 2012). Although, it is not known whether the bidirectional promoter of gene pairs, PLCζ and CAPZA3, co-regulates expression, screening of the region may not be straightforward. However, since the work of Pan et al. (2013), it is clear that our knowledge of PLCζ at the genomic level, particularly in understanding its regulatory mechanism is very limited. Additionally, the introns need also to be considered as they are proposed to be involved in the mRNA
processing, essential to establish normal protein expression (reviewed by Chorev and Carmel, 2012).

**Figure 29.** Schematic representation of the bidirectional promoter shared between gene pairs, CAPZA3 and PLCζ. CAPZA3 contains only one exon whereas PLCζ consists of 15 exons (3-15 not shown), such that exon 1 and the majority of exon 2 are untranslated segments.

Yang et al. (2013) reported that 25-50% of disease-associated mutations were discovered within the exome regions but little is known of the non-coding regions and their association with clinical diagnostics. The human genome consists of 80% non-coding regions whose main function is to regulate gene expression (ENCODE Project Consortium, 2012). Mutations located within these regions disrupt function, and thus result in anomalous gene expression. The variants discovered in PLCζ have, for the most part, been found within the exonic regions of the gene and result in patients’ compromised fertility. Yet, the possible genetic contribution that may have led to the wide variation of PLCζ parameters observed by Grasa et al. (2008) and Kashir et al. (2013) has not been examine (Figure 30). It could be speculated that variants (mutations or SNPs) located within the non-coding regions of these patients have influenced their PLCζ expression and thus, their abnormal fertility phenotype. As briefly described by Yoon et al. (2008), it may be that a transcriptional factor or corresponding transcriptional factor binding site responsible for regulating PLCζ expression, may be mutated in patients suspected of
having OAD, thus resulting in their abnormal protein levels. Single nucleotide polymorphisms (SNPs) rarely cause a disorder with a pathological consequence but may be associated with a particular phenotypic diversity (Rahim et al., 2008). As is the case of PLCζ, it is possible that certain SNPs might influence gene expression and it may be worthwhile exploring the non-coding regions to identify potential crucial variants, which play a role in regulating PLCζ expression. Moreover, 50% of human genetic disorders were estimated to be due to an interrupted splicing pattern, signifying the importance of introns and their function at the genomic level (Ward and Cooper, 2010).

Figure 30. Immunofluorescent analysis in single sperm quantifying the mean relative total PLCζ fluorescence levels from control and infertile (OAD) patients. Asterisks (*) and hash tags (#, ##, ###, ##') represent statistically significant differences ($P \leq 0.05$) and statistically non-significant differences, respectively. Comparisons between groups are depicted by the symbols (e.g., # denotes comparison with one group, and ##' indicates comparison with another group). Data are shown as mean ±SEM. Reproduced from Kashir et al. (2013).
The MOAT, MOCA, PLCζ immunofluorescent analysis, and routine genetic screening are currently the most reliable and accessible methods to diagnose patients with sperm-related OAD conditions, although these tools are available only in limited clinics worldwide. An important point to consider is that the PLCζ immunofluorescent studies by Grasa et al. (2008), Heytens et al. (2009), Kashir et al. (2013), Nomikos et al. (2013), and in this thesis utilised a polyclonal antibody. Although the effectiveness of this antibody has been supported by peptide-blocking assays, there are potential concerns over its specificity and how this may impact upon experimental outcomes. One particular disadvantage is that polyclonal antibodies may amplify the signal from a target protein with low expression level. This could lead to inaccurate protein quantification, since the target protein interacts with more than one antibody molecule on multiple epitopes. In other words, there is a need to overcome these limitations, and an alternative approach is the generation of a specific monoclonal antibody. A prerequisite to the generation of a pure human recombinant PLCζ protein, which at the moment is still lacking in spite of the intense efforts at successful purification (this is described further in Chapter 4). A monoclonal antibody may provide a more robust and accurate diagnostic/prognostic assay for patients with sperm-related OAD conditions, and help elucidate the wide variation of PLCζ expression observed in fertile and infertile individuals.

The link between PLCζ and its role in infertility is becoming increasingly prominent. In terms of research, in the past it may have been laborious to consider screening the non-coding regions due to their large size and therefore could have been very expensive. However, with the array of current sequencing technologies and databases, targeted sequencing and tools for analysis have facilitated research to become more reliable, rapid, and cost-effective. Next generation sequencing is the preferred technology since it sequences DNA and RNA quicker and more cost-effectively than its conventional predecessor, Sanger sequencing. New high-throughput modern technologies have been
introduced which include Illumina, Roche, Ion torrent and SOLiD. There are different platforms of sequencing technologies but their main function is to sequence millions of small DNA fragments in parallel. The most distinguished feature of NGS is the ability to sequence the whole genome, however, this technology can also be applied for targeted sequencing, whereby only specific regions of the DNA are sequenced. **Figure 31** summarises the different methods of targeted DNA capture prior to sequencing and data analysis. Bioinformatic analyses assemble the DNA fragments together by mapping single reads to the human reference genome and databases contain tools, which can detect variants within the reads (Behjati and Tarpey, 2013). NGS has revolutionised genomic research and in line with reproductive medicine, has been employed to identify the specific roles of individual RNAs in sperm (Sendler et al. 2013). In light of this, it could provide a valuable platform to broaden our knowledge of the regulatory mechanisms involved with PLCζ expression and the causes of deficiency.

**Figure 31.** A summary of the commercially available custom designs for capturing targeted DNA NGS. SureSelect, HaloPlex, and Nextera are commonly used assays for library preparation and the capturing of targeted DNA, prior to NGS. These methods differ to the more established whole genome or exome sequencing as they focus on particular regions of the DNA making them more cost-effective and clinically pragmatic for laboratories. Reproduced and modified from Samorodnitsky et al. (2015).
3.1.1 Aims

1. To the best of our knowledge, Pan et al. (2013) were the only group to investigate the PLCζ promoter region in Chinese Holstein bulls or any other species. Although these authors failed to associate the variants detected with PLCζ expression or function, they provided preliminary data from which to begin exploring the non-coding regions (promoter and introns) of PLCζ in the human and the mechanism regulating its expression. The aim of this study was to seek possible association between the non-coding regions of PLCζ, and the significant variation of PLCζ expression observed in fertile and infertile patients (Grasa et al., 2008; Kashir et al., 2013). For this, I intended to use targeted NGS sequencing to detect variants encompassing two ROIs, the promoter and XY-linker of PLCζ. This study was outsourced to Source Bioscience (Nottingham, UK), and I was only responsible for determining the co-ordinates corresponding to the ROIs and the bioinformatics analyses following sequencing. Unfortunately, due to mapping failure, which led to the reads (sequences) being mapped onto a different gene, as opposed to PLCζ, an alternative method to detecting SNPs was devised. This involved using a SNP database (dbSNP) to collate information on variants discovered in PLCζ.

Hypothesis: SNPs located within PLCζ may influence expression of the resulting protein therefore, helping elucidate the wide variation of PLCζ parameters reported.

2. The second aim of this chapter was to investigate the cause of TFF experienced by two specific infertile patients, as recommended by their fertility clinic and selected for further study by myself, based upon pilot data. Preliminary analysis showed that these patients exhibited abnormal PLCζ expression parameters and further
assessment was carried out to elucidate the potential reasons for this. This involved routine PLCζ immunofluorescence analysis and genetic screening.

Hypothesis: Abnormal PLCζ features such as expression and/or genotype may be responsible for the patient’s infertile state.
3.2 Materials and methods

3.2.1 Sample acquisition and processing

Samples from infertile and control patients were acquired and processed as described in Section 2.2.1 (Chapter 2).

3.2.2 Immunofluorescence of sperm PLCζ

The method used for PLCζ immunofluorescent analysis followed that of Section 2.2.4 (Chapter 2).

3.2.3 Next generation sequencing (Illumina)

Following PLCζ immunofluorescent analysis, a number of patients were selected and categorised, according to their phenotypic PLCζ expression, prior to being subjected to NGS. NGS was performed to analyse variants from two ROIs; promoter and XY-linker of PLCζ, as they may account for the patient’s observed PLCζ expression.

3.2.3.1 Patient selection

Patients used in this study were suspected of having OAD from their respective clinics, and were selected and categorised for the NGS project, as described in Figure 32. Subsequent to PLCζ immunofluorescent analysis, the patients were categorised into case study 1 (potential problem with PLCζ) or 2 (no obvious problem with PLCζ). Case study 1 included patients who exhibited reduced PLCζ in total relative fluorescence and proportion compared to a fertile control. Case study 2 included patients who exhibited no significant difference in the aforementioned parameters when compared to a fertile control.
Figure 32. Patient selected and categorised for NGS. Following PLCζ immunofluorescent analysis, patients (n) were selected and categorised according to their phenotypic expression on two parameters of interest. Patients who exhibited reduced expression or no significant difference when compared to a fertile control were classified as “Case study 1” and “Case study 2”, respectively. “Miscellaneous” defined patients who exhibited an increase, decrease or no significant difference in the first parameter and a different outcome in the second parameter (i.e. Patient 70 displayed a significant reduction ($P<$0.05) in total levels of PLCζ and no significant difference ($P>$0.05) in proportion of sperm exhibiting PLCζ when compared to a fertile control).

3.2.3.2 Regions of interest (ROIs)

Targeted sequencing was the method used to identify variants within specific regions of PLCζ. Two ROIs were screened, ROI 1 and ROI 2, each comprising of 1840bp and 1852bp, respectively (Figure 33). The co-ordinates for each ROI were obtained from the web-based tool, UCSC genome browser (https://genome-euro.ucsc.edu/index.html), and the Human GRCh37/hg19 assembly was used, as requested by the outsourcing company (Source Bioscience, Nottingham, UK). “PLCZ1” and “CAPZA3” were the genes inserted into the search field and the accession number (NM_033123) was selected to obtain the co-ordinates for the ROI. CAPZA3 was included into this study as it shares a bidirectional promoter with PLCζ. The co-ordinates for ROI 1 and ROI 2 were 18,890,284-18,892,124 and 18,854,710-18,852,858, respectively, which were provided to the company for oligonucleotide probe design. The company then performed prerequisite processes, as
described in Figure 34, necessary to ensure effective and accurate sequencing and analysis.

Figure 33. Regions of interest (ROI) sequenced from patient samples. The detection of variants from the ROIs (red boxes including the intervening intronic sequences) may be responsible for the phenotypic diversity of PLCζ expression observed from both the infertile and control patients.

Figure 34. Schematic flow diagram describing the processes done by the company prior to NGS. Genomic DNA (gDNA) extracted from the patients had insufficient yield therefore, library preparation with Haloplex HS Custom designed kit was utilised, as opposed to the initial approach using SureSelect XT protocol. Red box indicates that I performed the analysis after receiving raw data from the company.
3.2.3.3 Oligonucleotide probe design and genomic DNA (gDNA) extraction

Prior to designing the probes, the company sent a confirmation that the probes would cover the ROIs, which was viewed using the Integrated Genome Viewer (IGV) application. Following confirmation, the company designed the probes using the SureSelect DNA Advanced Design Wizard, and extracted gDNA from each patient using the QIAamp DNA Investigator kit (Qiagen, UK), as described in the manufacturer’s protocol.

3.2.3.4 Library preparation

The company’s initial approach for preparing the libraries was the SureSelectXT protocol but the minimum gDNA yield required was ≥200ng, and the patients selected for NGS generated low yield. The protocol employed was therefore changed to the Haloplex HS Custom designed kit as the minimum gDNA was ≥50ng. The first step to library preparation involved the digestion of gDNA with 16 restriction enzymes (RE). The restriction enzymes were provided in two different coloured 8-well strip tubes (green and red). The first RE from each tube was combined (A) and this was repeated for the consecutive enzymes to create eight different master mixes (B to H). gDNA (≥50ng) from a patient sample was then split and added into each RE master mix. Each patient was subjected to their own RE master mixes (A-H) and the last few steps were repeated accordingly. In addition to the patient samples, an enrichment control DNA (ECD) supplied in the kit was treated like an individual sample. The ECD was included into the reaction to validate the double digestion protocol by using a High Sensitivity DNA Kit and the 2100 Bioanalyzer system with 2100 Expert Software. The reaction was then run using a thermal cycler, which involved incubation (37°C, 30min) and storing (4°C).

The second step for library preparation involved the selective hybridisation of the probes to gDNA fragment digests originating from target regions of the genome, and direct circularisation of these fragments. During hybridisation, Illumina paired-sequencing
adapters and degenerate molecular barcode sequences were incorporated into the targeted fragments. This was to allow the targeted fragments to bind to the sequencing flow cell and tracking of individual target amplicons during sequencing analysis. Each sample was also uniquely indexed. The subsequent step involved the removal of the hybridisation buffer, and the ligation of the circularised hybridisation products with DNA ligase. The targeted fragments, which contained biotin, were then captured on streptavidin beads. The beads were then washed with HS wash solution and NaOH (1M) to isolate the captured target libraries.

3.2.3.5 **PCR amplification, purification and library validation**

The captured target libraries were then amplified using the Herculase II Fusion DNA Polymerase kit (Agilent Technologies, USA) with optimal cycle conditions. This involved initial denaturation (98°C, 2min), followed by 30 cycles of denaturation, annealing, and elongation (98°C for 30s, 60°C for 30s, and 72°C for 1min, respectively). A final elongation step (72°C, 10min) was then followed and the amplicons were stored at 8°C. The amplified target DNA was then purified using AMPure XP beads. Each patient sample was then pooled and validated using the 2100 Bioanalyser system analysis, as described in **Section 3.2.3.4**. Samples with sufficient DNA libraries were then subjected to direct sequencing.

3.2.3.6 **Sequencing**

The instrument used for sequencing was the MiSeq platform with standard Illumina paired-end primers. As this was a targeted sequencing protocol, the read length was 75bp, which ensured maximum achievable coverage. The data output sent from the company was in the FASTQ format. This format provides per-base quality scores to each read.
3.2.4 NGS analysis

Dr. Donatien Chedom Fotso (from the Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Oxford, UK) helped with designing the pipeline for data analysis and validation of findings. The web-based platform, Galaxy (https://usegalaxy.org/), was used and this allowed for the FASTQ data to be manipulated and converted into a format that would map the reads into a reference genome assembly. The workflow to achieve this can be seen in Figure 35.

![Figure 35. Workflow for the preparation of FASTQ data prior to mapping on to a reference genome using web-based platform, Galaxy.](image)

3.2.5 SNP data collation

As a result of mapping failure (described in Section 3.3.6), an alternative method to identifying SNPs localised within the gene of interest, PLCζ, was using the SNP database (dbSNP; https://www.ncbi.nlm.nih.gov/projects/SNP/). This database includes annotations (i.e. previous reports, clinical significance and minor allele frequency) of each SNP.
3.2.6 Case study for two Patients with TFF

Two particular patient samples, 79 and 107, who had suffered from TFF were submitted to our laboratory from their respective clinics, Ninewells Hospital and Medical School (Dundee, Scotland) and Oxford Fertility (Oxford, UK). Exome sequencing for Patient 79 (performed in Dundee) showed no underlying genetic anomalies, which could have contributed to his infertile phenotype, thus leaving the clinic with very limited options for therapy, or even patient feedback. Since the patient was undergoing continuous semen monitoring, our laboratory performed three separate PLCζ immunofluorescent analyses (described in Section 2.2.4). Patient 79 submitted three samples at different time points: 13th April 2015, 25th March 2016, and 27th November 2016. The second analysis showed a significant improvement in PLCζ expression, and personal communication with the fertility consultant (Dr. Sarah Martins da Silva from Ninewells Hospital and Medical School) revealed that medications taken by the patient might have impacted on his PLCζ expression, although this was highly speculative. Therefore, a third immunofluorescent analysis was performed to support this proposition.

As for Patient 107, preliminary immunofluorescent analysis performed by a member of our group showed that this patient had an unusual PLCζ expression such that there was increase in both the total levels of PLCζ and proportion of sperm exhibiting PLCζ when compared to a fertile control. Out of the 32 patients I have investigated in this study, none has ever shown these combinations of parameters. I therefore performed a detailed immunofluorescent analysis to validate this (as described in Section 2.2.4). Due to time constraints on including this patient for NGS (Section 3.2.3), I opted instead to perform routine genetic screening (including promoter-exon 1 and XY-linker), as described in Sections 2.2.2.2, and 2.2.5. NGS may have detected potential variants in the intronic regions, which may be responsible for the PLCζ expression observed.
3.3 Results

3.3.1 Patient PLCζ immunofluorescent analysis

Table 8 summarises the immunofluorescent sperm analysis measuring the total levels of PLCζ and proportion of sperm exhibiting PLCζ of patients from different clinics. Patients were either control or potential OAD cases. A number of patients (specified on the table) were subjected for NGS, and this was according to the PLCζ expression from two parameters aforementioned. NGS patients were categorised into case study 1 or case study 2. Case study 1 included patients who exhibited a significant ($P<0.05$) reduction of PLCζ expression for both parameters when compared to a fertile control. Case study 2 included patients who exhibited no significant difference ($P>0.05$) when compared to a fertile control in the previously described parameters.

Table 8. Table illustrating the immunofluorescent sperm analysis from patients (control and potential OAD cases) from different clinics. The two parameters of interest were measured from each patient compared to a fertile control: total levels of PLCζ and proportion of sperm exhibiting PLCζ. The color highlight categorises the patient subjected for NGS. Blue: control, Gray: CS1, Orange: CS2. Asterisk (*) denotes that Dr. Suseela Yelumalai, a previous member of the Coward group, performed the immunofluorescent studies. The reduction or increase in both parameters were statistically significant ($P<0.05$), whereas the $P$ value for no significant difference was $>0.05$.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Total levels of PLCζ (a.u.)</th>
<th>Proportion of sperm exhibiting PLCζ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>62 (control)</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>66 (control)</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>83 (control)</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>88 (control)</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>95 (control)</td>
<td>Reduced</td>
<td>No significant difference</td>
</tr>
<tr>
<td>104 (control)</td>
<td>Increased</td>
<td>No significant difference</td>
</tr>
<tr>
<td>60 (case)</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>70 (case)</td>
<td>Reduced</td>
<td>No significant difference</td>
</tr>
<tr>
<td>71 (case)</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
</tbody>
</table>
Table 9. Continuation from Table 8.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Total levels of PLCζ (a.u.)</th>
<th>Proportion of sperm exhibiting PLCζ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>81 (case)</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>86 (case)</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>87 (case)</td>
<td>Reduced</td>
<td>No significant difference</td>
</tr>
<tr>
<td>90 (case)</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>92 (case)</td>
<td>No significant difference</td>
<td>Increased</td>
</tr>
<tr>
<td>113 (case)</td>
<td>Increased</td>
<td>Reduced</td>
</tr>
<tr>
<td>114 (case)</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>116 (case)</td>
<td>Reduced</td>
<td>No significant difference</td>
</tr>
<tr>
<td>118 (case)</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>119 (case)</td>
<td>Reduced</td>
<td>No significant difference</td>
</tr>
<tr>
<td>64 (case)</td>
<td>Reduced</td>
<td>No significant difference</td>
</tr>
<tr>
<td>68 (case)</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>72 (case)</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>76 (case)</td>
<td>Reduced</td>
<td>No significant difference</td>
</tr>
<tr>
<td>78 (case)</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>80 (case)</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>84 (case)</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>85 (case)</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>91 (case)</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>93 (case)</td>
<td>Reduced</td>
<td>No significant difference</td>
</tr>
<tr>
<td>94 (case)</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>98 (case)</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>99 (case)</td>
<td>Reduced</td>
<td>No significant difference</td>
</tr>
<tr>
<td>100 (case)</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
</tbody>
</table>
Table 10. Continuation from Table 8.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Total levels of PLCζ (a.u.)</th>
<th>Proportion of sperm exhibiting PLCζ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>101 (case)</td>
<td>Reduced</td>
<td>No significant difference</td>
</tr>
<tr>
<td>108 (case)</td>
<td>Reduced</td>
<td>No significant difference</td>
</tr>
<tr>
<td>110 (case)</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>112 (case)</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>PM (case)</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>25 (control)*</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>32 (control)*</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>33 (control)*</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>41 (control)*</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>47 (control)*</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>48 (control)*</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>58 (control)*</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>9 (case)*</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>15 (case)*</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>28 (case)*</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>34 (case)*</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>35 (case)*</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>49 (case)*</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>59 (case)*</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>21 (case)*</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>31 (case)*</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>53 (case)*</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
<tr>
<td>54 (case)*</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
</tbody>
</table>
The following Table 11 summarises the patients who were selected for NGS from Table 8. Briefly, these included 11 fertile controls, 11 CS1 patients, and 10 CS2 patients.

**Table 11.** A summary of the individuals subjected for NGS. These patients were selected according to their PLCζ phenotypic expression following immunofluorescent analysis.

<table>
<thead>
<tr>
<th>Fertile control (n=11)</th>
<th>Case study 1 (n=11)</th>
<th>Case study 2 (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>66</td>
<td>84</td>
<td>60</td>
</tr>
<tr>
<td>83</td>
<td>94</td>
<td>86</td>
</tr>
<tr>
<td>88</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>95</td>
<td>PM</td>
<td>85</td>
</tr>
<tr>
<td>25</td>
<td>9</td>
<td>91</td>
</tr>
<tr>
<td>32</td>
<td>15</td>
<td>98</td>
</tr>
<tr>
<td>33</td>
<td>28</td>
<td>21</td>
</tr>
<tr>
<td>41</td>
<td>34</td>
<td>31</td>
</tr>
<tr>
<td>47</td>
<td>35</td>
<td>53</td>
</tr>
<tr>
<td>48</td>
<td>49</td>
<td>54</td>
</tr>
<tr>
<td>58</td>
<td>59</td>
<td></td>
</tr>
</tbody>
</table>

### 3.3.2 Validation of oligonucleotide probe design

The company was provided with the co-ordinates for ROI 1 and ROI 2, and prior to design, validation was necessary to ensure that probes would specifically interact with the target regions. This was visualised using IGV, as seen in Figure 36 for ROI 1 (top panel) and ROI 2 (bottom panel), and the probes (blue) were shown to cover the target regions (green) successfully. A total of 1 and 2 probe groups were designed for ROI 1 and ROI 2, respectively. The probes were designed to yield as much coverage as possible and as for ROI 2, two probe groups were used to maximise coverage of this region. However, a small
region of ROI 2 failed to be covered (as seen by red arrow), as it was not possible to design probes of novel origin on some regions of the genome.

**ROI 1**

**ROI 2**

**Figure 36.** A screenshot depicting the probes designed to cover target regions. One probe group was created for ROI 1 (top panel, last blue line). Two probe groups were designed for ROI 2 (bottom panel, last blue lines). Red arrow indicates the region that failed to be covered by the probes.
3.3.3 Patient gDNA concentration following extraction

Following gDNA extraction, 12 patients were withdrawn from the project as a result of low yield. The remaining patients (n=20) with gDNA concentration of ≥50ng, as seen in Table 12, were processed for library preparation using the Haloplex HS Custom designed kit.

<table>
<thead>
<tr>
<th>Category (n)</th>
<th>Patient</th>
<th>gDNA concentration (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertile Control (5)</td>
<td>47</td>
<td>57.3</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>180.3</td>
</tr>
<tr>
<td></td>
<td>66</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>88</td>
<td>100.5</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>119.1</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>54.3</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>54.3</td>
</tr>
<tr>
<td></td>
<td>49</td>
<td>148.8</td>
</tr>
<tr>
<td></td>
<td>59</td>
<td>122.7</td>
</tr>
<tr>
<td></td>
<td>84</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>94</td>
<td>77.7</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>55.5</td>
</tr>
<tr>
<td>Case study 1 (7)</td>
<td>21</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>122.7</td>
</tr>
<tr>
<td></td>
<td>53</td>
<td>147.3</td>
</tr>
<tr>
<td></td>
<td>54</td>
<td>160.5</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>101.4</td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>63.6</td>
</tr>
<tr>
<td></td>
<td>91</td>
<td>78.9</td>
</tr>
<tr>
<td></td>
<td>98</td>
<td>138.3</td>
</tr>
<tr>
<td>Case study 2 (8)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.3.4 Validation of double digestion protocol

The first step of library preparation performed by the company involved the digestion of gDNA with 16 restriction enzymes to create a library of gDNA restriction fragments. The ECD samples (A to H) and unrestricted control DNA were subjected to analysis using the 2100 Bioanalyser system to confirm success of the double digestion protocol (Figure 37). The supplied ECD comprised genomic DNA and an 800bp PCR product, which contained the restriction sites for all the enzymes used in digestion. As seen from Figure 37, the
undigested control had bands at >7000bp and 800bp, whereas, the ECD samples had predominant bands at approximately 125, 225, and 450bp. These bands corresponded to the 800bp PCR product-derived restriction fragment. The bands from the ECD samples were observed at the expected molecular sizes indicating that the double digestion protocol had been successful.

![Figure 37](image)

**Figure 37.** Validation of the double digestion protocol using the 2100 Bioanalyzer system analysis. Lane 1: DNA ladder (50bp); Lanes 2 until 9: ECD samples A to H; Lane 10 Undigested ECD.

### 3.3.5 Validation of library preparation

In order to validate whether library preparation was successful, each patient sample was pooled, quantified, and analysed using the 2100 Bioanalyzer system. This step was important to ensure that an appropriate quantity of libraries were made prior to sequencing. Limited or extensive DNA may make it difficult to interpret the sequencing data as a result of poor resolution. In this study 10 out of 20 samples either had sufficient or insufficient libraries for sequencing, whereas, the remaining 10 would be suitable for sequencing following an additional cleanup step. A supplied positive control was also processed with the samples and satisfactory library preparation, characterised by a peak fragment between 225 and 540bp, can be seen on **Figure 38A.** **Figures 38B and 38C** showed traces from patient samples, which passed or failed library preparation, respectively. A prominent peak
was present at around 140-150bp from each trace, which denoted the presence of adapter dimers. **Table 13** summarises these observations from each patient.

![Graph A: Positive Control](image1)

**Figure 38.** Validation of library preparation using the 2100 Bioanalyzer system analysis. Trace (A) denotes the supplied positive control exhibiting satisfactory library preparation, (B) patients who have sufficient libraries, and (C) patients who have insufficient libraries. bp: base pair, FU: fluorescent units

**Table 13.** A summary of patient libraries authorised and withdrawn for NGS following library validation.

<table>
<thead>
<tr>
<th>Library validation</th>
<th>Patient (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Passed</td>
<td>Control: 2</td>
</tr>
<tr>
<td></td>
<td>CS1: 2</td>
</tr>
<tr>
<td></td>
<td>CS2: 1</td>
</tr>
<tr>
<td>Suitable for sequencing</td>
<td>Control: 2</td>
</tr>
<tr>
<td></td>
<td>CS1: 3</td>
</tr>
<tr>
<td></td>
<td>CS2: 5</td>
</tr>
<tr>
<td>Failed</td>
<td>Control: 1</td>
</tr>
<tr>
<td></td>
<td>CS1: 2</td>
</tr>
<tr>
<td></td>
<td>CS2: 2</td>
</tr>
</tbody>
</table>
3.3.6 Mapping of sequencing reads to a reference genome

NGS produced raw data in the FASTQ format which underwent a pipeline of analysis to ensure that the reads produced were of high mapping quality. The reads were first mapped onto the reference genome, Human GRCh37/hg19 assembly, as this was used to locate coordinates for the ROIs (requested by the company, as described in Section 3.2.3.2). From Figure 39, it can be seen that the reads did not map onto either of the ROI, and instead mapped onto the gene upstream of PLCζ, phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing gamma polypeptide (PIK3C2G).

Figure 39. Mapping of reads on to the Human GRCh37/hg19 assembly using IGV. Reads were covered on two regions (top and bottom panels) of gene, PIK3C2G (blue box). The red and black arrows indicate the depth of coverage and reads, respectively.
To further validate the incorrect mapping, a single read was subjected to BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi); this confirmed a 100% match to PIK3C2G and not PLCζ, as expected (Figure 40).

Figure 40. Representative BLAST screen shot of a single read. This tool showed that the read had a 100% match to PIK3C2G and not PLCζ, as expected (red box).

3.3.7 **Alternative method to detect SNPs in PLCζ**

Due to the mapping failure with the NGS protocol, it was necessary to identify an alternative method to detect SNPs in PLCζ. This study has never been performed for PLCζ in the human or any other species. Data collation was performed using dbSNP, a public domain archive for an extensive collection of common and rare SNPs across 55 different organisms. As dbSNP is in the public domain, any individual could report variants thus, in this study I have filtered the search for SNPs sequenced and validated by the 1000 Genomes Project. The 1000 Genomes Project was introduced to build an understanding of genetic variation in the human population, and included over 2500 individuals from 26 populations (Birney and Soranzo, 2015).

Herein, a total of 2987 PLCζ SNPs were reported and validated by the 1000 Genomes Project. Figure 41 shows a representative SNP and its annotations from the database. The clinical significance of each SNP was not reported as the samples chosen for the 1000 Genomes Project were anonymous, and no associated clinical or phenotype data were collected from these participants. Therefore, it was not possible to identify the
fertility state of the individuals. However, it was clear that 2928 out of 2987 SNPs were localised within the intronic regions of PLCζ, and the remaining 59 SNPs were in the 5' prime untranslated region (Table 14). Due to time restrictions, it was not possible to locate each SNP precisely to its corresponding introns.

Table 14. Summary of the 2987 SNPs located within PLCζ identified by dbSNP searches. The SNPs were localised either in the intronic or the five prime untranslated (5'UTR) regions, and clinical significance was not available.

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Location (n)</th>
<th>Clinical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>2987</td>
<td>Intron (2928)</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>5'UTR (59)</td>
<td></td>
</tr>
</tbody>
</table>
3.3.8 Case study for patients with TFF

Preliminary PLCζ immunofluorescence analyses performed for Patients 79 and 107, who had experienced TFF, showed unusual PLCζ expression parameters. These patients were then subjected to our routine PLCζ immunofluorescence to validate the initial observations. The initial plan to include Patient 107 for the NGS study was not possible due to time restrictions, and therefore PLCζ exonic screening was performed instead. The information of each patient can be seen in Table 15.

Table 15. Sample submission and clinical history of Patients 79 and 107.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Location</th>
<th>Sample submitted</th>
<th>Fertilisation cycle</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>79</td>
<td>Dundee</td>
<td>13th April 2015</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>25th March 2016</td>
<td>0/2 (IVF)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>27th November 2016</td>
<td></td>
<td></td>
</tr>
<tr>
<td>107</td>
<td>Oxford</td>
<td>9th May 2016</td>
<td>0/1 (IVF) 0/1 (ICSI)</td>
<td>Normal sperm analysis parameters</td>
</tr>
</tbody>
</table>

3.3.8.1 Patient 79

Exome screening for Patient 79, which was performed at the Ninewells Hospital and Medical School (Dundee, Scotland), revealed no underlying genetic anomalies (data not shown). Three complete PLCζ immunofluorescent analyses were performed for Patient 79, such that each was from a different sample provided at different time points (Table 15). Figure 42 denotes the first immunofluorescent staining for Patient 79. The total levels of PLCζ (5.1 a.u; Figure 42, left panel) and proportion of sperm exhibiting PLCζ from...
Patient 79 (27.3%; Figure 42, right panel) were significantly lower ($P<0.05$) when compared to a fertile control (12.6 a.u, 91%; Figure 42).

![Figure 42](image)

*Figure 42.* Immunofluorescent analysis of total levels (left panel) and proportion of sperm exhibiting PLCζ (right panel). This analysis compared sperm from patient 79 and a fertile control. Asterisk (*) indicates a significant difference ($P<0.05$). The left and right panels represent the data as mean±SEM and mean percentages, respectively.

Figure 43 represents the second immunofluorescent staining for Patient 79, almost one year later after his first analysis. The total levels of PLCζ (14.2 a.u; Figure 43, left panel) and proportion of sperm exhibiting PLCζ from Patient 79 (86.5%; Figure 43, right panel) showed no significant difference ($P>0.05$) when compared to a fertile control (15.7 a.u, 96.9%; Figure 43).

![Figure 43](image)

*Figure 43.* The second immunofluorescent analysis showing total levels (left panel) and proportion of sperm exhibiting PLCζ (right panel). The analysis compared sperm from patient 79 and fertile control. Hash tag (#) indicates no significant difference ($P>0.05$). The left and right panels represent the data as mean±SEM and mean percentages, respectively.

Figure 44 denotes the final immunofluorescent staining for Patient 79, eight months after his second analysis. The total levels of PLCζ (12.8 a.u; Figure 44, left panel) were significantly lower ($P<0.05$) when compared to the fertile control (18.8 a.u).
However, no significant difference ($P>0.05$) was observed for the proportion of sperm exhibiting PLC$\zeta$ from Patient 79 (98%; Figure 44, right panel) when compared to a fertile control (95.9%; Figure 44).

**Figure 44.** The third immunofluorescent analysis showing total levels (left panel) and proportion of sperm exhibiting PLC$\zeta$ (right panel). The analysis compared sperm from patient 79 and fertile control. Asterisk (*) indicates a significant difference ($P<0.05$) whereas, hash tag (#) denotes no significant difference ($P>0.05$). The left and right panels represent the data as mean±SEM and mean percentages, respectively.

In summary, almost one year after Patient 79’s first PLC$\zeta$ immunofluorescent analysis, there was an improvement in both parameters (Figure 45 and 46). Significant recovery was particularly observed in the proportion of sperm exhibiting PLC$\zeta$ following Patient 79’s first assessment (Figure 46). Prior to providing his second sample, the patient had discontinued taking fexofenadine (antihistamine) and started vitamin C supplements following the recommendation of Patient 79’s consultant. To confirm that the discontinuation of the antihistamine or consumption of vitamin C might have an impact on PLC$\zeta$ expression, a third analysis was performed. The final investigation, which occurred eight months after his second test, showed that there was a continual improvement in the proportion of sperm exhibiting PLC$\zeta$. However, fluorescent intensity of protein level remained the same when compared to the second analysis (Figure 45).
Figure 45. Timeline showing the immunofluorescent analysis of PLCζ total levels from Patient 79 when compared to a fertile control. The analyses were performed at three different dates following each sample submission.

Figure 46. Timeline showing the immunofluorescent analysis of proportion of sperm exhibiting PLCζ from Patient 79 when compared to a fertile control. The analyses were performed at three different dates following each sample submission.
3.3.8.2 Patient 107

As for patient 107, immunofluorescent analysis measuring the total levels of PLCζ (18.8 a.u; Figure 47 left panel) and proportion (%) of sperm exhibiting PLCζ (98%; Figure 47 right panel) were significantly higher ($P<0.05$) when compared to a fertile control (8.2 a.u, 79.8%; Figure 47). This confirmed the preliminary analysis (data not shown), which showed that there was an increase in both PLCζ parameters.

![Figure 47. Immunofluorescent analysis of total levels (left panel) and proportion of sperm exhibiting PLCζ (right panel). The analysis compared sperm from patient 107 and a fertile control. Asterisk (*) indicates a significant difference ($P<0.05$). The left and right panels represent the data as mean±SEM and mean percentages, respectively.](image)

As I had never encountered a patient showing this combination of parameters before, the ensuing step was to include Patient 107 in the NGS study to detect variants in his non-coding regions as these may have influenced the increased PLCζ expression observed. However, due to time restrictions imposed on this thesis, and while waiting for the outcome of the first NGS trial, I decided to perform our routine genetic screening instead to identify variants which may have impacted upon his PLCζ expression and/or the possible cause for his TFF. Exonic PLCζ screening for Patient 107 revealed a base change from thymine (T) to adenine (A) at position 578 of the ORF sequence. This corresponded to the substitution of a valine residue (V or Val) for glutamic acid (E or Glu) in the X catalytic domain at position 193 (V193E) of the amino acid sequence, PLCζ$^{V193E}$. Chromatograms produced from Sanger sequencing of the forward and reverse strands displayed green and red peaks at this specific position, corresponding to bases A and T,
respectively (Figure 48). This might indicate that Patient 107 is heterozygous for variant, PLCζ\textsuperscript{V193E}, such that the patient exhibits either the normal (valine) or abnormal (glutamic acid) residue at this specific position, therefore the patient may have sperm of normal and mutant population.

![Figure 48](image)

**Figure 48.** Representative chromatogram peaks denoting the presence of a variant from Patient 107. Patient 107 exhibited bases A and T at this specific position from both the forward and reverse strand.

A three-dimensional model of PLCζ\textsuperscript{V193E} was generated based on the crystal structures of human PLCβ2 to understand the effect of the variant on PLCζ structure and function. V193E correlated to a leucine residue (Leu) at position 350 (Leu350) of PLCβ2. The side chain of Leu350 was exposed on the surface of the catalytic domain of PLCβ2 and was part of an interface between the catalytic and PH domains (Figure 49). A mutation present at Leu350 in PLCβ2 could disrupt the interaction between the two domains, however, it is unknown as to whether it would affect protein function. As PLCζ lacks the PH domain, Val193 is likely to be exposed on the protein surface. Moreover, valine is hydrophobic and its exposure on the surface may suggest a potential intermediate interaction between PLCζ and other unknown molecules to regulate protein folding and stability. The change from Val to Glu, a negatively-charged hydrophilic residue may possibly alter the interaction of PLCζ at this specific position.
Figure 49. Three-dimensional model of PLCβ2 displaying the interaction between the X catalytic and PH domains. The exposed Leu350 (red circle) side chain on the surface of the X catalytic domain interacts with the side chains present on the PH domain. Therefore, the presence of a variant, instead of Leu350, may disrupt the interaction between the two domains. As PLCζ lacks the PH domain, Val193 may be exposed on the protein surface to interact with other molecules, which regulate PLCζ stability and folding. PLCζ\textsuperscript{V193E} may therefore obstruct this interaction, resulting in abnormal PLCζ function.

Due to time constraints, functional studies were not performed and thus, it was not possible to associate Patient 107’s TFF to the presence of PLCζ\textsuperscript{V193E}. Theoretically, this variant may be attributed to Patient 107’s fertility state as the structural effects suggest that it influences the folding and stability of PLCζ. Furthermore, it is unlikely that the variant influenced his increased PLCζ expression, as the effect of PLCζ\textsuperscript{V193E} seems to be more inclined on structural features, as opposed to regulating expression. Additionally, no genetic anomalies were identified in this patient when screening the PLCζ promoter-exon 1 segment and thus, future work should include screening of the intronic regions. The study on this particular patient is still at its preliminary stages but it is an exciting prospect as it could be the sixth PLCζ exonic mutation discovered in another patient suspected of having OAD.
3.4 Discussion

The wide variation of $\text{PLC}\zeta$ expression observed in fertile and infertile individuals remain an enigma in male reproductive research. Grasa et al. (2008) were the first to report this disparity and then Kashir et al. (2013) made similar observations. A more recent study by Yelumalai et al. (2015) yielded comparable findings, however, these authors further demonstrated that total levels and localisation patterns of sperm exhibiting $\text{PLC}\zeta$ were significantly correlated to fertilisation rates following treatment by ICSI but not by IVF. It can therefore be proposed that quantifying the total levels and localisation patterns of $\text{PLC}\zeta$ may already represent an effective prognostic and diagnostic marker for failed ICSI cases. Ferrer-Vaquer et al. (2016) also reported a significant variation of total $\text{PLC}\zeta$ levels in fertile controls and patients with TFF. Although the patients with TFF used in this study showed lower total $\text{PLC}\zeta$ levels, 6 out 12 fertile controls exhibited similar $\text{PLC}\zeta$ expression to these patients but had proven fertility. Nevertheless, the reason for such variation has yet to be determined and while it may be that a genetic factor is responsible, mutations in the $\text{PLC}\zeta$ exonic regions have only been discovered in OAD patients, not in fertile patients. It is difficult to understand why some fertile control patients exhibit no significant difference in $\text{PLC}\zeta$ expression to that in patients suspected of having OAD. It is logical to assume that other regions of the DNA sequence may be responsible for such variation, and that these may include the non-coding fragments such as the promoter and introns.

Pan et al. (2013) were the first and only research group to explore the promoter region of $\text{PLC}\zeta$ in Chinese Holstein bulls or any other species. These authors discovered variants, which influenced semen quality traits, and thus fertilisation outcome. However, they failed to associate their findings specifically to downstream processes such as $\text{PLC}\zeta$ expression or $\text{Ca}^{2+}$ oscillation-inducing ability. It was still however, interesting that the promoter region of $\text{PLC}\zeta$ was being explored as knowledge of its regulatory mechanisms
remains very limited. In addition to the promoter, there is a distinct lack of research in the intronic regions of PLCζ.

Abnormal PLCζ expression is highly distinctive in patients with globozoospermia and while this feature is limited to that of the infertile condition (Dam et al., 2007; Taylor et al., 2010), recent reports have shown that the heterogeneity of PLCζ expression extends similarly to normozoospermic sperm. According to the World Health Organisation (WHO), normozoospermia refers to sperm with normal parameters (concentration, percentage of progressive motility and morphology) equal to, or above, lower reference limits (WHO, 2010). Three recent cases have reported that patients with normozoospermic sperm and a history of repeated low fertilisation, or TFF, had reduced levels or abnormal PLCζ profiles (Lee et al., 2014; Chithiwala et al., 2015; Durban et al., 2015). The underlying mechanism for this variation in expression, and the reason as to why some males suffer from PLCζ deficiency while others do not, remains entirely speculative, but it represented 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 PLCζ, which may be involved in the regulatory activity of its expression, particularly promoter and intronic DNA.

Following the discovery of PLCζ genetic anomalies, which have affected fertilisation outcome in patients suspected of OAD (Heytens et al., 2009; Kashir et al., 2012; Pan et al., 2013; Escoffier et al., 2016; Ferrer-Vaquer et al., 2016), clinical cases involving PLCζ SNPs have been gaining increasing attention over the past two years. Schrmpf et al. (2014) performed a genome-wide association study (GWAS) in Hanoverian warmblood stallions and discovered that PLCζ along with 7 other genes were a locus for equine fertility. As a result of the role of PLCζ as the SOAF, the authors focused their study on the gene and identified 45 SNPs. Out of the 45 SNPs genotyped, three were strongly associated with fertility and located within the intronic regions of PLCζ.
(g.45595060G>T, g.45599001G>A, and BIEC2-952439). These SNPs did not influence the coding sequence of PLCζ but the authors proposed that they might alter regulatory functions by disrupting intronic microRNA (miRNA). miRNAs are responsible for gene silencing and are therefore able to downregulate protein production by terminating translation or destabilising mRNAs (Lee et al., 2006). Schrimpf et al. (2014) proposed that SNPs localised in miRNAs could disrupt its function thus, resulting in phenotypic diversity of PLCζ expression levels. Aside from the PLCζ variants discovered by Pan et al. (2013) and Schrimpf et al. (2014) in the bovine and equine model, respectively, Durban et al. (2015) were the first to identify three intronic PLCζ SNPs from a normozoospermic patient with recurrent TFF by screening the PLCζ exonic and their surrounding 50bp intronic regions. The authors proposed that these variants; one previously reported by Yoon et al. (2008), and two novel SNPs that had not been associated with a disease previously, might have led to the observed reduction in PLCζ expression in this patient. However, the precise reasons that underlie this abnormal PLCζ expression were not explored. It should be noted that the studies by Schrimpf et al. (2014) and Durban et al. (2015) were published at the time this DPhil commenced. Although the SNPs discovered by Schrimpf et al. (2014) and Durban et al. (2015) were not investigated experimentally and further research is necessary to support these claims, the authors have suggested that altered transcription, translation, or turnover, were possible mechanisms. This postulate concurs with that of Yoon et al. (2008), though only recently has research begun to focus upon the non-coding regions of PLCζ. Yoon et al. (2008) speculated that the abnormal levels, or absence of, PLCζ in their infertile patients might have been due to variants localised within transcription factors, therefore affecting the transcriptional process of regulating PLCζ expression.

The aim of this part of my thesis was to detect and analyse variants from two ROIs: one encompassing CAPZA3, the promoter and upstream-untranslated fragments of PLCζ, while the other encoded for the whole of the PLCζ XY-linker segment. The subjects of this
investigation were selected on the basis of two parameters from their \(\text{PLC}_{\zeta}\) immunofluorescent studies: total levels of \(\text{PLC}_{\zeta}\), and the proportion of sperm exhibiting \(\text{PLC}_{\zeta}\). Patients were categorised into case study 1, case study 2, and control groups. Case study 1 (\(n=11\)) included patients who exhibited a significant \((P<0.05)\) reduction of \(\text{PLC}_{\zeta}\) expression for both parameters when compared to a fertile control. Case study 2 (\(n=10\)) included patients who exhibited no significant difference \((P>0.05)\) in the aforementioned parameters when compared to a fertile control. The controls used in this study (\(n=11\)) were donors who had proven fertility. These patients were initially subjected to NGS for the detection of variants, which may have been responsible for the phenotypic diversity observed between them.

The NGS project started with a total of 32 patients and following gDNA extraction, only 20 patients had sufficient gDNA \((\geq 50\text{ng})\) to participate on to the library preparation step using the Haloplex HS Custom design kit. The insufficient yield of gDNA may have been due to genomic material being trapped on the FTA card matrix and additionally, some of these samples had been stored in the laboratory for a number of years and this too may have hindered the release of DNA (Ahmed et al., 2011). Following library preparation, only 15 out of 20 patients passed this process. A possible reason for this was that the gDNA extracted from the FTA cards might have been degraded or contaminated thus, affecting the outcome of library preparation. This was further confirmed by the positive control included in the kit, which was processed with the samples, as this produced a satisfactory library indicating that the poor performance was linked to the input sample quality. These 15 patients were then subjected to NGS to detect variants within the ROIs.

In this study, it was evident that the reads were not correctly mapped onto the target ROIs, and instead mapped on to the gene directly upstream of \(\text{PLC}_{\zeta}\), \(\text{PIK3C2G}\). This was the result of an error within the company, which is still being investigated. The company designed the oligonucleotide probes and validation was sent to ensure that the ROIs were
covered, and this was confirmed prior to starting the project. It was therefore clear that the misdirection occurred following the probe validation stage and unfortunately there was no way of knowing of this error during the time of gDNA extraction until sequencing. The approach of targeted sequencing was considered due to its low cost, low turnaround time to completion, and its ability to process a high number of samples. Future work may therefore involve whole exome sequencing to ensure more accurate findings, although the number of samples would need to be lower than that of targeted sequencing. As a result of this experience, it was not possible to elucidate the wide variation of PLCζ expression observed in fertile and infertile individuals. Following the identification of specific variants within each category, the variants could have been investigated and characterised by using web-based tool “MutationTaster”. This tool predicts the effect of all intronic variants on DNA and protein expression. Additionally, previous reports on any of the variants detected could have been determined using dbSNP.

Although the NGS experiment failed to achieve the aim of this chapter, it did not prevent further investigation of SNPs localised within PLCζ. I identified and collated, for the first time in human PLCζ research, over 2500 SNPs using dbSNP. The SNPs were filtered to include only variants validated by the 1000 Genomes Project. No clinical or phenotypic data were available from the database as the participants involved in the 1000 Genomes Project were anonymised. However, it was apparent that a majority were situated in the intronic regions of PLCζ. While it is established that SNPs rarely cause a disorder with a pathological consequence, they have been reported to be associated with a particular phenotypic diversity (Rahim et al., 2008). Thus for PLCζ, certain SNPs found in the non-coding sequences might influence gene expression. Schrimpf et al. (2014) and Durban et al. (2015) came to similar conclusions as they detected SNPs in intronic DNA and suggested that these may have appeared to alter PLCζ expression.
Introns were initially viewed as “junk DNA”, and lacking function in the eukaryotic genome (Chow *et al*., 1977; Berk *et al*., 1977). However, as reviewed by Chorev and Carmel, (2012), introns are now considered to represent crucial prerequisites to achieve normal protein expression. These segments are proposed to be multi-functional and substantially involved in all steps of mRNA processing: initiation, termination, and the regulation of transcription, genome organisation and alternative splicing. Intron-bearing genes in both humans and plants have increased protein expression when compared to intronless genes. This is due to intron-bearing genes generating more mRNA, although the mechanism involved in this phenomenon has yet to be determined (Gruss *et al*., 1979; Buchman and Berg, 1988; Le Hir *et al*., 2003; Akua *et al*., 2010). As a result, it could be hypothesised that genetic anomalies within intron-bearing genes might mediate the regulation of mRNA production and therefore, influence expression of the final protein. From the findings collated from dbSNP, it appears that PLCζ introns are susceptible to SNPs and therefore may have the ability to influence protein expression. This could be a plausible explanation to account for the significant variation of PLCζ expression seen in fertile and infertile patients. Although further research is necessary, one method to support this hypothesis could be using the aforementioned tool “MutationTaster” to identify the effect of each SNP on PLCζ DNA and protein level.

An important note, which has been stated numerous times in this thesis, was the use of the polyclonal antibody for PLCζ immunofluorescent studies. There are potential concerns over its specificity, and while generating a monoclonal antibody may be ideal for a more robust and effective diagnostic/prognostic marker for patients suspected of having OAD, its production is still under progress. A recent study by Kashir *et al*. (2016) described the use of antigen unmasking/retrieval (AUM) protocols to improve the visualisation of PLCζ immunofluorescence using polyclonal antibodies. Aldehyde fixation is commonly used for sample preparation but creates protein cross-linkages, which may
prevent the antibody from fully engaging with the epitopes (D’Amico et al., 2009). AUM
treatment has been shown to break down these cross-linkages (Leong and Leong, 2007;
D’Amico et al., 2009). It may therefore be prudent that current analysis employs this
technique as Kashir et al. (2016) implied a risk of misdiagnosis in its absence, and most
importantly, this may represent a more effective diagnostic tool for PLCζ-related OAD
cases in fertility clinics (Kashir et al., 2016). However, investigations are currently being
performed by Dr. Xin Meng (from the Coward laboratory) to support the claims that the
proposed method by Kashir et al. (2016) is an improved assay. These include testing
against existing methodology and appropriate peptide-blocking analysis, which were not
performed by Kashir et al. (2016).

The main aim of this chapter was to seek an association between the phenotypic
diversity of PLCζ expression in fertile and infertile patients. Although this conclusion
could not be reached as a result of NGS failure, it was still of interest to investigate the
potential correlation between abnormal PLCζ features in the two patients with TFF (79 and
107). Exome screening of Patient 79 by collaborators at Ninewells Hospital and Medical
School (Dundee, Scotland) showed no underlying genetic anomalies, which could have
resulted in the patient’s infertile state. However, exome sequencing covers only exons of
the patient’s whole genome, and variants located within the non-coding regions may have
played a role in this patient’s infertile condition. The second immunofluorescent staining
for Patient 79, which was done one year after his first analysis showed an improvement in
the total levels of PLCζ and the proportion of sperm exhibiting PLCζ. This was of interest
since no patients in our laboratory have re-submitted their samples before so it was
necessary to investigate the cause of these different PLCζ expression patterns. A third
sample was therefore requested and eight months after his second investigation,
immunofluorescent analysis showed that there was a continual improvement in the
proportion of sperm exhibiting PLCζ, however, total levels of PLCζ were similar
compared to the second analysis. It is unclear how this may have occurred but it may be hypothesised that discontinuing antihistamine medication and/or taking vitamin C supplements may have influenced the production of PLCζ. However, this is highly speculative although it may be possible that an external factor such as the administration of medications may exert effects on protein production but how this may have regulated PLCζ expression in the sperm remains unknown. Vitamin C has been closely associated with fertility as it is an antioxidant and has been claimed that supplementation with this compound increases semen quality (Salas-Huetos et al., 2017), and may therefore, have improved PLCζ expression. One interesting study has shown that the vitamin A derivative, retinoic acid, plays a crucial role in spermatogenesis, and its absence results in the reduction or complete loss of differentiating germ cells (Hogarth et al., 2013). However, replenishing the diet with retinoic acid reinitiates spermatogenesis (Hogarth et al., 2013). Although Patient 79 was shown to have normal semen analysis parameters, the antihistamine medication may have influenced the expression of particular proteins, including PLCζ. Further to this, vitamin C supplementation may have lead to the recovery of PLCζ expression levels as complete spermatogenesis occurs approximately every 70 days (Amann, 2008), thus accounting for the improvement observed the year following his first investigation. A plausible way to rescue OAD could therefore be supplementation with vitamin C.

As for Patient 107, initial immunofluorescent sperm staining, performed for training by Dr. Xin Meng (a new DPhil candidate from the Coward group) showed that he had higher levels of total PLCζ and the proportion of sperm exhibiting PLCζ when compared to a fertile control was also higher. This particular combination of PLCζ expression parameters had not been observed during my DPhil and therefore, I repeated the immunofluorescent analysis to confirm this. The results were consistent the second time and the initial plan was to include Patient 107 for the NGS study to discover possible
anomalies in his non-coding regions as these may have influenced the increased PLCζ expression observed. However, due to time restrictions, it was decided that our routine genetic screening would be performed to identify variants, which may have impacted his PLCζ expression and/or the possible cause for his TFF. No genetic abnormalities were observed when screening his promoter-exon 1 region, and thus the introns may be responsible for the increased expression. However, exonic screening identified a novel variant, PLCζV193E, in exon 6 corresponding to the X catalytic domain. The double peaks shown in the chromatogram indicate that the patient may be heterozygous for PLCζV193E but more research such as mutation detection in single sperm would be required to confirm this. A predicted three-dimensional model revealed that the side chain of PLCζV193E is exposed on the surface of the catalytic domain and could affect the interaction between PLCζ and other molecules. Due to time constraints, no further characterisation of PLCζV193E was possible but future work could test the function of PLCζV193E. This would involve the microinjection of PLCζV193E cRNA or protein into human oocytes to deduce the Ca²⁺ oscillation-inducing activity of Patient 107. This finding would be exciting, as it would comprise the second novel variant detected during the duration of this DPhil. This is consistent with the notion that the association of PLCζ with male infertility is gradually being acknowledged as a result of patients suspected of having OAD requesting routine screening protocols.

The main objective of this chapter was to search for a correlation between specific variants on the non-coding regions of PLCζ, and the significant variation of PLCζ expression observed in fertile and infertile patients (Grasa et al., 2008; Kashir et al., 2013; Yelumalai et al., 2015; Ferrer-Vaquer et al., 2016). Although this aim was not met, it has expanded our knowledge of advanced sequencing and the efforts placed on each stage to ensure downstream processes are regulated at optimal conditions. This awareness will be
useful for future projects so as to avoid the troubleshooting, which took place in this current study.

Moreover, since Kashir et al. (2016) reported an improvement for visualising PLCζ during immunofluorescent studies, it is clear that further investigations are required prior to incorporating AUM protocols into the existing method. Further to this, an antibody that is monoclonal in nature may overcome the potential disadvantages of the current methods. However, its production requires a pure hrPLCζ, which at the present time remains a significant challenge for researchers and is the subject of the next chapter.
3.5 Key findings

The aim of this chapter was to search for an association between the phenotypic diversity of PLCζ expression observed in fertile and infertile patients. The initial method used to accomplish this was NGS to screen for variants at specific regions of PLCζ, which may have influenced PLCζ expression in patients suspected of having OAD. However, as a result of mapping failure from the NGS protocol, an alternative method was employed which involved collating PLCζ SNPs from dbSNP. Using appropriate filters so as to not include invalidated variants, over 2500 SNPs were localised within PLCζ. The majority of these SNPs were intronic, and as introns are crucial elements for regulating gene expression, it could be hypothesised that certain SNPs might alter PLCζ protein levels.

As this chapter focused upon a potential causative factor of the wide variation in PLCζ immunofluorescent parameters, two interesting patients with TFF, 79 and 107, were investigated. An improvement in the second and third PLCζ immunofluorescent analyses was observed from Patient 79 when compared to his initial investigation. This improvement may have been the result of an external factor such as the discontinuation and/or administration of certain medications. As for Patient 107, the reason for his increased PLCζ expression when compared to a fertile control was not determined as his promoter-exon 1 segment showed no underlying genetic anomalies. However, a novel variant, PLCζ^{V193E}, may have played a role in the patient’s infertile condition. PLCζ^{V193E} was predicted to disrupt the interaction between the X catalytic domain and other molecules responsible for regulating PLCζ stability and folding.
3.6 Future work

- An attempt to troubleshoot, validate, and re-analyse samples with NGS – pending sample availability.

- An alternative method would be to carry out a GWAS using two groups of participants: patients suspected of having OAD and fertile controls. This type of study would locate genetic variations (SNPs) from each individual’s genome, and those detected more frequently in patients suspected of having OAD. As GWAS involves whole genome sequencing from a large cohort of participants (particularly fertile controls), cost and acquiring the appropriate number of samples may be a limitation but might overcome the issues which may arise during targeted sequencing.

- As a result of time restrictions, only the predicted three-dimensional model of PLCζ\(^{V193E}\) detected from Patient 107 was characterised. The next step would be to perform mutation detection in single sperm, as this would confirm the heterozygosity of PLCζ\(^{V193E}\). Further to this, a functionality test involving the injection of PLCζ\(^{V193E}\) cRNA or protein into human oocytes would be carried out to test \(\text{Ca}^{2+}\) oscillation-inducing activity. As Patient 107 was shown to exhibit increased PLCζ levels when compared to a fertile control, NGS on the non-coding regions, with particular interest in the introns, might elucidate the observed expression.

- As for the 2987 variants discovered in dbSNP, future work would identify the specific location of each SNP and categorise them in a manner that would facilitate the following step (i.e. each intron, promoter, untranslated 3 prime). This would be to use the web-based tool “MutationTaster” to characterise the effects of SNPs on PLCζ DNA and protein expression.
Chapter 4: Production of a human recombinant PLCζ protein
4.1 Introduction

OAD is characterised by the absence or abnormal release of Ca\(^{2+}\) oscillations upon fertilisation, and while the aetiology of this condition remained idiopathic for a long period of time, critical evidence has now associated the condition with abnormal PLC\(_{\zeta}\) parameters (Heindryckx et al., 2005; Yoon et al., 2008; Kashir et al., 2010; Yeste et al., 2016). OAD is suspected following a repeated number of failed ICSI cycles, and sperm-related OAD cases can be identified from MOAT and MOCA, particularly, patients categorised in group 1 (low MOAT activating capacity) (Heindryckx et al., 2005; Yoon et al., 2008; Heindryckx et al., 2008; Vanden Meerschaut et al., 2012; Vanden Meerschaut et al., 2013). Subsequently, the patient’s sample (buccal, blood or sperm) can be subjected to PLC\(_{\zeta}\) genetic screening and immunocytochemistry to determine the possible role of PLC\(_{\zeta}\) deficiency in the infertile phenotype (Yoon et al., 2008; Heytens et al., 2009; Kashir et al., 2012; Durban et al., 2015; Escoffier et al., 2016; Ferrer-Vaquer et al., 2016). These investigations, however, are only available in certain clinics.

The first line of treatment for OAD is the use of AOAs, the most common of which are calcium ionophores (A23187 or ionomycin) (Yanagida et al., 2008; Nasr-Esfahani et al., 2010). Calcium ionophores are administered concomitantly with ICSI and are accessible from only a limited number of clinics worldwide. AOAs are believed to exert cytotoxic, mutagenic, and epigenetics effects on the developing embryo. This stems from the calcium release profile, which comprises a single transient as opposed to the oscillations presented during normal oocyte activation (Nasr-Esfahani et al., 2010). The topic of AOAs, and their safety, has become very controversial with more focus on the generation of an alternative, safer, and endogenous therapeutic option, namely a hrPLC\(_{\zeta}\) (For review, see Amdani et al., 2013). Yoon et al. (2008) were the first to suggest the use of PLC\(_{\zeta}\) mRNA for OAD therapy, particularly in infertile patients with low PLC\(_{\zeta}\) expression but this was not feasible clinically, however, moderate success has now been
achieved in generating a hrPLCζ. **Figure 50** describes the history of the production of a hrPLCζ and it is clear that failure in its generation for clinical purposes outweighs success, and no advancements have been made since the publication of Nomikos and colleagues in 2013.

**Figure 50.** Timeline describing the pursuit of human recombinant PLCζ protein (hrPLCζ) production in mammalian and bacterial expression systems. The methodology performed by each author is specified along with functionality and clinical viability following hrPLCζ generation.

Only gradual progress has been made in producing a reliable and clinically safe hrPLCζ, as this requires numerous optimisations. PLCζ cRNA seemed to be an effective treatment option as it was able to induce Ca^{2+} oscillations indistinguishable from normal fertilisation when injected into aging human oocytes, and the authors also reported successful parthenogenesis leading to blastocysts (Rogers *et al.*, 2004). However, employing this method clinically might result in an unfavourable outcome to the developing embryo due to uncontrollable endogenous transcriptase activity in the oocyte.
That same year, Kouchi et al. (2004) expressed a mouse recombinant PLCζ protein from an insect cell line, which was used to validate critical properties of PLCζ when compared to PLCδ1. These were the ability of PLCζ to induce Ca^{2+} oscillation-inducing activity (indistinguishable to normal fertilisation) and high Ca^{2+} sensitivity using the enzymatic assay, PIP2 hydrolysing activity. From then onwards, more modifications were made and the preferred method for hrPLCζ production is now via a bacterial expression system, as seen in Figure 50 (Grasa et al., 2008; Yoon et al., 2012; Nomikos et al., 2013).

Grasa and colleagues were the first to attempt the production of hrPLCζ expressed by a bacterial cell line in 2008. Although the authors validated the protein’s identity as PLCζ using immunoblot analysis (Figure 51), functionality of the hrPLCζ was not tested (Grasa et al., 2008).

Yoon et al. (2012) successfully expressed hrPLCζ via E. coli and a His-tag fusion system, and were the first to investigate the functional properties of their recombinant protein. However, there were concerns over the abnormal Ca^{2+} release profile when injected into human oocytes, and the resulting yield of the protein was very low. Further to this, an excessively high concentration of hrPLCζ was required to induce Ca^{2+} oscillations, making it clinically unreliable (Yoon et al., 2012). The subsequent hrPLCζ produced by Nomikos et al. (2013) has been deemed to be the most successful, as of yet. A bacterial expression
system was applied, similar to that of Yoon et al. (2012), and their methodology also differed in that Nomikos et al. (2013) utilised a Nus-A tag bound to WT PLCζ. This hrPLCζ generated Ca^{2+} oscillations indistinguishable to normal fertilisation (Figure 52), and the authors were also able to rescue OA ability of mouse oocytes previously administered with mutant cRNA (PLCζ^{H398P} and PLCζ^{H233L}). This authenticated PLCζ as a powerful therapeutic agent. However, Nomikos et al. (2013) failed to show Ca^{2+} oscillation-inducing activity upon removal of the tag. Thus, it can be assumed that the effective mode of action observed and stability of the hrPLCζ is influenced by the tag. This would make the hrPLCζ clinically unreliable as the presence of the tag eliminates the endogenous nature of PLCζ. Patients may therefore be reluctant to utilise it as OAD therapy as the outcome, favourable or otherwise, has yet to be determined.

Figure 52. Ca^{2+} oscillation-inducing activity of hrPLCζ produced by Nomikos et al. (2013) in mouse and human oocytes. hrPLCζ injected into mouse and human oocytes displayed characteristic Ca^{2+} release profile to normal fertilisation, as seen in the top and bottom traces, respectively. The middle trace denotes the microinjection of the tag, Nus-A, alone in mouse oocytes. Reproduced and modified from Nomikos et al. (2013).

Kashir et al. (2011a) successfully created an active recombinant PLCζ protein via a mammalian expression system. A variant of the human embryonic kidney cell line, which expresses a mutant SV40 large T-antigen (HEK293T) was transfected with a relevant vector, linked to WT PLCζ and a C-terminal His-tag. Cell lysates were harvested since
His-tag facilitates purification, and subsequent tests were carried out to identify the protein and test its functionality. The authors identified the recombinant protein to be PLCζ as immunoblotting revealed a band of around 70kDa - the expected size of expressed PLCζ protein. Further to this, microinjection of WT hrPLCζ and mutant (PLCζH398F) recombinant protein into mouse oocytes either induced Ca²⁺ oscillations or failed to evoke any activity, respectively (Kashir et al., 2011a). Although these results were very encouraging, its form as a non-purified mammalian cell lysate prevented this product from being viable in the clinics but did provide further evidence that PLCζ is the necessary SOAF to induce the events following fertilisation. Since the work of Kashir et al. (2011a), no further attempts have been made in generating hrPLCζ using a mammalian expression system although this might prove to be more advantageous than the standard bacterial expression system, particularly for therapeutic purposes in the future. Furthermore, mammalian cell systems have the ability to induce PTMs, which may be crucial for endogenous PLCζ activity (Kurokawa et al., 2007; Cooney et al., 2010).

Studies in both the porcine and mouse models have demonstrated the need for PLCζ to undergo spontaneous proteolysis (Kurokawa et al., 2007). Using porcine sperm, these authors showed that sperm fractions (SF), cytosolic (SFC) and high-pH soluble (SFpH), both induced PLCζ-like activity and Ca²⁺-oscillations when injected into mouse oocytes. However, the sperm fractions differed such that SFC and SFpH contained or lacked PLCζ, respectively. Further investigation into the SFpH fraction revealed that it contained N- or C-terminal fragments of PLCζ, which were proposed to have been cleaved and then formed stable complexes capable of retaining PLCζ activity. To consolidate these findings, Kurokawa et al. (2007) co-expressed two complimentary mPLCζ cRNAs (residues 1-361 and 361-647), derived from the cleavage of the XY-linker region. Co-injection of these cRNAs induced Ca²⁺-oscillations in mouse oocytes, indistinguishable to those triggered by WT mPLCζ cRNA. These authors therefore suggested that PLCζ may
initially interact with proteases present in the oocyte prior to achieving enzymatic activity, although the process in humans has yet to be determined (Kurokawa et al., 2007). In addition to proteolytic cleavage, the bovine model has revealed other necessary PTMs in PLCζ for the establishment of enzymatic activity (Cooney et al., 2010). The authors produced a bovine recombinant PLCζ protein, but did not observe Ca\textsuperscript{2+} oscillation-inducing activity when a range of protein concentrations (1pg – 10pg) was injected into either bovine or mouse oocytes. It was assumed that as the recombinant protein was made via an \textit{E. coli} expression system, PTMs could not occur, and the absence of \textit{in vivo} activity was therefore observed (Cooney et al., 2010). Since this study, advancements in protein purifications, and the production of recombinant PLCζ protein in other species have overcome this lack of \textit{in vivo} activity but no follow-up study has investigated the requirement for PTMs in mammalian PLCζ. It is therefore advisable that future studies consider both proteolysis and PTMs to understand the mechanisms regulating PLCζ activity and whether they occur in hPLCζ. In addition to PTM activity, the mammalian expression system allows for precise protein folding and relevant enzymatic activity to occur – features essential for full protein biological function (Khan, 2013). However, the low yield and increased expense of producing a high quantity and quality recombinant protein with a mammalian system, when compared to its prokaryotic counterpart, limits its wider application (Khan, 2013).

Along with a safer and reliable therapeutic requirement for PLCζ-related OAD cases, hrPLCζ production is necessary for the formation of a monoclonal antibody and preliminary crystallisation studies; currently lacking in the field. An alternative method to overcome the potential specificity issue that comes with using a polyclonal antibody for current immunofluorescent analysis is the generation of a monoclonal antibody. This could provide a more powerful and accurate diagnostic/prognostic assay for patients suspected of having OAD, and widespread speculation of the precise localisation pattern of PLCζ in
human sperm (Grasa et al., 2008; Kashir et al., 2013). As for crystallisation, this would allow for the production of a high-resolution PLCζ protein three-dimensional structure. At present, only a predicted three-dimensional structure of PLCζ has been created via the established structure of PLCδ1, due to its strong homology with PLCζ (Heytens et al., 2009). This model has helped understand the functional effects of genetic variants in PLCζ (Heytens et al., 2009; Kashir et al., 2012a). These findings, however, are still based on a predicted model and the availability of a definitive structure would elucidate the effects of the variants on PLCζ structure function. This can be achieved by X-ray crystallography which could help identify the structural attributes of PLCζ which are presently absent or inconclusive; the precise shape and domain structure, the conformational changes PLCζ undergoes during oocyte activation and its interaction with other macromolecules in the oocyte, to evaluate PTMs, and facilitate the development of structural-based drugs to regulate PLCζ activity and function (Smyth and Martin, 2000; Acharya and Lloyd, 2005).

However, in order to achieve these goals, the progressive rate as to which the hrPLCζ is produced needs to be established. It is therefore necessary to optimise a methodology that could overcome the limitations of previously generated hrPLCζ in order to create a large amount of an active, purified and efficient hrPLCζ for both clinical and research purposes.
4.1.1 Aims

Preliminary work performed by Kashir et al. (unpublished) generated hrPLCζ using a bacterial cell line transformed with a vector fused to a Glutathione S-transferase tag (GST tag). This protocol was first described in Grasa et al. (2008), however, the authors failed to further validate protein identity and test functionality of their hrPLCζ. Additionally, the hrPLCζ produced by Kashir et al. (unpublished) had drawbacks as a result of inclusion body formation and thus, reduced yield. The initial aim of this study therefore was to optimise the experimental conditions in order to achieve sufficient expression of hrPLCζ and confirm its identity. While this was successful (as described in the following sections), assessing functionality failed, as evidenced by the absence of a Ca²⁺ release profile when the hrPLCζ was microinjected into mouse oocytes. Consequently, an alternative method was used for generating hrPLCζ via a mammalian cell line. When this was carried out, no further advancements were made in the creation of a hrPLCζ since Kashir et al. (2011a), however, as described in the previous section, progress is fundamental in order to replace the potentially hazardous use of AOAs for OAD treatment. The aim was therefore to adopt a more effective approach, leading to the secretion of a purified form of hrPLCζ.

Hypothesis: Utilising a different form of gene fusion technology may allow for the secretion and purification of the first mammalian-expressed hrPLCζ, which could be used in downstream clinical applications and represent a more viable option than the bacterial-derived hrPLCζ.
4.2 Materials and methods

Two expression systems were used during this study, bacterial and mammalian. The first method using a bacterial cell line, successfully isolated and identified the hrPLCζ. However, the following step involving testing its functionality by microinjecting into mouse oocytes failed. An alternative technique was therefore considered, which involved expressing hrPLCζ via a mammalian cell line. Due to time constraints, functionality was not investigated, however the protein purified was established as PLCζ by LCMS. A summary of the materials and methods is shown in Figure 53.

Figure 53. The schematic flow diagram depicts the protocols used to achieve production of a human recombinant PLCζ protein. Two different expression systems were attempted during this study; bacterial and mammalian. The latter was used following the failure of functionality from the protein expressed in bacteria.
4.2.1 Production of a hrPLCζ via a bacterial cell line

4.2.1.1 Transformation of competent E. coli cells

A pGEX-PLCζ-WT molecular construct (Figure 54), described by Grasa et al. (2008), was transformed into BL21 Star™ (DE3) pLysS chemically competent E. coli cells (Life Technologies, UK). The competent cells and construct were incubated on ice (30min) then heat-shocked in a water bath (42°C, 30s), and immediately transferred back to ice. Cells were then cultured in 600μl super optimal broth with catabolite repression media (SOC; Life Technologies, UK) on a shaker (37°C, 1h, 200rpm). Transformants were spread onto agar plates containing ampicillin (100μg/ml; Sigma-Aldrich, UK) and chloramphenicol (17μg/ml; Sigma-Aldrich, UK), and then incubated overnight (37°C).

Figure 54. The 4.9kb pGEX-PLCζ-WT construct utilised for the production of a hrPLCζ in a bacterial expression system. The elements of the vector, which facilitate isolation and purification, are also described.

4.2.1.2 Bacterial cell culture, protein expression, and protein purification

Successful transformants were cultured in 100ml Luria Broth medium (LB; Sigma-Aldrich, UK) containing ampicillin (100μg/ml) and chloramphenicol (17μg/ml), and incubated overnight on a shaker (37°C; 200rpm). Overnight cultures were diluted 1:100 with fresh LB medium containing the relevant antibiotics described above and incubated until OD_{600} (optical density), measured using a spectrophotometer, reached 0.6-0.7. In
order to increase the resulting protein concentration, bacterial cell culture was repeated 12 times to give a total of 12x1L culture. GST-fusion protein expression was then induced by
the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.1mM; Sigma-Aldrich,
UK) to each 1L culture and incubated at 16°C for 18h. Cells were then harvested by
centrifugation (8°C, 15min, 4500g), and the pellet resuspended in PBS (Oxoid, UK).
Phenylmethanesulfonyl fluoride (PMSF; 200mM; Sigma-Aldrich, UK) and
ethylenediaminetetraacetic acid (EDTA; 1mM; Sigma-Aldrich, UK) were added into the
suspensions, and sonicated 4 x 30s on ice. Triton-X-100 (1%; Sigma-Aldrich, UK) was
added, and the suspensions harvested by centrifugation (8°C, 30min, 9000g). The
supernatant and pellet were retained for purification and analysis, respectively.

The soluble GST-fusion proteins were purified by affinity chromatography through
a column packed with glutathione sepharose beads (600μl; GE Healthcare Life Science,
UK). Two columns were prepared for the purification of PLCζ-WT and PLCζ-WT tagged
with GST (GST-PLCζ-WT). The columns were washed with PBS followed by PBS + 1%
Triton-X-100, and the supernatant added into each column (supernatant before and after
application onto the beads were collected for protein analysis described in Section 4.2.1.3).
The column for the purification of PLCζ-WT was washed with TNE buffer (50mM Tris-
HCl, pH 8; 150mM NaCl; 10mM EDTA) and then with TNED buffer (50mM Tris-HCl,
pH 8; 150mM NaCl; 10mM EDTA; 1mM DTT). TNED + PreScission protease enzyme
(600μl; GE Healthcare Life Science, UK) was added into the column and incubated
overnight on a slow shaker (RT; 50rpm)). The eluate was collected, and the column
washed twice with TNED buffer (600μl) to collect two additional eluates. The beads were
also recovered for protein analysis. As for the isolation of GST-PLCζ-WT, the column was
washed with PBS following addition of the supernatant, and subsequently eluted with
glutathione elution buffer (600μl; Sigma-Aldrich, UK). Elution was repeated twice to
obtain three eluates in total for protein analysis.
4.2.1.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and protein identification by coomassie blue and silver staining

NuPAGE loading buffer and NuPAGE sample reducing agent (Life Technologies, UK) were added to the samples (eluates, supernatant before, supernatant after, pellet and beads), and along with the Novex sharp pre-stained protein standard ladder (Life Technologies, UK), were incubated at 101°C for 5 min. The samples and ladder were then loaded into a Novex Bis-Tris pre-formed 10% gel with MES-SDS running buffer (Life Technologies, UK) and left to run at 160 V (1 h). The gel was washed twice with ultra-pure water and stained with coomassie blue (1 hr; Life Technologies, UK), then destained overnight with ultra-pure water. The Pierce Silver Stain Kit (Thermo Fisher Scientific, UK) was used following the manufacturer’s protocol, to detect the concentration of proteins less than 1 ng. The gel was viewed using the GelDOC-IT TS imaging system (Bio-Rad, UK), and bands localised at the expected molecular weight (PLCζ-WT: ~70 kDa, GST-PLCζ-WT: ~90 kDa) were excised and submitted for liquid chromatography mass spectrometry (LCMS) to validate protein identity (courtesy of Professor Benedikt Kessler, Nuffield Department of Medicine, University of Oxford, Oxford, UK). LCMS is an analytical technique that combines LC, which separates components of a sample (i.e. protein), and MS to create and detect charged ions. This method is used to provide information on the molecular identity, weight, and structure of the particular sample components (Pitt, 2009). Briefly, LCMS involves the digestion of protein into peptides by trypsin the followed by matching to an established protein sequence (PLCζ1 accession number: Q86YW0) obtained from UniProt (www.uniprot.org).
4.2.1.4 Mouse oocyte collection, culture, protein microinjection, and Ca\textsuperscript{2+}
imaging

Following validation of protein identity, the eluted products were concentrated and Ca\textsuperscript{2+} was removed with Ca\textsuperscript{2+}-free exchange buffer (120mM KCl, 20mM HEPES, pH 7.4) using the Vivaspin 20 VS2001 ultrafiltration unit with a 30kDa molecular weight cut off (Appleton Woods, UK) following the manufacturer’s protocol. The concentration of the cleaved (14.6μg/ml) and non-cleaved (30.9μg/ml) proteins was measured using Qubit.

Miss. Goli Ardestani performed the ensuing microinjection protocol (from the Department of Veterinary and Animal Sciences, University of Massachusetts Amherst, USA).

Female mice (CD-1, 6 to 10-weeks-old) were stimulated with pregnant mares serum gonadotropin (PMSG; Sigma, USA), and after 46 to 48 hours, hCG (5IU) was administered for ovulation. MII oocytes were then collected from the oviducts 13 to 14 hours after hCG injection. Cumulus cells were removed with bovine testis hyaluronidase (0.1%; Sigma, USA). All animal procedures were performed according to research animal protocols approved by the University of Massachusetts Institutional Animal Care and Use Committee. The media used for collection was HEPES-buffered Tyrode’s lactate solution (TL-HEPES) containing 5% heat-treated fetal calf serum media.

Ca\textsuperscript{2+} was measured using the Ca\textsuperscript{2+} sensitive dye, Fura-2-acetoxymethyl ester (Fura 2-AM; Molecular Probes, USA). MII oocytes were loaded with Fura-2AM supplemented with 0.02% pluronic acid (1.25uM; Molecular Probes, USA) and incubated (RT, 20min). Oocytes were placed in micro-drops of TL-HEPES on a monitoring glass bottom dish (MatTek Corporation, USA) under mineral oil. Oocytes were monitored simultaneously using an inverted microscope (Nikon Instruments, USA) equipped with fluorescence detection. Fura 2-AM was excited between 340nm and 380nm by a filter wheel (Ludl Electronic Products Ltd, USA), and fluorescence was captured every 20s. Oocytes were monitored in Ca\textsuperscript{2+} containing TL-HEPES (2mM).
Injected oocytes were transferred to drops containing KSOM under mineral oil at 37°C in a humidified atmosphere of 5% CO₂ (EmbryoMax KSOM Medium (1X) w/ 1/2 Amino Acids; Millipore, USA)). PLCζ proteins were centrifuged at 13000rpm for 5 minutes, and the top 1–2μl was used to prepare micro-drops from which glass micropipettes were loaded by aspiration. PLCζ was delivered into MII oocytes by pneumatic pressure (PLI-100 picoinjector; Harvard Apparatus, Cambridge, Massachusetts, USA). The estimated concentration of protein injected was ~15μg/ml and each oocyte received 5–10pl (approximately 1–3% of the total oocyte volume). Oocytes that survived the injection were immediately transferred for Ca²⁺ monitoring.

4.2.2 Production of a hrPLCζ via a mammalian cell line

As a result of the failure to demonstrate functionality of the bacterial-expressed hrPLCζ, as characterised by the absence of Ca²⁺ oscillation inducing activity, an alternative method was considered. This involved production of hrPLCζ using a mammalian cell line.

4.2.2.1 Cloning of PLCζ for the incorporation into pHLsec vector

Full length cDNA for human PLCζ was amplified by PCR (forward primer with restriction site for *Agel*: 5’-TATACGGGTATGGAAATGAGATGGTTTTTG-3’; reverse primer with restriction site for *KpnI*: TATGGTACCTCTGACGTACCAAACATA-3’). The PCR conditions involved an initial denaturation (94°C, 4min), followed by 10 cycles of denaturation, annealing, and elongation (94°C for 10s, 58°C for 60s, and 72°C for 2min, respectively). A second round of 20 cycles included further denaturation, annealing, and elongation (94°C for 15s, 58°C for 30s, and 72°C for 2min, respectively), followed by a final extension step (72°C, 7m) prior to storing the amplicons at 4°C to await further analysis. The resulting amplicons were analysed, purified, and measured using the methods described in Section 2.2.2.2, Section 2.2.2.3, and Section 2.2.2.1, respectively.
The construct used in this study, pHLsec, was based on the ~4.2kb pLEXm vector backbone (as described in Aricescu et al., 2006). The elements of the vector can be seen in Figure 55. The insert (amplicon) was incorporated into the vector, and digested for 2h with appropriate restriction enzymes (AgeI and KpnI). The insert was then ligated using the Rapid DNA dephosphorylation and ligation kit (Roche, UK), according to the manufacturer’s protocol.

**Figure 55.** The pHLsec-PLCζ-WT construct utilised for the production of hrPLCζ expressed in a mammalian cell line. The features of the vector, which facilitate isolation and purification, are described.

### 4.2.2.2 Transformation of competent E. coli cells and plasmid DNA analysis by restriction digestion

Ligated products were then transformed into TOP10 chemically competent *E. coli* cells (Life Technologies, UK), following the same method as in Section 4.2.1.1. However, the transformants were spread onto agar plates containing only ampicillin (100μg/ml; Sigma-Aldrich, UK). The next day, three colonies were cultured separately in 100ml LB medium containing ampicillin (100μg/ml) and incubated overnight on a shaker (37°C; 200rpm).
Plasmid DNA was then extracted using the QIAprep Spin Miniprep kit (Qiagen, UK), following the manufacturer’s protocol. Single and double digestion with the aforementioned restriction enzymes was performed to ensure that the insert was incorporated into the vector. The products were analysed on a 1% gel using agarose gel electrophoresis (Section 2.2.2.2). The band presented at the expected molecular weight (~1.8kb) was extracted using Qiagen gel extraction kit (Qiagen, UK), in accordance to the manufacturer’s protocol, and submitted for Sanger sequencing to verify its identity as PLCζ. Following validation in which the sequence obtained showed 100% similarity to PLCζ ORF, as described in Section 2.2.2.3, an additional transformation was performed to ensure sufficient DNA for transfection in Section 4.2.2.3. Transformed colonies were cultured in 100ml LB medium containing ampicillin (100μg/ml) and incubated overnight on a shaker (37°C; 200rpm). Plasmid DNA was then extracted using the QIAprep Spin Midiprep kit (Qiagen, UK), following the manufacturer’s protocol. DNA concentration was measured as described in Section 2.2.2.1.

### 4.2.2.3 Mammalian cell culture and DNA transfection

HEK293T cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; consisting of 10% fetal bovine serum, 5μl/500ml penicillin, 5mg/ml streptomycin, 2mM L-glutamine and sodium pyruvate; Sigma-Aldrich, UK). At 70-80% cell confluency, the pHLsec-PLCζ-WT vector was transfected into the cells using the jetPEI transfection kit (Polyplus-transfection, France), following the manufacturer’s protocol. The cells were left to incubate at 37°C for 5 days. Optimal supernatant collection for highest protein expression occurs within four to six days of incubation, as described in Falkenhagen et al. (2016) and Güler-Gane et al. (2016). Following the first day of incubation, the medium was replaced with fetal bovine serum-free DMEM. The supernatant was then collected from each flask on the last day of incubation and stored at -80°C until protein purification.
**4.2.2.4 Protein purification**

PMSF (200mM) and EDTA (1mM) were added to the supernatant, and harvested by centrifugation (4500g, 30min, 8°C). The resulting supernatant (FC-tagged soluble proteins) was purified by affinity chromatography packed with protein-A sepharose beads (600μl; GE Healthcare Life Science, UK). The column was firstly washed with PBS prior to adding the supernatant to the column, which was then washed with PBS again. Human rhinovirus (HRV) 3C protease enzyme and HRV 3C reaction buffer (Thermo Fisher Scientific, UK) were added to the column and left to incubate overnight on a slow shaker (RT; 50rpm). The eluate was collected and protein analysis was performed as described in Section 4.2.1.3. Detection was carried out using only silver staining prior to sending the excised band for LCMS.
4.3 Results

4.3.1 Production of hrPLCζ via a bacterial cell line

The construct used for the expression of hrPLCζ via bacterial cell lines was described by Grasa et al. (2008). The authors performed an immunoblot analysis using anti-human-PLCζ antibody with their hrPLCζ and confirmed the presence of a band at ~100kDa (GST: 26kDa, PLCζ-WT: 70kDa; as seen in Figure 56). In this study, human PLCζ bound to a GST tag was expressed in E. coli and purified by affinity column chromatography. Purification of PLCζ-WT and GST-PLCζ-WT was facilitated by the cleavage of protease enzyme and glutathione elution buffer, respectively. The cleaved and non-cleaved elutes were then subjected to SDS-PAGE, and protein detection with either coomassie blue or silver stain. The expected band size for the cleaved product, 70kDa (PLCζ-WT), was not present in the gel (Figure 56A). However, a prominent band at ~90kDa was seen from the pellet, which corresponded to the protein molecular weight of GST-PLCζ-WT (GST 26kDa + PLCζ-WT 70kDa). The same band was also observed from Figure 56B, in both the pellet and non-cleaved protein, consistent with the protein molecular weight of GST-PLCζ-WT. A band at ~60kDa has previously been ascribed to a Chaperone protein by the Coward group. Chaperone proteins, and the protease systems, are known to be involved in proteostasis. This includes mediating the regulation of protein synthesis, folding, unfolding, and turnover (Kim et al., 2013; Saibil, 2013). Chaperoning activity ensures that proteins attain their native and biologically active conformations. The identity of the chaperone protein seen in Figure 56 has yet to be determined but the most common forms found in the eukaryotic system are heat shock proteins (HSP60, HSP70, HSP90, and HSP100; Kim et al., 2013; Saibil, 2013).
Figure 56. SDS-PAGE (10%) gels with coomassie blue staining. Purification of (A) cleaved and (B) non-cleaved proteins. Bands highlighted with Red arrow: GST-PLCζ-WT; Black arrow: Chaperone protein. SBP: supernatant before purification; SAP: supernatant after purification.

The potential presence of GST-PLCζ-WT in the pellet was seen in both gels (Figure 56), therefore the cleaved and non-cleaved proteins were subjected to silver stain, as the concentration may have been too low to be able to detect PLCζ-WT using coomassie blue. From Figure 57, the expected band sizes for the cleaved and non-cleaved proteins were observed at ~70kDa and ~90kDa, respectively. These bands were excised and submitted for LCMS for protein validation. LCMS confirmed the cleaved and non-cleaved proteins as PLCζ and GST-PLCζ, respectively. These were deduced from the protein scores, which was 2232 and 317 for PLCζ and GST-PLCζ, respectively. The protein score
calculates the matches made by the peptides (band digested by trypsin) to the given protein (in this case PLCζ), and a higher score signifies a more confident match.

**Figure 57.** SDS-PAGE gel (10%) with silver staining. Purification of the cleaved and non-cleaved proteins prior to validation, as bands were observed on the expected molecular weight, ~70kDa and ~90kDa, respectively. Bands highlighted with the white arrow: excised and subjected to LCMS.

### 4.3.1.1 Microinjection of bacterial-expressed hrPLCζ into mouse oocytes to investigate Ca²⁺ oscillation-inducing activity

As LCMS identified both the cleaved and non-cleaved proteins as PLCζ, the following step was used to test its functionality by microinjecting each protein into mouse oocytes. Mouse oocytes have been the model cell used for testing functionality of PLCζ; both cRNA and recombinant proteins (Heytens *et al*., 2009; Kashir *et al*., 2012a; Nomikos *et al*., 2013). The resulting microinjections into mouse oocytes from both PLCζ-WT (n: 13) and GST-PLCζ (n: 7) failed to induce any Ca²⁺ oscillation-inducing activity (**Figure 58**). The protocol was repeated twice to confirm what was observed at the first attempt.
Figure 58. The microinjection of hrPLCζ into mouse oocytes. The protocol was performed to investigate the Ca^2+ oscillation-inducing activity of PLCζ-WT (top panel) and GST-PLCζ-WT (bottom panel).
4.3.2 Production of hrPLCζ via a mammalian cell line

As the bacterial-expressed hrPLCζ was non-functional, it was decided that an alternative method was used, in which hrPLCζ was expressed in a mammalian cell system. This was considered to be more likely to succeed than its prokaryotic counterpart, particularly for its use in future OAD therapy.

4.3.2.1 Cloning of PLCζ for the incorporation into pHLsec vector

Full length cDNA for human PLCζ was amplified by PCR and visualised using agarose gel electrophoresis. Figure 59 showed a band at ~1.8kb, which corresponded to the expected molecular weight of PLCζ. Furthermore, the single and double digestion protocol was performed to validate the identity of the insert following ligation and transformation.

A single digestion reaction produced a single band at ~6.0kb, which was consistent with the molecular mass of the linearised plasmid DNA (Figure 60), whereas the double digestion reaction generated bands at ~1.8kb and ~4.0kb, correlating to that of PLCζ and pHLLsec vector, respectively (Figure 60). The purified amplicons were submitted for
Sanger sequencing to further validate the identification of the samples and analysis showed a 100% similarity to the PLCζ ORF sequence.

![Representative 1% agarose gel showing the single and double digestion reactions with restriction enzymes, AgeI and KpnI. The red arrow indicates a band from the double digestion reaction which, corresponds to the WT-PLCζ-ORF molecular mass of ~1.8kb.](image)

**Figure 60.** Representative 1% agarose gel showing the single and double digestion reactions with restriction enzymes, *AgeI* and *KpnI*. The red arrow indicates a band from the double digestion reaction which, corresponds to the WT-PLCζ-ORF molecular mass of ~1.8kb.

### 4.3.2.2 Synthesis of hrPLCζ using a mammalian cell line

HEK293T cells were transfected with the relevant vector containing PLCζ-WT and a secretion signal sequence, which would release the protein into the supernatant. The supernatant was then collected and purified by affinity column chromatography. The presence of the FC-tag facilitated cleavage from the protein by 3C protease. The identity of the eluate was assessed by SDS-PAGE and silver stain. The band of interest from the eluate at 70kDa can be seen in **Figure 61**, and was consistent with the molecular mass of PLCζ-WT. The chaperone protein was also present at 60kDa. The band, which corresponded to PLCζ-WT, was excised and subjected to LCMS. LCMS provided a robust identification that the submitted band was PLCζ, and the score was 142. An important point is that each sample subjected for LCMS, both bacterial- and mammalian-expressed hrPLCζ, was submitted at different times as a purification of the former hrPLCζ was
achieved first. The LCMS experiment performed would therefore produce a variable score, and thus the values observed from the bacterial-expressed hrPLCζ (2232 and 317) cannot be compared with the score generated from hrPLCζ expressed by the mammalian cell line. However, further discussion of the results with a member of Professor Benedikt Kessler’s team indicated that the score was sufficient to identify the protein as PLCζ, and it was suggested that increasing the protein concentration by culturing more mammalian cells would most likely improve the protein score. Due to time restrictions, the functionality of the mammalian-expressed hrPLCζ was not investigated in this thesis.

Figure 61. SDS-PAGE (10%) with silver staining for hrPLCζ expressed by a mammalian cell line. Band highlighted with Red arrow: excised and subjected to LCMS; White arrow: Chaperone protein.
4.4 Discussion

The only available treatment option for patients with OAD involves the use of AOAs, commonly calcium ionophores such as A23187 or ionomycin. A number of live births have been established using this approach, although the technique remains the centre of controversy as a result of potential mutagenic, cytotoxic, and epigenetic threats to the developing embryo. Fertility clinics offering AOAs only offer treatment in extreme cases – globozoospermia or PLCζ deficiency (Yoon et al., 2008; Heytens et al., 2009; Vanden Meerschaut et al., 2014; Lee et al., 2014; van Blerkom et al., 2015). These concerns remain theoretical, as no experimental evidence in their support has been reported. In particular, it is evident that these agents do not evoke physiological oscillations but instead, trigger a single Ca^{2+} transient from all intracellular stores, even those not involved in oocyte activation.

Santella and Dale, (2015) criticised a comprehensive prospective multi-center study performed by Ebner et al. (2015), which suggested that AOA using A23187 improved the fertilisation rate of patients with previous poor fertilisation outcome using ICSI. Santella and Dale, (2015) outlined a number of effects of potential Ca^{2+} ionophores on cell homeostasis, and may influence gene expression in the long-term. Additionally, these authors also believed that the successful pregnancies reported by Ebner et al. (2015) were simply the result of the injected spermatozoa, as opposed to the injected Ca^{2+} ionophores. In agreement with this, a recent commentary released by Ebner et al. (2016) proposed that the use of Ca^{2+} ionophores may disrupt the epigenetic reprogramming of the genome and thus, may lead to an altered gene expression pattern. The general consensus is that AOAs are still “experimental” and that trials using a larger cohort of patients are a prerequisite to corroborate the clinical safety of these agents. Although it is currently a theoretical risk, Santella and Dale, (2015) have pointed out the importance of understanding the safety of AOAs prior to its use with utmost confidence if taken on board by more fertility clinics.
van Blerkom and colleagues also raised concerns about the clinical use of AOAs, and expressed disappointment that the HFEA had allowed this approach (van Blerkom et al., 2015). The authors warranted that more research is needed, with particular focus on the single ionophore-induced Ca\(^{2+}\) transient, as this may create undesirable downstream consequences. Ozil and Huneau, (2001) have shown that changes in the Ca\(^{2+}\) release profile in rabbit oocytes can have detrimental effects, which are not manifested during the preimplantation stages but during organogenesis. It is therefore imperative to generate an alternative safer therapy. An ideal form would be an agent that mimics the activity of endogenous PLCζ, namely a human recombinant PLCζ protein (hrPLCζ).

Optimising the appropriate method for protein purification requires several important factors: expression system, fusion protein, tag localisation within the gene of interest, IPTG concentration, incubation time and temperature. The expression system employed is dependent upon what the resulting recombinant protein will be used for which may include industrial, therapeutic, and basic research applications. Typically, the expression system is one that maintains the protein’s endogenous form and activity upon production. The most widely used system is bacterial, due to its relatively simple physiology, short generation time, large-scale product and therefore, cost effectiveness. However, the disadvantage of this system is that it may lead to improper protein folding which may compromise the activity of the protein. Further to this, due to the simplicity of prokaryotic genetic modifications, this system lacks the enzymes necessary to carry out PTMs, which are vital for protein function thus making this an unfavorable option for therapy (Khan, 2013). It is therefore more suitable for applications in the laboratory or on an industrial scale.

When choosing the expression system, it is crucial that the appropriate fusion protein/tag, enzyme for cleavage and tag location are selected as this facilitates purification. Gene fusion technologies have overcome limitations such as reduced yield,
increased production of inclusion bodies and low solubility. Tag location also impacts upon the activity and structure of the protein, such that Malhotra, (2009) suggested that tags should be localised at the N-terminus, instead of the C-terminus end of the target protein. Such a location provides efficient translation initiation, and that the tag can be readily removed without leaving additional residues at the N-terminal region.

Grasa et al. (2008) were the first to attempt the production of hrPLCζ expressed by a bacterial cell line. These authors generated their hrPLCζ using a GST tag, due to its small size (26kDa), which could easily be cleaved by 3c protease upon purification. Although the authors were able to validate the protein’s identity as PLCζ, functionality of the hrPLCζ was not tested (Grasa et al., 2008). Yoon et al. (2012) utilised a His-tag fused to the N-terminal region of PLCζ to create their hrPLCζ, and while the His-tag is commonly used, due to its small size and ease of elution via imidazole (Costa et al., 2014), the potential degradation of histidine residues reduces the affinity of the tagged protein which may decrease yield (Malhotra, 2009). The Nus-A tag used by Nomikos et al. (2013), also bound to the N-terminal region of PLCζ, generated a relatively pure, stable, soluble and effective protein. As reviewed by Costa et al., (2014), the Nus-A tag improves solubility and stability of the fusion protein and this may be correlated with the tag’s intrinsically solubility and biological activity in E. coli. Moreover, this tag has the ability to slow down protein expression, allowing additional time for protein folding (De Marco et al., 2004). For this reason, the hrPLCζ created by Nomikos et al. (2013) resulted in Ca^{2+} oscillation-inducing activity within oocytes at a reasonable and clinically applicable concentration. Preliminary work by Kashir et al. (unpublished) was similar to that described by Grasa et al. (2008), however, the outcome was unfavourable as a result of inclusion body formation and therefore, reduced yield. As a result of reduced yield, the LCMS that was performed generated a protein score which was very low, and thus the identity of the protein was not confirmed. Further optimisations were therefore necessary to ensure that the hrPLCζ
generated was of a high quality and quantity. The first aim of this chapter was to therefore overcome the limitations encountered by Kashir et al. (unpublished). The hypothesis was that altering experimental conditions (incubation temperature, incubation time, and concentration of protein expression reagent) would generate a hrPLCζ of better quality and quantity than previously attempted.

In this present study, protein expression using *E. coli* and purification facilitated by a GST tag localised at the N-terminal region of PLCζ, LCSM confirmed that the purified products (cleaved and non-cleaved) were indeed PLCζ. The protein score value (2232) was sufficient to confirm the excised band as PLCζ. The protein score calculates the matches made by the peptides (band digested by trypsin) to the given protein (in this case PLCζ), and a higher score signifies a more confident match. As for the non-cleaved product, the protein score was not as high as the cleaved product, but the fact that PLCζ was detected within the band submitted provides a reliable identification. Further to this, there can be more focus on the cleaved protein as this represents PLCζ at its most endogenous form.

The materials used in this study were similar to that of Kashir et al. (unpublished), however, the methodology was optimised to prevent a similar (i.e. failed) outcome. Changes followed that of Nomikos et al. (2013) and these included incubation time, incubation temperature, and IPTG concentration. The conditions used by Kashir et al. (unpublished) were: 4 hours, 37°C, and 0.5mM IPTG concentration, whereas, the conditions used in this thesis were: 18 hours, 16°C, and 0.1mM IPTG concentration. These conditions provided a more favourable outcome, as a longer incubation time may have allowed for sufficient protein expression, and the lower temperature may have resulted in a slower growth rate. This was beneficial, particularly for complex proteins that require optimal conditions to fold properly during expression, and can therefore reduce the formation of inclusion bodies and increase yield. Furthermore, the lower IPTG concentration used in this study may have influenced the positive result observed. Yoon *et al*.
al. (2012) induced protein expression with IPTG concentration of 1mM, and incubation
time and temperature of 12 hours and 20°C, respectively. Although the incubation time and
temperature seemed ideal for bacterial growth, the high IPTG concentration may have
compromised the yield. Indeed, Larentis et al. (2014) showed that a higher IPTG
concentration reduced cell growth rate and soluble protein yield, which may have been due
to the production of toxic proteins. Furthermore, Sriubolmas et al. (1997) showed that
IPTG concentration, from 0.2 to 0.5mM, reduced the enzymatic activity of penicillin G
acylase and increased the presence of inclusion bodies. As PLCζ is an enzyme with a
precise mode of action, the excessive IPTG concentration used by Yoon et al. (2012) may
have resulted in the abnormal Ca^{2+} release profile and reduced yield. Both Larentis et al.
(2014) and Sriubolmas et al. (1997) suggested that an IPTG concentration of 0.025 to
0.1mM was adequate to induce expression of their respective recombinant protein, and this
was the approach used in the present study.

Following LCMS confirmation of protein identity, functionality of the cleaved and
non-cleaved proteins was tested by microinjection into MII mouse oocytes. The
concentrations were similar to those used by Nomikos et al. (2013). However no Ca^{2+}
oscillation-inducing activity was observed after the microinjections. The GST tag may
have interfered with the structure, activity, and function of PLCζ. Specifically, the tag may
have disrupted the folding of PLCζ; both in its presence, and absence, during expression.
Nomikos et al. (2013) showed that their hrPLCζ was able to trigger a Ca^{2+} release profile
indistinguishable from that of normal fertilisation. While this was an improvement on the
abnormal Ca^{2+} release pattern depicted in Yoon et al. (2012), Nomikos et al. (2013) failed
to produce results when microinjections were performed in the absence of the tag. If this
had been the case, it would make the hrPLCζ a strong candidate for clinical applications,
as it would be in its native state. It is plausible that both Yoon et al. (2012) and Nomikos et
al. (2013) failed to show their observations upon microinjection of their hrPLCζ without
their respective tags because the tag may have destabilised the protein, resulting in degradation, and therefore inactivity. This may or may not have been applicable in the present study as the hrPLCζ generated in the presence of GST also failed to induce Ca^{2+} traces upon microinjection. It could be argued that the tags used in this study as well as that in Yoon et al. (2012) and Nomikos et al. (2013) were not suitable in the generation of native hrPLCζ. It is well established that in recombinant protein technology, trial and error experiments are necessary to generate an optimised protocol in creating the protein of interest.

As a result of the failure in functionality, an alternative approach was considered which involved the production of hrPLCζ using a mammalian cell line. Kashir et al. (2011a) were the first to create an active hrPLCζ via a mammalian expression system, HEK293T cells. The cells were transfected with a relevant vector linked to WT PLCζ and a C-terminal His-tag, and cell lysates were harvested to test identity and functionality of the protein. Kashir et al. (2011a) identified PLCζ in the cell lysates via immunoblot analysis. Immunoblotting revealed a band of around 70kDa - the expected size of expressed human PLCζ protein. Further to this, the microinjection of cell lysates containing WT and mutant (PLCζ^{H398P}) into mouse oocytes induced Ca^{2+} oscillations but failed to evoke any activity, respectively (Kashir et al., 2011a). These results were very encouraging, however, its form as a non-purified mammalian cell lysate prevented it from being useable in the clinic, but again provided further evidence that PLCζ is the necessary SOAF to induce the events following fertilisation.

The second aim of this study was to utilise a different approach, which it was hoped, would result in the first production of a purified hrPLCζ expressed by a mammalian cell line. The hypothesis was to use a different gene fusion technology, to allow for the secretion and purification of a mammalian-expressed hrPLCζ. This study showed that hrPLCζ could be expressed and purified by a mammalian cell line, the fist such
demonstration. LCMS confirmed that the band submitted was PLCζ. However, due to time restrictions, the activity of the hrPLCζ could not be tested.

The aim of this part of the chapter was similar to that of Kashir et al. (2011a) - to generate hrPLCζ expressed via a mammalian cell line for applications beneficial to clinical and research settings. As for methodology, the only similarity was the use of HEK293T cells to culture and express hrPLCζ. HEK293T cells are commonly used as hosts for gene expression, given their high transfection efficiency, large-scale protein production capability, robust growth rate and low-cost (Baldi et al., 2005). Notable differences in this present study included additional sequences (secretion signal sequence and FC-tag) within the vector, as opposed to the His-tag used by Kashir et al. (2011a). The secretion signal sequence allowed for the cells to secrete a purified form of the target protein which was therefore collected as a supernatant following sufficient culture. Further to this, the FC-tag facilitated purification of hrPLCζ as it was easily cleaved by HRV 3C protease, generating pure PLCζ unlike that of the cell lysate created by Kashir et al. (2011a). The hrPLCζ synthesised in this present study makes it a promising candidate from which to develop new lines of research and this include its applications in OAD therapy and monoclonal antibody production. However, a significant amount of work is now necessary before applying it clinically and in research, and testing its functionality will be particularly important for therapeutic use. In the event that the hrPLCζ is contaminant-free and microinjection is successful, the next crucial steps would be to create a large-scale of the hrPLCζ for the translation to the clinic, PLCζ crystallisation studies, and the formation of a monoclonal antibody. Prior to translating the hrPLCζ into OAD therapy, it is important that a more reliable assay for diagnosing PLCζ-deficiency is introduced. This could be approached by the use of a monoclonal antibody as this may elucidate the wide variability of PLCζ expression in fertile and infertile patients, particularly total levels and localisation patterns within the sperm head. Further to this, factors that need to be considered before
utilising the hrPLCζ as OAD therapy include: injection into human oocytes, form of administration, storage, and clinical trials. The Coward laboratory has recently received ethical permission to perform microinjections into human oocytes, since it is well established that prior to this advancement, the mouse model has been widely utilised. It is also known that hPLCζ is more potent than its mPLCζ counterpart, and thus poses a concern when performing MOAT or MOCA due to the possible inaccuracy of patient diagnosis. Employing this method for testing functionality either using cRNA or recombinant proteins would therefore lead to a more accurate result. Ca²⁺ ionophores are currently being used clinically as a solution, such that following ICSI the oocytes are incubated in a media containing ionomycin or A23187. This could be applied similarly to hrPLCζ, although it will very important to consider the storage conditions of the hrPLCζ to avoid degradation or contamination. It may even be that if the optimal form of hrPLCζ is in a solid form, the issue of stability will be less of a concern. Clinical trials would be carried out to test the safety, effectiveness, and efficacy of the treatment before it could be approved for clinical availability.
4.5 Key findings

The initial aim of this chapter was to purify an active form of bacterial-expressed hrPLCζ using materials and conditions described by Grasa et al. (2008) and Nomikos et al. (2013), respectively. Grasa et al. (2008) validated their hrPLCζ as PLCζ using immunoblot analysis but failed to test activity by microinjecting into mouse oocytes. In this part of my research, I was able to purify and further confirm that the hrPLCζ produced by Grasa et al. (2008) was indeed PLCζ by using LCMS. The step which followed, involved testing the functionality of the hrPLCζ failed, as the protein failed to induce Ca^{2+} oscillations when microinjected into mouse oocytes. Therefore, an alternative approach was considered; expressing hrPLCζ in a mammalian cell line using a different gene fusion technology. While I was successful in confirming that the hrPLCζ generated from the mammalian cell line was PLCζ via LCMS, due to time restrictions I was not able to perform a similar functionality study. However, the ability of the hrPLC to be secreted from the vector and cells used, makes this the first purified mammalian-expressed hrPLC, in contrast to the hrPLC generated by Kashir et al. (2011a), which was in the form of cell lysates.
4.6 Future work

To determine the function of the mammalian-expressed hrPLCζ by microinjecting into human oocytes, and monitoring the Ca\(^{2+}\) oscillation-inducing activity. In the case that the Ca\(^{2+}\) release profile of the hrPLCζ is indistinguishable from that of normal fertilisation, and the injections are performed at an optimal concentration and high number of oocytes, the same conditions carried out for purification should be continued. This will allow for the scaling up of hrPLCζ purification and preparation for downstream clinical and research applications.
Chapter 5: Summary and concluding remarks
5.1 Summary

PLCζ is a sperm-specific protein, and both biochemical and clinical evidence supports its role as the SOAF. OA is a sequential event following fertilisation, and is vital for healthy embryo development. PLCζ initiates OA, characterised as a series of Ca^{2+} oscillations in mammalian species. Since the discovery of PLCζ in 2002, much attention has surrounded its clinical association with OAD, an infertile condition in which the distinctive Ca^{2+} profile following fertilisation is either absent or abnormal. Patients who have experienced recurrent fertilisation failure following ICSI are suspected of having OAD. For many years, OAD was categorised as an idiopathic factor in infertility, however, substantial evidence has causally linked this condition to dysfunctional forms of PLCζ. The causative factor for the abnormalities observed have generally been proposed to be of genetic origin but more research is warranted to support this claim. Additionally, some OAD patients are currently receiving treatment in the form of AOAs, which may have potential detrimental effects on the developing embryo. The main objective of this thesis was to conduct a set of experiments, explained briefly below from each chapter, to establish the association between PLCζ and OAD, and provide better clinical and diagnostic options for patients. Chapter 1 provides a comprehensive literature review of this exciting field.

5.2 Identification of a novel PLCζ variant from a patient suspected of having OAD

Chapter 2 of this thesis focused upon identifying novel variants in two particular regions of PLCζ, the promoter and the XY-linker, from five patients suspected of having OAD. The promoter region has never been explored in human PLCζ, and this was a novel approach to identify variants which might account for the patients’ fertility state. As for the XY-linker region, previous attempts in our laboratory had consistently failed to screen this
segment and so newly synthesised primers and optimal PCR conditions were applied for efficient amplification. A novel nonsense mutation in the XY-linker region, $\text{PLC}_\text{\zeta}^{K322\text{Stop}}$, was identified in Patient LR. This mutation results in the truncation of $\text{PLC}_\text{\zeta}$ to almost half of its WT molecular mass. NGS analysis confirmed the heterozygous nature of $\text{PLC}_\text{\zeta}^{K322\text{Stop}}$ in Patient LR’s sperm population. An additional variant, $\text{PLC}_\text{\zeta}^{H233L}$ (previously reported by Kashir et al. (2012a)), was also identified in Patient LR following complete $\text{PLC}\zeta$ exonic screening. Four individual sperm from this patient were mini-sequenced and shown to possess either $\text{PLC}_\text{\zeta}^{K322\text{Stop}}$ or $\text{PLC}_\text{\zeta}^{H233L}$. Determining the genetic mode of inheritance of these two variants was not possible as I was not able to obtain DNA from Patient LR’s parents. $\text{PLC}_\text{\zeta}$ immunofluorescent analysis showed that the proportion of sperm exhibiting $\text{PLC}_\text{\zeta}$ from Patient LR was significantly reduced when compared to a fertile control. Functional studies confirmed that cRNA which encoded for $\text{PLC}_\text{\zeta}^{K322\text{Stop}}$, failed to induce $\text{Ca}^{2+}$ oscillation-inducing activity when microinjected into mouse oocytes. Further to this, Patient LR’s OAD was rescued following the microinjection of $\text{PLC}_\text{\zeta}^{\text{WT}}$ cRNA into mouse oocytes, which had been administered $\text{PLC}_\text{\zeta}^{K322\text{Stop}}$. This further reinforces the possibility of using $\text{PLC}_\text{\zeta}$ as a novel therapeutic agent for OAD.

The discovery of $\text{PLC}_\text{\zeta}^{K322\text{Stop}}$ was an exciting finding, as $\text{PLC}_\text{\zeta}$ mutations are very rare. Prior to this study, only five mutations had been published, and two of these were reported from the same patient. These mutations, however, have all been identified in the exonic regions, thus highlighting our limited knowledge of the non-coding regions of $\text{PLC}_\text{\zeta}$. One particular conundrum in $\text{PLC}_\text{\zeta}$ research remains, and that is the wide variation of $\text{PLC}_\text{\zeta}$ parameters observed in fertile and infertile patients. It is logical to assume that the exonic mutations discovered thus far may have resulted in the patient’s OAD phenotype but it remains puzzling as to how fertile control patients with proven fertility can have similar levels of $\text{PLC}_\text{\zeta}$ to that of abnormal patients. The subsequent set of experiments I designed aimed to discover the association between the non-coding regions of $\text{PLC}_\text{\zeta}$ and
the variation in question, with the hypothesis that non-coding regions might contain regulatory elements responsible for controlling the expression of PLCζ.

5.3 Investigating the phenotypic diversity of PLCζ expression in infertile patients suspected of having OAD

The main aim of Chapter 3 was to investigate the link between the non-coding regions (promoter and introns) of PLCζ and the significant variation of PLCζ parameters observed in fertile and infertile patients. For the first time in human PLCζ research, NGS technology was employed to detect variants within two ROIs, the promoter and XY-linker. Patients were selected and categorised according to their phenotypic PLCζ expression. However, as a result of mapping failure, this part of the research was compromised. The explanation for this error appears to lie with the company involved and is being investigated. Since the probes were designed and validated to cover the ROIs, the error must have occurred downstream of this. Due to time restrictions, it was not possible to repeat this experiment, and moreover this would be highly dependent upon sample availability. As the reads were mapped onto PIK3C2G, it is not clear whether there is any correlation with PLCζ for use to be made of the NGS data obtained. The first study released on PIK3C2G reported its role in signaling pathways, which regulate cell proliferation, oncogenic transformation, and cell migration (Rozycka et al., 1998). Only sixteen publications have appeared for this gene and its main association has been with cancer (Reyes-Gibby et al., 2016). However, I will make every effort to utilise this data if possible.

An alternative study design, which had not been performed for human PLCζ, was then deployed to detect SNPs within PLCζ. Using dbSNP, I collated over 2500 PLCζ SNPs and these were filtered to variants reported and validated by the 1000 Genomes Project. No
clinical significance data were available for these SNPs, however, it was apparent that the 
majority of these SNPs were localised within intronic regions of the gene. Due to time 
restrictions, it was not feasible to determine the precise location of each SNP. However, 
the discovery that most PLCζ SNPs were intronic concurs with previous publications for 
other genes, which proposed that variants located within the regulatory elements (promoter 
or introns) might influence mechanisms responsible for regulating expression. It was then 
possible to speculate that the wide variation of PLCζ parameters observed in fertile and 
infertile patients may have resulted from variants situated in non-coding regions, as 
opposed to the translated elements.

As the emphasis in this chapter was to identify a link between patients suspected of 
having OAD and their PLCζ immunofluorescence analysis, two particular patients who 
had experienced TFF, 79 and 107, were investigated. As recommended by their fertility 
clinics, I assessed the possible cause of the patient’s TFF by performing PLCζ 
immunofluorescence analysis and/or genetic screening. For Patient 79, exome screening 
was carried out by the fertility clinic in Dundee and no genetic anomalies were detected. 
Three separate immunofluorescent analyses was therefore performed according to the day 
the sample was submitted. The first immunofluorescence evaluated showed a significant 
reduction in both the total level of PLCζ and proportion of sperm exhibiting PLCζ when 
compared to a fertile control. However, a significant improvement in both parameters was 
observed from Patient 79’s second analysis, one year later. Eight months after the second 
investigation, his final analysis showed that total levels of PLCζ remained the same while a 
continual improvement was exhibited in the proportion of sperm exhibiting PLCζ. We 
were then informed by the clinic that the patient had discontinued taking antihistamine and 
started vitamins C supplements after his first analysis. Vitamin C supplementation may 
increase semen quality, and although Patient 79 had normal semen analysis parameters, it 
is possible that this compound may have influenced the regulation of PLCζ expression,
although this is highly speculative at present. A follow-up to this patient could involve performing the MOAT or MOCA to test for his sperm-oocyte activating capacity. If these tests show that there is an improvement in fertilising ability, although earlier samples to compare are lacking, it is plausible that consuming vitamin C supplements may be an alternative way to rescue OAD. An approach to this could be to recommend such supplements to patients who are suspected of having OAD, and who have exhibited a significant reduction in \( \text{PLC}_\zeta \) expression following immunofluorescent analysis when compared to a fertile control. These patients would then be asked to resubmit a sample two to three months after their first investigation, as it takes approximately 70 days to complete spermatogenesis (Amann, 2008), to evaluate any improvements in \( \text{PLC}_\zeta \) expression.

As for Patient 107, no genetic anomalies were identified in the promoter-exon 1 segment, therefore, it could be that variants within his intronic regions may be responsible for the increased parameters observed. However, I discovered another novel mutation, \( \text{PLC}_\zeta^{V193E} \), following \( \text{PLC}_\zeta \) genetic screening (exonic, promoter and XY-linker regions). Chromatogram peaks indicated that the patient was heterozygous for the variant but due to time constraints, it was not possible to perform NGS for validation. A predicted three-dimensional model showed that \( \text{PLC}_\zeta^{V193E} \) disrupts the interaction between the X catalytic domain and other molecules responsible for protein folding and stability. As \( \text{PLC}_\zeta^{V193E} \) was discovered at the end of my DPhil, it was not possible to perform a functionality study to determine the effects of the mutation on \( \text{PLC}_\zeta \) activity. Nevertheless, this would be the second novel mutation detected during the period of my DPhil. This signifies that the association between \( \text{PLC}_\zeta \) and male infertility is gaining credibility by the public as an influx of patients suspected of having OAD are requesting our routine screening protocols.

However, another controversial topic within \( \text{PLC}_\zeta \) research involves the use of AOAs as a mode of therapy for OAD. Although these agents have resulted in successful births from the few clinics worldwide performing this practice, there are significant
concerns over its use. These include mutagenic, cytotoxic and epigenetic effects on the developing embryo. While this apprehensiveness is not based upon firm scientific or clinical evidence, there is global interest in creating an alternative and safer OAD therapy. Therefore, the aim of the next part of my thesis was to generate hrPLCζ for both clinical and research applications.

5.4 Production of a human recombinant PLCζ protein

Gradual progress has been made in this aspect of PLCζ research stemming from the fact that successfully purifying an active hrPLCζ is a challenge for researchers. Recombinant protein production has always been a trial and error procedure as optimal factors and conditions must first be elucidated; for example, the expression systems used, fusion tag, incubation time, and incubation temperature. In this thesis, an initial approach involved using a bacterial expression system and GST tag (26kDa) to purify hrPLCζ successfully. Validation using LCMS confirmed that the protein produced was indeed PLCζ, however, investigating its activity by microinjecting into mouse oocytes failed, characterised by the absence of Ca²⁺ transients. Therefore, an alternative method was considered.

The subsequent experiment involved using a mammalian cell line and a vector, which included elements that facilitated both secretion and purification. While purification and validation by both SDS-PAGE and LCMS was successful, I was not able to test the functionality of the hrPLCζ due to time restrictions. Therefore, future work would involve microinjecting the hrPLCζ into mouse oocytes to determine Ca²⁺ oscillation-inducing activity. Accomplishing this could disclose a plethora of clinical and research opportunities including OAD therapy, generation of a monoclonal antibody, and preliminary crystallisation studies. No published accounts have indicated progress in developing these applications, which may suggest that synthesising hrPLCζ of a high quality and quantity remains an arduous task in the field.
5.5 Concluding remarks

Collectively, the experimental findings in this thesis consolidated the presence of an association between PLCζ and OAD. Since the discovery of PLCζ in 2002 and up until the start of this thesis, only two novel exonic mutations had been discovered in the same infertile patient, concurring with the rarity of disease-causing mutations. However recently four additional PLCζ mutations, two published and two discovered during this study, were identified from patients suspected of having OAD. This is a clear indication that a genetic factor contributed to the dysfunctional forms of PLCζ reported. However, it would be premature to apply this statement to OAD generally as other factors, yet to be determined in the oocyte, may also be responsible for the condition. Therefore, it may be prudent that more fertility clinics introduce the PLCζ genetic screening protocol so as to identify the cause of patients with TFF. Patients are gradually being informed of TFF and OAD, and its possible association with PLCζ-deficiency, as can be seen in various Internet forums. In addition, patients unaffiliated with Oxford Fertility, and even from the USA, have contacted Dr. Kevin Coward about their condition and the availability of diagnostic testing. Several initiatives include starting new collaborations with fertility clinics across the world to introduce PLCζ genetic screening in patients suspected of having OAD. The more prominent the link between OAD and PLCζ-deficiency becomes, the more fertility clinics can be informed of this diagnostic test. Additionally, most clinics host regular patient information evenings, and this provide a great opportunity for them to be informed of the causative factors in OAD. It is also important that embryologists become aware of this condition, as this helps to spread information.

While progress has been established in investigating the coding-regions of PLCζ, it was obvious that our knowledge of the regulatory elements consisting of the promoter and introns was very limited. These elements are especially involved in regulating gene expression, and thus variants within these regions may influence the resulting protein. Due
to the NGS failure, it was not possible to relate the untranslated regions of PLCζ with the distinct variation in PLCζ immunofluorescence levels observed in fertile and infertile patients. However, using dbSNP, I concluded that variants within PLCζ were mainly confined to the intronic regions and it is possible to speculate that these SNPs may play a role in the phenotypic diversity of PLCζ expression. As a result, it is important that future studies investigate these non-coding regions so as to understand the regulatory mechanisms involved in expressing PLCζ. One feasible approach into investigating the non-coding regions may be to categorise the SNPs into their respective intronic locations and using online databases such as “MutationTaster” to understand the effect the variants on expression and protein level. The introns, which are shown to be most susceptible to abnormalities, may be involved in the regulation of expression. Therefore, by applying the sequences of these introns to online databases such as “Nsite” (www.softberry.com) may help to identify regulatory elements within the gene.

This thesis also attempted to resolve the controversial matter of the use of AOAs for OAD therapy by generating a purified and active hrPLCζ. The initial method using a bacterial cell line to express PLCζ was unsuccessful due to the absence of activity and an alternative approach was employed expressing hrPLCζ using a mammalian cell line. Due to time restrictions, the activity could not be tested but this study describes for the first time, a purified mammalian-expressed hrPLCζ. Previous work by Kashir et al. (2011a) successfully generated a mammalian-expressed hrPLCζ in the form of cell lysates, thus making it a non-viable option for OAD therapy or research applications. Therefore, the hrPLCζ purified during this study may be applied in both the clinical and research settings but it is imperative that a functional study, which exhibits the characteristic Ca^{2+} oscillations following microinjection, be performed prior to its use. The potential success of this experiment would allow for the scaling up of hrPLCζ purification and preparation. Sufficient purity and quantity of the hrPLCζ could be submitted for X-ray crystallography
to establish an accurate three-dimensional structure of PLCζ. Additionally, the hrPLCζ could be submitted to Covalab (France) for the generation of a monoclonal antibody to confirm the widespread speculation of the significant variation of PLCζ expression observed in fertile and infertile patients. Lastly, the hrPLCζ could be utilised as a potential therapy for OAD but several factors, which needs to be considered, include the appropriate storage conditions to prevent degradation or contamination, clinical administration to patients (as an injection or media), and of course the need for clinical trial before it could be available commercially.

In conclusion, it is evident that PLCζ plays a fundamental role in OA, and its association with OAD further signifies this. However, our lack of knowledge, particularly its regulatory mechanism or the complex nature of the protein to be made into a recombinant form hinders progress into translating PLCζ research clinically. With increasing public awareness and interest, and growing understanding among clinical embryologists, it is vital that scientific progress gathers pace to meet patient expectations.
Chapter 6: Commentary
Previous published materials and this thesis supports the notion of \( \text{PLC}\zeta \) and its fundamental role as the SOAF, as well as its strong association with male infertility (OAD). Although continuous progress has been made in \( \text{PLC}\zeta \) research, particularly in the clinical field, it is evident that our knowledge of the \( \text{Ca}^{2+} \) signaling pathway in the oocyte upon fertilisation remains limited. One such question is the \( \text{Ca}^{2+} \) release profile between mammalian and non-mammalian species, whereby a series of oscillations and a single transient is observed, respectively. There is no definite answer to this phenomenon but it was proposed that a single transient is not efficient for OA in mammals. Further to this, due to the complex physiology of mammals, the oscillations could be a safety measure for OA to proceed in an appropriate manner, thus for a healthy embryo development. Ozil et al. (2005) showed that mouse eggs activated by a single \( \text{Ca}^{2+} \) rise develop inefficiently when compared to eggs activated by \( \text{Ca}^{2+} \) oscillations, as observed by the PN formation and implantation success of either method. The nature of the oscillations itself poses many questions; what causes them and how are they maintained?

It is still unclear what causes \( \text{Ca}^{2+} \) to oscillate upon fertilisation but earlier studies suggested that it might be the feedback mechanism from IP\(_3\) production relevant to \( \text{PLC}\zeta \), and \( \text{Ca}^{2+} \) influx via the entry of extracellular \( \text{Ca}^{2+} \). \( \text{Ca}^{2+} \) is rapidly restored to its resting level following each single peak (Swann and Yu, 2008). The mechanism for removing \( \text{Ca}^{2+} \) involves intricate co-ordination between the sarcoplasmic/endoplasmic reticulum \( \text{Ca}^{2+} \) ATPases (SERCA) pumps, mitochondria, plasma membrane \( \text{Ca}^{2+} \) ATPase (PMCA) pumps and \( \text{Na}^{2+}/\text{Ca}^{2+} \) exchangers in the plasma membrane. The efflux of \( \text{Ca}^{2+} \) into intracellular stores causes the entry of extracellular \( \text{Ca}^{2+} \) to compensate for the loss and to sustain the oscillations (Swann and Yu, 2008). The mechanism for \( \text{Ca}^{2+} \) influx remains controversial, as it appears that an earlier proposed candidate, store-operated \( \text{Ca}^{2+} \) entry (SOCE), is not required for fertilisation-induced \( \text{Ca}^{2+} \) oscillations (Bernhardt et al., 2017). SOCE is important in maintaining cellular \( \text{Ca}^{2+} \) balance and two major components of
SOCE are STIM and ORAI proteins, which reside in the endoplasmic reticulum and plasma membrane, respectively (Prakriya and Lewis, 2015). STIM and ORAI proteins co-ordinate to induce Ca\(^{2+}\) influx to support the oscillations, in response to Ca\(^{2+}\) decrease in the endoplasmic reticulum. Knockout mice lacking STIM and ORAI proteins did not prevent Ca\(^{2+}\) influx thus indicating that SOCE is not necessary to maintain the oscillations (Bernhardt et al., 2017). Instead an alternative channel, TRPM7 (melastatin-related transient receptor potential) or a TRPM7-like channel, contributed significantly to both spontaneous Ca\(^{2+}\) influx and support post-fertilisation Ca\(^{2+}\) oscillations (Bernhardt et al., 2017). However, it is unlikely that TRPM7 functions alone to mediate the influx and therefore, future investigations may involve knocking out a combination of channels within the oocyte to fully understand the mechanisms of Ca\(^{2+}\) influx.

Another interesting candidate involved in Ca\(^{2+}\) signaling following fertilisation is the postacrosomal WW domain-binding protein (PAWP). In recent years, PAWP was involved in a controversy whereby Aarabi et al. (2012) claimed its dominant role as the SOAF, as opposed to PLC\(\zeta\). However, Satouh et al. (2015) refuted this claim as the authors demonstrated that knockout mice absent of PAWP continued to trigger Ca\(^{2+}\) release, therefore it may not play an important role in OA but it could be involved in Ca\(^{2+}\) signaling to maintain the oscillations, possibly in concert with TRPM7 channels.

In addition to the increase of Ca\(^{2+}\) within the ooplasm for activation, recent work by Duncan et al. (2016) using three parthenogenetic approaches (calcium ionomycin, ionomycin, and hPLC\(\zeta\) cRNA) observed the coordinated release of zinc into the extracellular space, termed “zinc spark”. Human eggs contain zinc transporters and cortically enriched zinc vesicles indicating a possible role of zinc during activation or embryo development. Duncan et al. (2016) also demonstrated that the zinc spark was more prominent in MII-arrested oocytes than in immature prophase I-arrested germinal vesicle-intact oocytes, which strongly suggests that this response was meiotic-stage dependent.
Additionally, the reduction of zinc in M-II arrested human eggs via treatment with a zinc-specific chelator, resulted in OA as the oocytes entered into the mitotic cell cycle (Duncan et al., 2016). This important discovery in human provides an early hallmark of OA, and that chelation of intracellular zinc is sufficient to induce OA. Furthermore, the use of intracellular zinc chelator treatment may potentially be applied in assisted OA in ART. Herein, our knowledge must be explored as it is clear that a multitude of events, not limited to just the release of Ca$^{2+}$ following fertilisation, are prerequisites to achieving oocyte activation.
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Appendix

Figure 62. Representative 2% agarose gel showing the PCR amplification of PLCζ promoter-exon 1. The red arrow indicate bands from PCR products corresponding to the PLCζ promoter-exon 1 molecular mass of 371bp.

Figure 63. Representative 1% agarose gel showing the PCR amplification of PLCζ XY-linker. The red arrow indicate bands from PCR products corresponding to the PLCζ XY-linker molecular mass of 1988bp.
Figure 64. Multiple alignment of the molecular construct utilised to create PLCζK322Stop cRNA. The raw data received from Sanger sequencing was compared to wild type PLCζ ORF. The red arrow indicates the site in which the variant was successfully inserted. The color highlight represents PLCζ domains. Yellow: EF hand, Green: X catalytic, Red: XY-linker, Blue: Y catalytic, Gray: C2.
Research output

Peer-reviewed articles in International journals


Yelumalai S, Yeste M, Jones C, Amdani SN, Kashir J, Mounce G, Da Silva SM, Barratt CL, McVeigh E, Coward K. Total levels, localization patterns and proportions of sperm exhibiting phospholipase C zeta (PLCζ) are significantly correlated with fertilization rates following intracytoplasmic sperm injection. Fertil Steril 2015; 104:561-568.


Book chapter


Oral presentation


Clinical association between phospholipase C zeta (PLCζ) and male infertility. Amdani SN. Green Templeton College, University of Oxford, 2016.

**Poster presentation**


What therapeutic solution can we offer to patients with recurrent total fertilisation failure but normal levels of phospholipase C zeta (PLCζ) protein? Jones C, Amdani SN, Mounce G, Malinauskas T, Child T, Coward K. Fertility Conference 2017, Edinburgh, Scotland.
