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# Artificial oocyte activation (AOA) and phospholipase C zeta (PLC $\zeta$ ) in assisted reproductive technology (ART)

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D.Phil. thesis

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## Abstract

Oocyte activation deficiency (OAD) is the main cause of total fertilisation failure/low fertilisation rate in assisted reproductive technology (ART). Phospholipase C zeta (PLC $\zeta$ ) is the dominant sperm-specific factor responsible for triggering oocyte activation in mammals. Abnormalities in PLC $\zeta$  have been linked with OAD. Artificial oocyte activation (AOA) is currently the only option for OAD in clinic but lacks reliable diagnostic approach and may cause abnormal Ca<sup>2+</sup> release in oocytes. Previous studies have attempted to synthesise recombinant human PLC $\zeta$  (rhPLC $\zeta$ ) protein but struggled to purify the protein or maintain functionality and stability. This thesis aimed to address these shortfalls by investigating the mechanism(s) of OAD and improving the applicability of PLC $\zeta$  as a diagnostic and therapeutic agent for male infertility. This thesis first compared the efficiency of two immunofluorescence methods to assess PLC $\zeta$  expression in male sperm (n=26 males) and demonstrated that our in-house assay showed the best labelling results when compared with a recently-published antigen-unmasking (AUM) method. I further developed our in-house PLC $\zeta$  assay into a clinical diagnostic assay, which targets on infertile candidates for the AOA treatment (i.e., Ca<sup>2+</sup> ionophores). Four out of five couples received AOA and achieved healthy live-births (fertilisation rate from 18.6% to 56.8%, p<0.001). Cut-offs for the key PLC $\zeta$  characteristics were also determined for the first time (mean PLC $\zeta$  levels: 15.57 arbitrary units [a.u.], and the proportion of sperm exhibiting PLC $\zeta$ : 71%). Globally, eleven mutations have been identified in the *PLC $\zeta$*  gene; however, the XY-linker that connects the main X and Y domains has received for less research attention. In addition, *CAPZA3* shares a bidirectional promoter with *PLC $\zeta$*  and is responsible for sperm morphology and function. In this thesis, Sanger sequencing and next-generation sequencing (NGS) were used to screen *PLC $\zeta$*  exons (n=61 males), and *PLC $\zeta$*  promoter, *XY-linker* and *CAPZA3* regions (n=33 males). Ten single polymorphisms (SNPs) in *PLC $\zeta$*  introns and three mutations in *CAPZA3* exon were identified, some of which may partially explain the causes for male infertility. In addition, this study attempted to synthesise a novel rhPLC $\zeta$  with uses of a pHLsec vector feathered with secretion signal sequence and further examined the functionality of the protein. Surprisingly, simple incubation of the protein with mouse oocytes triggered activation (22/52 developed to the 2-cell stage, p<0.001), which not only suggests that the protein may be functional, but also indicates the possible existence of a novel plasma membrane-bound mechanism that may act alongside the sperm factor.

**Key words:** artificial oocyte activation, oocyte activation deficiency, phospholipase C zeta, and PLC $\zeta$  deficiency

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## List of abbreviations

|                   |                                                           |
|-------------------|-----------------------------------------------------------|
| ANOVA             | analysis of variance                                      |
| AOA               | artificial oocyte activation                              |
| AOAs              | artificial oocyte activators                              |
| ART               | assisted reproductive technology                          |
| AT                | acidic Tyrode's solution                                  |
| a.u.              | arbitrary units                                           |
| AUC               | area under the ROC curve                                  |
| AUM               | antigen unmasking                                         |
| BSA               | bicinchoninic acid                                        |
| cAMP              | cyclic adenosine monophosphate                            |
| Ca <sup>2+</sup>  | calcium ions                                              |
| CAMKII            | calmodulin-dependent protein kinase II                    |
| Cas9              | CRISPR-associated protein 9                               |
| CASA              | computer-assisted sperm analyses                          |
| CDK1              | cyclin-dependent kinase 1                                 |
| CRISPR            | clustered regularly interspaced short palindromic repeats |
| CSF               | cytostatic factor                                         |
| Cyto D            | cytochalasin D                                            |
| DAG               | diacylglycerol                                            |
| DAPI              | 4',6-diamidino-2-phenylindole                             |
| DGW               | density gradient washing                                  |
| <i>E.coli</i>     | <i>Escherichia coli</i>                                   |
| EDTA              | ethylenediaminetetraacetic acid                           |
| ER                | endoplasmic reticulum                                     |
| FITC              | fluorescein isothiocyanate                                |
| h                 | hour(s)                                                   |
| FBS               | fetal bovine serum                                        |
| HBV               | hepatitis B virus                                         |
| HCl               | hydrochloric acid                                         |
| HCV               | hepatitis C virus                                         |
| HEK293T           | human embryonic kidney 293 cells                          |
| HFEA              | Human Fertilisation and Embryology Authority              |
| HIV               | human immunodeficiency viruses                            |
| HOCA              | human oocyte Ca <sup>2+</sup> analysis                    |
| HRV               | human rhinovirus                                          |
| FSH               | follicle-stimulation hormone                              |
| human PLCζ        | hPLCζ                                                     |
| ICSI              | intracytoplasmic sperm injection                          |
| IMSI              | intracytoplasmic morphologically selected sperm injection |
| InsP <sub>3</sub> | inositol 1,4,5-trisphosphate                              |
| IVF               | <i>in-vitro</i> fertilisation                             |
| KO                | knock-out                                                 |
| MAPK              | mitogen-activated protein kinase                          |

|                   |                                                                       |
|-------------------|-----------------------------------------------------------------------|
| MII               | meiosis II                                                            |
| min               | minute(s)                                                             |
| miRNA             | microRNA                                                              |
| MOAT              | mouse oocyte activation test                                          |
| MSOME             | motile sperm organelle morphology examination                         |
| mPLC $\zeta$      | mouse PLC $\zeta$                                                     |
| MPF               | M-phase promoting factor                                              |
| NGS               | next-generation sequencing                                            |
| NLS               | nuclear localisation signal                                           |
| OAD               | oocyte activation deficiency                                          |
| ORAI1             | Ca <sup>2+</sup> release-activated Ca <sup>2+</sup> channel protein 1 |
| PBS               | phosphate-buffered saline                                             |
| PIP <sub>2</sub>  | phosphatidylinositol-4,5-bisphosphate                                 |
| PIV               | particle image velocimetry                                            |
| PLC               | phospholipase C                                                       |
| PLC $\zeta$       | phospholipase C zeta                                                  |
| PN                | pronuclei                                                             |
| PKA               | protein kinase type A                                                 |
| PKC               | protein kinase C                                                      |
| rhPLC $\zeta$     | recombinant human PLC $\zeta$                                         |
| rmPLC $\delta$ 1  | recombinant mouse PLC $\delta$ 1                                      |
| rmPLC $\zeta$     | recombinant mouse PLC $\zeta$                                         |
| RNA               | ribonucleic acid                                                      |
| ROC               | receiver operating characteristic curve                               |
| ROI               | region of interest                                                    |
| RT                | room temperature                                                      |
| SCAF              | sperm cryptic activating factor                                       |
| SrCl <sub>2</sub> | strontium chloride                                                    |
| SNPs              | single nucleotide polymorphisms                                       |
| SERCA             | sarco/ER and plasma membrane Ca <sup>2+</sup> ATPase pumps            |
| SOAFs             | sperm-borne oocyte activating factors                                 |
| SOCE              | store operated calcium entry (SOCE)                                   |
| SSS               | secretion signal sequence                                             |
| STIM              | stromal interaction molecule                                          |
| TAE               | tris-acetate-ethylenediaminetetraacetic acid                          |
| TFF               | total fertilisation failure                                           |
| TPEN              | N,N,N',N'-tetrakis (2-pyridylmethyl) ethane 1,2-diamine               |
| WHO               | World Health Organization                                             |
| Zn <sup>2+</sup>  | zinc ions                                                             |
| 3D                | three-dimensional                                                     |
| 5'UTR             | 5'untranslated region                                                 |

## Chapter 1. Introduction

### 1.1 Human infertility and assisted reproductive technology (ART)

#### 1.1.1 Introduction - the definition, prevalence, and causes of human infertility

Fertility is the ability to achieve a clinical pregnancy and is a crucial factor in reproductive health. According to the international glossary published by the World Health Organization (WHO) in 2009, infertility is the failure to establish a clinical pregnancy after 12 months of regular, unprotected sexual intercourse (Zegers-Hochschild *et al.* 2009). Sterility refers to permanent infertility without time restriction, while subfertility refers to an interchangeable infertility condition. To achieve a broad global understanding in both medical and scientific communities, avoid confusion, and standardise the definition of infertilities, the WHO subsequently removed the term subfertility and expanded the definition of infertility to encompass not only a disease, but a 'disability' of impaired reproductive function (Zegers-Hochschild *et al.* 2017). The updated definition of infertility also covers a wider range of conditions affecting the capacity of an individual or couples to reproduce. The WHO further suggested that fertility treatment can be conducted after less than 1 year based on the clinical evidence from individual patients such as reproductive history, age and diagnostic testing (Zegers-Hochschild *et al.* 2017).

The incidence of infertility is rising as the global population grows. The number of affected couples in the world increased from 42.0 million in 1990 to 48.5 million in 2010 (Mascarenhas *et al.* 2012). However, it is difficult to calculate a precise figure because the prevalence of infertility differs among

developing and developed countries, and because the definitions of infertility, and the criteria use to estimate population-based data varies between different surveys. Nevertheless, infertility is now estimated to affect one in four couples in developing countries and one in six couples in western countries (Inhorn and Patrizio 2015; Polis *et al.* 2017; ESHRE 2018; Vander Borgh and Wyns 2018). Previous researchers have investigated the prevalence of infertility in both the female and male genders. In 2002, Rutstein and Shah reported that over 186 million females from developing countries were suffering from infertility (Rutstein and Shah 2016). Boivin *et al.* estimated that there was 72.4 million infertile females worldwide in 2007, based on the analysis of more than 150 million females from 25 populations (Boivin *et al.* 2007). More recently, Agarwal *et al.* analysed 16 systematic, or meta-analysis, reviews reporting male fertility data and determined that over 30 million were infertile (Agarwal *et al.* 2015). In summary, the estimated worldwide infertility rates for those at reproductive age are 13-15% for couples, 9% for females, and 2.5-12% for males (WHO 1983; Sharlip *et al.* 2002; Boivin *et al.* 2007; Esteves *et al.* 2011; Agarwal *et al.* 2015; ESHRE 2018; Vander Borgh and Wyns 2018).

The European Society for Human Reproduction and Embryology (ESHRE) reported that the causes of infertility were 20%-30% for sole-male factor, 20%-35% for sole-female factor, 25%-40% due to both partners, and 10-20% were idiopathic (ESHRE 2018). The Human Fertilisation and Embryology Authority (HFEA) reported that the most common reason for patients seeking *in vitro* fertilisation (IVF) treatment were male infertility (37%), female infertility (31%), and 32% for cases involving unexplained infertility (HFEA 2018a). The main causes of male factor infertility are testicular deficiency (such as



varicocele), post-testicular impairment and other genetic/molecular abnormalities that affect sperm production, quality and function (Esteves 2016). The quality of sperm, including concentration, motility, and morphology, is crucial for the successful development of an oocyte into a healthy viable embryo.

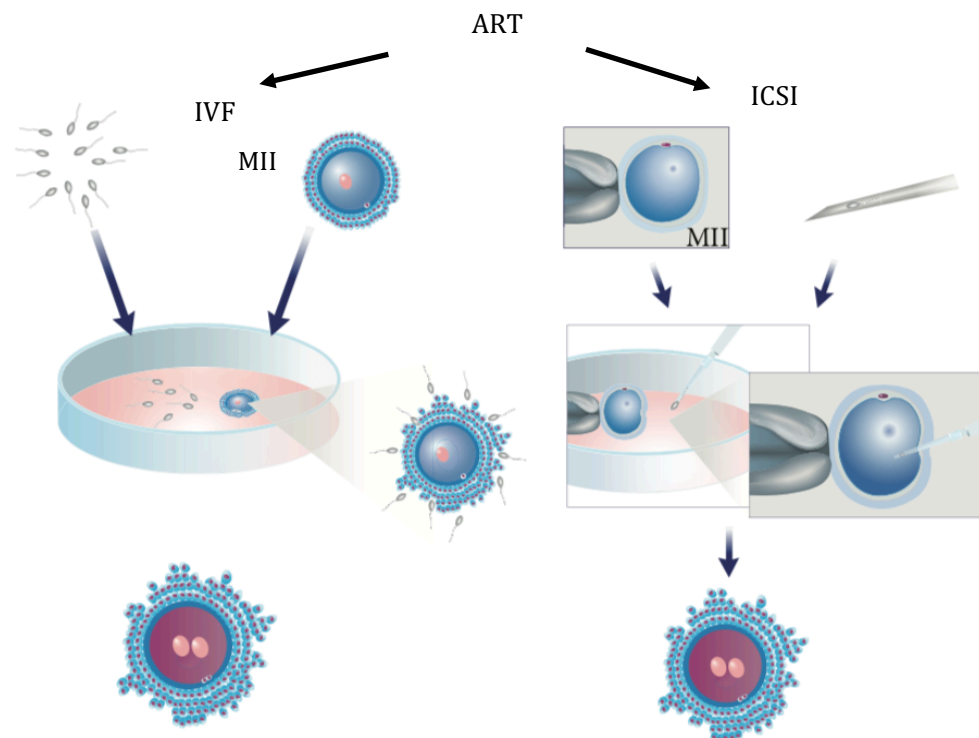
There are many causes of female-related infertility, including ovulatory disorders (such as polycystic ovary syndrome), tubal disease, and endometriosis (HFEA 2018a). Hormonal dysfunction, infections, lifestyle (obesity and smoking), age, and stress can also influence fertility in both genders (Stevenson *et al.* 2016). Current infertility treatments are mainly the state-of-the-art assisted reproductive technology (ART) with/without surgery (sperm retrieval from the epididymis or testis of varicocele or azoospermia males, laparoscopy or tubal surgeries for female reproductive tract disorders), and/or medicine (hormone stimulations/replacements, antibiotic treatment for infections causing infertility) (Stevenson *et al.* 2016).

### **1.1.2 Assisted reproductive technology (ART)**

Over recent decades, ART has significantly improved pregnancy outcomes for infertile couples. In 1978, Louise Brown was the first IVF baby born in the UK, which represented a milestone in medical science in terms of expanding our knowledge, revolutionizing clinical treatments for infertility, and providing hopes and opportunities for millions of families worldwide. Since then, ART has been applied globally and over 7 million babies have been born as a result. A recent worldwide report suggested that the estimated number of ART cycles performed each year is 2.4 million, resulting in approximately 500,000 babies born per year (ESHRE 2018).

ART initially aimed to help couples to overcome fallopian tube blockage, via the insemination of oocytes and sperm in a laboratory (*in vitro*) instead of in the female reproductive tract (*in vivo*). This was then combined with other techniques (such as micromanipulation) to solve more difficult cases of infertility, although the routine steps are similar: ovary hyperstimulation, extraction of mature oocytes, fertilisation *in vitro*, and the transfer of good-quality embryo(s) back to the uterus (DeVos *et al.* 2000). Additional techniques include zona drilling, zona thinning, assisted hatching, cryopreservation of sperm/oocytes/embryo, preimplantation genetic diagnosis and screening, and artificial oocyte activation (AOA).

There are two ways in which an oocyte can be fertilised *in vitro*: IVF and intracytoplasmic sperm injection (ICSI). In IVF cycles, an oocyte is inseminated with concentrated sperm with progressive motility, thus mimicking natural fertilisation. In ICSI treatment, an individual immobilised sperm is injected into a denuded oocyte (cumulus cells removed) using a micropipette and a micromanipulator (**Figure 1.1**)(ESHRE 2015). ICSI involves the direct injection of a single sperm into the ooplasm, bypassing the zona pellucida. This avoids problems associated with polyspermy, dysfunction of the sperm's acrosome reaction, and the immaturity of sperm following surgical retrieval (Palermo *et al.* 2017). ICSI also reduces the chances of virus transmission (human immunodeficiency viruses [HIV], hepatitis B virus [HBV], and hepatitis C virus [HCV]) and the exocytosis of cortical granules in cryopreserved oocytes (Ghetler *et al.* 2006; Vitorino *et al.* 2011). ICSI is also an effective option for couples who experience recurrent fertilisation failure with conventional IVF, although its use varies according to different national guidelines and local protocols.



**Figure 1.1 Schematic diagram of *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI).** In an IVF cycle, mature oocytes at the metaphase II of the meiosis (MII) are retrieved from ovaries guided by ultrasound. Oocytes are then inseminated with concentrated and motile sperm ( $\geq 10 \times 10^6/\text{ml}$  and  $\geq 30\%$ , respectively) in a culture dish. Sperm is then free to fertilise with an oocyte, thus mimicking natural fertilisation. Successful fertilisation is defined by assessing pronuclei and 2-cell formations. Embryo(s) at the 2-cell stage, or blastocyst stage, are then be transferred back to the female's uterus for further development. In ICSI treatment, an individual immobilised sperm is injected into a denuded oocyte (cumulus cells removed), using a micropipette and micromanipulator. ART: assisted reproductive technology. Reproduced from Bos-Mikich *et al.* (2016).

ICSI is currently the most common technique, accounting for two-thirds of all ART treatments performed worldwide, while conventional IVF accounts for only one-third (Calhaz-Jorge *et al.* 2017). However, in 2016, ICSI was performed for only 36% of ART cycles in the UK (HFEA 2018a). This may be due to the fact that ICSI can improve fertilisation rates but not pregnancy rates, compared to IVF alone (Johnson *et al.* 2013; Chambers *et al.* 2016). In 2014, the mean pregnancy rates per embryo transfer in European countries were 30% and 35% after ICSI and IVF, respectively (ESHRE 2018). Indeed, IVF and ICSI have largely improved pregnancy outcomes. The overall live-birth rate from ART cycles were 26% per fresh cycle and 28% per frozen cycle in the UK in 2016, and 40.1% per cycle in American women younger than 35 in 2013 (Hornstein 2016; HFEA 2018a). Earlier data suggested that the fertilisation rates in IVF and ICSI were ~60-70% and ~65-75%, respectively (Van Steirteghem *et al.* 1993; Wittmaack *et al.* 1994; De Croo *et al.* 2000; Swain and Pool 2008). In addition, a recent study suggested that IVF and ICSI methods were not different in terms of embryos reaching to blastocyst stage, if the patients' sperm exhibited progressive motility ( $\geq 1.0 \times 10^6/\text{ml}$ ) (Speyer *et al.* 2019). Despite these promising figures, fertilisation failure still occurs.

Understanding the reasons for fertilisation failure can further improve pregnancy outcomes, although the precise reasons for failure remain unclear. According to figures released by the HFEA in 2014, male factors attributed to 10% of failed IVF cases and 50.4% of failed ICSI cases, while unexplained reasons for failure accounted for 50.3% and 25.8% of cases, respectively (HFEA 2014). IVF failure may be due to dysfunction of sperm capacitation or the acrosome reaction, abnormalities during/after sperm binding or gamete fusion, or poor

gamete quality (Liu and Baker 2000; Rawe *et al.* 2000; Esfandiari *et al.* 2005; Combelles *et al.* 2010). Given that ICSI bypasses the potential barriers created by sperm binding and the sperm-zona pellucida interactions involved in normal fertilisation, this approach specifically addresses severe male factors, especially poor sperm motility. Accordingly, 50% of ICSI failures are due to male factors, such as abnormal sperm morphology and sperm fragmentation (Yavetz *et al.* 2001; Ito *et al.* 2009; Zhao *et al.* 2014a). In addition, in some cases, fertilisation can be rescued in ICSI-failed human oocytes after injection with fertile donor sperm (Yanagida 2004).

Complete fertilisation failure or total fertilisation failure (TFF), where all mature oocytes fail to fertilise in one cycle, occurred in 5-20% of IVF cycles and 1-3% of ICSI cycles (Liu and Baker 2000; Bhattacharya *et al.* 2001; Mahutte and Arici 2003; Vanden Meerschaut *et al.* 2014b). TFF recurrence affects ~30% of cases (Barlow *et al.* 1990). The principal cause of TFF in ICSI is oocyte activation failure, which is the failure of an oocyte to undergo further maturation, and now is commonly referred to as oocyte activation deficiency (OAD) (Weissman *et al.* 2002; Nikiforaki *et al.* 2016; Yeste *et al.* 2016a). Immunolabeling of 150 ICSI-failed human oocytes with  $\beta$  tubulins, a key component involved in oocyte spindle formation, showed that OAD caused 39.9% of ICSI failures (Rawe *et al.* 2000). In addition, OAD has been attributed to 15.1% of IVF failures, although the main cause of TFF in IVF is failed sperm penetration (55.5%). Other causes of TFF include pronuclear formation defects (19.2% in IVF vs 22.6% in ICSI), oocyte arrest in the mitotic metaphase (21.4% in IVF vs 13.2% in ICSI) (Rawe *et al.* 2000), and DNA decondensation (Aguila *et al.* 2017; Pabuccu *et al.* 2017).

## **1.2 Biological events and molecular mechanisms at oocyte activation**

### **1.2.1 Introduction and key events at oocyte activation**

Oocyte formation begins with the differentiation of oogonia from primordial germ cells into primary oocytes via mitosis (Virant-Klun 2015). The diploid primary oocyte is then arrested at the prophase I of the meiosis until ovulation (Mattiske *et al.* 2009). At ovulation, primary oocyte completes meiosis I and divides into a haploid secondary oocyte, which further initiates the meiosis II (MII) but remains arrested at the metaphase stage (Raven 2013). Oocyte arrestment at MII is a stable stage in which maternal ribonucleic acids (RNAs), proteins, and other cellular components are stored and await a trigger to undergo the final transition. This trigger is usually, but not always, fertilisation. The transition is accomplished by fundamental cellular changes and is commonly termed ‘oocyte activation’, which marks the development of a mature oocyte into an embryo (Heindryckx *et al.* 2008; Swain and Pool 2008; Ebner and Montag 2016; Yeste *et al.* 2016a; Karabulut *et al.* 2018).

Mammalian oocyte activation includes a series of fundamental embryonic events such as meiosis resumption, cortical granule exocytosis, pronuclei development, polyspermy blockage, sperm nucleus decondensation, maternal messenger ribonucleic acid (RNA) degradation, protein synthesis, and cytoskeleton rearrangement (Amdani *et al.* 2013). The morphological formation of the second polar body and pronuclei are the main indicators of successful oocyte activation (Rawe *et al.* 2000).

Recognising these morphological changes can help confirm oocyte activation in live cells. When oocyte activation is initiated, the meiotic spindle at the peripheral position rotates at a 90° angle, and the second polar body is

extruded (Brunet and Verlhac 2010). Extrusion of the second polar body requires a series of coordinating intracellular changes such as cytoskeleton rearrangement, chromatin configuration, and modifications in mitochondrial distribution (Yi *et al.* 2011; Luciano *et al.* 2012; Dalton and Carroll 2013), which indicates that the meiosis phase is completed. At this point, the oocyte is often referred to as an 'ovum' in humans.

The male pronucleus, containing genetic information, is introduced into the female ovum after sperm fusion. The paternal pronucleus then moves towards the maternal pronucleus and they progressively fuse together, forming a pronuclei and becoming diploid. Pronuclear formation represents successful oocyte activation, the completion of fertilisation and progression to the next stage of development.

These intracellular dynamics are inter-dependent and regulated by multiple mechanisms such as the ubiquitin–proteasome system (ubiquitin C-terminal hydrolases L) (Mtango *et al.* 2012), gap junction communications (Webb *et al.* 2002), microtubule-mediated mitochondrial distribution (Sun *et al.* 2001), phosphoinositide-mediated pathways (Amdani *et al.* 2016); cyclic adenosine monophosphate (cAMP) signalling (Viste *et al.* 2005; Vaccari *et al.* 2008), and multiple kinase factors such as M-phase promoting factor (MPF), mitogen-activated protein kinase (MAPK), protein kinase types A and C (PKA and PKC) and the G-protein coupled receptor (Tiwari *et al.* 2018).

### **1.2.2 Calcium oscillations at oocyte activation**

The main biochemical process underlying mammalian oocyte activation involves a series of oscillations in calcium ions ( $\text{Ca}^{2+}$ ) released from the

endoplasmic reticulum (ER) via the phosphoinositide-mediated pathway (Amdani *et al.* 2016; Swann 2018b). This pattern of  $\text{Ca}^{2+}$  release persists during fertilisation, but oscillation amplitude and frequency progressively decline, eventually ceasing at the time of pronuclei (PN) formation (Marangos *et al.* 2003; Ferrer-Buitrago *et al.* 2018b).

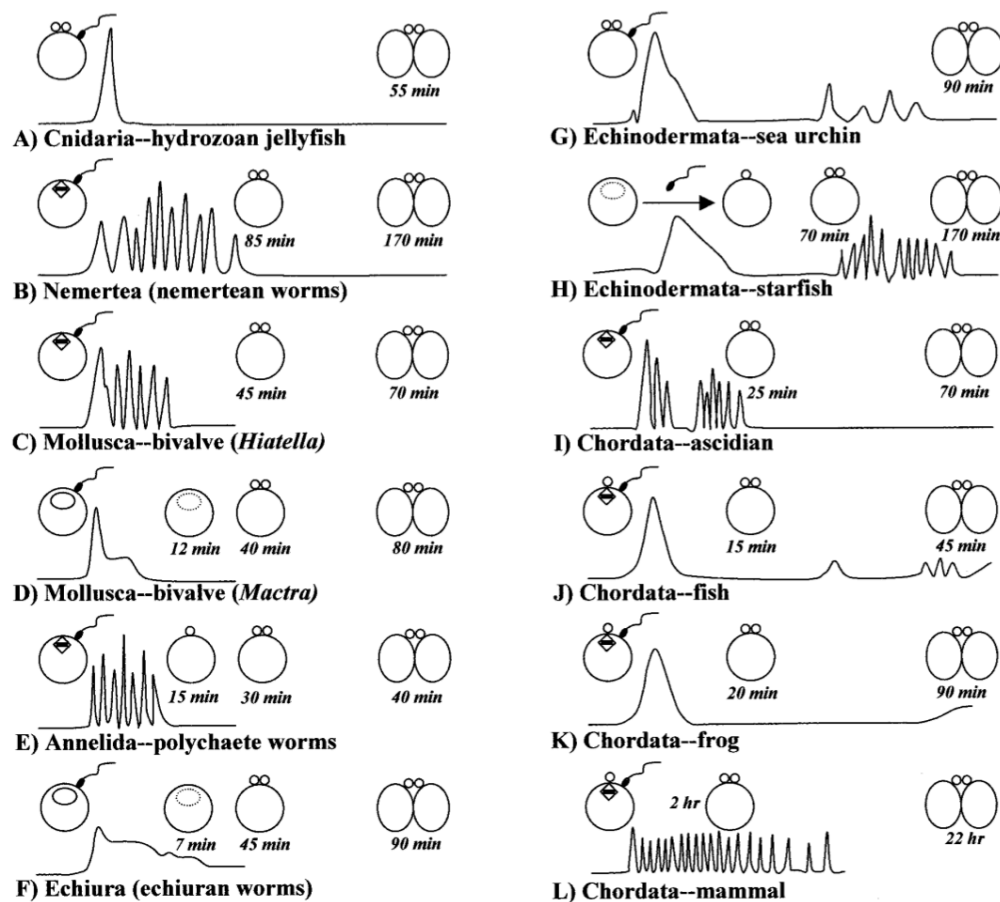
In the early 20<sup>th</sup> century, researchers were aware that ionic changes played a key role in initiating and supporting oocyte activation, and efforts had been made to identify the specific ion involved since the 1930s (Mazia 1937; Moser 1939). Significant progress was made in the 1970s, when Steinhardt *et al.* observed that intracellular  $\text{Ca}^{2+}$  release triggered echinoderm oocytes to undergo activation processes, but these events did not occur when they applied extracellular  $\text{Ca}^{2+}$ , sodium, or magnesium (Steinhardt and Epel 1974). Additional studies were subsequently carried out and identified that such  $\text{Ca}^{2+}$  release occurs in both mammalian and non-mammalian oocytes at fertilisation.

An increase in intracellular  $\text{Ca}^{2+}$  has now been identified as a universal event occurring at oocyte activation across different species, though its pattern may be single, repetitive, or involve sustained plateaus (**Figure 1.2**) (Jaffe 1983; Berridge 1993; Kashir *et al.* 2013a). Indeed, the oocytes of some species only exhibit a  $\text{Ca}^{2+}$  transient once at activation (such as cnidarians, echinoderms, frogs, and some murine species) (Stricker *et al.* 1992; Deguchi *et al.* 2005), whereas most mammalian oocytes exhibit repetitive  $\text{Ca}^{2+}$  elevations (commonly termed ‘ $\text{Ca}^{2+}$  oscillations’) (Stricker 1995; 1996; Ito *et al.* 2008b; Sensui *et al.* 2012). In addition, oocyte activation takes place at the point when oocytes are arrested, which varies across different species: prophase I in echiuran worms, metaphase I in ascidians, and at metaphase II in most mammals (**Figure 1.2**)

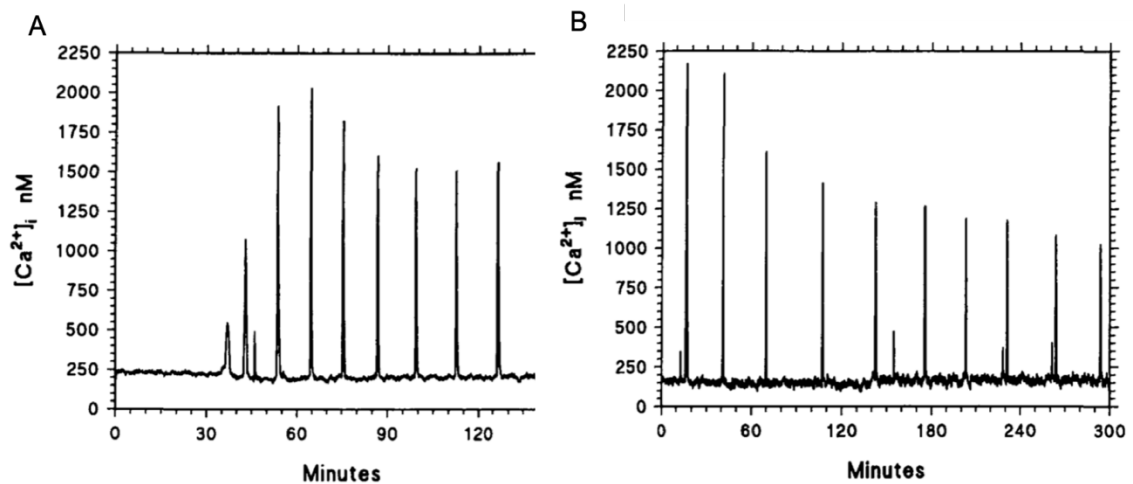


(Stricker 1999). Furthermore, the durations, frequencies, and amplitudes of  $\text{Ca}^{2+}$  oscillations show great diversity across different mammalian species (Kashir *et al.* 2013a; Nakai *et al.* 2016).  $\text{Ca}^{2+}$  oscillations occur every 10-35 min in human oocytes (i.e., 10-35 min intervals between individual  $\text{Ca}^{2+}$  rise), compared to every 10 min in mouse oocytes (Taylor *et al.* 1993; Miyazaki and Ito 2006).

The unique pattern of oscillatory  $\text{Ca}^{2+}$  release in human oocytes features in two types of increase: an initial tonic increase consisting of a rapid increase with a higher amplitude where lasts longer than subsequent increase; this then reaches a plateau and is followed by a series of oscillations. (Saunders *et al.* 2002; Halet *et al.* 2004; Norozi-Hafshejani *et al.* 2018). Taylor *et al.* studied the pattern of each  $\text{Ca}^{2+}$  peak using the photoprotein aequorin, a  $\text{Ca}^{2+}$ -sensitive imaging system. These authors found that fertilised human oocytes from IVF exhibited  $\text{Ca}^{2+}$  oscillations lasting 3-4 h with an amplitude of each peak up to 2.5  $\mu\text{M}$  and a frequency of every 10-35 min, which was the first direct measurement of  $\text{Ca}^{2+}$  changes in a human oocyte (**Figure 1.3**) (Taylor *et al.* 1993). They also observed that zona-free human oocytes exhibited oscillation patterns similar to zona-intact oocytes (**Figure 1.3**) (Taylor *et al.* 1993). Based on the distinct oscillatory patterns in human oocytes,  $\text{Ca}^{2+}$  imaging can be used to predict the capacity of oocyte activation in IVF/ICSI (Vanden Meerschaut *et al.* 2013).



**Figure 1.2 Temporal patterns of fertilisation-induced  $\text{Ca}^{2+}$  signals in protostome (B–F) and deuterostome (G–L) animals.** Normal state of oocyte maturation at fertilisation: prophase I-arrested (D, F); between germinal vesicle breakdown and polar body formation (H); metaphase I-arrested (B, C, E, I); metaphase II-arrested (J–L); pronuclear egg (A, G). Amplitudes, durations, and frequencies of calcium transients are not drawn to scale, and the timings of first cell cycle events are only approximate. Reproduced from Stricker (1999).

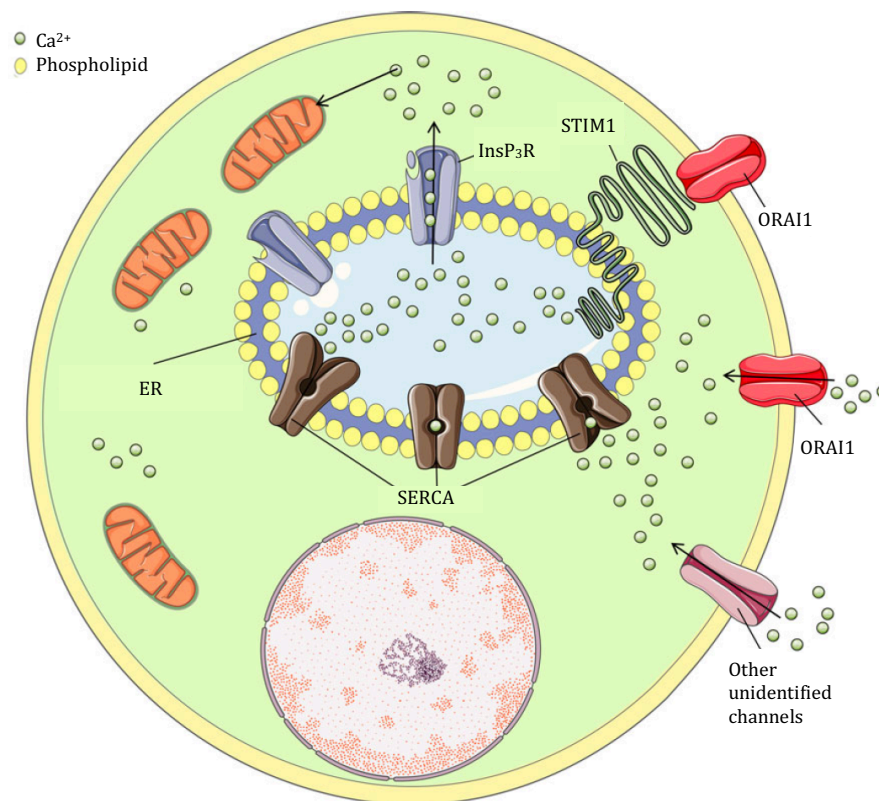


**Figure 1.3 Changes in intracellular  $\text{Ca}^{2+}$  concentrations in fertilising human oocytes.**  $\text{Ca}^{2+}$  oscillations were measured after microinjection of calcium-sensitive photoprotein aequorin into human zona-free oocytes (A) and zona intact oocytes (B). Sperm were added at time 0 in (A) and (B). Figures reproduced from Taylor *et al.* (1993).

The rise in  $\text{Ca}^{2+}$  at oocyte activation is fundamental in triggering most cellular events from fertilisation to embryogenesis. Therefore, efforts have been made to investigate the machinery included in  $\text{Ca}^{2+}$  release. Accumulating evidence indicates that this  $\text{Ca}^{2+}$  oscillatory pattern is initiated by a  $\text{Ca}^{2+}$  rise from the ER that is triggered by sperm-oocyte fusion via the  $\text{Ca}^{2+}$  signalling cascade, and is then accomplished by  $\text{Ca}^{2+}$  efflux and influx from ER membrane (Miyazaki *et al.* 1992; Miyazaki *et al.* 1993; Boulware and Marchant 2005; Yamasaki-Mann *et al.* 2010; Lopreiato *et al.* 2014; Stammers *et al.* 2015).

The widely accepted mechanisms underlying  $\text{Ca}^{2+}$  changes at oocyte activation is the ‘store-operated calcium entry’ (SOCE) (Kline and Kline 1992; Stammers *et al.* 2015). It is a protein system which operates  $\text{Ca}^{2+}$  entry in the most non-excitabile cells (cells do not generate action potentials) and are

composed of several gated-channels (Smyth *et al.* 2010). The major components of SOCE are 'sarco/ER and plasma membrane  $\text{Ca}^{2+}$  ATPase pumps (SERCA)', stromal interaction molecule (STIM), and  $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  channel protein 1 (ORAI1) (Smyth *et al.* 2010; Yeste *et al.* 2016a). These channels/molecules present in either ER membrane (SERCA), plasma membrane (ORAI1), or ER membrane and plasma membrane (STIM), and operate coordinately to maintain  $\text{Ca}^{2+}$  homeostasis in the oocyte (Yeste *et al.* 2016a) (**Figure 1.4**).



**Figure 1.4 Mechanisms involved in store-operated calcium entry (SOCE) for  $\text{Ca}^{2+}$  regulation.** ER: endoplasmic reticulum; SERCA: sarco/ER and plasma membrane  $\text{Ca}^{2+}$  ATPase pumps; STIM: stromal interaction molecule; and ORAI1:  $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  channel protein 1. Figures reproduced from Yeste *et al.* (2016a).

Indeed, ER is an important organelle which mediates  $\text{Ca}^{2+}$  oscillations, based on evidence that thapsigargin, an ER  $\text{Ca}^{2+}$  ATPase inhibitor, can deplete  $\text{Ca}^{2+}$  stores and thus yield reduced  $\text{Ca}^{2+}$  transients (Kline and Kline 1992). These authors further proposed that the oscillatory pattern is due to the periodic filling and emptying of  $\text{Ca}^{2+}$  stores (Kline and Kline 1992). In addition, a recent study on *Xenopus* oocytes suggested a 'pump-leak' mechanism involving SOCE as a 'pump' to provide  $\text{Ca}^{2+}$  influx and inositol 1,4,5-trisphosphate ( $\text{InsP}_3$ )-dependent substrate as a 'leak' to empty accumulated  $\text{Ca}^{2+}$ , therefore switching the  $\text{Ca}^{2+}$  release patterns between oscillatory and tonic (Courjaret *et al.* 2017).

$\text{InsP}_3$  is also vital in the mediation of  $\text{Ca}^{2+}$  signalling cascades (Miyazaki and Ito 2006; Parrington *et al.* 2019). Injection of  $\text{InsP}_3$  into mouse oocytes causes a rise in  $\text{Ca}^{2+}$  levels (Kline and Kline 1992), while downregulation of the  $\text{InsP}_3$  receptor ( $\text{InsP}_3\text{R}$ ) leads to  $\text{Ca}^{2+}$  oscillation failure in mouse and bovine oocytes (Brind *et al.* 2000; Malcuit *et al.* 2005). However, the key factor promoting  $\text{InsP}_3$  production and further stimulating  $\text{Ca}^{2+}$  signalling pathways remained unclear for a very long period; this will be introduced later. Nevertheless, it is at least clear that  $\text{InsP}_3$  is strongly involved in triggering  $\text{Ca}^{2+}$  release as a second messenger.  $\text{InsP}_3$  binds to its receptor,  $\text{InsP}_3\text{R}$ , on the ER surface, and this binding leads to conformational changes that open the ligand-gated channel, leading to the release of  $\text{Ca}^{2+}$  from the pores of the ER membrane via the channel (Carreras-Sureda *et al.* 2018). In line with this, a recent study applied sensitive fluorescence sensors to monitor  $\text{Ca}^{2+}$  and  $\text{InsP}_3$  changes during mouse oocyte activation and observed that delayed  $\text{InsP}_3$  peaks occur after each  $\text{Ca}^{2+}$  peak (Matsu-ura *et al.* 2019). These authors concluded that  $\text{Ca}^{2+}$ -induced

activity produces  $\text{InsP}_3$  and, in turn, sustained levels of  $\text{InsP}_3$  maintain the long-lasting  $\text{Ca}^{2+}$  oscillations (Matsu-ura *et al.* 2019).

The complexity of mammalian  $\text{Ca}^{2+}$  oscillation patterns indicates that multiple molecules and effectors are involved in this signalling cascade. Apart from  $\text{InsP}_3$ -mediated  $\text{Ca}^{2+}$  release, changes in PKC pathways have been reported to trigger early activation events such as second polar body excursion, cortical granule exocytosis, and the induction of small  $\text{Ca}^{2+}$  oscillations (Colonna *et al.* 1989; Ducibella 1996; Tatone *et al.* 2003; Yu *et al.* 2008). Stith *et al.* showed that PKC activity acts in synchrony with diacylglycerol (DAG), indicating that DAG contributes to PKC production (Stith *et al.* 1997). However, additional  $\text{Ca}^{2+}$  injection into *Xenopus* oocytes increases DAG levels, but the blockade of such  $\text{Ca}^{2+}$  does not stop DAG production. In addition, microinjection of PKC $\alpha$  cRNA into mouse oocytes does not increase  $\text{Ca}^{2+}$  levels (Madgwick *et al.* 2005). Halet *et al.* observed translocation of PKC between the cytosol and plasma membrane in living mouse oocytes, and noted that this was coordinated by changes in the intracellular  $\text{Ca}^{2+}$  levels (Halet *et al.* 2004). These authors proposed that PKC promotes  $\text{Ca}^{2+}$  influx through SOCE. Collectively, evidence suggests that the PKC pathway acts as a downstream factor that is both  $\text{Ca}^{2+}$  dependent and independent (Stith *et al.* 1997; Halet *et al.* 2004; Madgwick *et al.* 2005).

$\text{Ca}^{2+}$  oscillations also serve crucial roles in early embryo development, mainly through a downstream signalling pathway involving  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase (Backs *et al.* 2010). This pathway further regulates the expression of essential regulators such as MAPK and MPF (Ducibella *et al.* 2006; Miao *et al.* 2012). In addition,  $\text{Ca}^{2+}$  oscillations also activate the expression of calmodulin-dependent protein kinase II (CAMKII) by inhibiting the expression

of cytosolic factor (CSF) (Whitaker 1995; Yu *et al.* 2007). CAMII, together with other enzymes including DAG and PKC, belongs to a group of important transducers transferring  $\text{Ca}^{2+}$  signals to cellular responses (Swann and Yu 2008). In addition,  $\text{Ca}^{2+}$  contributes to developmental biology in the subsequent mammalian embryonic/foetal/postnatal development stages (Stewart and Davis 2019). For example,  $\text{Ca}^{2+}$  maintains the homeostasis required for cardiac and vascular development during week 3 of embryogenesis in humans (Ritchie *et al.* 2019).  $\text{Ca}^{2+}$  is transferred from the maternal to foetal circulation via the placenta and supports further development and mediates cytotoxicity (Baczyk *et al.* 2011).  $\text{Ca}^{2+}$  signalling is also critical in regulating the differentiation and proliferation of male prostate development after birth (Lloyd-Lewis *et al.* 2018).

Intracellular zinc ion ( $\text{Zn}^{2+}$ ) levels also play an important role in the biological events of oocyte activation. It is acknowledged that the meiotic progression of mammalian oocytes is accompanied by increased  $\text{Zn}^{2+}$  levels, and that this is important in supporting oocyte development beyond telophase I and establishing meiotic arrest (Zhao *et al.* 2014c). In particular, at oocyte activation, intracellular levels of  $\text{Zn}^{2+}$  are reduced and then released into the extracellular space through  $\text{Zn}^{2+}$ -enriched cortical vesicles; this occurs concurrently with the increase in intracellular  $\text{Ca}^{2+}$  (Kim *et al.* 2011; Zhao *et al.* 2014b; Que *et al.* 2019). Changes in  $\text{Zn}^{2+}$  levels during oocyte activation are in line with the triggering of exocytosis in mammalian oocytes by high intracellular  $\text{Zn}^{2+}$  levels. The  $\text{Zn}^{2+}$  transients at oocyte activation, termed the 'zinc spark', is more dependent on  $\text{Ca}^{2+}$  transients, possibly because these are regulated by downstream effectors of the  $\text{Ca}^{2+}$  signalling cascade (Kim *et al.* 2010; Suzuki *et al.* 2010). In addition,

the amplitude of the zinc spark is associated with embryo quality (Zhang *et al.* 2016).

### **1.3 Phospholipase C zeta (PLC $\zeta$ )**

#### **1.3.1 Sperm-borne oocyte-activating factors (SOAFs)**

Rigorous attempts were made to identify the stimulus/stimuli that induce(s) Ca<sup>2+</sup> release and oocyte activation. Ground-breaking evidence was occurred in the 1980s, when the microinjection of sperm extracts into sea urchin and ascidian oocytes caused them to undergo biological changes similar to those seen at normal fertilisation, such as cortical granule exocytosis and changes in Ca<sup>2+</sup> levels (Dale *et al.* 1985; Dale 1988). Subsequent studies revealed that microinjected sperm extracts caused fertilisation-like Ca<sup>2+</sup> increases, or oscillations, in oocytes from several different species (Swann 1990; Wilding and Dale 1998; Swann and Parrington 1999; Runft and Jaffe 2000) and triggered biological development events that occurred in synchrony with the changes in Ca<sup>2+</sup> levels (Kuretake *et al.* 1996; Stricker 1997; Kyoizuka *et al.* 1998; Perry *et al.* 2000). Furthermore, the fact that pig sperm extracts triggered mouse oocyte activation and that this was found to be mediated by InsP<sub>3</sub>R further confirmed that the stimulus/stimuli was/were sperm-derived (Wu *et al.* 2001).

However, which part of the sperm, and the precise mechanism of oocyte activation, were highly debatable initially. Three hypotheses were proposed initially (Oda *et al.* 1999; Kashir *et al.* 2012a), but accumulating evidence over the years has shifted strong support to the 'sperm factor' theory which suggested that a soluble factor was released from the sperm head into the oocyte during gamete fusion and triggered oocyte activation. Subsequent studies



suggested that the properties of the sperm-derived factor(s) would be proteinaceous and proteolytic (Perry *et al.* 1999; Perry *et al.* 2000). Potential candidates expressed in the sperm head sheath were referred to as sperm-borne oocyte-activating factors (SOAFs) (Amdani *et al.* 2015).

The dominant SOAF representative is phospholipase C zeta (PLC $\zeta$ ), which is expressed in the sperm head and can induce Ca<sup>2+</sup> release in oocytes (Parrington *et al.* 2002; Saunders *et al.* 2002). Other SOAF candidates included PAWP (post-acrosomal sheath WW domain-binding protein), truncated form of c-kit tyrosine kinase receptor (tr-kit) and citrate synthase (Yeste *et al.* 2016a). However, microinjection of PAWP-deficient sperm from a transgenic mouse still caused Ca<sup>2+</sup> oscillations in mouse oocyte (Satouh *et al.* 2015), and tr-kit was suggested to interact with oocytes via PLC $\gamma$ 1 that is commonly expressed in non-mammalian species (Sato *et al.* 2006). Therefore, with the exception of PLC $\zeta$ , these proteins became less convincing over the years, given by the fact that none of these candidates presented the characteristics responsible for triggering Ca<sup>2+</sup> release and activating mammalian oocytes (Sette *et al.* 1998; Harada *et al.* 2007; Nomikos *et al.* 2014a; Kashir *et al.* 2015; Freour *et al.* 2017).

### 1.3.2 Discovery and localisation of PLC $\zeta$

There is now substantial scientific and clinical evidence to support the fact that PLC $\zeta$  is the primary factor responsible for Ca<sup>2+</sup> release and initiation of oocyte activation (Amdani *et al.* 2013; Kashir *et al.* 2018; Kimura *et al.* 2018). **Figure 1.5** shows a schematic representation of how PLC $\zeta$  is thought to contribute to mammalian oocyte activation.

PLC $\zeta$  was first discovered in 2002. Saunders *et al.* identified a new form of PLC from mouse extracts (~74 kDa) which was different from previously identified isoforms ( $\beta$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$ ). These authors further synthesised PLC $\zeta$  cRNA from a mouse spermatid cDNA library and microinjected it into mouse MII oocytes, which enabled oocytes to undergo embryonic development in the absence of fertilisation (Saunders *et al.* 2002). Conversely, Ca<sup>2+</sup> activity in mouse oocytes was abolished following microinjection with PLC $\zeta$ -depleted sperm extract (Saunders *et al.* 2002). Grasa *et al.* later used immunoblotting to identify the sizes of PLC $\zeta$  in human sperm and sperm extract, which both showed a band at ~70 kDa, although minor bands with smaller molecular weights were also observed in human sperm (Grasa *et al.* 2008).

Since the discovery of PLC $\zeta$ , substantial evidence has confirmed the indispensable role of PLC $\zeta$  in oocyte activation. For example, the microinjection of sperm extracts containing PLC $\zeta$  into mouse oocytes elicited fertilisation-like Ca<sup>2+</sup> oscillations (Fujimoto *et al.* 2004). The amount of PLC $\zeta$  protein in mouse oocytes after PLC $\zeta$  cRNA injection was also coordinated with the amplitude of Ca<sup>2+</sup> release triggered at oocyte activation (Yoda *et al.* 2004). Accordingly, mouse sperm containing reduced levels of PLC $\zeta$  as a result of RNA interference showed premature and non-persistent Ca<sup>2+</sup> oscillatory patterns, indicating that PLC $\zeta$  is important in shaping Ca<sup>2+</sup> patterns (Knott *et al.* 2005). However, such modification did not cause these mice to be infertile, as female mice mating with PLC $\zeta$ -deficient males resulted in fewer and smaller pups (Knott *et al.* 2005).

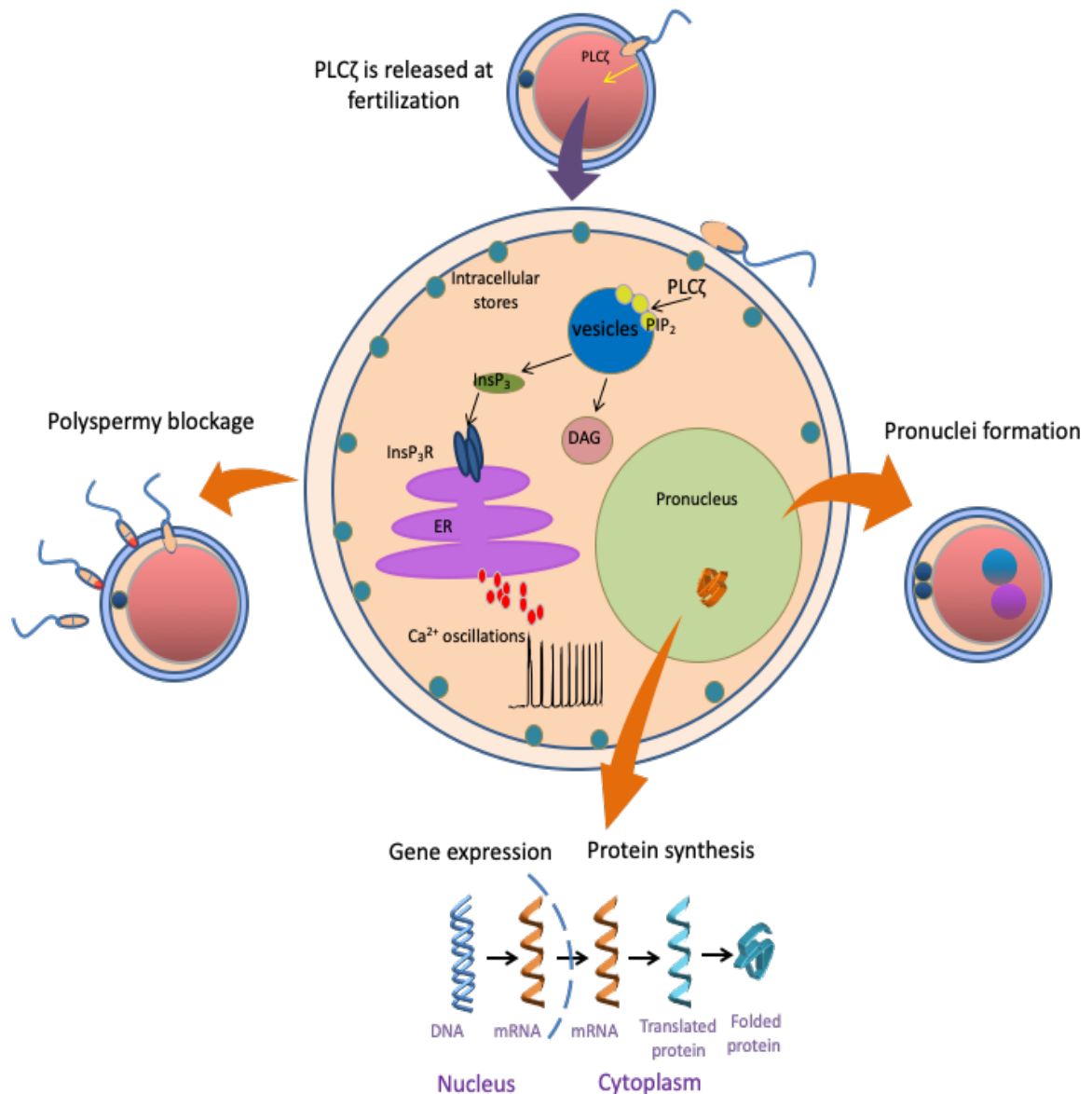
PLC $\zeta$  is an important physiological factor in triggering oocyte activation, but in recent years it has become debatable whether PLC $\zeta$  is the sole factor required for fertilisation, as PLC $\zeta$ -deficient mice do not seem to be sterile

(Hachem *et al.* 2017b; Nozawa *et al.* 2018). Indeed, PLC $\zeta$  is more necessary for monospermic fertilisation. Two recent studies applied a clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) approach to generate PLC $\zeta$  knock-out (KO) mice. Hachem *et al.* truncated exons 3 and 5 of the mouse PLC $\zeta$  gene, while Nozawa *et al.* deleted all coding exons of PLC $\zeta$  (Hachem *et al.* 2017b; Nozawa *et al.* 2018). Subsequent ICSI in both studies using mutant sperm into mouse oocytes did not trigger Ca<sup>2+</sup> oscillations nor oocyte activation. Interestingly, authors from both studies mated PLC $\zeta$  KO mice with wild-type female mice, and both obtained few, but viable, pups. Nozawa *et al.* also collected fertilised oocytes from females after mating with PLC $\zeta$  KO mice and found that fertilised oocytes exhibited abnormal oocyte activation (1 pronucleus), polyspermy, or were arrested at the pronuclei stage (Nozawa *et al.* 2018). As mentioned earlier, the ICSI procedure involves microinjection with sperm from oocyte membrane into cytoplasm, while normal fertilisation or IVF keeps the oocyte membrane intact. The differences between these two approaches may explain the different outcomes in the experiments. Nozawa *et al.* further proved that PLC $\zeta$  helped to block polyspermy in IVF (Nozawa *et al.* 2018).

A recent review hypothesised that a 'sperm cryptic-activating factor (SCAF)' is responsible for initiating Ca<sup>2+</sup> oscillations only in the absence of PLC $\zeta$  (Jones 2018). However, what exactly SCAF is, and how it activates an oocyte, remain unknown. Jones further proposed that the SCAF should meet two conditions: (1) its activity is somehow destroyed/diminished in ICSI and (2) that membrane-based fertilisation (i.e., IVF and normal fertilisation) is a prerequisite to the SCAF. Both tr-kit and PAWP have been suggested as potential candidates

for the SCAF (Jones 2018). Again, more data are needed to support this hypothesis.

Despite of conflicting debates, the overall evidence suggests that PLC $\zeta$  is the dominant physiological trigger of normal Ca<sup>2+</sup> oscillations, especially in monospermic fertilisation.

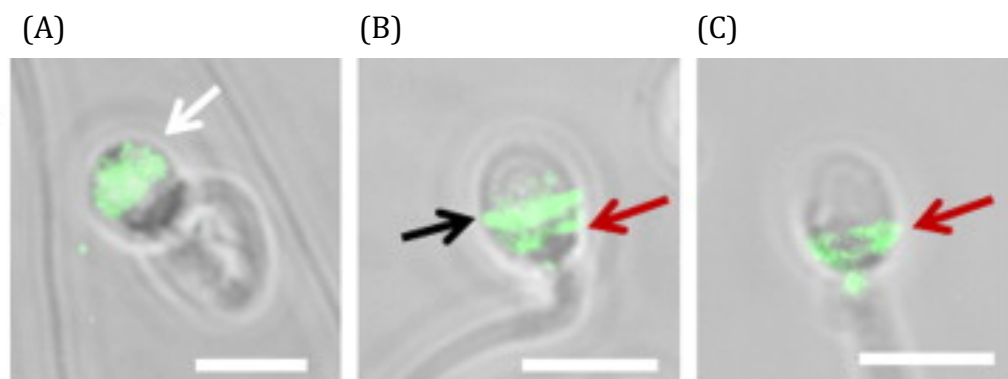


**Figure 1.5 Schematic diagram of the biological events that occur during PLC $\zeta$ -mediated oocyte activation.** PLC $\zeta$ : phospholipase C zeta; DAG: diacylglycerol; InsP<sub>3</sub>: inositol 1,4,5-trisphosphate; PIP<sub>2</sub>: phosphatidylinositol-4,5-bisphosphate. Reproduced from Yeste *et al.* (2016).

PLCζ was reported to be secreted from the epididymal epithelial cells and expressed in sperm during mammalian sperm maturation (Aarabi *et al.* 2012). Several studies have identified the expression of PLCζ in the sperm head of both mammalian (e.g., human, pigs, horse, and bull) and non-mammalian (e.g., fish and chickens) species (Coward *et al.* 2005b; Young *et al.* 2009; Coward *et al.* 2011; Kashir *et al.* 2013a; Sato *et al.* 2013). Furthermore, PLCζ has distinctive localisation patterns in different species. For instance, PLCζ is expressed in the acrosomal and equatorial regions of equine sperm (Sato *et al.* 2013) and in the equatorial or post-acrosomal regions of bull sperm (Yoon and Fissore 2007; Aarabi *et al.* 2012). In mouse and porcine sperm, PLCζ is found in the acrosomal and post-acrosomal regions, with additional expression in the tail of porcine sperm (Young *et al.* 2009; Nakai *et al.* 2011). Interestingly, immunofluorescence labelling of mouse and hamster sperm showed that the proportion of non-capacitated sperm expressing PLCζ in the acrosomal region was reduced after capacitation, while the proportion of that in the post-acrosomal region was increased. Evidence indicates that PLCζ expression patterns are associated with the acrosome reaction, and that PLCζ may play a role in the acrosome reaction (Young *et al.* 2009)

PLCζ expression patterns in human sperm were investigated with the aim of associating such patterns with human oocyte activation capacity. The perinuclear theca in the sperm head is a protein-condensed matrix surrounding the sperm nucleus and is crucial for maintenance of sperm head structure (Amdani *et al.* 2015). Immunofluorescent labelling revealed PLCζ expression in three regions of the perinuclear theca: acrosomal, equatorial, and post-acrosomal regions (Yoon and Fissore 2007; Grasa *et al.* 2008; Young *et al.* 2009).

PLC $\zeta$  protein is predominantly expressed in the equatorial region of human sperm. Equatorial localisation, or equatorial combined with acrosomal/post-acrosomal localisation is considered to be functional for oocyte activation (**Figure 1.6**) (Grasa *et al.* 2008). A subsequent study observed other PLC $\zeta$  localisation patterns in human sperm (acrosomal, post-acrosomal, acrosomal + post-acrosomal, acrosomal + equatorial + post-acrosomal, and acrosomal + equatorial + post-acrosomal) and reported that both infertile and fertile individuals exhibited significant variance in PLC $\zeta$  expression patterns, when compared to their counterparts (Kashir *et al.* 2013b). Occasionally, PLC $\zeta$  has also been reported in the tail or midpiece of human sperm (Grasa *et al.* 2008; Durban *et al.* 2015).



**Figure 1.6 Representative immunofluorescence images of PLC $\zeta$  in human sperm showing characteristic localisation patterns.** Sperm from male donor showed PLC $\zeta$  at acrosomal (A, white arrow), equatorial (B, black arrow) and post-acrosomal (B and C, red arrow) localisation. Scale bar represents 5  $\mu$ m. Reproduced from Kashir *et al.* (2013b).

Indeed, localisation of human sperm varies across different studies. A recent study observed a dominant acrosomal localisation over equatorial localisation in the sperm (Ferrer-Vaquer *et al.* 2016). Aarabi *et al.* reported PLC $\zeta$  in the acrosome and post-acrosome of non-permeabilised human sperm, and further debated whether PLC $\zeta$  localised to the sperm surface or the perinuclear theca area (Aarabi *et al.* 2012).

The inconsistency of PLC $\zeta$  localisation patterns across different studies could represent poor antibody specificity or disparity between immunological protocols, and/or sperm processing protocols. Our laboratory has optimised a standard 'in-house' protocol to best visualise and evaluate PLC $\zeta$  expression in mammalian sperm; this assay has proven to be sensitive, consistent and effective. This in-house method has been successfully applied to human sperm in many studies (Heytens *et al.* 2009; Young *et al.* 2009; Kashir *et al.* 2012c; Kashir *et al.* 2013a; Kashir *et al.* 2013b; Yelumalai *et al.* 2015; Yeste *et al.* 2016b). However, another group proposed that the investigation of PLC $\zeta$  localisation using immunolabelling could result in poor visualisation due to the low affinity of the synthesised polyclonal antibody to the antigens in some previous studies performed by other research group, and suggested that the antigen unmasking method might improve visualisation (Kashir *et al.* 2017).

PLC $\zeta$  localisation patterns also vary when detected under different conditions. For example, Grasa *et al.* found that the proportion of PLC $\zeta$  expressed in the post-acrosomal region of human sperm increased after capacitation and induction of the acrosome using ionophores, with a corresponding reduction in the acrosomal region (Grasa *et al.* 2008). Accordingly, Durban *et al.* subsequently found that the dominant PLC $\zeta$  localisation was in the acrosomal

region of acrosome-intact human sperm but then shifted to the midpiece after the acrosome reaction (Durban *et al.* 2015). Interestingly, PLC $\zeta$  immunofluorescence levels are significantly lower in cryopreserved fertile sperm than in fresh male sperm, indicating that the capacity of sperm to activate oocytes may be affected by cryopreservation (Kashir *et al.* 2011a; Moreau *et al.* 2019).

### 1.3.3 PLC $\zeta$ domain structure and function

The phospholipase C (PLC) family is an important group of proteins that regulate multiple cellular activities including proliferation, differentiation and pathological tumour invasion via the phosphoinositide-specific PLC signalling cascade (Lattanzio *et al.* 2013; Huang *et al.* 2018). As a new PLC isoform, PLC $\zeta$  shares certain domains and a common mechanism for signal transduction. It has been acknowledged that PLC $\zeta$  hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) to produce the second messengers DAG and InsP<sub>3</sub>; then, DAG activates the PKC pathway, while InsP<sub>3</sub> triggers Ca<sup>2+</sup> release from ER (**Figure 1.5**) (Kadamur and Ross 2013).

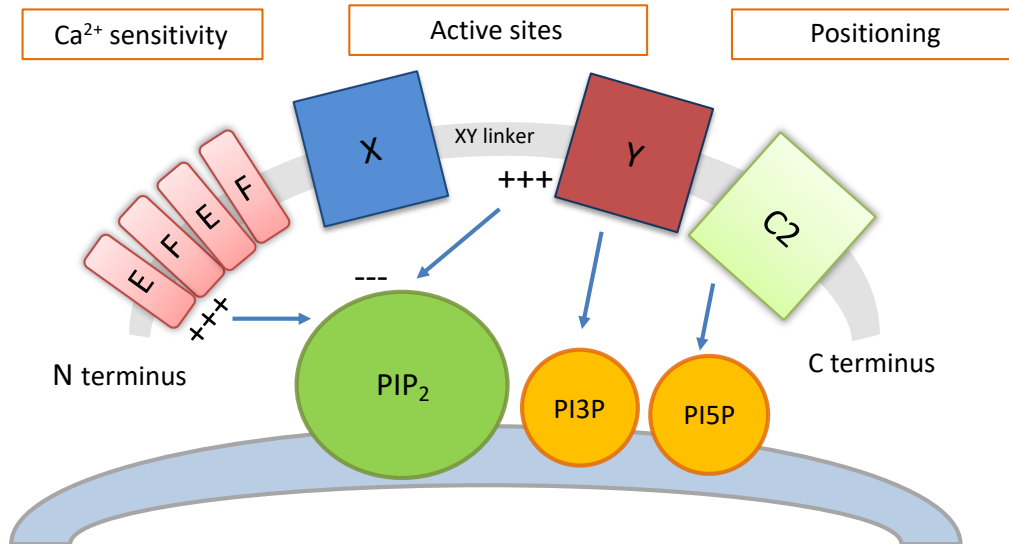
It has been unclear whether PLC $\zeta$  hydrolyses PIP<sub>2</sub> in the cytoplasm or plasma membrane. PIP<sub>2</sub> is a membrane-associated substrate, and depletion of PIP<sub>2</sub> changes the activities of phospholipase-based proteins, such as PLC isoforms (PLCs) (Huang *et al.* 2011). Accordingly, the hydrolytic activity of PIP<sub>2</sub> by PLC $\zeta$  was increased in detergent micelles after adding Ca<sup>2+</sup>, which indicated that Ca<sup>2+</sup> triggered PLC $\zeta$  to hydrolyse the PIP<sub>2</sub> (Huang *et al.* 2018). However, PLC $\zeta$  has not been detected in the plasma membrane of an activated oocyte. In contrast, some studies have suggested that PLC $\zeta$  targets its substrates in a



cytosolic manner by targeting PIP<sub>2</sub>-containing vesicles in mammalian oocytes (Yu *et al.* 2012; Nomikos 2015). However, if the vesicle is a cargo of the hydrolysis process, then Ca<sup>2+</sup> should be released from the cytoplasm, which would contradict early understanding that the Ca<sup>2+</sup> release process occurred via the plasma membrane (Amdani *et al.* 2015; Horn and Jaiswal 2018). One explanation is that the phosphoinositide signalling bypasses the plasma membrane completely (Yu *et al.* 2012). Accordingly, a recent study investigated PLCζ-induced intracellular PIP<sub>2</sub> distributions and accompanied Ca<sup>2+</sup> release, using a rhodamine-peptide probe that can bind to PIP<sub>2</sub> in a live cell (Sanders *et al.* 2018). These authors found PIP<sub>2</sub>-containing vesicles distributed throughout the entire cytoplasm of PLCζ cRNA-injected mouse oocytes. In addition, they demonstrated that PLCζ is more dependent upon cytoplasmic vesicles to trigger Ca<sup>2+</sup> oscillations (Sanders *et al.* 2018).

Since the discovery of PLCζ, numerous studies have been conducted to investigate PLCζ protein structure and associated functions at oocyte activation. At the protein level, PLCζ is most similar to PLCδ1; the similarity of these two isoforms is 33% (Saunders *et al.* 2002). To date, 14 PLCs have been identified in 6 subfamilies (β, δ, γ, ε, ζ, and η). PLCζ shares common domains with other PLCs, including two pairs of elongation-factor (EF) domains at the N-terminus, X and Y catalytic domains connected by an XY-linker, and a PKC-homology type II domain at the C-terminus (C2 domain) (schematic representation is shown in **Figure 1.7**) (Swann *et al.* 2007; Nomikos *et al.* 2011a; Nomikos *et al.* 2011b; Nomikos *et al.* 2015). However, PLCζ lacks a common pleckstrin homology (PH) and Src homology (SH) domain, making it the smallest PLC isoform (~70 kDa in human and ~74 kDa in mouse) (Saunders *et al.* 2002; Nomikos *et al.* 2017a). To

fulfil the functional role of PLC $\zeta$  at oocyte activation, all structural components are expected to operate nominally.



**Figure 1.7 Schematic representation of PLC $\zeta$  domain structure and function.** EF domains maintain the sensitivity of PLC $\zeta$  to Ca<sup>2+</sup>; XY-linker and C2 domains are responsible for the protein targeting on phospholipase-containing substrate; X and Y domains form the active sites that regulate the hydrolysis activity of PLC $\zeta$ . PLC $\zeta$  targets on negatively charged (---) PIP<sub>2</sub>, via electrostatic interactions with positively charged (+++) EF domain and XY linker. PIP<sub>2</sub>: phosphatidylinositol-4,5-bisphosphate; PI3P: phosphatidylinositol-3-phosphate; and PI5P: Phosphatidylinositol 5-phosphate. Figures reproduced from Nomikos *et al.* (2017).

The catalytic X and Y domains form active sites which are responsible for the enzymatic activities of PLC $\zeta$ ; the sequences of these domains are highly conserved across all PLCs (Kashir *et al.* 2012a). Accordingly, mutagenesis of these conserved residues affect the ability of PLC $\zeta$  to hydrolyse PIP<sub>2</sub> and results in the failure of PLC $\zeta$  to generate Ca<sup>2+</sup> oscillations (Ellis *et al.* 1998; Swann *et al.* 2007).

The region connecting the X and Y domains is a charged region, known as the XY-linker. It is worth noting that this region plays a significant role in PLC $\zeta$  activity, including its ability to trigger the Ca<sup>2+</sup> cascade by hydrolysing PIP<sub>2</sub> at activation. The negatively charged XY-linkers of PLC  $\beta$ ,  $\delta$ , and  $\epsilon$  inhibit the hydrolysis of PIP<sub>2</sub> with electrostatic repulsion (Hicks *et al.* 2008). In contrast to the negatively charged XY-linker in other PLCs, that of PLC $\zeta$  is positively charged, which could help PLC $\zeta$  anchor to the negatively-charged phospholipase-based substrate. Indeed, mutagenesis of three lysine residues related to the positively charged features of the PLC $\zeta$  XY-linker affected the binding affinity of PLC $\zeta$  mutant protein to liposomes containing PIP<sub>2</sub> (Nomikos *et al.* 2011a). Furthermore, Nomikos *et al.* deleted 12 amino acids from the PLC $\zeta$  XY-linker, or replaced those with that of PLC $\delta$ 1, then synthesised a PLC $\zeta$  cRNA mutant. Microinjection of these mutants significantly reduced Ca<sup>2+</sup> oscillations in mouse oocytes from 25 spikes to 3.4 or 5.3 spikes every 2 h (Nomikos *et al.* 2011b). Collectively, evidence indicates that the XY-linker of PLC $\zeta$  is a distinctive structure from the other PLCs and functionally regulates PLC $\zeta$  enzymatic activity via the hydrolysis of PIP<sub>2</sub>. Notably, PLC $\zeta$  cRNA mutants only diminished, not completely abolished, the Ca<sup>2+</sup>-inducing activity of PLC $\zeta$ , thus indicating the existence of other mechanisms regulating PLC $\zeta$  hydrolysis activity (Nomikos *et al.* 2011a; Nomikos *et al.* 2011b).

In mice, the XY-linker is also associated with translocation of PLC $\zeta$  to the nuclei, possibly through nuclear localisation signals (Larman *et al.* 2004; Kuroda *et al.* 2006). Myc-tagged PLC $\zeta$  was detected in the nucleoplasm of the pronuclei of the activated oocytes (Larman *et al.* 2004). Proteins that are larger than 60 kDa can be imported into the nuclear membrane via the nuclear localisation

signal (NLS), which contains a group of basic amino acid sequences (Dingwall and Laskey 1991). Interestingly, PLC $\zeta$  contains NLS sequences in the XY-linker region (374-381 amino acids), and microinjection of mutant PLC $\zeta$  cRNA lacking XY-linker sequences failed to cause nuclear import. Correspondingly, the replacement or point mutation of some of these regions also abolished nuclear translocation (Kuroda *et al.* 2006). Given this evidence, the PLC $\zeta$  XY-linker may play a role in nuclear translocation, possibly by regulating the NLS, and it is very possible that abnormalities in this region will have functional consequences.

The EF domains help PLC $\zeta$  maintain high sensitivity to Ca<sup>2+</sup> (Kouchi *et al.* 2005; Nomikos *et al.* 2005; Nomikos *et al.* 2015). In studies of PLC $\zeta$  sensitivity, maximum concentrations of Ca<sup>2+</sup> triggering the PLC-PIP<sub>2</sub> hydrolytic activity were assessed, and the 'half maximal effective concentration' (EC<sub>50</sub>) was used to reflect sensitivity to Ca<sup>2+</sup>. Nomikos *et al.* showed that PLC $\zeta$  is ~100 times more sensitive to Ca<sup>2+</sup> than PLC $\delta$ 1, with an EC<sub>50</sub> of 50-80 nM for PLC $\zeta$  and 6  $\mu$ M for PLC $\delta$ 1 (Nomikos *et al.* 2005). Deletion of a pair, or all EF domains, increased EC<sub>50</sub> values to 10 and 300 fold, respectively (Nomikos *et al.* 2005). Conversely, Kouchi *et al.* found that deletion of a pair of EF domains at the N terminus did not significantly reduce Ca<sup>2+</sup> sensitivity, whereas two point mutations resulted in an EC<sub>50</sub> that was doubled (Kouchi *et al.* 2005). These authors also found that deletion of 3 EF domains increased EC<sub>50</sub> by ~12 fold (Kouchi *et al.* 2005). To investigate if EF domains are entirely responsible for the high sensitivity of PLC $\zeta$  to Ca<sup>2+</sup> on their own, the same research group synthesised a chimeric model using mouse PLC $\zeta$  (mPLC $\zeta$ ); these authors substituted the EF domains with those of rat PLC $\delta$ 1. Accordingly, the chimeric protein showed lower sensitivity

to  $\text{Ca}^{2+}$  than wild-type mouse PLC $\zeta$ , indicating that PLC $\zeta$ 's high sensitivity to  $\text{Ca}^{2+}$  is predominantly mediated by EF domains (Theodoridou *et al.* 2013).

A subsequent study replaced the EF domain from human PLC $\zeta$  (hPLC $\zeta$ ) with corresponding mPLC $\zeta$  and obtained a chimera protein (hPLC $\zeta$ /mEF). These authors observed that the sensitivity of hPLC $\zeta$ /mEF to  $\text{Ca}^{2+}$  was reduced by 3-fold, which indicated that the role of the EF domain in PLC $\zeta$  sensitivity may vary across species (Nomikos *et al.* 2014b). Indeed, the potency of PLC $\zeta$  in inducing  $\text{Ca}^{2+}$  oscillations is known to be species-specific (Bedford-Guaus *et al.* 2011); hPLC $\zeta$  is more potent than mPLC $\zeta$ . A previous study injected mPLC $\zeta$  cRNA and hPLC $\zeta$  cRNA (20-fold less) into mouse oocytes and showed that hPLC $\zeta$  cRNA induced higher  $\text{Ca}^{2+}$  oscillation frequencies than mPLC $\zeta$  cRNA (Nomikos *et al.* 2014b). These authors further proposed that EF domains are related to the potency of PLC $\zeta$ , because replacement of the EF domains in hPLC $\zeta$  with those of mPLC $\zeta$  yielded reduced frequency but increased amplitude of  $\text{Ca}^{2+}$  oscillations (from 46.2 to 36.4 spikes per 2 h and from 7.6 to 51.5 counts per second, respectively) (Nomikos *et al.* 2014b).

EF domains may also play a regulatory role in PLC $\zeta$  enzymatic activity. Deletion of the entire EF domain resulted in complete loss of the  $\text{Ca}^{2+}$ -inducing activity of PLC $\zeta$  (Kouchi *et al.* 2005). Accordingly, a subsequent study revealed that mutation of the cationic-containing EF domain and XY-linker influenced PLC $\zeta$  binding affinity to  $\text{PIP}_2$  (Nomikos *et al.* 2015). In contrast, when the EF domains of mPLC $\zeta$  were substituted with those of PLC $\delta$ 1, there was similar enzymatic activity to wide-type PLC $\zeta$  (Theodoridou *et al.* 2013). Overall, the available evidence suggests that EF domains maintain the high sensitivity of

PLC $\zeta$  to Ca<sup>2+</sup>, whereas overall PLC $\zeta$  activity may require other domains to act cooperatively.

The C2 domain is conserved in all PLCs, but its exact physiological role remains unclear. It has been suggested that the C2 domain is required for PLC $\zeta$  catalytic activity, because its deletion leads to the failure of PLC $\zeta$  protein to cause hydrolysis of its substrate (Kouchi *et al.* 2005). Similarly, Theodoridou *et al.* synthesised PLC $\zeta$ / $\delta$ 1C2 cRNA encoding PLC $\zeta$  in which the C2 domain was substituted with the PLC $\delta$ 1 C2 domain. Furthermore, the microinjection of this cRNA into mouse oocytes failed to cause Ca<sup>2+</sup> release (Theodoridou *et al.* 2013). Conversely, Nomikos *et al.* argued that ~70% of the hydrolytic activity of PLC $\zeta$  is retained after C2 domain deletion (Nomikos *et al.* 2005). Nevertheless, the injection of a cRNA encoding for C2-deleted PLC $\zeta$  into mouse oocytes resulted in the complete loss of Ca<sup>2+</sup> oscillations (Nomikos *et al.* 2005). In line with this, PLC $\zeta$  cRNA encoding a point mutation in the C2 domain caused failure of Ca<sup>2+</sup> oscillations in mouse oocytes, and its corresponding protein was less effective than wild-type in triggering Ca<sup>2+</sup> oscillations (Nomikos *et al.* 2017b). Combining all of the available evidence, the C2 domain may play a physiological role in Ca<sup>2+</sup>-inducing activity, but not hydrolytic activity. Indeed, a liposome binding assay in micelles indicated that the C2 domain interacted with PIP<sub>3</sub> and PIP<sub>5</sub>, instead of PIP<sub>2</sub> (Kouchi *et al.* 2005; Nomikos *et al.* 2017b). This further suggested a regulatory role of the C2 domain by binding to subcellular substrate, but not PIP<sub>2</sub>.

Collectively, PLC $\zeta$  domains may have different enzymatic functions and act cooperatively at oocyte activation. Any abnormality in PLC $\zeta$  protein/genes or its ability to release Ca<sup>2+</sup> will therefore cause OAD.

#### **1.3.4 The association between PLC $\zeta$ abnormalities and infertility**

OAD has been increasingly linked to deficient levels and abnormal localisation patterns of PLC $\zeta$  protein in sperm. Studying the expression of PLC $\zeta$  protein in human sperm can help us to understand their ability to trigger oocyte activation. Yoon *et al.* were the first to clinically link OAD with PLC $\zeta$ . In this study, the authors injected human sperm from patients with a history of ICSI-failure into mouse oocytes, which resulted in a lack of Ca<sup>2+</sup> oscillations and oocyte activation failure. Further investigation revealed that such sperm exhibited absent/abnormal PLC $\zeta$  expression or abnormal PLC $\zeta$  localisation, and also that the co-injection of PLC $\zeta$  mRNA into mouse oocytes successfully rescued oocyte activation failure (Yoon *et al.* 2008). Further investigations showed that PLC $\zeta$  expression levels help to evaluate OAD. Using an immunofluorescence technique, Kashir *et al.* found that the relative intensity of PLC $\zeta$  in infertile patients diagnosed with OAD was significantly lower than fertile controls and that there was significant variability in PLC $\zeta$  expression across different individuals (Kashir *et al.* 2013b).

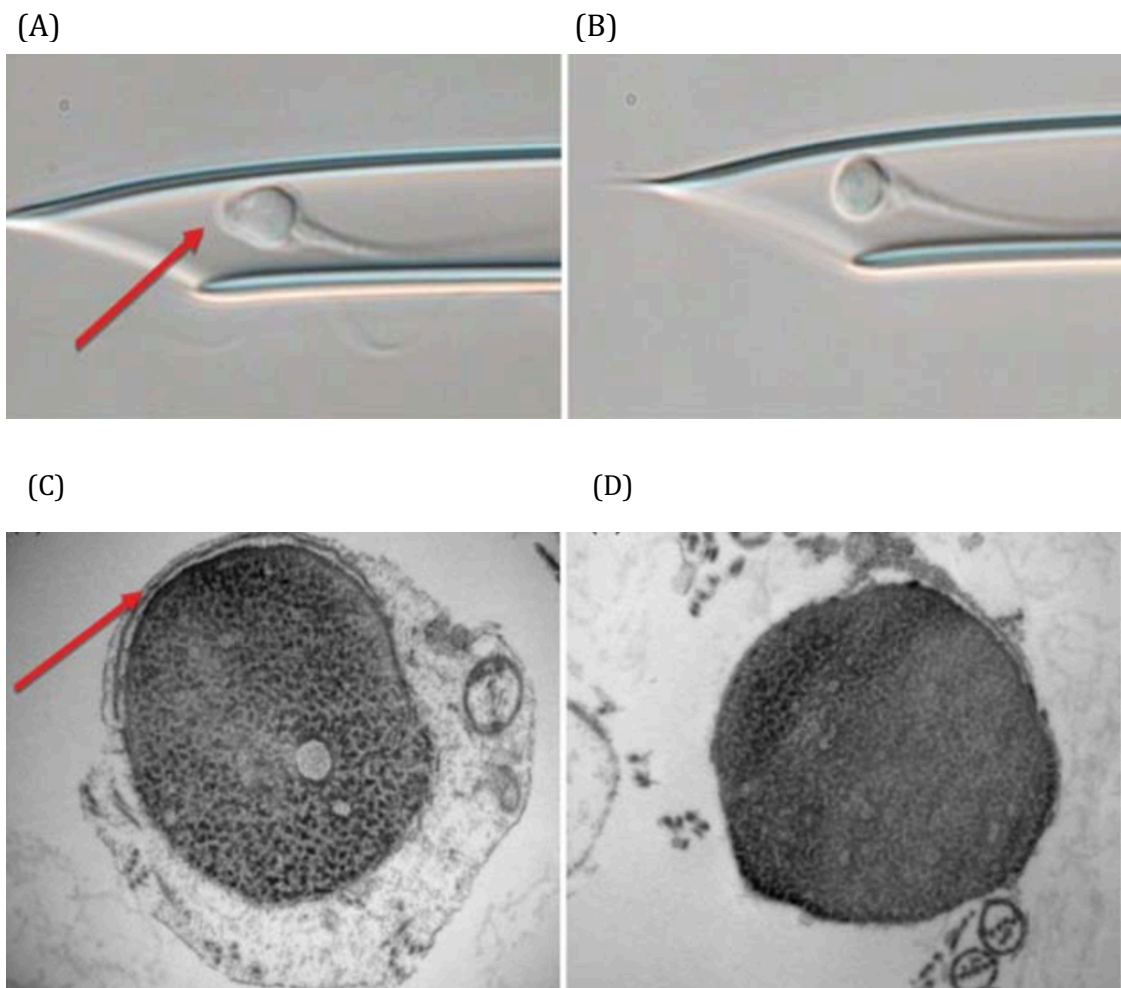
Localisation patterns of PLC $\zeta$  in sperm have also been associated with oocyte activation. As mentioned above, PLC $\zeta$  exhibits characteristic localisation patterns mainly in three areas of sperm head: acrosome, equatorial, and post-acrosome (see 'Introduction, Section 1.3.2'). Equatorial localisation of PLC $\zeta$  is predominant in human sperm and remains consistent during sperm capacitation and the acrosomal reaction (Grasa *et al.* 2008). In contrast, PLC $\zeta$  expression in the acrosome of the sperm head may be related to acrosome reaction mediation rather than the capacity to trigger oocyte activation (Grasa *et al.* 2008). Because the acrosomal reaction involves acrosomal exocytosis,

where a group of proteins participate in enzymatic activities such as depolarization and phosphorylation (Quevedo *et al.* 2019), PLC $\zeta$  from the acrosome is unlikely to remain intact following the acrosomal reaction. Interestingly, immunofluorescence labelling of normozoospermic sperm from a patient with TFF showed that PLC $\zeta$  localisation patterns changed after the acrosomal reaction and were different from those in fertile controls (Durban *et al.* 2015).

PLC $\zeta$  has also been investigated in a particular population of individuals with globozoospermia. Globozoospermia is a rare cause of male infertility, affecting 0.1% of infertile men in which sperm lack the acrosome (i.e., round-headed sperm) (Berruti and Paiardi 2011). Microinjection of human globozoospermic sperm into mouse oocytes failed to trigger oocyte activation or induce Ca<sup>2+</sup> oscillations (Heytens *et al.* 2009). In addition, immunoblotting showed significantly lower PLC $\zeta$  mRNA and protein levels in sperm from globozoospermic males compared to fertile males (Tavalaee and Nasr-Esfahani 2016). Sermondade *et al.* used motile sperm organelle morphology examination (MSOME) to study globozoospermic sperm morphology and identified that 1% of these sperm exhibited a small acrosome bud (**Figure 1.8**). Subsequent ICSI cycle using globozoospermic sperm with the small acrosomal bud helped globozoospermia patients achieve live-birth without the use of AOA (Sermondade *et al.* 2011). Given this, Kashir *et al.* used immunofluorescence labelling and demonstrated that globozoospermic male sperm either lacked PLC $\zeta$  completely or exhibited abnormal punctate patterns. They further identified that almost half (43%) of sperm exhibiting acrosomal bud morphology expressed PLC $\zeta$  in the sperm head (Kashir *et al.* 2012c). Finding



acrosomal buds in sperm heads with the use of MSOME can further guide clinical treatment when selecting a sperm for ICSI and can therefore improve the likelihood of selecting live sperm containing PLC $\zeta$ .



**Figure 1.8 Globozoospermic sperm exhibiting a small acrosome bud.** Representative images of globozoospermic sperm with (A) acrosome bud (red arrow) and (B) no acrosome, visualised during motile sperm organelle morphology examination (MSOME) under a light microscopy at x 10,000 magnification. Representative images of globozoospermic sperm with (C) acrosome bud (red arrow) and (D) no acrosome, visualised using transmission electron microscopy. Reproduced from Sermondade *et al.* (2011).

The PLC $\zeta$  gene shares a bi-directional promoter with the capping protein muscle Z-line, alpha 3 (CAPZA3). CAPZA3 contributes to spermatid development and is responsible for the maintenance of sperm structure (Coward *et al.* 2011). One potential reason for infertile males with abnormal sperm morphology exhibiting aberrant PLC $\zeta$  levels and localisation patterns may be due to abnormalities in the shared promoter. Therefore, it is worth investigating whether the shared promoter contains genetic variations which might influence downstream protein expression and sperm morphology (Kashir *et al.* 2012c).

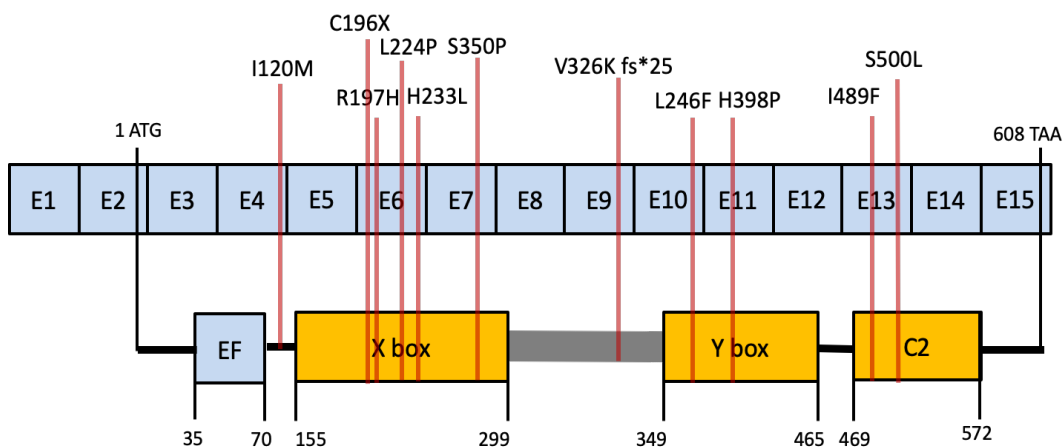
Most notably, PLC $\zeta$  levels, localisation patterns, and the proportion of human sperm exhibiting PLC $\zeta$ , significantly correlate with ICSI fertilisation rates, indicating that PLC $\zeta$  is a promising biomarker for diagnosing OAD and predicting the outcome of ICSI (Yelumalai *et al.* 2015; Nazarian *et al.* 2019). Similar to what was found in human, a recent study reported that higher PLC $\zeta$  protein levels in stallion sperm contributed to better cleavage (i.e., formation of 2-cell) rate in embryos, after injection of such sperm into bovine oocytes (Amoroso-Sanches *et al.* 2019). However, another study proposed that PLC $\zeta$  levels and localisation were not correlated with fertilisation outcome (Ferrer-Vaquer *et al.* 2016). Collectively, there are more existing evidence suggests that PLC $\zeta$  is a practical clinical biomarker which can be used to assess oocyte activation ability, except for the only one sceptical study.

The PLC $\zeta$  gene consists of 15 exons between introns, and is located on the reverse strand of chromosome 12 (Gat and Orvieto 2017). Abnormalities/alterations in the transcription or translation of PLC $\zeta$  are potential causes for the failure of fertilisation or oocyte activation (Yeste *et al.* 2016a). In fact, previous quantitative analysis showed that PLC $\zeta$  mRNA

expression levels in human sperm correlated to their fertilisation outcome, and that male patients with total fertilisation failure had significantly lower expression of PLC $\zeta$  mRNA than fertile males (Aghajanpour *et al.* 2011).

A total of 11 mutations have been identified in the human PLC $\zeta$  gene, and ten of these were shown to disrupt the structure and function of the protein.

**Figure 1.9** summarises the mutations that have been identified in human PLC $\zeta$  genes and their locations in corresponding protein domains.



**Figure 1.9 Mutations that have been identified in human PLC $\zeta$  exons.** Positions of PLC $\zeta$  mutations in exons and secondary protein structures are highlighted in red line. I120M: isoleucine replaced with methionine; however, this mutation does not affect protein structure. C196X, cysteine to undetermined amino acid; R197H: arginine to histidine at residue 197; L224P: leucine to proline at residue 224; H233L: histidine to leucine at residue 233; S350P, serine to proline; V326K fs\*25: a frame shift caused by the deletion of nucleotides A and G, resulting in the generation of a stop codon and substitution of valine to lysine; L246F, leucine to phenylalanine; H398P: histidine to proline at residue 398; I489F: isoleucine to phenylalanine at residue 489. Exon sizes are not shown to scale. Figure reproduced from Escoffier *et al.* (2015), Ferrer-Vaquer *et al.* (2016) and Torra-Massana *et al.* (2019).

Several of these mutations were extensively studied with biological experiments to confirm whether protein function was impaired at the physiological levels.

The first mutation of PLC $\zeta$  was found in exon 11 in an infertile patient, causing a substitution of histidine to proline at residue 398 (H398P) within the Y domain (Heytens *et al.* 2009). In addition, a mouse mutant *PLC $\zeta$*  equivalent to human PLC $\zeta^{\text{H398P}}$  was generated (PLC $\zeta^{\text{H345P}}$ ) and was unable to hydrolyse PIP<sub>2</sub>, suggesting that this mutation caused dysfunctional PLC $\zeta$  enzymatic activity (Nomikos *et al.* 2017b). Kashir *et al.* proposed that H398P caused instability of the PLC $\zeta$  protein which may be the reason for the low proportion of sperm expressing PLC $\zeta$  and reduced mean PLC $\zeta$  levels in sperm from PLC $\zeta^{\text{H398P}}$  patients (Kashir *et al.* 2011b).

The second mutation identified in *PLC $\zeta$*  led to the substitution of histidine to leucine (H233L) within the X catalytic domain. Interestingly, H398P and H233L were identified in the same infertile patient (Kashir *et al.* 2011c). Further inheritance analysis showed that these mutations were present on different alleles and that PLC $\zeta^{\text{H233L}}$  was inherited via the maternal lineage (Kashir *et al.* 2011c). Subsequent genotyping of single sperm from the infertile patient possessing H398P and H233L revealed that sperm exhibited either PLC $\zeta^{\text{H398P}}$  or PLC $\zeta^{\text{H233L}}$  but not PLC $\zeta^{\text{H398P+H233L}}$ , which confirmed that the two mutations are heterozygous (Kashir *et al.* 2012b). In addition, transfection of PLC $\zeta^{\text{H233L+H233L-}}$  containing plasmid DNA with human embryonic kidney cells (HEK293T) showed that the fluorescence level of recombinant mutant protein was significantly lower than PLC $\zeta^{\text{H398P}}$  and PLC $\zeta^{\text{H233L}}$ , indicating that a combination

of these mutations (i.e., PLC<sup>H398P+H233L</sup>) may influence the stability of the protein more than a single mutation (Kashir *et al.* 2012b).

Subsequently, a missense mutation was identified in an exonic region of *PLCζ* in normozoospermic brothers who experienced total fertilisation failure in ICSI. Whole-exome sequencing showed that a mutation caused a substitution of isoleucine with phenylalanine (I489F) in exon 13 (Escoffier *et al.* 2015). Further three-dimensional (3D) modelling of PLCζ revealed that this mutation may interfere with downstream sites in the C2 domains and may alter their conformation (Escoffier *et al.* 2015). A liposome-binding assay further showed that mouse PLCζ<sup>I489F</sup> protein exhibited reduced binding activity to PIP<sub>3</sub> and PIP<sub>5</sub>. However, the hydrolytic activity of PLCζ<sup>I489F</sup> to PIP<sub>2</sub> was unchanged, suggesting that the I489F mutation may impair PLCζ targeting to the membrane instead of influencing PLCζ enzymatic activity (Nomikos *et al.* 2017b).

Since the discovery of the first three mutations, more genetic screening studies have been carried out to investigate potential abnormalities in the *PLCζ* exons of infertile males, and eight novel mutations were identified in six exons of *PLCζ*, corresponding to the X, Y, C2 domains and the XY-linker; namely, R197H (arginine to histidine), S500L (serine to leucine), I120M (isoleucine to methionine), L224P mutation (leucine to proline), V326K fs\*25 (a frame shift caused valine to lysine), C196X (cysteine to undetermined), S350P (serine to proline) and L246F (leucine to phenylalanine) (Ferrer-Vaquier *et al.* 2016; Dai *et al.* 2019; Torra-Massana *et al.* 2019). Notably, the V326K fs\*25 mutation is the only mutation situated in the XY-linker that have been identified, the deletion of two nucleotides caused a frame shift to a stop codon, resulting in a truncated form of the XY-linker and lacks of Y and C2 domains (Torra-Massana *et al.* 2019).

In line with this, microinjection of the mutant PLC $\zeta$  cRNA into human *in-vitro* matured oocytes caused total fertilisation failure, which indicates the deleterious effect of this mutation and also shows that the XY-linker plays an indispensable role in oocyte activation.

Although most previous work has focused on PLC $\zeta$  exons, some researchers have suggested to investigate the introns or promoter region of PLC $\zeta$  (Kashir *et al.* 2012c; Amdani *et al.* 2013; Amdani *et al.* 2015). Previously, two genetic variations, located within the core promoter of PLC $\zeta$ , were identified in Chinese Holstein bulls, and were suggested to affect semen quality (Pan *et al.* 2013). However, thus far, no genetic mutation has been found in the promoter of human PLC $\zeta$ . One nucleotide change was found in the CAPZA3 promoter region of DNA from a globozoospermic patient; the opposite strand was located in the vicinity but outside of the PLC $\zeta$  promoter (Javadian-Elyaderani *et al.* 2016). This base change was predicted to influence the transcription binding site of regulatory factor X 4, a testis-specific DNA-binding protein (Javadian-Elyaderani *et al.* 2016).

Other preliminary investigations have extended to intronic PLC $\zeta$ . One clinical study, involving normozoospermic patients with repeated fertilisation failure, identified three single nucleotide polymorphisms (SNPs) in intronic PLC $\zeta$  regions (Yoon *et al.* 2008). Similarly, three SNPs were found in a patient with TFF; however, one of these SNPs was also found in a fertile control, indicating that such SNPs may not impair fertility (Durban *et al.* 2015). In addition, a genomic study, involving all exons and introns of horse PLC $\zeta$  genes, revealed 47 polymorphisms, 3 of which were associated with horse fertility,

possibly via regulation of transcriptional binding sites or microRNAs (Schrimpf *et al.* 2014).

Collectively, these data show that the abnormal expression of the *PLCζ* gene, and problems associated with protein translation, can cause a number of adverse effects on the ability of PLCζ to trigger oocyte activation, thus leading to OAD. Furthermore, this also indicates that PLCζ can be used as a plausible diagnostic marker for predicting OAD.

## **1.4 Artificial oocyte activation (AOA) in ART**

### **1.4.1 Introduction**

Currently, the only possible clinical option for OAD rescue is AOA, a technique offered to couples who experience fertilisation failure after ICSI. Globally, AOA has been used in a small number of clinics over the last two decades, although it was developed from a parthenogenesis technique dating back to the 1930s, with evidence that rabbit oocytes could be activated after 2 h of heat treatment or incubation with butyric acid and hypertonic solutions (Pincus and Enzmann 1936). Parthenogenesis is a form of asexual reproduction that is very rare in mammals because of the absence of one of the parental genome (Graham 1974). Parthenogenesis can stimulate an unfertilised oocyte to develop to the blastocyst stage and even to an early foetal stage (Kaufman *et al.* 1977) and has been applied in research to study the physiological and genetic development of mammalian embryos, somatic cell cloning or pluripotent differentiation of stem cells (Bos-Mikich *et al.* 2016). In contrast, AOA in clinical ART involves sperm and is normally carried out after ICSI.

AOA involves the use of various mechanical, physical, or chemical stimuli to artificially create a source of  $\text{Ca}^{2+}$  (Vanden Meerschaut *et al.* 2014b). Mechanical activation works by vigorous aspiration of the oocyte cytoplasm before injecting sperm; as a result, the structure of the ER is disrupted, thus resulting in  $\text{Ca}^{2+}$  release (Ebner *et al.* 2004). Physical activation applies electrical charges to proteins in the lipid bilayer of the cell membrane. The movement of charged proteins forms pores that allow extracellular  $\text{Ca}^{2+}$  from the culture medium to translocate, ultimately leading to increased  $\text{Ca}^{2+}$  levels (Egashira *et al.* 2009; Zhang *et al.* 2017).

Chemical activation is more common and uses a stimulus/stimuli to either (1) alter plasma membrane permeability as a result of triggering intracellular organelles to release  $\text{Ca}^{2+}$  ( $\text{Ca}^{2+}$  ionophores and ethanol), (2) directly increase intracellular  $\text{Ca}^{2+}$  levels (strontium chloride ( $\text{SrCl}_2$ )), or (3) inhibit protein/kinase synthesis (cycloheximide or puromycin) (Morgan and Jacob 1994; Yanagida *et al.* 2008; Kharche and Birade 2013).

Studies using a variety of AOA methods have reported improved fertilisation rates in mammalian oocytes (Heytens *et al.* 2008; Bonte *et al.* 2019). However, there is no standard activation protocol because responses to such stimuli are species-specific. Furthermore, the concentrations of chemicals, incubation times, and the use of post-treatment(s) may vary by species. Regardless of which methods are used, this technique can activate oocytes effectively in a range of animals, including mouse, rabbit, cattle, goat, horse and cow (Nagai 1987; Stice *et al.* 1994; Uranga *et al.* 1996; Onger *et al.* 2001; Ozil and Huneau 2001; Fernandes *et al.* 2014). Protocols for activating human and mouse oocytes are described in **Table 1.1** and have been reviewed by Yanagida



*et al.* (2008), Vanden Meerschaut *et al.* (2014b) and Ferrer-Buitrago *et al.* (2017).

This will be further introduced in Chapter 5.

**Table 1.1 Summary of artificial oocyte activation (AOA) protocols for human and mouse oocytes**

| Activation agent  | Species | Ca <sup>2+</sup> response                           | Protocol                                                                     |                                                       |
|-------------------|---------|-----------------------------------------------------|------------------------------------------------------------------------------|-------------------------------------------------------|
|                   |         |                                                     | #1                                                                           | #2                                                    |
| Ionomycin         | Mouse   | Single transient                                    | 10 μM for 10 min (2 exposures, 30min interval)                               | 2 μg/mL cytoD for 3 h                                 |
|                   |         |                                                     | 10 μM for 10 min (2 exposures, 30min interval)                               | 2 μg/mL cytoD for 4 h                                 |
|                   |         |                                                     | 10 μM for 10 min (2 exposures, 30min interval)                               | Mouse wobbler sperm                                   |
|                   | Human   |                                                     | 10 μM for 6 min (1 exposure)                                                 | 2 mM 6-DMAP for 3 h                                   |
|                   |         |                                                     | 10 μM for 10 min (2 exposures, 30min interval) + 0.1 mol/L CaCl <sub>2</sub> | Human <i>globozoospermic</i> sperm                    |
|                   |         |                                                     | 10 μM for 10 min (2 exposures, 30min interval)                               | Human sperm with low or complete failed fertilization |
| Calcimycin        | Mouse   |                                                     | 5 μM for 5 min (1 exposure)                                                  | 1 μg/mL cytoD for 5 h                                 |
|                   |         |                                                     | Ready-to-use solution for 15 min (1 exposure)                                | 2 μg/mL cytoD for 4 h                                 |
|                   |         |                                                     | Ready-to-use solution for 15 min (1 exposure)                                | Mouse wobbler sperm                                   |
|                   | Human   |                                                     | 5 μM for 5 min (1 exposure)                                                  | 2 mM 6-DMAP for 3 h                                   |
|                   |         |                                                     | Ready-to-use for 15 min (1 exposure)                                         | Human <i>globozoospermic</i> sperm                    |
|                   |         |                                                     |                                                                              |                                                       |
| Ethanol           | Mouse   |                                                     | 8.6% for 5 min (1 exposure)                                                  | 0.5 μg/mL cytoD for 5–7 h                             |
|                   |         |                                                     | 7% for 7 min (1 exposure)                                                    | 2 μg/mL cytoD for 2 h                                 |
|                   |         |                                                     |                                                                              |                                                       |
|                   | Human   | 8% for 10 min (1 exposure)                          | nd                                                                           |                                                       |
|                   |         | 7% for 8–12 min (1 exposure)                        | nd                                                                           |                                                       |
|                   |         |                                                     |                                                                              |                                                       |
| SrCl <sub>2</sub> | Mouse   | 7% for 6 min (1 exposure)                           | Human donor sperm                                                            |                                                       |
|                   |         | 10 mM for 2 h (1 exposure)                          | 2 μg/mL cytoD for 2 h                                                        |                                                       |
|                   |         | 10 mM for 4 h (1 exposure)                          | 2 μg/mL cytoD                                                                |                                                       |
|                   | Human   | 10 mM for 1 h (1 exposure)                          | Human sperm with low or complete failed fertilization                        |                                                       |
|                   |         |                                                     |                                                                              |                                                       |
|                   |         |                                                     |                                                                              |                                                       |
| PLC $\zeta$       | Mouse   | 10 mM for 20min (1 exposure)                        | 2.5 mM 6-DMAP for 1.5 h                                                      |                                                       |
|                   |         | cRNA injection: 0.02 mg/mL                          | 2 μM cytoD for 4 h                                                           |                                                       |
|                   |         | Human recombinant protein injection: (0.0167 mg/mL) | 5 μg/mL cytoB for 6 h                                                        |                                                       |
|                   | Human   | cRNA injection: 0.1 μg/mL                           | 2 μM cytoD for 2 h                                                           |                                                       |
|                   |         | 10 μg/mL for 5–24 h (1 exposure)                    | /                                                                            |                                                       |
|                   |         |                                                     |                                                                              |                                                       |
| Puromycin         | Human   | 100 μg/mL for 7–8 h (1 exposure)                    | /                                                                            |                                                       |
|                   |         |                                                     |                                                                              |                                                       |
|                   |         |                                                     |                                                                              |                                                       |
| Roscovitine       | Mouse   |                                                     | 50 μM for 8 h (1 exposure)                                                   | Mouse sperm                                           |
| Cycloheximide     | Mouse   |                                                     | 20 μg/mL for 4 h (1 exposure)                                                | 2 μg/mL cytoD for 2 h                                 |
| TPEN              | Mouse   |                                                     | 100 μM for 45 min (1 exposure)                                               | 5 μg/mL cytoB for 6 h                                 |
|                   | Human   |                                                     | 50 μM for 30 min (1 exposure)                                                | /                                                     |

AOA protocols were applied variously by different research groups. Human/mouse oocytes received AOA via incubation/microinjection with various activators with/without the presence of sperm. CytoD: cytochalasin D; cytoC: cytochalasin C. Table reproduced from Ferrer-Buitrago *et al.* (2018).

### 1.4.2 Chemicals for AOA

Commonly used chemical reagents for AOA are  $\text{Ca}^{2+}$  ionophores, including calcimycin (i.e., A23187) and ionomycin (Nikiforaki *et al.* 2016). It was first demonstrated in the 1970s that calcimycin-stimulated hamster oocytes could reach the 2-cell stage, but exhibited abnormal morphology (Steinhardt *et al.* 1974). Subsequent studies confirmed that calcimycin and ethanol caused parthenogenetic activation in aged human oocytes (Winston *et al.* 1991) and normal mouse oocytes (Uranga *et al.* 1996). The first report of the use of calcimycin in human oocytes was in 1995; calcimycin was applied after ICSI, which resulted in a live birth (Hoshi *et al.* 1995). Subsequent studies further explored the efficacy of chemical AOA in patients with a low fertilisation rate, repeated fertilisation failure, or previous TFF (**Appendix Table 1**). These clinical case studies revealed that ICSI, supplemented with AOA treatment, significantly improved fertilisation rates and pregnancy outcomes. However, it should be noted that earlier studies included rather small numbers of patients, and that the patient selection criteria varied between studies, thus hindering comparison (**Appendix Table 1**).

Notably,  $\text{Ca}^{2+}$  ionophores were shown to improve fertilisation outcomes for normozoospermic males with repeated fertilisation failure (Eldar-Geva *et al.* 2003) and also resolve some forms of male factor-related OAD such as teratozoospermia (Nasr-Esfahani *et al.* 2008; Li *et al.* 2019b), azoospermia (Ahmady and Michael 2007; Ebner *et al.* 2012) and sperm with amorphous and tapered heads (Moaz *et al.* 2006). Calcimycin can also overcome suspected female-related OAD (Tesarik *et al.* 2002). In addition,  $\text{Ca}^{2+}$  ionophores help globozoospermic males achieve clinical pregnancies and live births (Tesarik *et*

*al.* 2002; Heindryckx *et al.* 2005; Taylor *et al.* 2010; Karaca *et al.* 2015; Kochhar and Ghosh 2018).

In 2014, one ready-to-use AOA, GM508, was commercialised for use in ART. GM508 not only incorporates calcimycin, but also includes nutrients that are required for embryo development, including lactate and pyruvate, and thus is easier for clinic use. Studies have shown the success of GM508 in cases of fertilisation failure in ICSI or abnormal sperm, as demonstrated by improved cleavage rate, blastocyst formation, fertilisation rate, pregnancy outcomes, and live births in subsequent studies (Ebner *et al.* 2015a; Ebner *et al.* 2015b; Nicopoullos *et al.* 2015; Karabulut *et al.* 2018). In addition, GM508 and calcimycin were suggested to improve the further development of embryos arrested at the pronuclear stage (Ebner *et al.* 2015b). In contrast, two studies reported that GM508 did not improve the fertilisation rates and pregnancy rates for male patients with teratozoospermia nor females with diminished ovarian reserve (Aytac *et al.* 2015; Aydinuraz *et al.* 2016).

As the use of AOA increased in the clinical setting, subsequent studies reported fertilisation and pregnancy outcomes for larger numbers of ICSI/AOA-treated patients (**Appendix Table 2**). Multiple comparisons across different studies can help to evaluate the association between AOA treatment and patient fertilisation outcomes. A recent systematic review analysed 14 studies using  $\text{Ca}^{2+}$  ionophores for patients who experienced fertilisation failure due to male- or female-related defects (Murugesu *et al.* 2017). Meta-analysis of these 14 articles suggested that  $\text{Ca}^{2+}$  ionophores significantly improved clinical pregnancy and live birth rates, as well as fertilisation, cleavage, blastocyst, and implantation rates (Murugesu *et al.* 2017).

Besides  $\text{Ca}^{2+}$  ionophores, other chemical activators such as  $\text{SrCl}_2$ , puromycin, and  $\text{Zn}^{2+}$  chelators have also shown activation ability in human oocytes. Several studies reported that  $\text{SrCl}_2$  treatment improved fertilisation outcomes in patients with previous low fertilisation rates and/or resulted in live births (Yanagida *et al.* 2006; Kyono *et al.* 2012; Yoon *et al.* 2013; Fawzy *et al.* 2018; Norozi-Hafshejani *et al.* 2018).  $\text{SrCl}_2$  was also found to rescue pregnancy outcomes in patients who had fertilised oocytes in previous ICSI-AOA ( $\text{Ca}^{2+}$  ionophores) but no pregnancies (Kim *et al.* 2014). In another study, Fawzy *et al.* further indicated that both  $\text{SrCl}_2$  and calcimycin significantly improved the fertilisation and pregnancy rates of infertile patients, and suggested that the justification of which AOA method to apply should depend on the origins of OAD; calcimycin and  $\text{SrCl}_2$  are more effective for male- and female-related OAD, respectively (Fawzy *et al.* 2018). However, another study argued that  $\text{SrCl}_2$  was not the trigger of  $\text{Ca}^{2+}$  oscillations (Lu *et al.* 2018). This was because no  $\text{Ca}^{2+}$  release or abnormal  $\text{Ca}^{2+}$  oscillations was observed in  $\text{SrCl}_2$ -treated human oocytes, instead, subsequent treatment using ionomycin caused a large  $\text{Ca}^{2+}$  rise in those oocytes that were previously failed in AOA with the use of  $\text{SrCl}_2$  (Lu *et al.* 2018). Another laboratory confirmed a total lack of  $\text{Ca}^{2+}$  oscillations when human oocytes were incubated with  $\text{SrCl}_2$  medium (Swann 2018a).

An earlier study revealed that puromycin parthenogenetically activated human oocytes (De Sutter *et al.* 1992). This was followed by the report of successful pregnancies after puromycin was combined with the use of calcimycin, but this did not confirm the efficiency of puromycin alone (Murase *et al.* 2004). In addition, another AOA chemical, the  $\text{Zn}^{2+}$ -specific chelator N,N,N',N'-tetrakis(2-pyridylmethyl) ethane-1,2-diamine (TPEN), was also

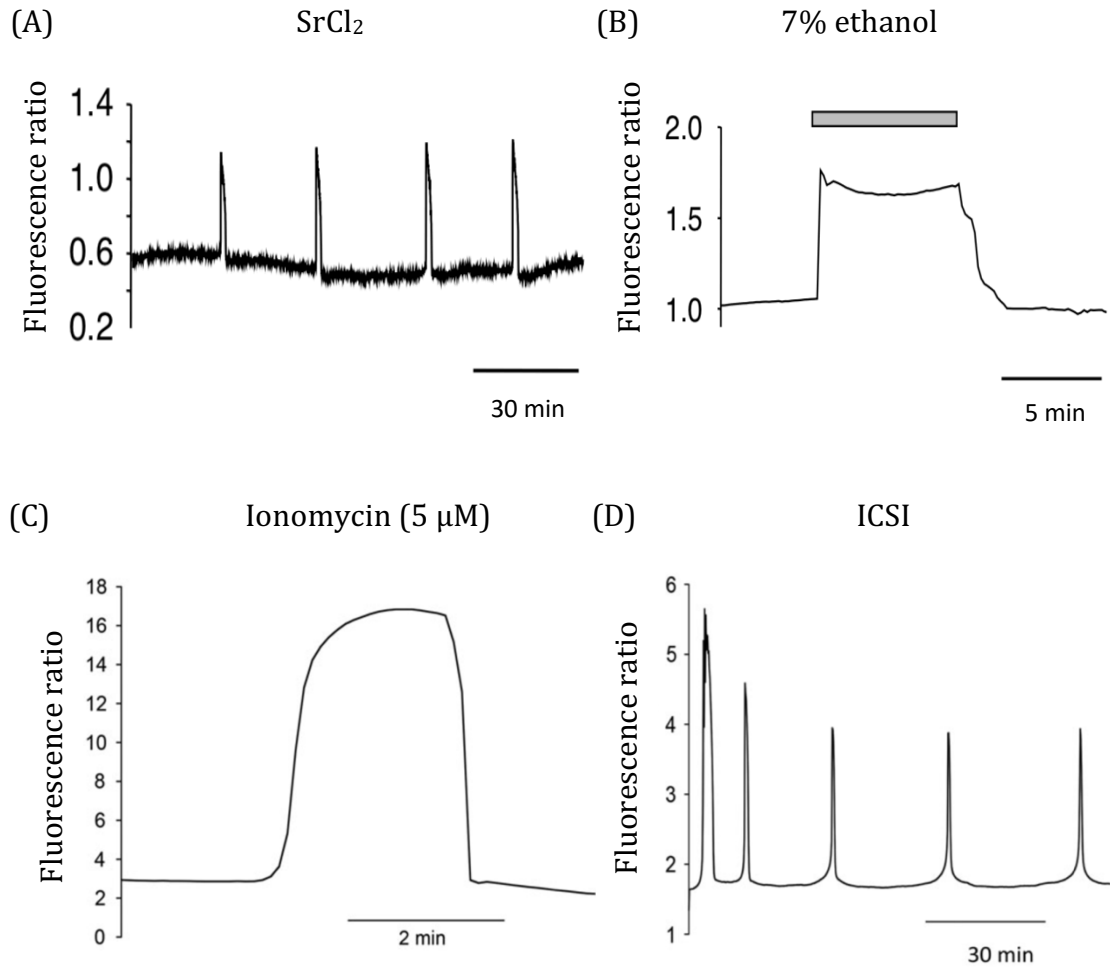
shown to trigger human MII oocytes to undergo fertilisation-like cytoplasmic changes, such as cytoskeleton rearrangement (Duncan *et al.* 2016). Accordingly, TPEN was found to be effective in causing pig oocyte activation (Uh *et al.* 2019). To date, no study has shown the activation effect of TPEN in improving clinical pregnancy outcomes. Further investigation is therefore needed.

Apart from clinical studies, laboratory-based studies have also confirmed the beneficial effect of AOA chemicals. For example, Nakagawa *et al.* used calcimycin and then puromycin, to activate unfertilised human oocytes that lacked pronuclei and a second polar body 18 h after ICSI. These oocytes then showed restored fertilisation ability, and a normal diploid set of chromosomes was observed in fertilised oocytes (Nakagawa *et al.* 2001). Capalbo *et al.* further reported that  $\text{Ca}^{2+}$  ionophore-activated human MII oocytes exhibited normal segregation of maternal chromosomes but may experience issues with second polar extrusion (Capalbo *et al.* 2016). In addition, calcimycin was found to induce a higher activation rate than  $\text{SrCl}_2$  in 3-day-old unfertilised human oocytes (Lu *et al.* 2006; Liu *et al.* 2014) and fertilised human oocytes (Norozi-Hafshejani *et al.* 2018). Nikiforaki *et al.* evaluated the efficiencies of calcimycin and ionomycin in activating human and mouse oocytes with regard to their abilities to induce  $\text{Ca}^{2+}$  release and concluded that ionomycin is more effective than calcimycin (Nikiforaki *et al.* 2016).

Collectively, a large number of clinical studies have reported that AOA successfully improves cleavage, fertilisation, and pregnancy rates in ART.

### 1.4.3 Concerns raised in relation to the use of AOA in ICSI

Despite the beneficial effects of AOA treatment, its use has become a source of much debate. Firstly, it is well-known that AOA chemicals cause a single prolonged  $\text{Ca}^{2+}$  increase instead of oscillatory patterns (**Figure 1.10**) (Rinaudo et al. 1997; Nikiforaki et al. 2016; Lu et al. 2018). Functional  $\text{Ca}^{2+}$  release at oocyte activation requires periodic frequency, appropriate amplitude, and an adequate number of oscillations. However, AOAs that are lipid-soluble ionophores cause only a single  $\text{Ca}^{2+}$  transient when passing through the membrane, but fail to maintain oscillations, possibly due to the lack of an endogenous mechanism. At normal fertilisation, intracellular  $\text{Ca}^{2+}$  proteolyses cyclin B, reduces cyclin-dependent kinase 1 (CDK1) activity, and inactivates MPF, which collectively lead to the resumption of MII oocytes (Ducibella *et al.* 2002). A single  $\text{Ca}^{2+}$  transient may be insufficient to sustain those disruptions and result in oocyte re-arrestment (Swann 2018a). To complement this insufficiency, ionophore-based parthenogenesis in mammals is usually combined with 6-dimethyl aminopurine or cytochalasin B or D, which belong to a group of protein synthesis inhibitors or kinase polymerization inhibitors (Heytens *et al.* 2008; Canel *et al.* 2010). The same principal has been used in human experiments; however the outcome was not as effective as in other mammals (Nikiforaki *et al.* 2016).



**Figure 1.10  $\text{Ca}^{2+}$  release patterns in mouse oocytes after artificial oocyte activation (AOA).** The frequency of  $\text{Ca}^{2+}$  oscillations was increased in  $\text{SrCl}_2$ -treated oocytes (A), while single  $\text{Ca}^{2+}$  transients were observed in ethanol- and ionomycin-treated oocytes (B and C), when compared to those occurred after ICSI (D). Reproduced from Sanusi *et al.* (2015) and Rogers *et al.* (2006).

Secondly, the efficacies of chemical AOA on human oocyte activation were inconsistent when compared across different studies. In an early investigation, calcimycin induced activation in only 16% of unfertilised oocytes (a total of 108). Tesarik and Sousa showed that 88% of oocytes did not exhibit spontaneous fertilisation with an absence of pronuclear formation after ICSI-AOA treatment (Tesarik and Sousa 1995). Rinaudo *et al.* also found that calcimycin failed to activate oocytes (Rinaudo *et al.* 1997).

Indeed, the effects of an AOA strategy are not universal. In contrast to most studies examining the benefits of AOA, one suggested that AOA-treated embryos have reduced quality (Aydinuraz *et al.* 2016). Furthermore, AOA did not improve fertilisation rates for sperm with bent necks (Moaz *et al.* 2006) or azoospermic sperm aspirated from testicles (Borges Jr *et al.* 2009a). In addition, fertilisation outcomes after AOA treatment varied in studies that investigated the same population. GM508 improved the fertilisation rate in males with teratozoospermia who experienced low fertilisation (<50%) to as high as 82.5% (Aydinuraz *et al.* 2016). In a similar study, ICSI supplemented with ionomycin resulted in a fertilisation rate between 57.8% and 63.4% (Nasr-Esfahani *et al.* 2008). In addition, several studies all recruited couples with a history of TFF, however, calcimycin-assisted ICSI differentially improved the fertilisation rates to 41.6% (Montag *et al.* 2012), 67% (Fawzy *et al.* 2018) and 78% (Heindryckx *et al.* 2005).

Notably, AOA protocols vary between IVF units, with significant differences in reagents, incubation times, and supplement use. Patient responses to the same protocol may also be discrepant due to individual factors. In addition, patient selection criteria differ, for example, the cut-off for 'low



fertilisation' of couples in previous IVF/ICSI treatments varied between 20 - 50% of total fertilised oocytes (**Appendix Table 1 and 2**). Collectively, these factors limited analysis of the association between AOA and ICSI-AOA outcomes such as fertilisation and early embryonic development. Interestingly, a meta-analysis of 14 randomised controlled trials of AOA calculated risk ratios and 95% confidence intervals and concluded that the available evidence was insufficient to support the conclusion that AOA is beneficial to fertilisation-failed couples. Furthermore, 13 out of 14 studies showed a high risk of bias for patient selection, performance and detection (Sfontouris *et al.* 2015).

Thirdly, the short- and long-term safety of babies born as a result of AOA is another concern. Few studies have reported outcomes for children born following ICSI-AOA. The overall rates of AOA-born infants were not significantly different from ICSI-born infants in terms of birth defects (Kyono *et al.* 2012), early-neonatal death rate, fetal sex ratio (Li *et al.* 2019a), language development (D'haeseleer *et al.* 2014), or neonatal and neural development (Vanden Meerschaut *et al.* 2014a; Miller *et al.* 2016).

However, Van Blerkom *et al.* expressed concern over the miscarriage rate of AOA-treated patients and congenital malformation rates of AOA-treated infants (Van Blerkom *et al.* 2015). Birth defects were not detected in infants at delivery but were subsequently found in 14.3% of toddlers (Van Blerkom *et al.* 2015). In one study of neonatal and neural development in children born via AOA aged 3-10 years, language development was slower than in peers born naturally, and AOA-born children were more likely to have autism spectrum disorders (Vanden Meerschaut *et al.* 2014a). Malformation with anal atresia was also identified in an AOA-treated infant (Ebner *et al.* 2015a). In line with this

case, an IVF clinic suggested that AOA-born twins exhibit a higher rate of major malformations than AOA-born singletons, such as unilateral hip and knee dislocation and imperforate anus (Mateizel *et al.* 2018). Furthermore, one child of a set of AOA-born twins died from severe renal dysplasia and pulmonary hypoplasia (Mateizel *et al.* 2018). In addition, Miller *et al.* reported six birth defects in AOA-born children (Miller *et al.* 2016).

Unfortunately, we still know little about the downstream events of AOA-induced events in oocytes and embryos. But one thing we should bear in mind is that physiological  $\text{Ca}^{2+}$  elevation, and the trigger for  $\text{Ca}^{2+}$  signals, is linked with not only early embryo developmental events, but with embryo implantation and long-term development, possibly by the regulation of chromatin remodelling and the epigenetic reprogramming of gene expression (Ducibella *et al.* 2006). Epigenetic changes refer to heritable changes in gene function without affections in DNA sequence (Sepulveda-Rincon *et al.* 2016). Methylation is an epigenetic dynamic that is responsible for gene silencing and gene transcription, and is crucial for early mammalian embryo development (Torres-Padilla *et al.* 2007).

Defective patterns of  $\text{Ca}^{2+}$  release induced by chemical AOA may interfere with embryo development through epigenetic reprogramming and subsequently alter gene expression, impair protein synthesis, ultimately exerting epigenetic, mutagenic and/or cytotoxic effect(s) on embryogenesis and embryo development (Ozil and Huneau 2001). It is evident that the placentas of IVF/ICSI-assisted offspring showed a significant reduction of DNA methylation at H19 than those conceived naturally, indicating that ART can affect DNA methylation (Nelissen *et al.* 2013). In line with this, H3K9 dimethylation was

decreased in ICSI-treated human oocytes at the pronuclear stage with low morphology grade and increasing fragmentation of blastomeres (Qiao *et al.* 2010). The use of AOAs in ICSI may be faced with the same problem, or even worse in the long run. After all, these AOAs, which are exogenous reagents, do not replicate the calcium patterns which occur during natural fertilisation. Although oocyte activation can be rescued, AOAs might interfere with long-term embryo development through epigenetic changes on gene expression.

In addition, van Blerkom *et al.* were concerned whether the ionophore-induced single  $\text{Ca}^{2+}$  transient can influence other developmental issues, such as mitochondrial metabolism, or other embryo physical-related pathways (Van Blerkom *et al.* 2015). Santella *et al.* also proposed that non-physical oocyte activation may interrupt cell homeostasis as a result of abnormal gene expression although this has not yet been confirmed (Santella and Dale 2015). Nevertheless, many molecular mechanisms relating to oocyte activation remain unknown, which can be associated with oocyte activation and can be altered by the abnormal  $\text{Ca}^{2+}$  profiles, which leads to developmental-lethal. Collectively, long-term follow-up data would be paramount in informing clinicians about the immediate and delayed risks associated with the AOA approach.

#### **1.4.4 The urgency for guidelines to justify the use of AOAs and the need for an endogenous therapeutic reagent for OAD**

Given the wide-ranging concerns on the safety of AOA, it is of great importance to seek innovative endogenous treatments for OAD. Currently, the HFEA recognises that using AOA may lead to aneuploidy during embryogenesis (Bhak *et al.* 2006), and has thus recommended that treatment with AOA should

be limited to infertile patients with fertilisation failure, and only where a significant male factor problem has been identified with evidence, such as PLC $\zeta$  deficiency (HFEA 2018b). Indeed, despite of the fact that AOA rescues fertilisation failure relating to OAD, the use of AOA should not be a routine procedure in ART; AOA should be confined to a situation where male-related infertility is a significant problem. Unfortunately, the diagnostic applicability of AOA in clinical practice remains unclear. Most IVF clinics carry out AOA mainly based on patient history of fertilisation failure after ICSI, or suspected OAD (Tesarik *et al.* 2002; Montag *et al.* 2012; Nicopoullos *et al.* 2015). Considering this, van Blerkom *et al.* were worried that AOA treatment might become more frequently used in ICSI, to ensure pronuclear formation and to eliminate potential fertilisation failure (Van Blerkom *et al.* 2015). Therefore, it is imperative to have standard guidelines for helping clinical decision-making on AOA. Although the HFEA reinforces that the use of AOA should only be considered when infertility problem is demonstrated with evidence (HFEA 2019), no study has yet correlated PLC $\zeta$  deficiency with AOA justification. Previous attempts have been made to associate successful fertilisation with PLC $\zeta$  mRNA, RNA, or protein levels in human sperm (Aghajani *et al.* 2011; Kashir *et al.* 2013b; Abadi *et al.* 2016). These indicate that PLC $\zeta$  has potentials to provide useful evidence for the justification of AOA in clinic, as required by the HFEA.

There are several other methods that have been experimentally applied to evaluate sperm ability for oocyte activation, such as heterologous injection of human sperm into mouse oocytes, the mouse oocyte activation test, MOAT (Heindryckx *et al.* 2008), the measurement of calcium levels in human oocytes

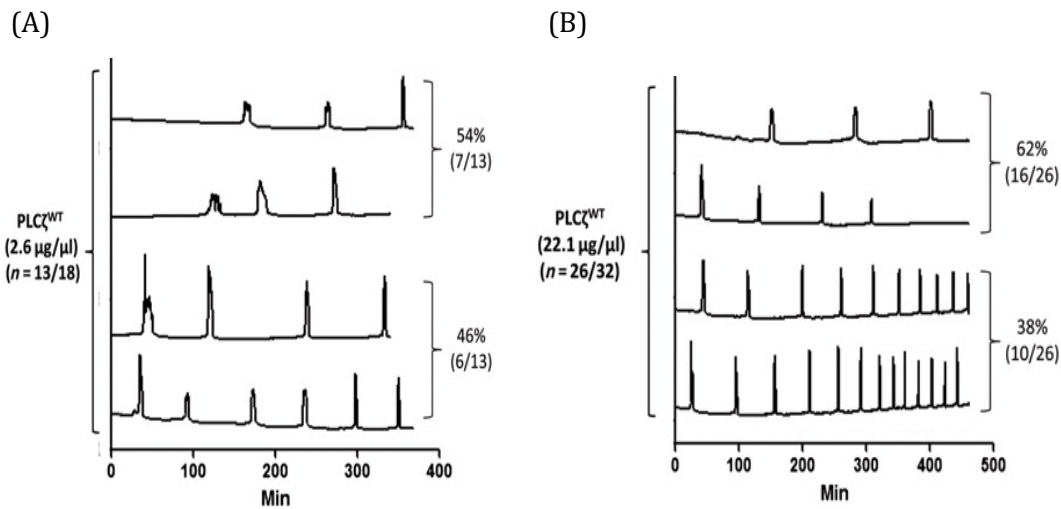
upon human sperm injection (Ferrer-Buitrago *et al.* 2018c), or the assessment of sperm centrosomal function (Terada *et al.* 2009). Sperm DNA integrity, in particular, has been associated with successful pregnancy in IVF/ICSI, although its applicability as a diagnostic tool requires further research (Collins *et al.* 2008; López *et al.* 2013).

In addition, electrical charges upon the sperm membrane can also help select PLC $\zeta$ -containing sperm (Chan *et al.* 2006; Khakpour *et al.* 2019). Chan *et al.* applied a voltage of  $\sim +2$  to  $+4$  kV to create an electrical charge between the sperm membrane and the surrounding environment, which could select negatively-charged live sperm with better morphology and higher motility (termed as zeta method) (Chan *et al.* 2006). Khakpour *et al.* further associated the zeta method with PLC $\zeta$  expression in sperm (Khakpour *et al.* 2019). These authors found that the zeta method selected sperm which expressed higher PLC $\zeta$  levels than the control sperm. However, sperm selected from this method also showed an overall reduction in the proportion of sperm containing PLC $\zeta$  (Khakpour *et al.* 2019). Unfortunately, these authors did not investigate whether the zeta method could help improve fertilisation outcome.

Taking all together, it is imperative to determine a diagnostic approach with which to predict sperm capacity in oocyte activation and thus helping clinical decision-making on AOA treatment. Apart from this, attempts have been made to identify an appropriate therapeutic treatment as an alternative option for OAD. In particular, PLC $\zeta$  has been proposed as an endogenous therapeutic agent to alleviate OAD-related fertilisation failure. Indeed, human PLC $\zeta$  cRNA was previously shown to activate IVF/ICSI-failed oocytes to blastocyst stage (Rogers *et al.* 2004). However, exogenous cRNA can be incorporated into the

embryonic genome, thus interfering with embryogenesis (Spadafora 2004). Consequently, PLC $\zeta$  cRNA is not a feasible clinical option. Therefore, the development of a stable, reliable, and endogenous recombinant human PLC $\zeta$  protein (rhPLC $\zeta$ ) would be an excellent step forward.

Attempts have been made to synthesise rhPLC $\zeta$ , which will be introduced in detail in Chapter 5. In brief, Kashir *et al.* were the first to produce an active but non-purified form of rhPLC $\zeta$  from a mammalian cell line (Kashir *et al.* 2011b). Microinjection of rhPLC $\zeta$ -containing cell lysate successfully triggered Ca<sup>2+</sup> oscillations with variable amplitude and frequency patterns, which enabled mouse oocytes to form pronuclei with satisfying activation rates (72.2 – 81.2%, **Figure 1.11**)(Kashir *et al.* 2011b). However, these authors were unable to purify the protein from the cell lysates, and the use of a non-purified protein is not acceptable for clinical purpose in terms of biosafety.



**Figure 1.11  $\text{Ca}^{2+}$  oscillation patterns of mouse oocytes injected with rhPLC $\zeta$ -containing lysates from HEK293T cells.** The rhPLC $\zeta$  was injected into mouse oocytes at (A) low concentrations (2.6  $\mu\text{g}/\mu\text{l}$ ) and (B) higher concentrations (22.1  $\mu\text{g}/\mu\text{l}$ ). Good activation outcomes were observed in both protein concentrations (13/18 and 26/32, respectively). Reproduced from Kashir *et al.* (2011b).

Later, Yoon *et al.* synthesised a purified form of rhPLC $\zeta$  from *Escherichia coli* (*E.coli*) that contained an N-terminal His-tag (Yoon *et al.* 2012). Microinjection with the tagged protein successfully triggered repetitive  $\text{Ca}^{2+}$  transients in both human and mouse oocytes, but these oscillatory patterns varied enormously. Microinjection of this rhPLC $\zeta$  also enabled human and mouse oocytes to undergo embryonic development. However, the amount of protein used to activate mouse oocytes was approximately 1000 times more than the normal physiological level, which is problematic for commercialization and routine ART. Furthermore, this protein was not viable for clinical use because bacterial expression systems are not efficient in synthesising

recombinant protein and because the procedure is laborious and costly (Yoon *et al.* 2012).

In 2013, Nomikos *et al.* produced a Nus-A tagged rhPLC $\zeta$ ; microinjection with this construct rescued fertilisation-like Ca<sup>2+</sup> oscillations in mouse oocytes previously injected with PLC $\zeta$ <sup>H398P</sup> and PLC $\zeta$ <sup>H233L</sup> cRNA (Nomikos *et al.* 2013). Subsequently, Nus-A tagged rhPLC $\zeta$  generated from the same method was compared with other AOA chemicals (Ca<sup>2+</sup> ionophore and SrCl<sub>2</sub>), and Nus-A tagged rhPLC $\zeta$  was proven to be better at triggering Ca<sup>2+</sup> oscillations (Sanusi *et al.* 2015). However, Nomikos *et al.* also reported that the enzymatic activity of this recombinant protein declines readily, and that the protein requires the Nus-A tag to stabilise the activity (Nomikos *et al.* 2013). Furthermore, no evidence was provided to demonstrate that the activity and function of this protein remained after the removal of the tag. This create a problem for the clinic with regards to the biosafety of injecting an exogenous tag into a human embryo. Since then, no study has been reported in successful synthesis of functional rhPLC $\zeta$  which showed demonstrated evidence in oocyte activation.

Collectively, existing studies on the production of rhPLC $\zeta$  confirm the capability of this protein to alleviate OAD and supports its therapeutic potential as the best option for treating OAD. However, early studies explored the therapeutic role of PLC $\zeta$  at the experimental stage, and rhPLC $\zeta$  has not yet been approved for clinical purpose. More work is needed in order to produce a pure, stable and functional rhPLC $\zeta$  which is clinically viable. This requires that the protein expression system features a high yield, good efficiency and the capability of allowing post-translational modifications. These issues will be addressed in Chapter 5.



## 1.5 DPhil aims

The general aim of my thesis was to develop better clinical tools with which to provide patients with the best chances of conception via ART, focusing particularly upon the diagnosis and treatment of OAD. My research was mainly composed of four projects which are inherently linked by calcium signalling mechanisms evoked by the sperm at the time of gamete fusion.

**Aim 1:** To optimise a laboratory assay for clinical diagnostic purposes (Chapter 2). In this section of my thesis, I compared a PLC $\zeta$  diagnostic assay developed in the Coward Laboratory ('in-house' method) which has been used clinically for many years with an antigen unmasking (AUM)" method which was recently published and questioned the validity of the Coward Laboratory assay.

*Hypothesis: The in-house method shows good efficacy and reliability in visualising of PLC $\zeta$  levels, when compared to the AUM method, and should remain as the main assay for evaluating PLC $\zeta$  expression.*

**Aim 2:** To investigate PLC $\zeta$  protein levels in human sperm and its diagnostic role for OAD (Chapter 3). Here, I investigated the applicability of PLC $\zeta$  in assisting the clinical decision-making process on the use of AOA. Previous studies by our and other research groups have identified the association between PLC $\zeta$  expression in sperm and male infertility, especially OAD. I aimed to enhance the diagnostic potential of PLC $\zeta$  by developing a targeted and individualised approach with which infertile patients can benefit from AOA treatment.

*Hypothesis: The in-house assay is efficient and accurate in identifying PLCζ abnormalities and can inform clinical decision making in the treatment of couples with male-factor infertility.*

**Aim 3:** To investigate genetic mechanisms of male infertility that are correlated to PLCζ (Chapter 4). This aim featured several objectives, as given below. I first used Sanger sequencing to identify novel mutations in the PLCζ exons. Given the fact that PLCζ deficiency is intimately related to OAD, it is imperative to investigate the underlying mechanism(s) responsible for defective forms of PLCζ protein expression. Another objective in this section of my thesis was to expand genetic screening into the *XY-linker* region and the promoter regions responsible for PLCζ expression, by performing next-generation sequencing (NGS) on the PLCζ gene from genomic DNA extracted from 30 males with characterised state of PLCζ expression. The last objective was to screen the *CAPZA3* gene and to investigate whether abnormalities in this gene might link with problems with PLCζ, as *CAPZA3* shares a bidirectional promoter with PLCζ and is responsible for sperm function and morphology.

*Hypothesis: Abnormality(ies) in the investigated regions may affect protein expression and function, and therefore impair the activation ability of sperm.*

**Aim 4:** To investigate the therapeutic role of PLCζ as an alternative option for AOAs. Here, I aimed to evaluate early embryogenic events in mouse oocytes activated by AOAs (existing therapeutic option) and 'in-house' rhPLCζ generated using a novel vector, pHLsec, from a mammalian cell line (novel therapeutic

option) (Chapter 5). To achieve these, I evaluated a series of existing AOA protocols (e.g., ionomycin and the commercially-available AOA, 'GM508) in the activation efficiency of mouse oocytes. I also synthesised and characterised rhPLC $\zeta$  in activating mouse oocytes. Kashir *et al.* (2011b) used the pTracer vector and synthesised rhPLC $\zeta$  from the mammalian cell line HEK293T but failed to purify the protein. Previous work by Coward's group has successfully inserted PLC $\zeta$  full-length human cDNA into the pHLsec vector which contains a Fc-fusion site, a secretion signal and a 3C protease cleavage site. The rhPLC $\zeta$  can therefore be secreted to the culture medium via the secretion signal and be purified via the cleavage site. These represent a novel unpublished approach to synthesis rhPLC $\zeta$ . In my thesis, I also attempted to evaluate the functionality of rhPLC $\zeta$  by using physiological indications, including cytoplasmic cellular movements and morphological changes in mouse oocytes.

*Hypothesis: the novel pHLsec vector allows rhPLC $\zeta$  to be generated and purified from the HEK293T cell line, and rhPLC $\zeta$  shows overall better activation efficiency in mouse oocytes when compared to existing AOAs.*

## Chapter 2. Optimisation of an 'in-house' laboratory assay for clinical diagnostic purposes

### 2.1 Introduction

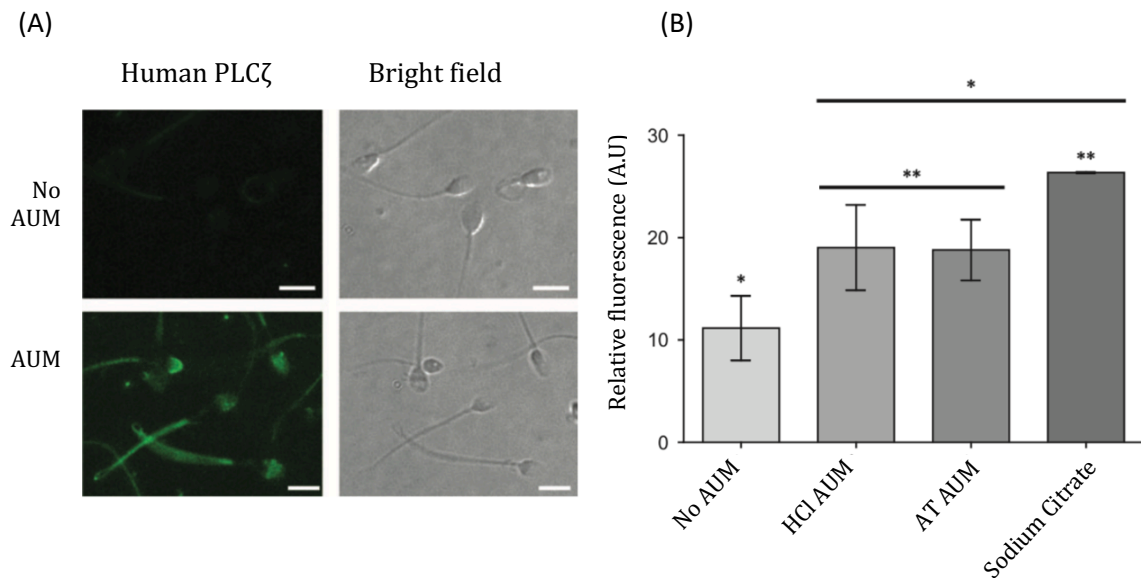
A substantial body of evidence has shown that PLC $\zeta$  plays a crucial role in initiating Ca<sup>2+</sup> oscillations and oocyte activation (Coward *et al.* 2005a; Coward *et al.* 2011; Ito *et al.* 2011; Yu *et al.* 2012; Kashir *et al.* 2013a). Immunofluorescence analysis has revealed that PLC $\zeta$  is predominantly expressed in the equatorial segment of the human sperm, but is also expressed in the acrosome, post-acrosome, or a combination of these locations (Grasa *et al.* 2008; Kashir *et al.* 2013b; Yelumalai *et al.* 2015; Ferrer-Vaquer *et al.* 2016; Yeste *et al.* 2016b).

Although PLC $\zeta$  immunofluorescence analyses have shown that PLC $\zeta$  relative fluorescence (RF) intensity in human sperm varies considerably across individual males, the levels of PLC $\zeta$  are overall significantly lower in OAD patients than in fertile males (Heytens *et al.* 2009; Kashir *et al.* 2013b). Accordingly, infertile males exhibiting globozoospermic or teratozoospermic sperm have been shown to have reduced PLC $\zeta$  levels (Kashir *et al.* 2012c; Azad *et al.* 2018). Most notably, PLC $\zeta$  RF intensity in sperm has been significantly correlated with fertilisation rates after ICSI (Yelumalai *et al.* 2015; Nazarian *et al.* 2019), although Ferrer-Vaquer *et al.* stated that PLC $\zeta$  expression was not associated with fertilisation outcomes (Ferrer-Vaquer *et al.* 2016). Collectively, evidence suggests that immunofluorescence labelling can help investigate PLC $\zeta$  expression in sperm and indicates a diagnostic role of PLC $\zeta$  for male infertility.

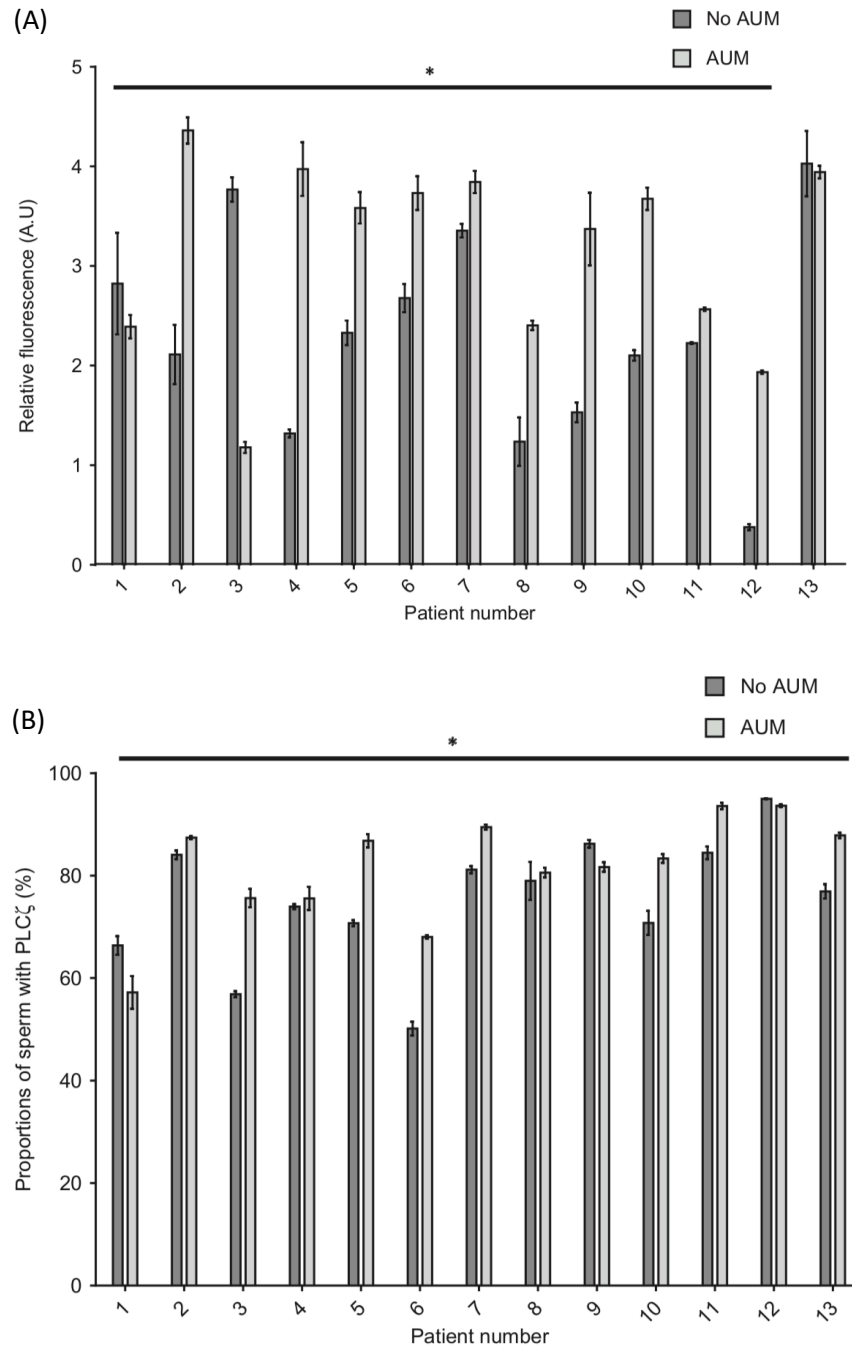
Based on the immunofluorescence technique, our laboratory previously optimised a standard “in-house” labelling protocol to visualise and evaluate PLC $\zeta$  expression in human sperm. This assay has been used consistently in a range of publications (Grasa *et al.* 2008; Heytens *et al.* 2009; Kashir *et al.* 2011a; Kashir *et al.* 2013b; Yelumalai *et al.* 2015; Yeste *et al.* 2016b) and has been validated by numerous peptide blocking experiments (Grasa *et al.* 2008; Yoon *et al.* 2008; Young *et al.* 2009). However, in a recent study, Kashir *et al.* proposed that inadequate specificity between the PLC $\zeta$  antibody and its antigens hindered visualisation efficacy in PLC $\zeta$  immuno-analysis (Kashir *et al.* 2017). These authors incubated mouse, pig, and human sperm with hydrochloric acid (HCl), acidic Tyrode’s solution (AT), or heated sperm with sodium citrate, and reported that these methods can help to unmask PLC $\zeta$  antigens, thereby improving RF intensity in PLC $\zeta$  immuno-analyses (**Figure 2.1**). They further proposed that this antigen unmasking (AUM) method may help to reduce the chances of misdiagnosed PLC $\zeta$  deficiency in clinical settings. However, this study also showed that individual fertile donors exhibited variable PLC $\zeta$  level changes in sperm after receiving AT-AUM methods (**Figure 2.2**).

It is critical to choose the best method for assessing PLC $\zeta$  levels in males, particular given the importance of PLC $\zeta$  deficiency is linked with OAD. In addition, the HFEA recommends the use of AOAs in clinic should with justified reasons and only for patients with demonstrated evidence for OAD, such as PLC $\zeta$  deficiency (HFEA 2018b). This guideline highlights the importance of determining the best labelling method for assessing PLC $\zeta$  expression, which could serve as a diagnostic approach for future clinical diagnosis. Currently, there is no such established assay.

Collectively, this aspect of my work aimed to compare the efficiency of our in-house assay and the new AUM method, with regards to PLC $\zeta$  visualisation in sperm from both fertile and infertile males.



**Figure 2.1 Immunofluorescence PLC $\zeta$  levels in human sperm before and after antigen unmasking (AUM).** Representative images of PLC $\zeta$  in sperm with/without AUM are shown in (A); all three AUM methods (HCl, AT and Sodium Citrate with heating) were shown to significantly increase total mean PLC $\zeta$  levels in sperm, when compared to sperm without AUM treatment (B). Scale bar=5  $\mu$ m. AT: acidic Tyrode's solution. \* denotes  $p < 0.05$  and \*\* denotes significant differences between sodium citrate and HCl AUM groups, and between sodium citrate and AT AUM groups, as demonstrated by one-way ANOVA. Reproduced from Kashir *et al.* (2017).



**Figure 2.2 Comparison of the effect of acidic Tyrode's solution on improving mean PLC $\zeta$  levels and the proportion of PLC $\zeta$  in sperm from fertile individual donors.** With AUM method, ten males exhibited significantly increased PLC $\zeta$  levels ( $p < 0.05$ ), whereas two males showed significantly decreased PLC $\zeta$  levels ( $p < 0.05$ ), and one male did not show changes in PLC $\zeta$  levels (A). With AUM method, eight males showed significantly increased proportion of sperm exhibiting PLC $\zeta$  ( $p < 0.05$ ), whereas two males showed significantly decreased ( $p < 0.05$ ), and two males showed no changes in proportion of sperm exhibiting PLC $\zeta$  (B). \* represents  $p < 0.05$  between AUM and non-AUM groups, determined by the Student's t-test. Reproduced from Kashir *et al.* (2017).

## **2.2 Materials and methods**

### **2.2.1 Ethics and patients**

The frozen sperm samples from 14 donors were obtained from VivaNeo Sperm Bank (Germany). Fresh sperm samples were also obtained from nine infertile patients and three fertile donors from Oxford Fertility (UK). All patients and donors provided written informed consent. The study was approved by the National Research Ethics Service (reference number: 10/H0606/65).

Eligibility criteria for males from Oxford Fertility were as followed. Infertile males had to have at least one of the following features: (1) abnormal sperm head morphology (i.e., either grossly abnormal, 100% globozoospermic, or pin-shape/enlarged/diminished head), (2) experienced total fertilisation failure, low fertilisation rate (<50%), or recurrent fertilisation failure in previous ICSI cycles. Exclusion criteria included severe oligozoospermia where the number of sperm identified on the staining glass slide were less than 100 and hence insufficient for testing with our PLC $\zeta$  assay. Fertile control subjects included: (1) men who had previously fathered a child via natural conception within 10 years prior to the time of consent, or (2) men who had normal parameters in semen analysis according to 5<sup>th</sup> WHO guidelines (WHO 2010), and whose sperm was able to fertilise an oocyte using IVF.

### **2.2.2 Sperm preparation**

The fresh ejaculation samples from 12 males (9 infertile and 3 fertile) acquired from Oxford Fertility were subjected to density gradient washing (DGW) and computer-assisted sperm analyses (CASA). The samples were incubated at 37°C to liquefy prior to processing.



The frozen sperm samples from VivaNeo Sperm Bank were thawed before undergoing DGW. In brief, vials containing frozen semen were removed from the container and held by hand until they thawed. Thereafter, the vials were uncapped, transferred to a microcentrifuge tube, and placed at room temperature (RT) for 30 min to allow the sperm to regain motility. Subsequently, DGW was performed using 40/80 PureSperm medium (Nidacon International, Mölndal, Sweden) according to the manufacturer's protocol, which has been described previously (Kashir *et al.* 2013b). CASA was performed and then sperm were centrifuged at 800 g for 3 min followed by fixation with 4% (w/v) formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 10 min. Excess fixative was then removed and fixed sperm were stored at 4°C.

### **2.2.3 Antibody purification**

A rabbit polyclonal antibody was synthesised by Cova-lab (Villeurbanne, France), with peptides derived from two human PLC $\zeta$  amino acid sequences (C-ETHERKGSDKRGDN, and C-RESKSYFNPSNIKE-coNH<sub>2</sub>; Accession Number: AF532185). The antibody was purified using immunogenic peptides binding to an agarose-containing column according to the manufacturer's instructions (SulfoLink Kit, Pierce Biotechnology, Rockford, IL, USA). The specificity of the antibody was tested with peptide blocking test, as described in Grasa *et al.* (2008).

### **2.2.4 In-house immunofluorescence labelling**

In-house labelling was performed as previously described publications (Grasa *et al.* 2008; Heytens *et al.* 2009; Kashir *et al.* 2011a; Kashir *et al.* 2013b;

Yelumalai *et al.* 2015; Yeste *et al.* 2016b). In brief, first, 50  $\mu$ L of fixed sperm was loaded onto 0.01% (w/v) poly-L-lysine-coated hydrophobic glass slides (Sigma-Aldrich). After 30 min, the sperm were permeabilised with 0.5% Triton X-100 in phosphate-buffered saline (PBS) overnight at 4°C. After washing three times with PBS, the sperm samples were incubated with 3% bovine serum albumin (BSA; Sigma-Aldrich) in PBS at RT to prevent non-specific antigen binding. The sperm were subsequently probed with 25  $\mu$ g/mL FITC-tagged rabbit anti-PLC $\zeta$  antibody in 0.05% BSA-PBS and incubated overnight at 4°C. The glass slides were then washed with PBS three times and incubated with 5  $\mu$ g/mL AlexaFluor-488-conjugated goat anti-rabbit antibody (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at RT. The glass slides were then washed three times with PBS and mounted with Vectashield H-1200 medium containing DAPI (Vector Laboratories, Burlingame, CA, USA).

The glass slides were then observed at 40x magnification using a fluorescence microscope equipped with a FITC filter (Eclipse 80i, Nikon, Tokyo, Japan). To confirm visualisation efficacy, images were also captured with a FV1000 confocal microscope (Olympus, Sir William Dunn School of Pathology, University of Oxford). Sperm samples were analysed using a bright-field channel. Cell nuclei were identified by DAPI labelling, and PLC $\zeta$  RF intensity was observed after 400 ms of exposure in the FITC channel. Multiple fields of view were captured, and the illumination settings remained the same for each experiment.

### **2.2.5 Antigen unmasking (AUM) methods**

The main difference between the AUM method (Kashir *et al.* 2017) and our in-house method is that after permeabilisation in the AUM method, either one of three different AUM reagents were applied to the glass slides to unmask the antigens, namely, HCl, AT, and heating with sodium citrate. After AUM, 100 mM of Tris (pH 8.5) was applied to the sperm as a neutralisation buffer. In this study, the reagents (HCl and AT) were the same as in the AUM study (Kashir *et al.* 2017). According to the AUM study, the other differences between the in-house and AUM methods are the BSA concentration used for blocking non-specific antigens (3% and 5%, respectively), and the BSA concentration in PBS buffer used for antibody incubation (0.05% and 5%, respectively).

Differences between the in-house and AUM labelling methods are summarised in the **Table 2.1**.

**Table 2.1 Differences between immunofluorescence labelling of PLC $\zeta$  in human sperm using in-house and antigen-unmasking (AUM) protocols.**

| Procedure         | In-house method                 | Antigen unmasking (AUM) method                                                                                                                                                                                                                                                                                                                         |
|-------------------|---------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Antigen unmasking | No                              | <p>Sperm received one of the following treatments:</p> <ul style="list-style-type: none"> <li>• HCl (1M, pH=0.1-0.5),</li> <li>• Acidic tyrode's solution (AT, pH=2.5-3),</li> <li>• Heating for 7 min with sodium citrate (10mM, 0.05% Tween 20, pH=6.0);</li> </ul> <p>Then, sperm was incubated with neutralization buffer (100mM Tris, pH=8.5)</p> |
| Blocking agent    | 3% BSA                          | 5% BSA                                                                                                                                                                                                                                                                                                                                                 |
| Primary antibody  | 25 $\mu$ g/ml, in 0.05% BSA-PBS | 25 $\mu$ g/ml, in 5% BSA-PBS                                                                                                                                                                                                                                                                                                                           |
| Second antibody   | 5 $\mu$ g/ml, in 0.05% BSA-PBS  | 5 $\mu$ g/ml, in 5% BSA-PBS                                                                                                                                                                                                                                                                                                                            |

Main differences between the in-house and AUM protocols are the uses of AUM reagents, and the BSA concentration for blocking unspecific antigens and antibody preparation. BSA: bovine serum album; HCl: hydrogen chloride; PBS: phosphate-buffered saline

### 2.2.6 Specificity test

Peptide blocking was performed to investigate antigenic specificity after both methods (i.e., AUM and in-house). Peptides with amino acid sequences corresponding to PLC $\zeta$  sequences (C-RESKSYFNPSNIKE-coNH<sub>2</sub> and C-ETHERKGS DKRGDN) were produced (Covalab, Lyon, France), which ensured that the anti-hPLC $\zeta$  antibody would specifically bind to them. An excess of the two peptides (4 mg in total) was then incubated with BSA for 1.5 h on ice with rough shaking. All labelling procedures in the peptide blocking assays were the same as those used in the immunofluorescence labelling assays, except that the sperm were incubated with primary anti-PLC $\zeta$  antibody diluted in BSA medium containing the peptides.

### 2.2.7 Study design

This part of my study included five experiments comparing the labelling efficacy of in-house and AUM methods (**Table 2.2**). The five comparative experiments used frozen sperm from fertile donors (experiments 1 and 5), fresh-fixed sperm from fertile donors (experiment 2), and fresh-fixed sperm from infertile males (experiments 3 and 4), respectively. In experiments 1, 3 and 5, sperm from an individual male were equally distributed into four groups, which were then stained with the in-house, AT-AUM, HCl-AUM, and non-AUM methods, respectively. The non-AUM method refers to sperm labelled following the AUM protocol in the absence of AUM reagents. In experiment 2, HCl was chosen as the AUM reagent, as HCl was suggested as the second most efficient method with which to improve RF intensity (Kashir *et al.* 2017). According to Kashir *et al.*, the most effective AUM method involved heating sperm with

sodium citrate for 7 min (Kashir *et al.* 2017). I chose not to heat sperm with sodium citrate as I found that heating damaged the sperm, and no fluorescence was observed afterwards. In experiment 4, AUM reagents (HCl and AT) were used in the in-house method. Sperm from every infertile/fertile individual were equally distributed to different groups.

**Table 2.2 Study design of each experiment.**

| <b>Experiment number</b> | <b>Sperm information</b>                                                                                   | <b>Groups</b>                                      |
|--------------------------|------------------------------------------------------------------------------------------------------------|----------------------------------------------------|
| Experiment 1             | Frozen sperm from fertile donors (n=14)                                                                    | (1) in-house, (2) AT-AUM, (3) HCl-AUM, (4) non-AUM |
| Experiment 2             | Fresh-fixed sperm from fertile donors (n=3)                                                                | (1) in-house, (2) HCl-AUM, (3) non-AUM             |
| Experiment 3             | Fresh-fixed sperm from infertile males (n=9)                                                               | (1) in-house, (2) AT-AUM, (3) HCl-AUM, (4) non-AUM |
| Experiment 4             | Fresh-fixed sperm from infertile males (n=3)                                                               | (1) AT-in-house, (2) HCl-in-house, (3) in-house    |
| Experiment 5             | Specificity testing of binding between PLC $\zeta$ antigen and antibody, Frozen sperm from a fertile donor | (1) in-house, (2) AT-AUM, (3) HCl-AUM, (4) non-AUM |

AUM: antigen unmasking; AT: acidic Tyrode's solution.

### 2.2.8 Sperm analysis and statistical analysis

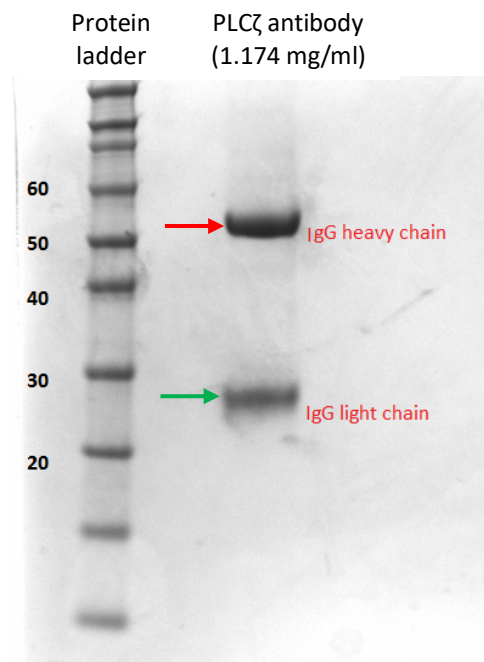
At least 100 cells were analysed per slide using ImageJ (National Institutes of Health, Bethesda, MD, USA). After performing bright-field microscopy, the sperm heads were circled, and only those with normal morphology and attached tails were selected for analysis. The RF intensity of sperm heads exhibiting PLC $\zeta$  was then quantified using the region of interest (ROI) tool in ImageJ. The mean RF intensities in the ROIs for each experiment were calculated and compared between labelling methods.

Statistical analysis was conducted using Prism software 5.0 (GraphPad, San Diego, CA, USA). Normality test of PLC $\zeta$  RF intensity was conducted with D'Agostino & Pearson omnibus test, and statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons, or Kruskal-Wallis test where appropriate. Differences were considered to be statistically significant at  $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.001$ . Data are expressed as mean  $\pm$  S.E.M, unless stated otherwise.

## 2.3 Results

### 2.3.1 Antibody purification

To begin with, I purified a stock of anti-human PLC $\zeta$  polyclonal antibody to be used throughout my thesis work. Specificity of the antibody has been previously verified in Grasa *et al.* (2008). **Figure 2.3** shows the IgG heavy chain and light chain of the PLC $\zeta$  antibody, which indicated that the PLC $\zeta$  antibody in the present study had been successful purified. Purified PLC $\zeta$  antibody (1.174 mg/ml) was then diluted for the subsequent immunofluorescence labelling (25  $\mu$ g/ml, in accordance with all other publications).



**Figure 2.3 Analysis of anti-human PLC $\zeta$  (anti-hPLC $\zeta$ ) antibody by SDS-PAGE (10% gel) with Coomassie blue staining.** Red arrow and green arrow indicate the heavy chain and the light chain of anti-hPLC $\zeta$  antibody, respectively.

### **2.3.2 Experiment 1: Does AUM improve PLC $\zeta$ visualisation efficiency in frozen sperm from fertile donors?**

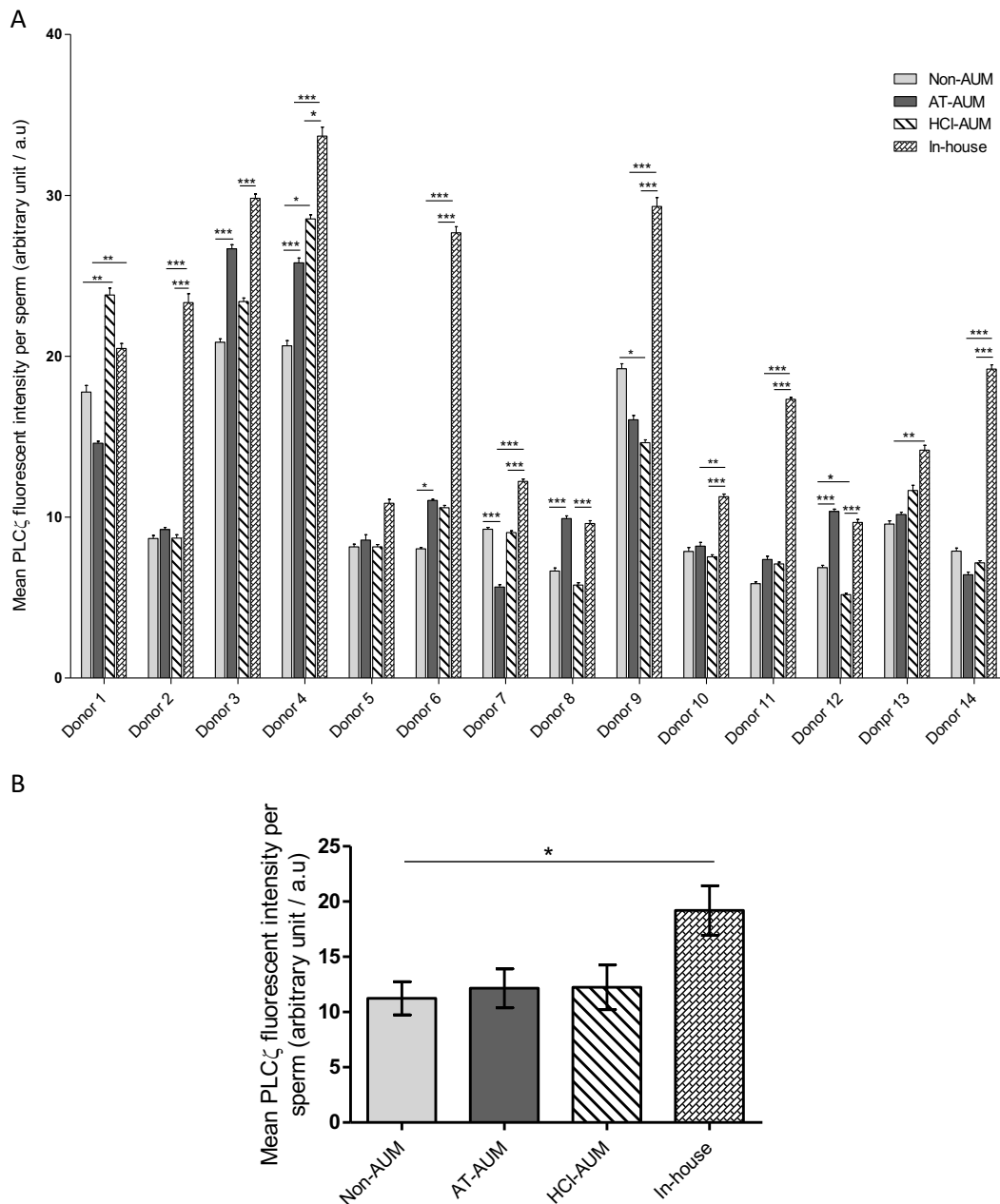
Comparison of PLC $\zeta$  RF intensities in AUM-treated (AT or HCl) and non-AUM-treated sperm from each individual allowed us to test whether the detection of PLC $\zeta$  changed according to the reagent used. **Figure 2.4 A** shows that PLC $\zeta$  RF intensity varied between the HCl-AUM, AT-AUM, non-AUM and in-house methods. Of the 14 donors, 6 showed a significant increase in PLC $\zeta$  levels in the AT- or HCl- treated group compared to the non-AUM group ( $p < 0.001$ , 0.01 or 0.05). PLC $\zeta$  levels were increased in 8 out of 14 samples in the HCl-AUM group compared to the non-AUM group (donors 1-6, 11, and 13); of there, two were significantly increased (donors 1 and 4,  $p < 0.01$  and 0.05, respectively). AT was

more efficient in improving PLC $\zeta$  RF intensity than HCl; of the 14 donors, 10 showed higher PLC $\zeta$  levels in the AT-AUM group compared to the non-AUM group (donors 2-6, 8, and 10-13), of which 5 were significantly different (donors 3, 4, 6, 8, and 12,  $p < 0.001$  or 0.05). Nevertheless, of the 14 males, HCl-treated sperm from 6 donors (donors 7-10, 12, and 14) and AT-treated sperm from 4 males (donors 1, 7, 9, and 14) showed reduced PLC $\zeta$  RF intensity compared to the non-AUM group, and one from each group was statistically significant (donors 7 and 9,  $p < 0.001$  and 0.05, respectively).

Comparison between the in-house and AUM methods showed that the former exhibited better visualisation efficacy regarding PLC $\zeta$  RF. The in-house method led to significantly higher PLC $\zeta$  levels in 13 out of 14 males compared to either the AT-AUM or HCl-AUM method (**Figure 2.4 A**, except donor 5). Notably, 8 out of 14 males showed significantly higher PLC $\zeta$  levels in sperm stained with the in-house method than any of the AUM methods (**Figure 2.4 A**, donors 2, 4, 6, 7, 9-11, and 14,  $p < 0.001$ , 0.01 or 0.05). Only three donors had lower PLC $\zeta$  levels in sperm stained with the in-house method compared to those with HCl or AT treatment, and neither difference was significant (**Figure 2.4 A**, donors 1, 8 and 12).

I also investigated the overall mean PLC $\zeta$  level in each of the four groups. The overall mean levels of PLC $\zeta$  in the AUM-treated (HCl and AT) groups were higher than in the non-AUM group (**Figure 2. B**, RF AT-AUM:  $12.38 \pm 1.89$  and HCl-AUM:  $12.51 \pm 2.16$  vs. non-AUM:  $11.44 \pm 1.61$  arbitrary units [a.u.]), but these differences were not significant. However, overall, the in-house group exhibited a significantly higher RF intensity compared to the other groups (RF in-house:  $18.87 \pm 2.39$  [a.u.],  $p < 0.05$ ).



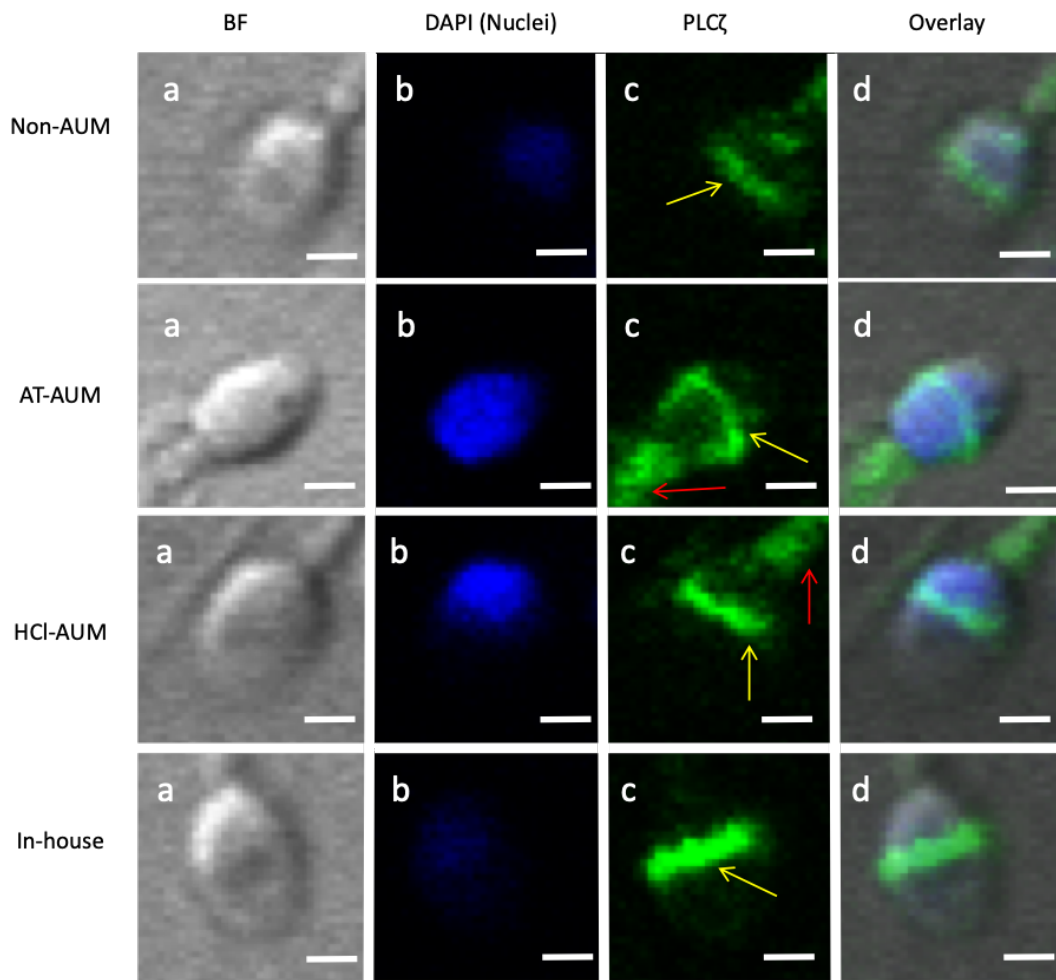


**Figure 2.4 Comparison of PLC $\zeta$  visualisation efficiency in frozen sperm from fertile donors between ‘in-house’ and AUM methods. (A)** Mean PLC $\zeta$  levels in frozen sperm from 14 fertile individual donors. PLC $\zeta$  RF intensity was compared between AUM and in-house methods, and between methods with and without AUM reagents. **(B)** Overall mean PLC $\zeta$  levels in frozen sperm from a total of 14 fertile donors. AT: acidic Tyrode’s solution. Data represent mean  $\pm$  S.E.M, \*\*\*  $p < 0.001$  and \*\*  $p < 0.01$ , and \*  $p < 0.05$  with one-way ANOVA and Bonferroni correction.

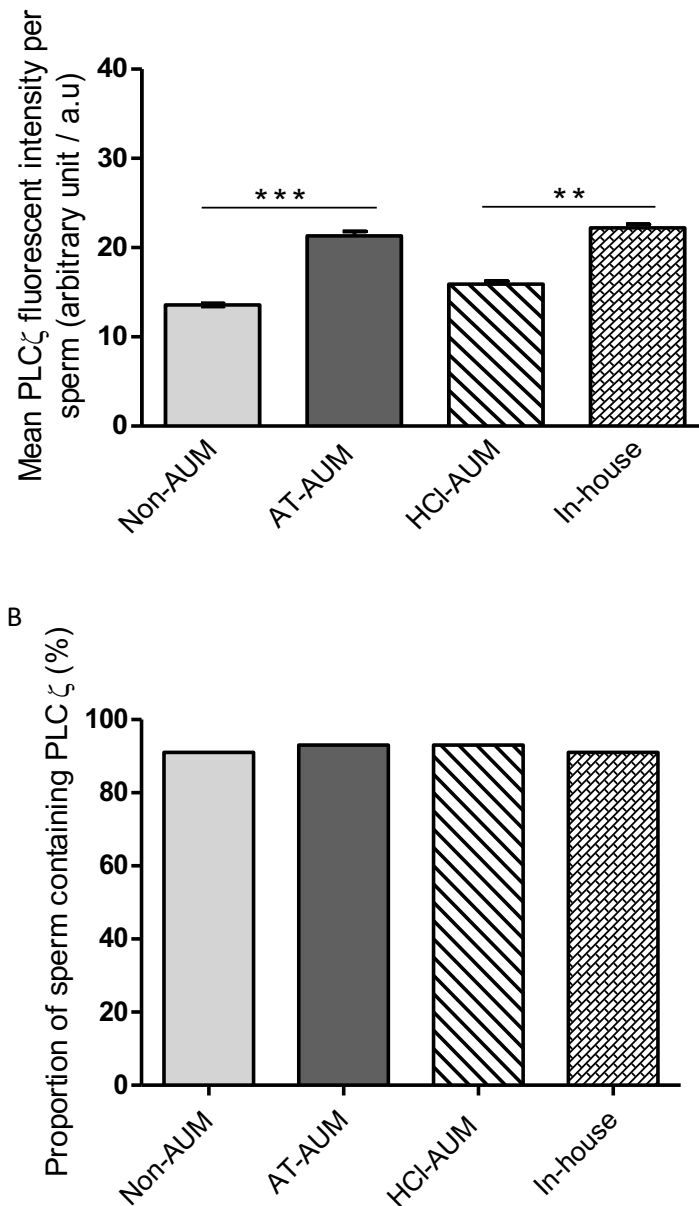
In addition, I used confocal microscopy to confirm the efficiency of visualisation. **Figure 2.5** shows representative images of PLC $\zeta$  visualised in frozen sperm from donor 3, who had shown increased PLC $\zeta$  RF intensity in AT-AUM treated sperm than in non-AUM-treated sperm under normal microscopy (**Figure 2.4 A**, donor 3,  $p < 0.001$ ). Under confocal microscopy, the RF intensity was stronger in the midpiece of AUM-treated sperm than in non-AUM-treated sperm and in-house-stained sperm (**Figure 2.5**). This midpiece localisation of PLC $\zeta$  was not observed under fluorescence microscopy.

The overall comparison results of mean PLC $\zeta$  level in sperm stained with different methods were similar when using confocal microscopy, except that confocal microscopy provided better resolution and yielded stronger RF intensity (**Figure 2.6 A**). Briefly, AT-AUM significantly improved PLC $\zeta$  levels in sperm compared to the level in the non-AUM group (RF AT-AUM:  $21.32 \pm 0.51$ , non-AUM:  $13.57 \pm 0.27$  [a.u.],  $p < 0.001$ ), HCl-AUM also increased PLC $\zeta$  level but this was not statistically significant (RF HCl-AUM:  $15.91 \pm 0.34$  [a.u.]). The in-house method remained the most efficient method compared with the other methods, with a significant difference between the in-house and HCl-AUM methods (RF in-house:  $22.23 \pm 0.39$  vs. HCl-AUM:  $15.91 \pm 0.34$  [a.u.],  $p < 0.01$ ).

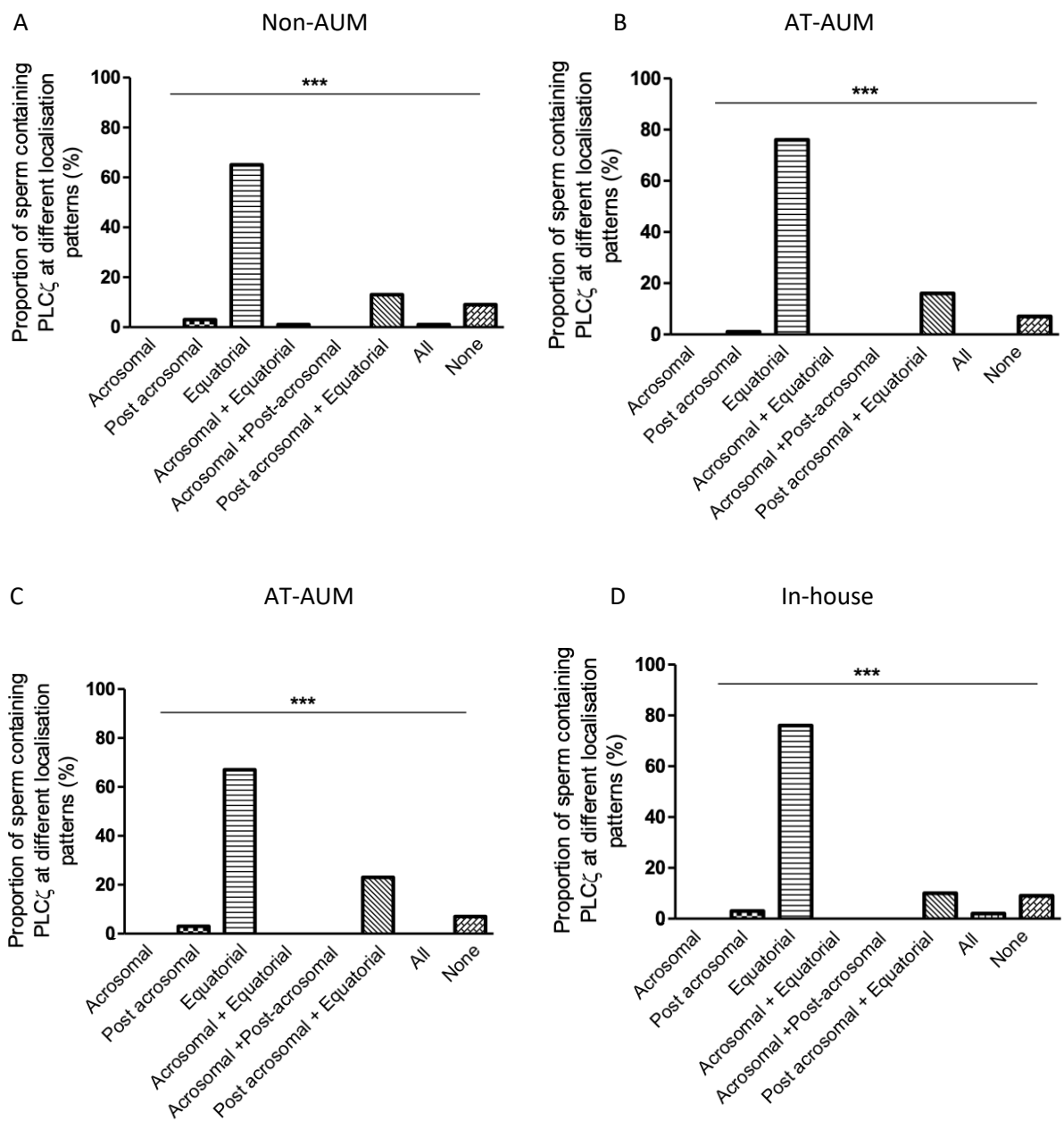
I also analysed the proportion of PLC $\zeta$  expressed in the sperm heads and PLC $\zeta$  localisation patterns under confocal microscopy; using the sample from donor 3 (**Figure 2.6 B**). Proportions of sperm expressing PLC $\zeta$  were similar when compared between different labelling methods (**Figure 2.6 B**). The distribution of different PLC $\zeta$  localisations in sperm heads were also similar, i.e., dominant equatorial PLC $\zeta$  localisations in all labelling methods (**Figure 2.7 A-D**).



**Figure 2.5 Confocal microscopy visualisation of PLCζ in frozen sperm from a representative fertile donor (donor 3).** The in-house method yielded sperm exhibiting better PLCζ relative fluorescence (RF) intensity in the equatorial segment of the sperm head, compared to antigen unmasking (AUM) methods. Extensive PLCζ RF intensity was observed in the midpiece of AUM-treated (AT or HCl) sperm (red arrows). Yellow arrows indicate PLCζ. Representative sperm images for (a) bright-field microscopy, (b) 4'-6-diamidino-2-phenylindole (DAPI), (c) fluorescein isothiocyanate (FITC)-PLCζ labelling, and (d) overlay showing sperm expressing PLCζ. AT: acidic Tyrode's solution. 40x magnification. Scale bar = 5 μm.



**Figure 2.6 Comparison of PLC $\zeta$  visualisation efficiency in frozen sperm from a fertile donor between ‘in-house’ and AUM methods.** Images were obtained under a confocal microscope. **(A)** Overall mean RF intensity of PLC $\zeta$  in sperm from donor 3. Data represent mean  $\pm$  S.E.M **(B)** Proportions of sperm exhibiting PLC $\zeta$  from donor 3. Data represent mean percentage. \*\*\* p<0.001 and \*\* p<0.01, with one-way ANOVA and Bonferroni correction.

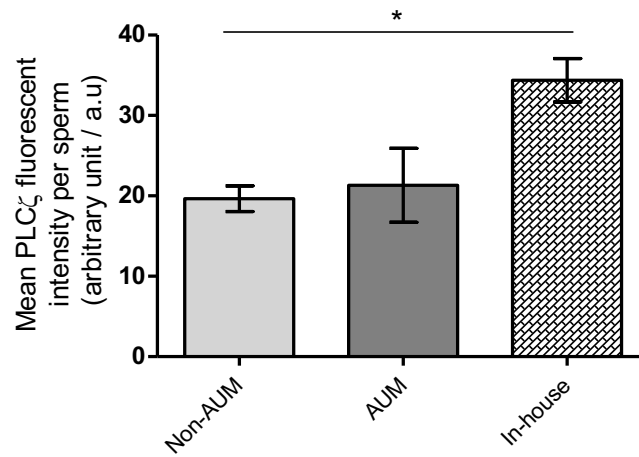


**Figure 2.7 Comparison of PLC $\zeta$  localisation in frozen sperm from fertile donor 3 between 'in-house' and AUM methods.** PLC $\zeta$  localisation was classified into eight patterns and compared between sperm stained with (A) non-AUM, (B) AT-AUM, (C) HCl-AUM and (D) in-house methods. AT: acidic Tyrode's solution. Data represent mean percentages. \*\*\* p<0.001 with one-way ANOVA and Bonferroni correction.

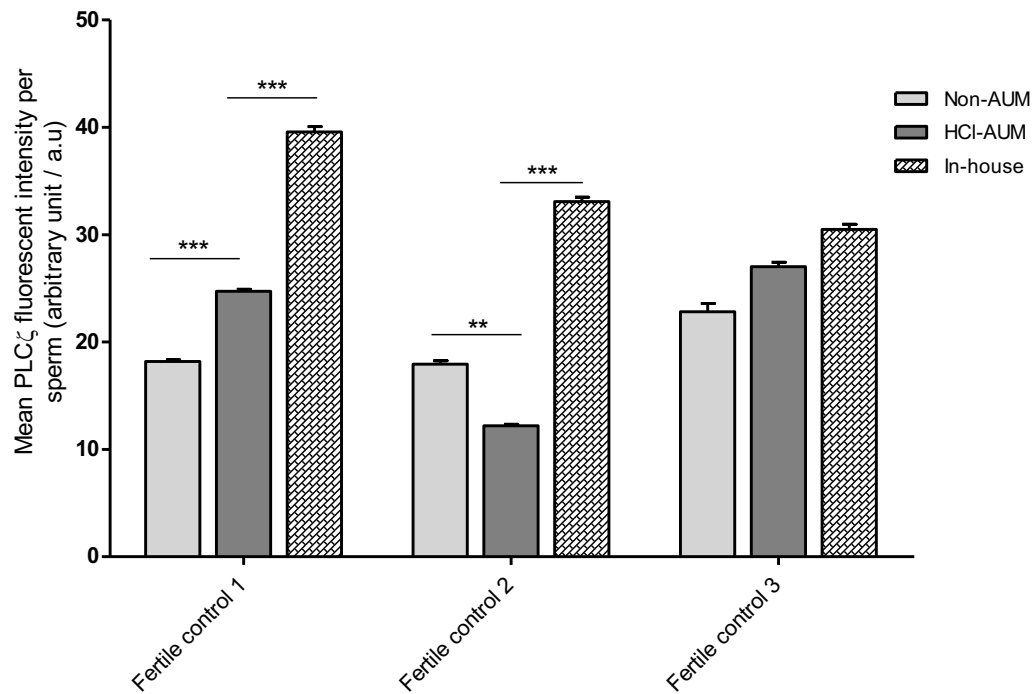
### 2.3.3 Experiment 2: Does AUM improve PLC $\zeta$ visualisation efficiency in fresh-fixed sperm from fertile donors?

The visualisation efficiency of PLC $\zeta$  was also investigated using fresh-fixed sperm from fertile donors. HCl-treated sperm from three fertile donors exhibited increased PLC $\zeta$  RF intensity compared to non-AUM-treated sperm (**Figure 2.8 A**, RF HCl-AUM:  $21.32 \pm 4.61$  vs non-AUM:  $19.65 \pm 1.58$  [a.u.]). However, the in-house method led to significantly highest RF intensity in fresh-fixed sperm than the other methods (**Figure 2.8 A**, RF in-house:  $34.38 \pm 2.70$  [a.u.],  $p < 0.05$ ). PLC $\zeta$  levels in sperm from individuals subjected to different treatment is shown in **Figure 2.8 B**.

A



B



**Figure 2.8 Comparison of PLC $\zeta$  visualisation efficiency in fresh-fixed sperm from fertile donors between ‘in-house’ and AUM methods (n=3).** HCl was used for antigen unmasking (AUM). (A) Overall mean levels of PLC $\zeta$  in sperm from three fertile donors. (B) Mean PLC $\zeta$  levels in sperm from individual fertile donors. AT: acidic Tyrode’s solution. Data represent mean $\pm$  S.E.M. \*\*\* p<0.001 and \*\* p<0.01, and \* p<0.05 with one-way ANOVA and Bonferroni correction.

### 2.3.4 Experiment 3: Is the in-house method efficient in assessing PLC $\zeta$ levels in infertile males?

As the aim of the AUM method is to improve poor visualization of PLC $\zeta$  in sperm, I also examined the AUM method using sperm from nine infertile males. These patients attended Oxford Fertility seeking treatment, and in-house analyses showed that they had significantly reduced PLC $\zeta$  levels in their sperm compared to fertile controls. In the present study, the overall mean PLC $\zeta$  level in AT- and HCl-treated sperm from the nine patients was higher compared to the level in the non-AUM group, but this was not significant (**Figure 2.9 A**, RF AT-AUM:  $6.86 \pm 1.07$ , HCl-AUM:  $7.21 \pm 0.81$  vs. non-AUM:  $6.49 \pm 0.87$  [a.u.]). However, the in-house method showed a significantly higher PLC $\zeta$  RF intensity than both the HCl-AUM and AT-AUM methods (RF in-house:  $11.55 \pm 1.51$  [a.u.],  $p < 0.05$ ).

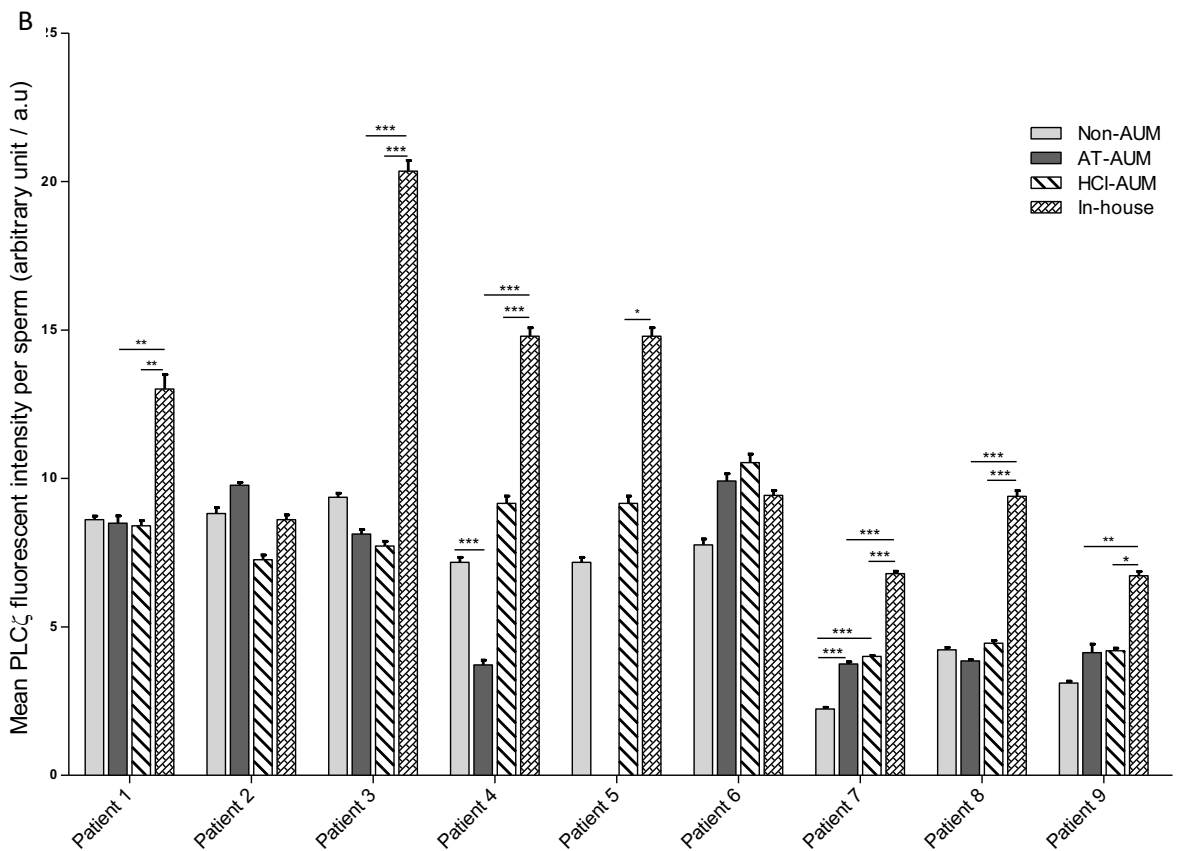
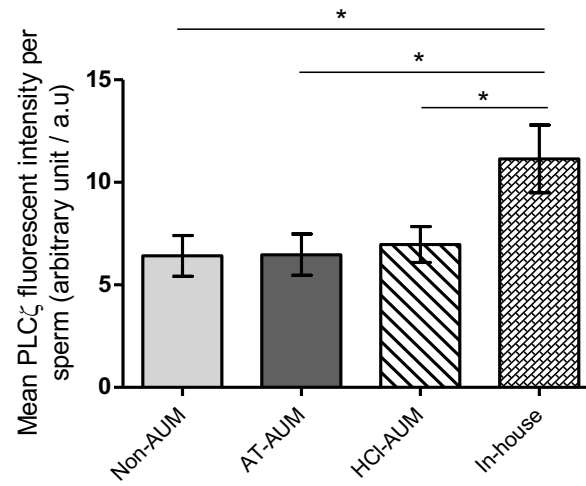
The efficiency of the AUM (AT and HCl) and in-house methods for evaluating RF intensity was also compared using sperm from nine individual patients (**Figure 2.9 B**). The AUM method did not achieve consistent visualisation efficiency of PLC $\zeta$  in sperm from infertile males. To be precise, of the nine patients, seven showed increased PLC $\zeta$  levels after AT or HCl treatment compared to the non-AUM group (**Figure 2.9 B**, patients 2, and 4-9); however, only one was significant (patient 7,  $p < 0.001$ ). Meanwhile, AT- and HCl-AUM methods also caused reduced PLC $\zeta$  levels in sperm from five out of nine patients, when compared to non-AUM method; one was statistically significant (patient 4,  $p < 0.001$ ) and four were not significant (patients 1, 2, 3, and 8, **Figure 2.9 B**).

Compared to these inconsistencies in PLC $\zeta$  levels across individual patients caused by the AUM method, the in-house method achieved good and consistent visualisation; seven of the nine infertile males exhibited significantly



higher PLC $\zeta$  RF intensity after in-house labelling compared to the other labelling methods (**Figure 2.9 B**, patients 1, 3-5, and 7-9,  $p < 0.05$ ). The other two patients showed lower PLC $\zeta$  RF intensity in sperm after in-house labelling compared to AT-AUM and/or HCl-AUM labelling; this difference was not statistically significant (**Figure 2.9 B**, patients 2 and 6).

A

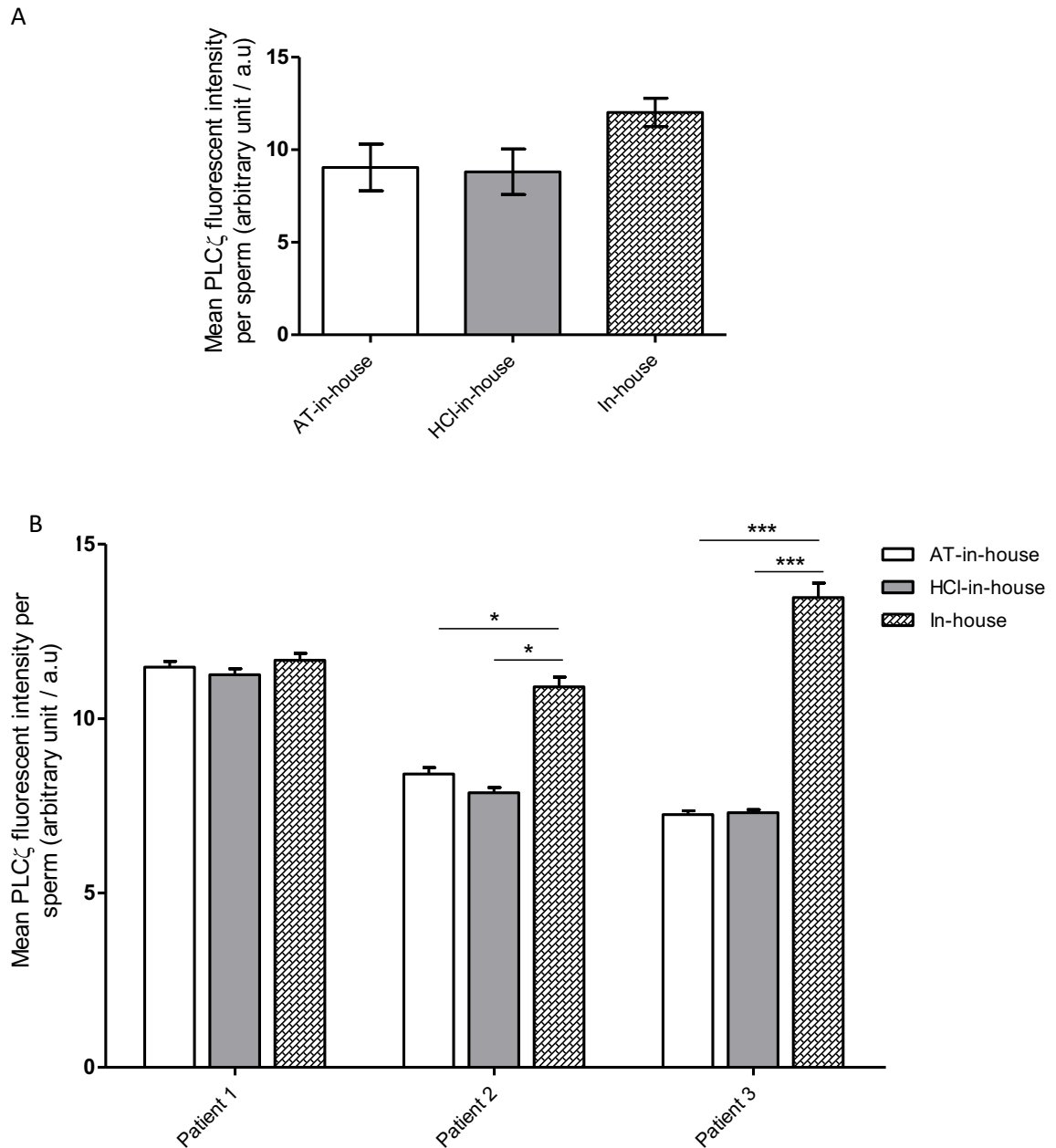


**Figure 2.9 Comparison of PLC $\zeta$  visualisation efficiency in fresh-fixed sperm from infertile males between ‘in-house’ and AUM methods (n=9).** (A) Overall mean levels of PLC $\zeta$  in sperm from nine infertile males. (B) Mean levels of PLC $\zeta$  in sperm from nine infertile males. AT: acidic Tyrode’s solution. Data represent mean  $\pm$  S.E.M. \* p < 0.05 with one-way ANOVA and Bonferroni correction.

### 2.3.5 Experiment 4: Should AUM be incorporated with in-house method?

As mentioned earlier, the concentrations of BSA were different in the in-house and AUM methods (Section 2.2.5 and **Table 2.1**). Briefly, 3% BSA and 0.05% BSA were used in the in-house method, to block non-specific antigens and prepare antibody solutions, while 5% BSA was used throughout in the AUM method. To investigate whether the RF intensity for PLC $\zeta$  in sperm is improved by acidic AUM reagents (AT and HCl), I applied these reagents to sperm while following the in-house protocol.

Following the in-house protocol, the overall mean PLC $\zeta$  RF intensity in sperm from the three infertile males was not significantly improved after HCl or AT treatment (**Figure 2.10 A**). Instead, the in-house method without AT/HCl led to the highest overall PLC $\zeta$  RF intensity, although this difference was insignificant (**Figure 2.10 A**,  $p=0.15$ ). Of the three males, two showed significantly reduced PLC $\zeta$  sperm levels after exposure to AT or HCl (**Figure 2.10 B**, patients 2 and 3,  $p<0.05$  and  $p<0.001$  respectively).

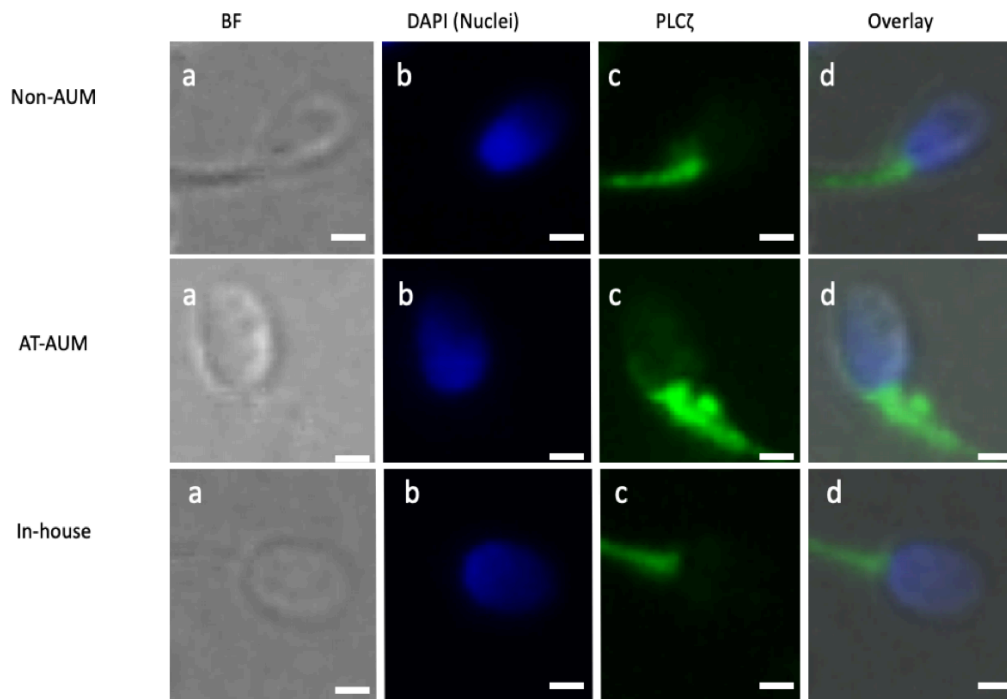


**Figure 2.10 Investigation of the applicability of antigen unmasking (AUM) reagents using the in-house method.** (A) Overall mean relative fluorescence (RF) intensity of PLC $\zeta$  in sperm from infertile males (n=3). (B) Mean PLC $\zeta$  RF intensity in fresh-fixed sperm from individual patients. AT: acidic Tyrode's solution. \*\*\* p<0.001 and \* p<0.05 with one-way ANOVA and Bonferroni correction. Data represent mean  $\pm$  SEM.

### 2.3.6 Experiment 5: Does AUM increase the chances of non-specific binding?

Based on the finding that AUM increased PLC $\zeta$  RF intensity in the sperm, I investigated antibody binding specificity, as this was not performed in the previous AUM study (Kashir *et al.* 2017). AT- and HCl- treated sperm exhibited visible fluorescence even after peptide blocking, as compared to the in-house method (**Figure 2.11**). Notably, peptide blocking was performed on sperm from a fertile donor who previously exhibited significantly increased PLC $\zeta$  RF intensity after AT treatment compared to non-AUM labelling (**Figure 2.4 A, donor 12,  $p < 0.001$** ), which indicates that the increased RF intensity of PLC $\zeta$  was non-specific.

In addition, strong RF intensity in the sperm midpiece after peptide blocking was observed (**Figure 2.11**), indicating that the fluorescence signal in the midpiece was due to non-specific binding. However, the irregular shape of the sperm midpiece limited the further quantification analysis of the RF intensity.



**Figure 2.11 Comparison of specific antigen-antibody binding between 'in-house' and AUM methods, using PLC $\zeta$  peptides for peptide blocking (PB).** Representative sperm images (donor 12) for (a) bright-field microscopy, (b) 4'-6-diamidino-2-phenylindole (DAPI) labelling, (c) fluorescein isothiocyanate (FITC)-PLC $\zeta$  labelling, and (d) overlay after PB. The in-house method labelled sperm lacked PLC $\zeta$  after PB, while AT-treated sperm exhibited strong relative fluorescence (RF) intensity after PB. AT: acidic Tyrode's solution. 40x magnification. Scale bar = 5  $\mu$ m.

## 2.4 Discussion

This study compared labelling efficacy between our established in-house method and a recently published AUM method (Kashir *et al.* 2017). Results showed that the in-house method stained antigen targets in sperm with good specificity and consistently exhibited the best overall visualisation efficacy, indicating that AUM is not necessarily a feasible and reliable method for PLC $\zeta$  immuno-analysis. Furthermore, this study further highlighted that the AUM method is not the most viable tool for clinical assessments.

PLC $\zeta$  is the key factor responsible for initiating oocyte activation by inducing Ca<sup>2+</sup> oscillations. Thus, assessing PLC $\zeta$  expression can help investigate the oocyte activation ability of infertile males and determine their chances of successful fertilisation (Aghajanpour *et al.* 2011; Yelumalai *et al.* 2015). To accurately reflect the realistic levels of PLC $\zeta$  in patients, it is critical to ensure visualisation efficacy after labelling. I found that labelling sperm using our established in-house method was effective, without any additional treatment, which is in line with the results of previous studies conducted by our research group (Grasa *et al.* 2008; Heytens *et al.* 2009; Kashir *et al.* 2011a; Kashir *et al.* 2012c; Yelumalai *et al.* 2015; Yeste *et al.* 2016b). Other research groups have used immunofluorescence to study human PLC $\zeta$  expression levels using the same antibody and have also observed good antibody–antigen affinity in sperm from fertile donors without the need for antigen retrieval (i.e., without reversing epitope masking) (Durban *et al.* 2015; Ferrer-Vaquer *et al.* 2016; Azad *et al.* 2018; Khakpour *et al.* 2019; Moreau *et al.* 2019; Nazarian *et al.* 2019).

Kashir *et al.* recently suggested that poor specificity between the antibody and masked PLC $\zeta$  antigens resulted in poor visualisation efficacy

(Kashir *et al.* 2017). These authors speculated that such poor affinity may lead to the miscalculation of PLC $\zeta$  levels in sperm from males attending IVF clinics, thereby negatively affecting subsequent clinical decisions. In the present study, the PLC $\zeta$  RF intensity in AUM-treated sperm from fertile donors was higher than in non-AUM treated sperm following their AUM protocol. Although this increase was not statistically significant, the trend is in line with previous results (Kashir *et al.* 2017). It is worth mentioning that Kashir *et al.* used fresh-fixed fertile human sperm in the AUM study, while my study used both frozen and fresh-fixed sperm. The two preservation methods may cause variable PLC $\zeta$  RF intensities; frozen sperm can exhibit lower RF intensity than fresh-fixed sperm (Kashir *et al.* 2011a; Moreau *et al.* 2019). Nevertheless, we should consider whether AUM is necessary for labelling sperm, and if there is a possibility that this technique may improve clinical decision making.

The purpose of AUM in the immunochemistry labelling technique is to improve the accessibility of target antigens to the antibody and therefore improve immunofluorescence levels (Robinson and Vandr  2001; Leong and Leong 2007). Antigen retrieval has been previously used for formalin- and formaldehyde-fixed tissues (Shi *et al.* 1995; Pileri *et al.* 1997; Sarnat *et al.* 1998). However, antigen retrieval tends to be widely applied in cellular nuclei analyses, where the target structures are condensed, or in histochemical staining, where the tissue is embedded in both formalin or paraffin, thus leading to cross-linkages that can cause epitope masking (Shi *et al.* 1991; Shi *et al.* 1993; Li and O'Neill 2012; 2017). Furthermore, it is well-known that AUM cannot retrieve all antigens, and the justification for applying this method should depend on the exact localisation of the target protein. For instance, AUM by heating was



effective on the study of the early human fetal nervous system (Sarnat *et al.* 1998), and an immunological study of human fetal and adult ovaries (Abir *et al.* 2004). However, the soluble cytosolic localisation of PLC $\zeta$  in the perinuclear theca of the sperm head indicates that AUM is not necessary (Fujimoto *et al.* 2004).

It should be noted that Kashir *et al.* also applied the AUM method to methanol-fixed sperm and suggested that the AUM reagents optimally improved the visualisation efficacy particularly in methanol-fixed sperm (Kashir *et al.* 2017). In this study, the fixative was 4% formaldehyde, as this is widely used for research purposes, and methanol was not used because the mechanism underlying AUM involves breaking down the cross-links mostly induced by aldehyde fixation. Aldehyde-containing fixative can lead to a reaction between the aldehyde and groups of amino acids in the target antigen, which involves the formation of cross-linkages and stabilises the target antigen. The good affinity of some amino acids (e.g. tyrosine and arginine) to aldehyde may lead to the masking of epitopes (Leong and Leong 2007; Thavarajah *et al.* 2012). This is why the aldehyde-fixed tissue/cells may have poor visualisation efficacy.

However, it is unlikely that aldehyde-induced cross-linkage affects PLC $\zeta$  labelling. First, cross-linking, in theory, takes place randomly. Second, PLC $\zeta$  is mainly composed of leucine and isoleucine, and lacks the amino acids that have high affinity to aldehyde, thus aldehyde-based fixation should not influence labelling outcomes. Third, the effects of cross-linking are likely to be limited to alterations of a few protein epitopes or subtle three-dimensional conformational changes (Leong and Leong 2007). Notably, the current study used an antibody produced by the Coward Laboratory, which was raised against

two human PLC $\zeta$  amino acid sequences to bind different epitopes of the PLC $\zeta$  protein and provide good visualisation, suggesting that it is unlikely that aldehyde fixation masks PLC $\zeta$  antigens in sperm.

Interestingly, formaldehyde-fixed sperm were incubated with HCl or AT in experiment 4 using the in-house protocol, but this did not improve PLC $\zeta$  RF intensity (**Figure 2.10 A**). This indicates that these acidic reagents (which are recommended for the AUM method) are not effective in breaking down cross-linkages, or they are not the key factors that increase PLC $\zeta$  RF intensity.

It is critical to ensure the accuracy of labelling results, as these data can be used to make clinical decisions. Immunofluorescence analyses of PLC $\zeta$  are based on data generated from signals emitted by a fluorescent-tagged antibody that conjugates with the target antigen. Therefore, specific binding between the antigen and antibody is of the utmost importance. This study examined the specificity of antibody–antigen binding after AUM, which is a unique part of this study. The peptides contained amino acid sequences corresponding to PLC $\zeta$  sequences, so they bound to the anti-human PLC $\zeta$  antibody and hindered any further antibody binding with the PLC $\zeta$  proteins in the test samples. Strikingly, fluorescence signals from AUM-treated sperm heads were observed after peptide blocking, indicating that some of the observed signals after AUM reflect non-specific binding, which was not considered by Kashir *et al.* (2017). Successful immunochemical labelling requires both good antibody affinity to antigens and high antibody specificity. The antibodies used in the current study and the AUM study (Kashir *et al.* 2017) were both polyclonal; using monoclonal antibodies would further improve binding specificity.

The reliability and consistency of the immuno-analysis results is also important. The AUM labelling using frozen/fresh-fixed sperm from fertile/infertile males all showed variable responses to the different AUM reagents (**Figure 2.4 A, 2.8 B, 2.9 B, 2.10 B**). For example, compared to the non-AUM method, the AT-AUM method significantly increased PLC $\zeta$  levels in sperm from fertile donor 12, while HCl-AUM method significantly decreased the RF intensity in the same donor (**Figure 2.4 A**, donor 12,  $p < 0.001$  and  $p < 0.5$ , respectively). The goal of assessing PLC $\zeta$  levels in patients attending IVF clinics is to provide evidence regarding PLC $\zeta$  deficiency-induced OAD, without artificially increasing signals; this then guides decision making regarding subsequent AOA treatment (Nazarian *et al.* 2019). Inconsistent responses to AUM reagents would cause difficulty in clinical settings when deciding which acid reagent to use and make the assay more complicated to perform.

Furthermore, in our laboratory, PLC $\zeta$  analyses of infertile males are performed alongside identical analysis of a fertile donor (Yelumalai *et al.* 2015). Therefore, it is meaningless to apply the AUM method in clinical settings, as our conclusions regarding PLC $\zeta$  levels were drawn by comparing two individuals (one fertile, one infertile). The good immunoreactivity in sperm from fertile donors in the current study indicates that poor PLC $\zeta$  levels observed in the sperm of infertile males is not due to an ineffective immunofluorescence technique. Indeed, dysfunction of regulatory genes or transcriptional/translational factors can impair the corresponding protein expression. Several studies have found that genetic variations in PLC $\zeta$  exons can influence the proportion of sperm expressing PLC $\zeta$  (Kashir *et al.* 2011b), protein enzymatic activity (Nomikos *et al.* 2017b), or stability (Kashir *et al.* 2012b).

Interestingly, a recent paper reported that PLC $\zeta$  mutations were not associated with PLC $\zeta$  protein levels in sperm (Torra-Massana *et al.* 2019). The underlying mechanisms responsible for impairing PLC $\zeta$  protein levels in sperm from infertile males remains unknown and needs to be further investigated.

I also investigated the factors underlying the increased PLC $\zeta$  RF intensity in the AUM study. As mentioned earlier, the difference between the in-house and AUM methods does not only relate to the use of acidic AUM reagents; different BSA concentrations were also used. Comparing the visualisation efficacy between the in-house and AUM methods without acid reagents (i.e., non-AUM) can help clarify whether the BSA buffer concentrations influence the labelling outcome. Strikingly, a significant difference between the in-house and non-AUM groups was observed (**Figures 2.4 B, 2.8 A, and 2.9 A**). This indicates that the increased PLC $\zeta$  RF intensity observed for the AUM method may be at least partially due to the BSA concentrations used. I therefore hypothesise that the significant increase in PLC $\zeta$  RF intensity in AUM-treated sperm reported by Kashir *et al.* (Kashir *et al.* 2017) may be influenced by the BSA-lowered baseline of PLC $\zeta$  RF intensity before AUM reagents were applied, as the in-house method used 3% BSA and 0.05% BSA-PBS while the AUM method used 5% throughout.

Indeed, many attempts have been made to assess PLC $\zeta$  expression, mainly by analysing PLC $\zeta$  mRNA, RNA levels (Aghajanpour *et al.* 2011; Nomikos *et al.* 2013; Kamali-Dolat Abadi *et al.* 2016; Khakpour *et al.* 2019), or by analysing PLC $\zeta$  protein levels with immunoblotting (Gonzalez-Castro *et al.* 2018a) and cell sorting (Gonzalez-Castro *et al.* 2018b; Khakpour *et al.* 2019). Alternative approaches for investigating oocyte activation ability include the MOAT test (which involves the microinjection of human sperm into mouse

oocytes), and imaging  $\text{Ca}^{2+}$  level changes in human oocytes upon the injection of human sperm (Heindryckx *et al.* 2008; Ferrer-Buitrago *et al.* 2018c). Among these approaches, the immunofluorescence labelling of PLC $\zeta$  protein in sperm is an attractive method with which to quantitatively analyse PLC $\zeta$  protein levels. The advantages of immunocytochemical labelling are that it does not require special equipment, which is easier for clinical diagnostic purpose, and that the protocol of immunolabelling is simple and easy to follow. In addition, the emitted signals after antibody binding can be quantified. Therefore, applying our in-house PLC $\zeta$  assay is realistic for both research and clinical purposes and can provide reliable results in a consistent manner.

However, immunocytochemical labelling has some limitations. First, it is not a real-time method and evaluates overall PLC $\zeta$  levels in sperm from individual males, which requires a certain amount of sperm on the staining glass slides (at least 100 sperm in the current study). This means that patients with certain infertile conditions, such as azoospermia, may not have a sufficient number of sperm in their samples. In addition, the potential loss of protein during the immunocytochemical procedure cannot be avoided due to the need to wash the sperm with PBS and permeabilise the sperm membrane. Our research group is currently optimising the in-house protocol so that it is more stable, reliable, and sensitive. To achieve these goals, efforts are being made to synthesise a monoclonal antibody to replace the current polyclonal antibody. Chapter 3 further focuses on applying our in-house PLC $\zeta$  RF intensity assay as a diagnostic and prognostic tool in the investigation of PLC $\zeta$  expression in sperm from infertile males, in order to assist clinicians with decision making related to AOA treatment.

## 2.5 Conclusions

This study aimed to compare the efficiency of immunofluorescence labelling between our established in-house method and a published AUM method for visualising PLC $\zeta$  levels in human sperm. I conducted comparisons using frozen and freshly-fixed sperm from fertile donors and showed that the in-house labelling method is more effective than the AUM method regardless of the sperm preservation method. In addition, this study investigated the potential diagnostic role of AUM in clinical settings. In sperm from infertile males with reduced PLC $\zeta$  levels (as identified by the in-house method), the AUM method failed to improve PLC $\zeta$  RF intensity, indicating that it is not superior for clinical settings. Intriguingly, antibody–antigen binding specificity assays showed that the increased PLC $\zeta$  RF intensity in sperm after the AUM method may be due to the overexposure of antibody to non-specific antigens. Overall, this study demonstrated the good efficacy and reliability of our established in-house method compared to the published AUM method and supports the continued use of our in-house assay for clinical screening.

## 2.6 Key findings

- The AUM method did not improve PLC $\zeta$  visualisation efficiency in frozen/fresh-fixed sperm from fertile donors nor fresh-fixed sperm from infertile males
- AUM reagents are unnecessary and should not be incorporated into the in-house method
- The in-house method is efficient in assessing PLC $\zeta$  levels in sperm and showed good efficacy and reliability for clinical diagnostic purposes

- The AUM method increases the chances of non-specific binding, and is therefore unreliable and inapplicable for clinical settings

## **Chapter 3. The use of PLC $\zeta$ analysis to identify candidates for artificial oocyte activation (AOA): a case series of clinical pregnancies and a proposed algorithm for patient management**

### **3.1 Introduction**

Worldwide, ICSI was used in 66% of non-donor IVF cycles between 2008 and 2010 (Dyer *et al.* 2016), while in the UK it accounted for only 36% of cycles in 2016 (HFEA 2018a). Despite ensuring the direct injection of an individual sperm into one oocyte, TFF, where all mature oocytes fail to fertilise, remains a problem and occurs in 5-10% of conventional IVF cycles and 1-3% of ICSI cycles (Mahutte and Arici 2003; Vanden Meerschaut *et al.* 2014b). According to figures released by the HFEA in 2014, male factor is associated with 10% of failed IVF cases and 50.4% of failed ICSI cases (HFEA 2018a). The principal cause of TFF is oocyte activation failure, known as OAD (Yeste *et al.* 2015). OAD can result from female- or male-related factors. The main reason leading to OAD is a deficiency in PLC $\zeta$  (Saunders *et al.* 2002). Other causes contributing to OAD include pronuclear formation defects, oocyte arrest in the mitotic metaphase, or sperm DNA fragmentation (Rawe *et al.* 2000; Aguila *et al.* 2017).

Currently, the only viable clinical option for the rescue of OAD is AOA, which involves the use of various mechanical, physical or chemical stimuli to artificially induced Ca<sup>2+</sup> release (Vanden Meerschaut *et al.* 2014b), and has been successful in triggering oocyte activation in humans (Vanden Meerschaut *et al.* 2014a; Ebner *et al.* 2015a). ICSI supplemented with AOA especially benefits



males whose sperm exhibits abnormal morphology or in poor conditions, such as asthenozoospermia, teratozoospermia, globozoospermia, or severe oligoasthenoteratozoospermia (**Appendix Table 1 and 2**). A recent retrospective study suggested that AOA significantly increased the fertilisation and pregnancy outcomes of patients who previously experienced fertilisation failure in ICSI (Bonte *et al.* 2019). In line with this, several studies have reported satisfying results after the use of AOA in ICSI, such as improved rates of PN formation, cleavage rate, and blastocyst formation (Nakagawa *et al.* 2001; Isachenko *et al.* 2010; Darwish and Magdi 2015; Ebner *et al.* 2015b).

Among AOA methods, chemical AOA is more widely used, such as ionomycin, calcimycin, and  $\text{SrCl}_2$ . In 2012, Ebner *et al.* reported the first use of a ready-to-use solution incorporating  $\text{Ca}^{2+}$  ionophores, GM508, for azoospermic and cryozoospermic males and reported a total of 32 healthy children born as a result of GM508 (Ebner *et al.* 2012). Few IVF clinics in U.K. have been authorised to use GM508 for patients in recent years, although the HFEA used to forbid its use. Admittedly, AOA is becoming increasingly recognised by more and more authorities/clinics as an additional treatment for fertilisation failure. Worldwide, GM508 has now been applied in an effort to treat some infertile conditions, such as TFF or oligoasthenoteratozoospermia, and has been shown to improve fertilisation outcomes (Ebner *et al.* 2015a; Nicopoullos *et al.* 2015; Karabulut *et al.* 2018; Bonte *et al.* 2019). Indeed, the commercially-produced GM508 saves time for IVF embryologist to prepare, and the procedure is simple (incubation for 5 min followed by a double wash). However, GM508 was not effective for all infertile male conditions (Aytac *et al.* 2015; Aydinuraz *et al.*

2016), which highlights that specific guidelines are essential for the justification of AOA in a clinical setting.

Indeed, despite of the fact that AOA can rescue the devastating situation of fertilisation failure, Van Blerkom *et al.* expanded concerns that AOA may become a routine technique in ICSI, regardless of whether a male infertility factor was involved or not (Van Blerkom *et al.* 2015). Similar to what has occurred with ICSI, which has become a very common treatment in ART, Van Blerkom *et al.* worried that AOA may be used more frequently in clinic than it should, with the aim of guaranteeing at least the presence of PN formation (Van Blerkom *et al.* 2015). Undoubtably, achieving good numbers of PN is not the end-point when defining the success in ART. Moaz *et al.* observed that ionomycin significantly increased the fertilisation outcomes of infertile males whose sperm heads showed abnormal morphology (tapered and amorphous heads) but failed to increase cleavage rate in subsequent development (Moaz *et al.* 2006). Furthermore, Santella and Dale argued that non-physical activation can affect cell homeostasis and may cause epigenetic issues (Santella and Dale 2015). Therefore, careful decisions should be made based on validated evidence indicating that patients may benefit from AOA treatment.

Unfortunately, most IVF clinics carry out AOA mainly based on patient history of IVF/ICSI failure, such as recurrent fertilisation failure, TFF, low fertilisation rate, or suspected OAD (Vanden Meerschaut *et al.* 2012; Nicopoullos *et al.* 2015). AOA was initially linked only with severe infertile conditions; TFF and recurrent TFF (Eldar-Geva *et al.* 2003; Chi *et al.* 2004), but has now been expanded to a wider range of infertile/subfertile conditions, such as low (20 - 50%) fertilisation rate (Ebner *et al.* 2015a; Fawzy *et al.* 2018; Li *et al.* 2019b). In

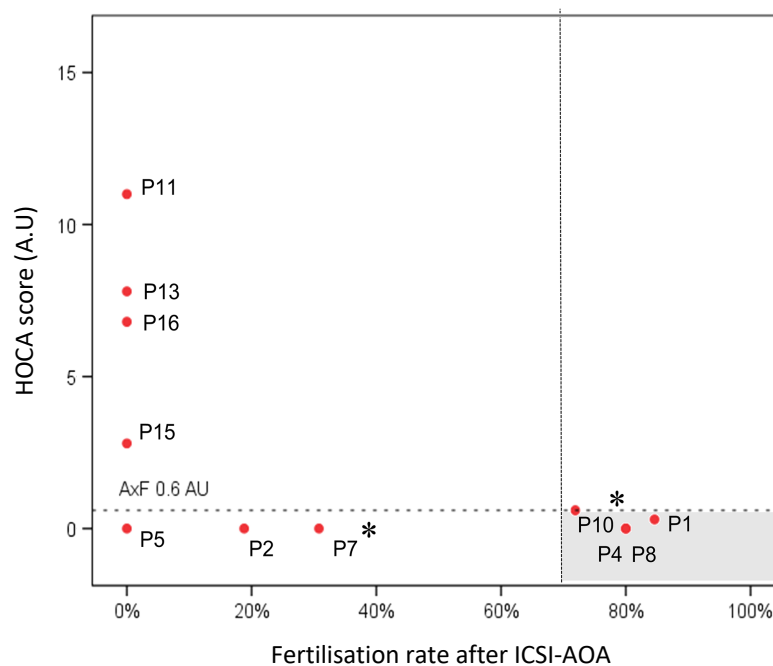
the absence of a standard to justify the use of AOA, AOA may be used unnecessarily, which may exaggerate the effect of AOA in achieving successful fertilisation. Santella and Dale suspected that the successful fertilisation reported in some AOA studies was due to the careful selection of embryos with good quality in repeated trials, and not the ionophores (Santella and Dale 2015). In fact, the HFEA recommended that treatment with AOA should be limited to infertile patients and only if a significant male factor has been identified (HFEA 2019), such as PLC $\zeta$  deficiency (HFEA 2018b).

Unfortunately, the diagnostic applicability of AOA in clinical practice remains unclear. One test, referred to as the MOAT, can identify whether OAD is male- or female-related. The MOAT, a form of heterologous ICSI, involves the injection of human sperm into mouse oocytes and evaluates sperm activation capacity by the 2-cell formation (Heindryckx *et al.* 2005; Heindryckx *et al.* 2008). The application of AOA resulted in successful pregnancies in patients who experienced OAD caused by a male factor (Heindryckx *et al.* 2005). These authors further categorised patients based on activation rates in the MOAT and found that AOA significantly improved fertilisation and pregnancy rates in the OAD group (Heindryckx *et al.* 2008). However, these authors found that AOA also improved the fertilisation outcomes of the MOAT group without activation problems, indicating that the sensitivity of MOAT needs to be further verified (Heindryckx *et al.* 2008). Indeed, human sperm are more potent than mouse sperm in triggering oocyte activation (Nomikos *et al.* 2014b), which may limit the accuracy of MOAT in predicting AOA outcomes. A subsequent study investigated the Ca<sup>2+</sup> oscillatory patterns in each MOAT group and found that

MOAT activation capacity was correlated with the amplitude and frequency of the  $\text{Ca}^{2+}$  oscillations (Vanden Meerschaut *et al.* 2013).

More recently, a study used human oocytes that were matured *in-vitro* for oocyte activation  $\text{Ca}^{2+}$  analysis (HOCA) (Ferrer-Buitrago *et al.* 2018c). Based on donor oocytes responses to  $\text{Ca}^{2+}$  induced by patient sperm, the HOCA showed good sensitivity but average specificity (1.00 and 0.57, respectively) in predicting patient AOA outcomes (**Figure 3.1**). Notably, some oocytes from donors were matured *in-vitro* and were then vitrified. It has been established that freeze-thawing may result in changes in  $\text{Ca}^{2+}$  release subsequently (Nikiforaki *et al.* 2013). Collectively, the laboratory-based MOAT (or HOCA) can identify male-related OAD and serve as a prognostic tool for evaluating AOA outcomes, although its efficiency needs to be further improved. Furthermore, such tests remain possible only for clinics that have access to laboratory facilities, qualified personnel and are also to source human and animal oocytes.

In addition, acrosomal-related tests, such as the total acrosin test, can also provide information for AOA justification (Nasr-Esfahani *et al.* 2010). However, the acrosomal test may relate more to sperm function during the acrosome reaction, not at oocyte activation.



**Figure 3.1 Human oocyte  $\text{Ca}^{2+}$  analysis (HOCA) associated with patient ICSI/AOA outcomes.** HOCA classified patient sperm activation ability according to the amplitude and frequency of  $\text{Ca}^{2+}$  release in donor oocytes (Y-axis) against fertilisation outcomes in ICSI/AOA (X-axis). HOCA showed good sensitivity in identifying eligible patients for AOA (grey area). However, patients whose sperm were shown to have good activation ability in HOCA (P11, 13, 15 and 16) experienced total fertilisation failure in ICSI/AOA, thus indicating the poor specificity of HOCA. Reproduced from Ferrer-Buitrago *et al.* (2018b).

It was reported previously that the PLC $\zeta$  content of ICSI patients is significantly lower than fertile controls, in terms of the total levels and the proportion of sperm expressing PLC $\zeta$ . We further found that PLC $\zeta$  parameters significantly correlate to fertilisation rates after ICSI (Yelumalai *et al.* 2015). A recent study showed that AOA significantly improved the fertilisation rates of PLC $\zeta$ -deficient patients compared with non-deficient ones (Nazarian *et al.* 2019). However, this previous study only evaluated the proportion of sperm expressing PLC $\zeta$  and defined <60% and >85% as PLC $\zeta$ -deficient and PLC $\zeta$  non-deficient,

respectively, without justification (Nazarian *et al.* 2019). Nevertheless, overall evidence supports the fact that PLC $\zeta$  can serve as a prognostic and diagnostic marker in ICSI/AOA.

The present study aimed to use our in-house PLC $\zeta$  assay (tested and verified in Chapter 2) to investigate PLC $\zeta$  protein expression in patients with a history of fertilisation failure following IVF/ICSI, and to identify patient eligibility criteria for AOA treatment. Furthermore, we present the first four successful ICSI-AOA cases in men with clear evidence of PLC $\zeta$  deficiency as identified by our assay.

## **3.2 Materials and methods**

### **3.2.1 Patients and ethical approvals**

Written informed consent was obtained from all participants. Forty-three infertile male patients and 13 fertile donors were recruited from Oxford Fertility, UK, between October 2016 - April 2019. Eligibility criteria for case study subjects has been described in Chapter 2 (Section 2.2.1). Participants' fertilisation history was recorded either by our fertility centre, or other IVF centres in the UK previously involved in their care.

According to PLC $\zeta$  expression in sperm, infertile patients were divided into three groups: (1) PLC $\zeta$ -deficient group, including patients who showed significantly lower mean PLC $\zeta$  levels in sperm, and significantly lower proportion of sperm exhibiting PLC $\zeta$ , when compared to those of fertile controls; (2) PLC $\zeta$ -reduced group, including patients who showed either a significantly lower mean PLC $\zeta$  levels in sperm, or a significantly lower proportion of sperm exhibiting PLC $\zeta$ , when compared to those of fertile controls; and (3) PLC $\zeta$ -

normal group, including patients who showed no significant difference in the aforementioned two parameters when compared to fertile controls. Patients from the PLC $\zeta$ -deficient group were offered clinical advice on AOA treatment.

### **3.2.2 Sperm preparation, antibody, immunofluorescence labelling and quantitative analyses**

Semen samples were collected after at least 3 days of abstinence. Ejaculated semen from all 56 patients and controls were received and subjected to DGW. The protocol for DGW, antibody production and purification were the same as those described in Chapter 2 (Section 2.2.2 and 2.2.3). The present study in this chapter used in-house immunofluorescence labelling method for assessing PLC $\zeta$  expression in sperm, which has been described in Chapter 2 (Section 2.2.4).

### **3.2.3 Ovarian stimulation, ICSI-AOA, embryo culture and transfer**

The protocol used for ovarian stimulation has been described elsewhere (Ebner et al. 2012). Women received a long stimulation protocol using a GnRH agonist with 400  $\mu$ g nafarelin (Syneral). After nafarelin administration for three weeks, the status of pituitary repression was confirmed when serum estradiol levels were lower than 150 pmol/L. Women then received 125~375 IU recombinant FSH (rFSH) (FSH, Merck-Serono). Ultrasound was subsequently performed to observe follicular response. When the leading follicle reached  $\geq 18$  mm diameter, ovulation induction was performed by using 250  $\mu$ g of  $\beta$ -hCG (Merck Serono, Germany). Oocyte collection was carried out 36 h later, followed by IVF or ICSI as previously described (Yelumalai et al. 2015).

Following ICSI, oocyte activation was performed as described elsewhere (Aytac *et al.* 2015). Five female patients whose partners showed PLC $\zeta$  deficiency underwent ICSI-AOA using protocols with additional GM508 (Cult-Active, Gynemed, Germany) treatment. Briefly, GM508 was first equilibrated at 37.5 °C with 5% CO<sub>2</sub> for 4 h before use. Immediately after ICSI, oocytes were exposed to 200  $\mu$ L GM508 for 15 min, followed by three thorough washes in 500  $\mu$ L culture medium. Injected oocytes were then transferred to 30  $\mu$ L sequential culture media and were cultured until a cleavage check was performed (16-20 h after ICSI). Embryo quality was checked and scored 43-35, 67-69, and 91-93 hrs post-ICSI (i.e., Days 2, 3 and 4), following ESHRE guidelines (Embryology 2011). Top- and medium-quality Day 5 blastocysts were then transferred (Gardner scoring system, **Figure 3.2**) (Gardner *et al.* 2007), and remaining blastocysts were cryopreserved for future use.

Fertilisation rate was calculated by the number of oocytes forming pronuclei divided by the number of oocytes injected. Pregnancy was defined as a rise in  $\beta$ -hCG at 14 days after embryo(s) transfer. Clinical pregnancy was defined as the presence of a fetal heartbeat on ultrasound ~4 weeks after embryo transfer. Pregnancy and clinical pregnancy rates were calculated per initiated cycle. Live birth rate was defined as the number of deliveries that resulted in at least one live born baby expressed per initiated cycle.





**Figure 3.2 Gardner grading system for blastocyst selection according to the evaluation on the inner cell mass (ICM) and trophectoderm morphology.** Representative images showing the good quality (Grade 4AA) blastocyst that recommended for embryo transfer. Images of the same blastocyst was taken at different phases of focus (A-C). The surrounding cells formed a cohesive of epithelium (A), and ICM has well developed to multiple cells (B and C). Reproduced from Gardner *et al.* (2007).

### 3.2.4 Statistical analysis

The mean levels of PLC $\zeta$  in sperm from each patient were quantified as 'PLC $\zeta$  immunofluorescence intensity' and compared to those of a matched fertile control at similar age and similar sperm parameters according to 5<sup>th</sup> WHO criteria. Fertile controls were used in more than one PLC $\zeta$  analysis. Parametric assumption was tested with Prism 5.0 (Graph-Pad, CA, USA), and normality of the data was confirmed by the D'Agostino & Pearson omnibus normality test. Statistical PLC $\zeta$  analysis was performed using the Mann-Whitney test or Student's t-test (fluorescence PLC $\zeta$  levels), and Bonferroni's multiple comparisons by one-way ANOVA (localisation of PLC $\zeta$ ). The proportion (%) of sperm exhibiting PLC $\zeta$  first underwent arcsine transformation, in order to stabilize variances, and was then analysed by the Student's t-test. Medcalc software (Version 18.6) was used to determine a receiver operating

characteristic curve (ROC), area under the ROC curve (AUC), and Youden's index analysis, to indicate the cut-off values for PLC $\zeta$  analysis. Data was expressed as mean  $\pm$  S.E.M unless otherwise stated, and considered statistically significant where  $p < 0.05$ ,  $p < 0.01$  or  $p < 0.001$ .

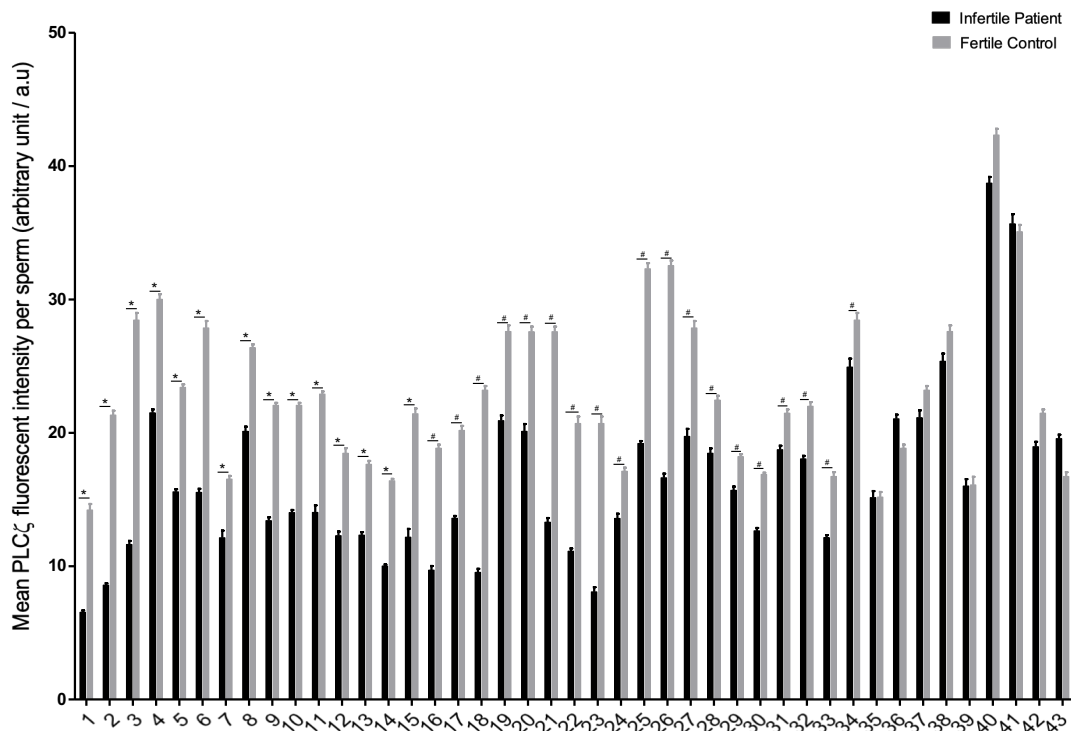
### **3.3 Results**

#### **3.3.1 Patient characteristics**

No significant age difference was identified between patients ( $35.8 \pm 4.3$  years) and controls ( $38.0 \pm 5.0$  years) ( $p = 0.1105$ ). Semen analysis of all 43 patients are shown in **Appendix Table 3**.

#### **3.3.2 PLC $\zeta$ protein levels and localisation pattern analysis in infertile patients**

In total, 34 of 43 patients showed significantly lower levels of PLC $\zeta$  in sperm ( $p < 0.05$ ), and/or significantly lower proportions of sperm exhibiting PLC $\zeta$  ( $p < 0.05$ ) than fertile controls. Among these 34 patients, 15 showed significant PLC $\zeta$  reduction in both parameters; I termed this condition as 'PLC $\zeta$  deficiency' (patients 1-15, **Figure 3.3**). PLC $\zeta$  parameters of all 43 patients are shown in **Table 3.1**.



**Figure 3.3 The total levels of PLCζ in sperm from 43 individual males, as compared to a matched fertile control.** \* denotes a patient's sperm exhibited significant PLCζ deficiency (i.e., significantly lower mean PLCζ level and proportion of exhibiting PLCζ), # denotes that the patient's sperm showed a significantly lower mean PLCζ level ( $p < 0.05$ ), or proportion of sperm containing PLCζ, compared to fertile control. Each number on the X-axis represents an infertile patient. Data are expressed as mean  $\pm$  SEM and are demonstrated by Student's *t*-test or Mann-Whitney test. A larger version of this figure can be seen in Appendix figure 4.

**Table 3.1 PLCζ parameters in 43 infertile males.**

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

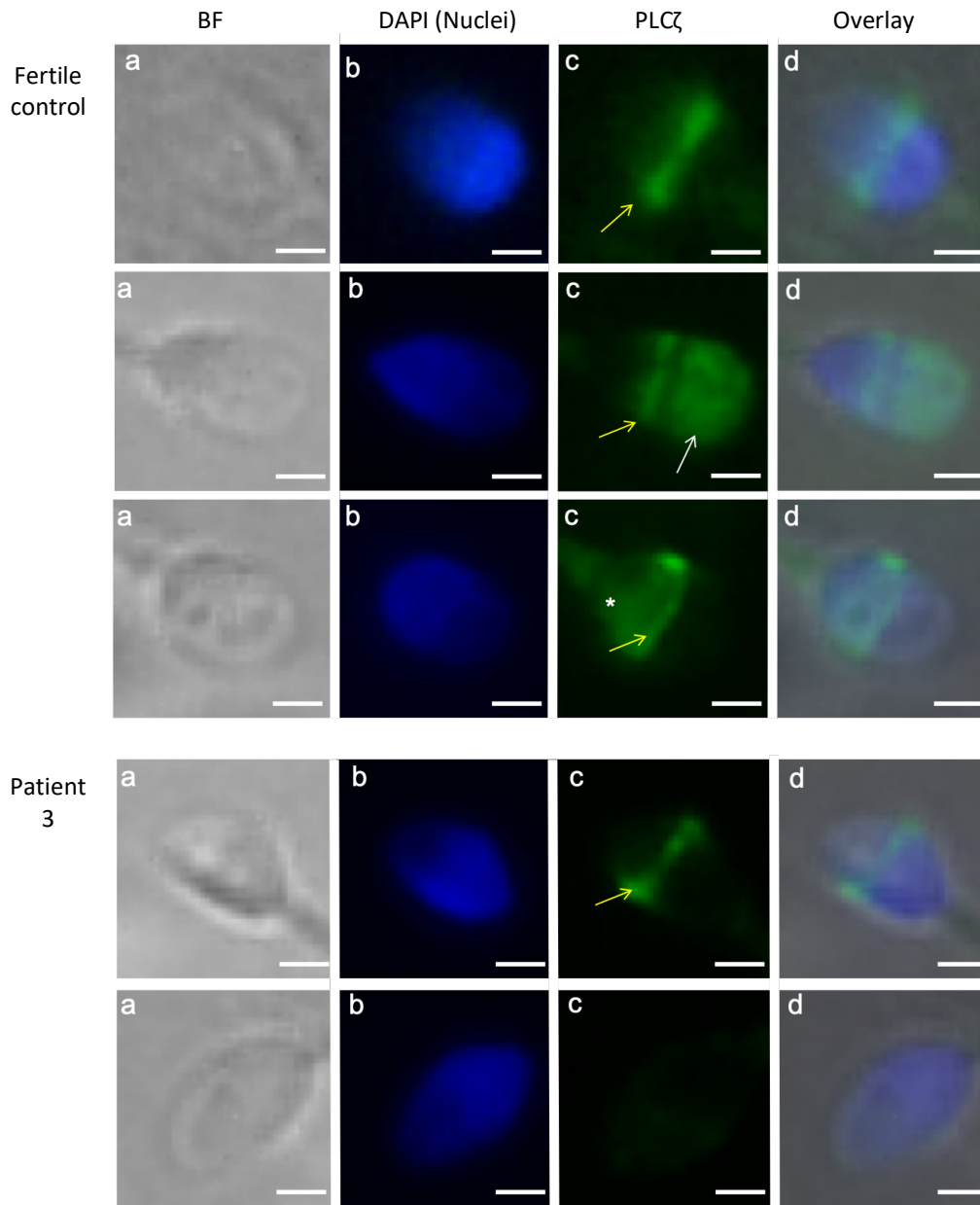
|    |    |                     |                      |
|----|----|---------------------|----------------------|
| 37 | No | 21.13 (17.18-25.08) | 92.61 (83.83-101.40) |
| 38 | No | 25.36 (21.31-29.41) | 98.26 (95.63-100.90) |
| 39 | No | 16.01 (12.36-19.66) | 92.75 (83.73-101.80) |
| 40 | No | 38.71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

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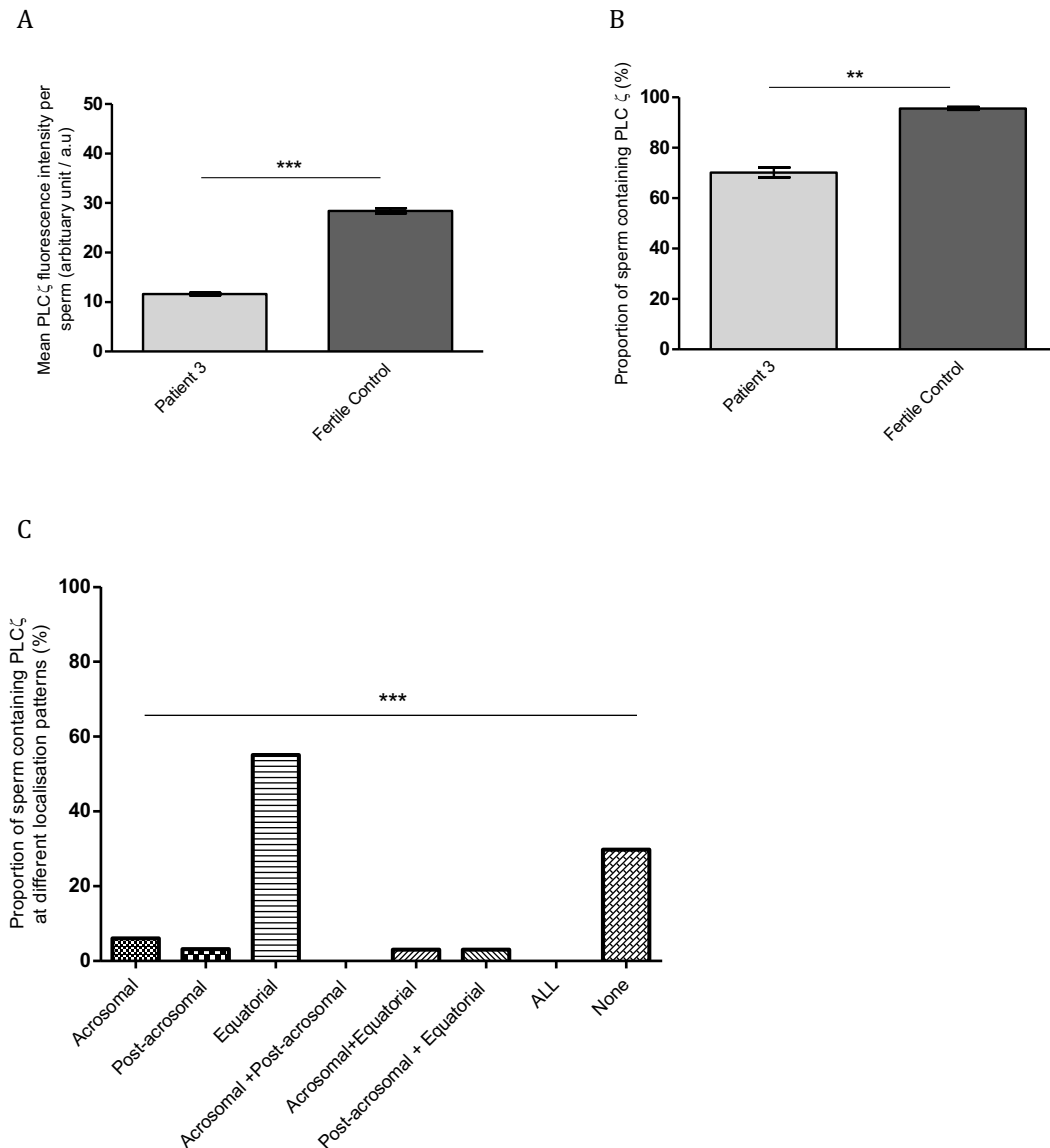
a.u: arbitrary units; 95% CI: 95% confidence interval.

**Figure 3.4** is representative of the immunofluorescence in-house analysis of a PLC $\zeta$ -deficient sample; in this case, patient 3. All 15 PLC $\zeta$ -deficient patients showed significantly lower mean PLC $\zeta$  levels than a fertile control respectively ( $p < 0.05$ ), and a significantly lower proportion of sperm exhibiting PLC $\zeta$  compared to a fertile control ( $p < 0.05$ ). **Figure 3.5 A** and **B** showed an example of PLC $\zeta$  deficiency in an infertile patient's sperm, as revealed by our in-house analysis. In addition, one-way ANOVA and Bonferroni's post-hoc test revealed that PLC $\zeta$  was predominantly located in the equatorial region of patient 3 sperm ( $p < 0.05$ ) (**Figure 3.5 C**).

In addition, 2 globozoospermic patients showed PLC $\zeta$  deficiency (Patient 2, RF:  $8.58 \pm 0.14$  vs. fertile control  $21.33 \pm 0.32$  arbitrary units [a.u], and Patient 4, RF:  $21.48 \pm 0.25$  vs. fertile control  $28.45 \pm 0.53$  [a.u]). Further review of patient history revealed that 1 PLC $\zeta$ -deficient male (patient 1, RF  $6.54 \pm 0.14$  vs. fertile control  $14.21 \pm 0.45$  [a.u]) had undergone orchidectomy at age 14 and presented with a 4-year history of infertility.

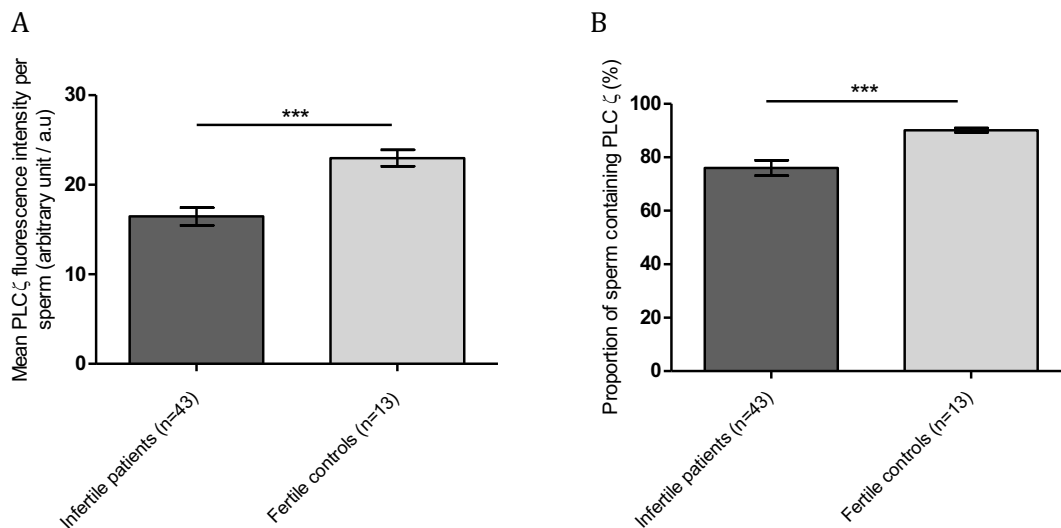


**Figure 3.4 Representative immunofluorescence images of a sperm from patient 3 and a fertile control exhibiting PLC $\zeta$  at different localisations.** Images were captured under (a) Bright-field, (b) DAPI (4',6-diamidino-2-phenylindole), (c) FITC- PLC $\zeta$ , and were merged as (d) Overlay. First three lanes represent PLC $\zeta$  localisations in fertile sperm. PLC $\zeta$  was dominantly expressed in sperm equatorial regions (yellow arrow), equatorial combined with acrosomal/post-acrosomal expression was also observed in the fertile control. PLC $\zeta$  acrosomal and post-acrosomal localisations were observed in sperm (White arrow and asterisk, respectively). The bottom two lanes showed immunofluorescence PLC $\zeta$  in sperm from infertile patient 3. Images were captured at 40x magnification, FITC filter (wavelength 488nm) and at 400ms exposure. Scale bar = 5 $\mu$ m.



**Figure 3.5 An example of in-house PLC $\zeta$  assay of a PLC $\zeta$ -deficient patient (patient 3).** **(A):** Mean total PLC $\zeta$  levels in sperm from patient 3 and a fertile control. **(B):** Proportion of sperm exhibiting PLC $\zeta$  in patient 3 and a fertile control. (B and C): Data are expressed as mean  $\pm$  SEM. \*\*\* denotes statistically significant differences at  $p < 0.001$  and \*\* denotes statistically significant differences at  $p < 0.01$ , as demonstrated by the Mann Whitney test. **(C):** Proportion of sperm exhibiting PLC $\zeta$  at different localisation patterns in patient 3. Patient 3's sperm predominantly expressed equatorial localisation; however, overall, patient 3's sperm did not contain sufficient sperm. \*\*\* denotes statistically significant differences as demonstrated by a one-way ANOVA test ( $p < 0.001$ ). Data are expressed as mean percentages.

I also compared PLC $\zeta$  levels between the 43 infertile patients and the 13 fertile controls. The mean total protein level of PLC $\zeta$  in sperm from the infertile group was significantly lower ( $p<0.001$ ) than that of the fertile control group (**Figure 3.6 A**, RF:  $16.41\pm0.96$  and  $22.85\pm0.90$  [a.u], respectively). Furthermore, the mean proportion of sperm expressing PLC $\zeta$  in the infertile group was significantly lower ( $p<0.001$ ) than that of the control group (**Figure 3.6 B**,  $78.80\pm2.82$  and  $89.99\pm1.06$  [a.u], respectively).



**Figure 3.6 Comparison of the quantitative immunofluorescence analysis of PLC $\zeta$  between infertile (black) and fertile (grey) males. (A):** Comparison between the mean total PLC $\zeta$  level in the infertile and fertile groups. **(B):** Comparison between the mean proportion of sperm exhibiting PLC $\zeta$  in infertile and fertile patients. “n” represents the total number of PLC $\zeta$  analyses. Data are expressed as mean  $\pm$  SEM, \*\*\* denotes a significant difference at  $p<0.001$  demonstrated by the Mann Whitney test (A), and the Student’s *t*-test (B).



### 3.3.3 Outcomes following ICSI-AOA

**Table 3.2** shows the IVF/ICSI outcomes of infertile patients who showed PLC $\zeta$  deficiency, reduced PLC $\zeta$  (significantly lower mean PLC $\zeta$  levels in sperm or significantly lower proportion of sperm exhibiting PLC $\zeta$ ) and normal PLC $\zeta$  characteristics when compared to fertile controls. Of the 15 PLC $\zeta$ -deficient patients, 5 opted to undergo ICSI/AOA following clinician advice (patients 3-7); all 5 couples achieved fertilised oocytes following ICSI/AOA. Fertilisation rate of the PLC $\zeta$ -deficient patients was significantly improved from 18.6% in ICSI cycles to 56.8% in ICSI-AOA cycles (**Table 3.2**). The overall pregnancy rate in ICSI-AOA was 60.0% per started cycle, with a clinical pregnancy rate of 40.0%.

The clinical outcomes of the 5 patients who received ICSI/AOA is summarised in **Table 3.3** and will be discussed on a case-by-case basis. Of these, 4 had live-births (patients 3, 5, 6, and 7), leading to a live-birth rate of 40.0 % per started cycle. Although patient 4 did not achieve a clinical pregnancy after ICSI/AOA, the couple experienced biochemical pregnancy and had 2 good-quality blastocysts frozen.

**Table 3.2 ICSI and ICSI-AOA outcomes in the PLCζ deficiency group, and ICSI outcomes in the PLCζ-reduced and PLCζ normal groups.**

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

Patient age was expressed as Mean ± Standard Deviation (M ± SD); n represents the number of cycles; 2pn/n represents the number of cells with pronuclei out of the total number of injected oocytes. \*\*\* denotes a significant difference at  $p < 0.001$  between ICSI/AOA and ICSI cycles in PLCζ deficiency group. Pregnancy: positive serum β-hCG levels at 14 days after embryo transfer. Clinical pregnancy: the presence of a fetal heartbeat on ultrasound ~4 weeks after embryo transfer. Pregnancy rate and clinical pregnancy rate were determined per initiated cycle. Live birth rate was defined as the number of deliveries that resulted in at least one live born baby expressed per initiated cycle.

**Table 3.3 PLCζ-deficient patient information and IVF/ICSI outcomes (n=15).**

| No. | Male age at date of sample | Female age at date of sample | IVF/ICSI history |                                 | AOA treatment |                                                      | Pregnancy outcome           |
|-----|----------------------------|------------------------------|------------------|---------------------------------|---------------|------------------------------------------------------|-----------------------------|
|     |                            |                              | Cycles (n)       | Fertilisation rate % (2pn/n)    | No. of ET (n) | Fertilisation rate % (2pn/n) in ICSI if received AOA |                             |
| 1   | 38                         | 36                           | 1                | 16.7 (2/12) (ICSI)              | 2             |                                                      | Chemical pregnancy          |
| 2   | 37                         | 35                           | 2                | 0.0 (0/6) (IVF)                 |               |                                                      | N. A                        |
|     |                            |                              |                  | 0.0 (0/4) (ICSI)                |               |                                                      | N. A                        |
| 3   | 34                         | 32                           | 1                | 0.0 (0/5) (IVF)                 |               | 37.5 (3/8) (ICSI)                                    | 2 AOA live-birth, singleton |
| 4   | 25                         | 21                           | 1                | 20.8 (5/24) (ICSI)              | 1             | Cycle 1 37.5 (2/7)                                   | 2 Negative                  |
|     |                            |                              |                  |                                 |               | Cycle 2 27.3 (6/22)                                  | 1 Chemical pregnancy        |
| 5   | 36                         | 38                           | 1                | 0.0 (0/12) (IVF)                |               | 75.0 (15/20) (ICSI)                                  | 2 AOA-live birth, singleton |
| 6   | 27                         | 28                           | 2                | 50.0 (4/8) (IVF)                | 2             | 100.0 (8/8)                                          | 2 Negative                  |
|     |                            |                              |                  | 22.2 (2/9) (ICSI)               | 2             |                                                      | 2 AOA-live birth, singleton |
| 7   | 36                         | 35                           | 1                | 0.0 (0/9) (IVF)                 |               |                                                      | 1 Negative                  |
|     |                            |                              |                  |                                 |               | Cycle 1 66.7 (8/12)                                  | 1 Chemical pregnancy        |
|     |                            |                              |                  |                                 |               |                                                      | 1(FET) Negative             |
|     |                            |                              |                  |                                 |               | Cycle 2 72.7 (8/11)                                  | 2 AOA-live birth, singleton |
| 8   | 32                         | 25                           | 0                | Round heads and small acrosomes |               |                                                      |                             |
| 9   | 34                         | 34                           | 1                | 0 (0/2) (IVF)                   |               |                                                      |                             |
| 10  | 36                         | 40                           | 1                | 0 (0/9) (ICSI)                  |               |                                                      |                             |
| 11  | 42                         | 34                           | 1                | 0 (0/5) (IVF)                   |               |                                                      |                             |
| 12  | 42                         | 35                           | 1                | 0 (0/10) (ICSI)                 |               |                                                      |                             |
|     |                            |                              |                  | Severe oligoasthenozoospermia   |               |                                                      |                             |
| 13  | 34                         | 38                           | 2                | 14.3 (1/7) (ICSI)               | N. A          |                                                      |                             |
| 14  | 32                         | 29                           | 1                | 20 (1/5) (ICSI)                 | 1             |                                                      | Negative                    |
| 15  | 32                         | N. A                         | 5                | 30.3 (10/33)                    | 1x twice ET   |                                                      | Negative                    |
|     |                            |                              |                  | Frozen cycle                    | 1             |                                                      | Negative                    |

IVF/ICSI history may be displayed as mixed cycles performed by different IVF clinics. Abbreviations: N. A: not applicable; TFF: total fertilisation failure; IVF: *in-vitro* fertilisation; ICSI: Intracytoplasmic sperm injection, ET: embryo transfer; FET: frozen embryo transfer. Chemical pregnancy: human chorionic gonadotropin was detected after embryo transfer; however, ultrasound showed no fetal heart rate at week 6. Pregnancy rate, chemical pregnancy rate and clinical pregnancy rate and live birth rate were determined per initiated cycle.

Here I describe the first series of cases of successful AOA treatments carried out at Oxford Fertility on PLC $\zeta$ -deficient patients who were diagnosed by our PLC $\zeta$  assay.

### **Case 1**

Patient 3 presented at an age of 34 years with a normal semen analysis; sperm concentration was 15 million/ml, sperm motility A+B was 46% and normal morphology (6%). His female partner was 32 years old and had previously conceived with a different partner. The couple had experienced failure of one previous IVF cycle (0 of 5 fertilised oocytes). Following a diagnosis of male PLC $\zeta$  deficiency (PLC $\zeta$  RF:  $11.62 \pm 0.26$  vs. fertile control  $28.44 \pm 0.53$  [a.u], 70.23% of sperm containing PLC $\zeta$  vs. 95.64% in fertile control), the female underwent a long stimulation protocol and 13 oocytes were collected. ICSI was subsequently carried out on 8 mature oocytes with supplementary calcimycin (GM508) treatment. Three of the 8 oocytes were successfully fertilised and developed into blastocysts; 2 good quality blastocysts were transferred resulting in a successful singleton pregnancy. The remaining blastocyst was cryopreserved. This represented the first pregnancy in Oxford following treatment with ICSI-AOA, with a healthy male baby born in September 2018 (weight: 4.3 kg).

### **Case 2**

Patient 5 presented at an age of 36 years, with a history of fertilisation failure in 1 previous IVF cycle (0 of 12 fertilised oocytes). Our analysis indicated that his sperm was deficient in PLC $\zeta$  (RF:  $15.57 \pm 0.19$  vs. fertile control  $23.40 \pm 0.22$  [a.u], 71.00% of sperm containing PLC $\zeta$  vs. 94.00% in fertile control). In the subsequent

cycle with GM508, sperm concentration was low (12 million/ml), but the other semen parameters were normal (sperm motility A+B 54% and 6% normal morphology). Fifteen of 20 oocytes were fertilised. Of these, 2 embryos were cryopreserved, and 2 embryos were transferred, resulting in the birth of a healthy male baby in December 2018 (weight: 4.4 kg).

### **Case 3**

Patient 6 (27 years old) had two previous failed IVF/ICSI cycles before undergoing ICSI-AOA (4 of 8 fertilised oocytes in IVF, and 2 of 9 in ICSI). On each occasion, two embryos were transferred but did not result in pregnancy. In-house analysis revealed PLC $\zeta$  deficiency (RF:  $15.53 \pm 0.26$  vs. fertile control  $27.85 \pm 0.52$  [a.u], 45.00% of sperm containing PLC $\zeta$  vs. 90.00% in fertile control). At the time of receiving GM508 treatment, patient 6 showed normal sperm concentration (52 million/ml) and sperm motility (A+B 58%), but abnormal sperm morphology (3%). Following ICSI/AOA, 8 of the 8 oocytes retrieved were successfully fertilised. Of these, 2 were transferred and 2 were cryopreserved. No pregnancy was observed at the first transfer attempt, however the subsequent transfer of 2 frozen embryos resulted in a healthy singleton live-birth.

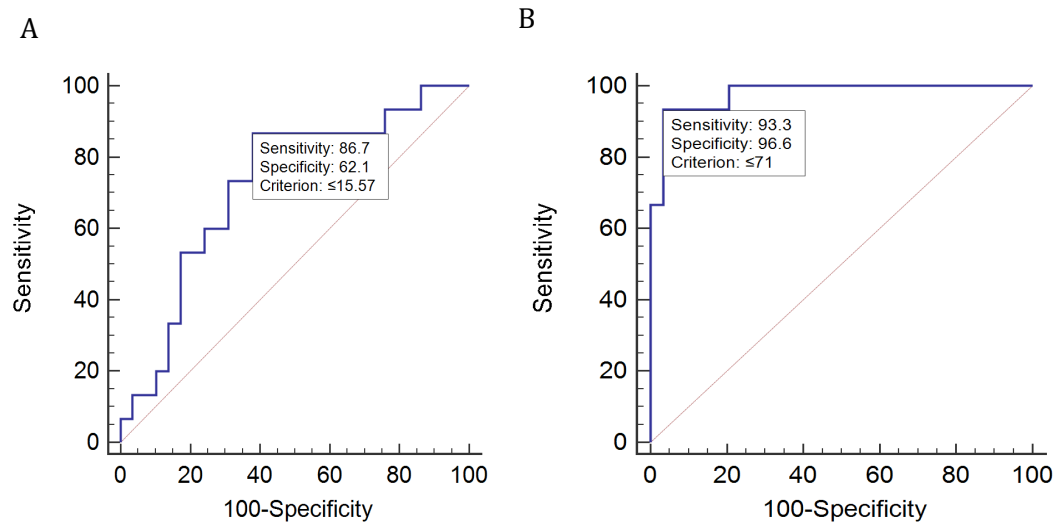
### **Case 4**

Patient 7 was 36 years old at the time of treatment. Our group has previously identified a point mutation in exon 6 of the PLC $\zeta$  gene in patients 7's brother. Patient 7 exhibited PLC $\zeta$  deficiency (relative fluorescence:  $12.12 \pm 0.54$  vs. fertile control  $16.51 \pm 0.25$  [a.u], 70.53% of sperm containing PLC $\zeta$  vs. 97.00% in fertile control), and had no fertilization in a previous IVF cycle (0 of 9 fertilised oocytes). In the

subsequent cycle using GM508, he exhibited normal semen parameters (sperm concentration 88 million/ml, sperm total motility A+B 55% and 6% normal morphology), and 8 of 12 oocytes fertilised; one good-quality blastocyst was transferred and 2 were frozen. However, no pregnancy was observed. Subsequent frozen embryo transfers resulted in no pregnancy and a missed miscarriage at 6 weeks. Following a second attempt with AOA, 8 of 11 oocytes were fertilised and two blastocysts were transferred, resulting in a live female infant (birth weight: 4.2 kg).

#### **3.3.4 Diagnostic accuracy of PLC $\zeta$ analysis to predict AOA applicability in infertile male patients**

The diagnostic accuracy of PLC $\zeta$  analysis to determine eligibility for AOA was evaluated. Cut-off values were determined using the Youden index and justified AUC (Greiner *et al.* 2000), based on the PLC $\zeta$  data of all 56 males (43 infertile and 13 fertile males). The Youden index indicated that the cut-offs associated with OAD were 15.57 a.u for mean PLC $\zeta$  level (AUC: 0.722,  $P < 0.01$ ), and 71% for proportion of sperm containing PLC $\zeta$  (AUC: 0.977,  $p < 0.001$ ) (**Figure 3.7**).

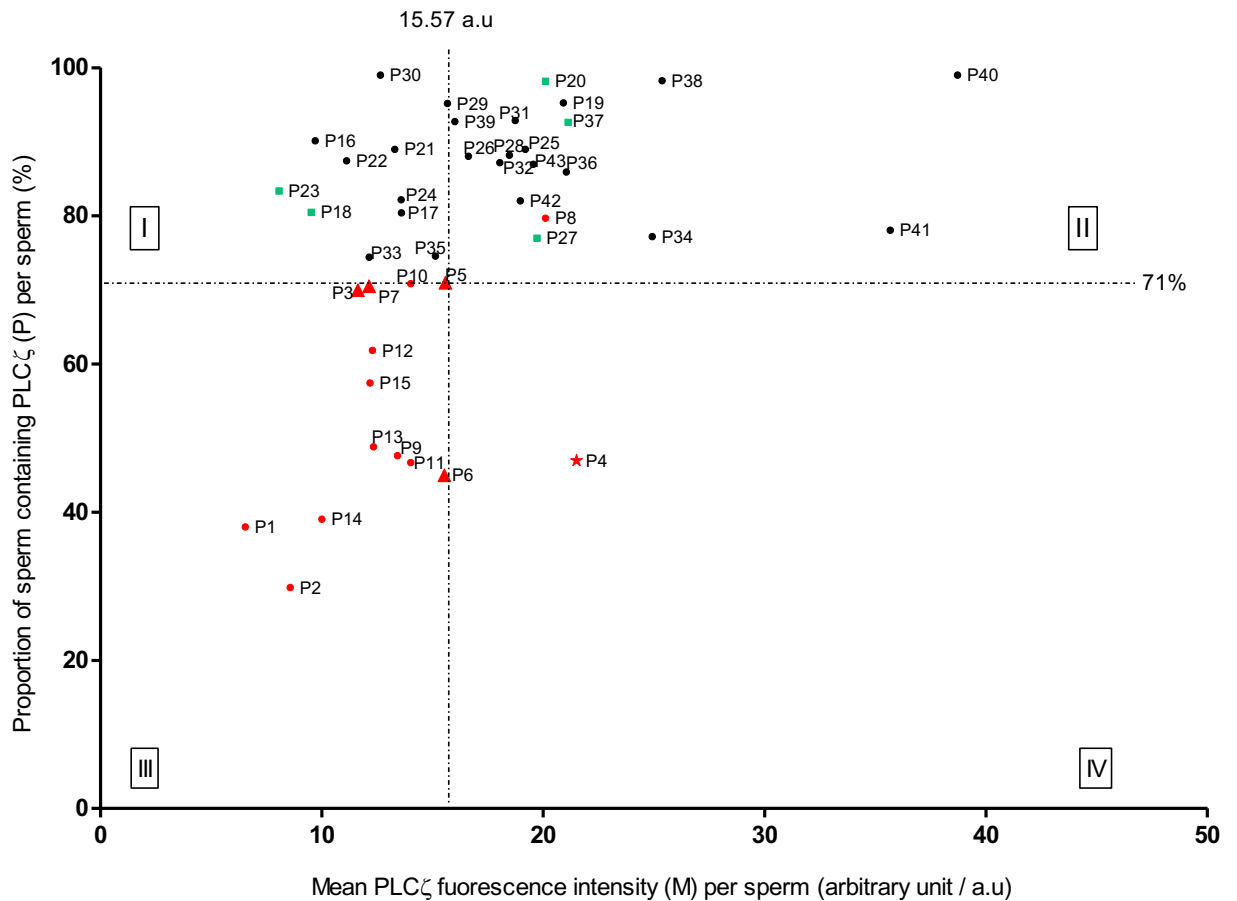


**Figure 3.7 Cut-offs for (A) mean PLCζ level and (B) proportion of sperm containing PLCζ to identify infertile males for AOA treatment.** Cut-offs were calculated according to the receiver operating characteristic curve (ROC) and the area under the ROC curve (AUC). Cut-offs were chosen when the corresponding sensitivity and specificity both reached the best values, as calculated using Youden's index. The value of AUC was 0.722 for the mean PLCζ level ( $P < 0.01$ ) and 0.977 for the proportion of sperm containing PLCζ ( $p < 0.001$ ), thus verifying the accuracy of the cut-offs.

I then plotted a graph of mean PLCζ level in sperm (M, X-axis) against the proportion of sperm containing PLCζ (P, Y-axis), using data from individual infertile males (**Figure 3.8**). Cut-off points divided these patients into four categories; (I) patients with  $M < 15.57$  a.u, but  $P > 71\%$ ; (II) patients with  $M > 15.57$  a.u and  $P > 71\%$ ; (III)  $M \leq 15.57$  a.u and  $P \leq 71\%$  (PLCζ deficiency) and (IV)  $M > 15.57$  a.u, but  $P < 71\%$ .

Strikingly, 13 of the 15 men with PLCζ deficiency in our cohort were in category III (**Figure 3.8**). Moreover, all 4 cases of successful ICSI-AOA pregnancies were in this category. Patients in categories I and IV were those who had either form of defective PLCζ (low mean PLCζ levels or low percentage of sperm containing PLCζ) but not overall PLCζ deficiency. Their IVF history (patient 16 - 43) is summarised in **Table 3.4**. Further follow-up revealed that patients 18 and 23, with category I PLCζ reduction, had both experienced previous live births with their respective current partners without AOA (**Figure 3.8 and Table 3.4**). Furthermore, our analysis revealed that patient 4 (globozoospermia) with PLCζ deficiency was in category IV. The couple then received AOA treatment, which yielded 2 fertilised oocytes from 7, although no pregnancy was observed following the transfer of the 2 embryos. Subsequent ICSI-AOA achieved 6 fertilised oocytes from 22. One embryo was transferred, and only biochemical pregnancy was observed. Category II included patients who exhibited similar (or better) levels of PLCζ parameters when compared to fertile controls. In line with this, patients 20, 27 and 36 from category II have experienced live births/on-going pregnancy without AOA (**Figure 3.8 and Table 3.4**). However, patient 8 was in category III and PLCζ analysis identified that he may have PLCζ deficiency.





**Figure 3.8 The categorisation of infertile males by mean PLCζ level in sperm (X-axis) against the proportion of sperm containing PLCζ (Y-axis).** The vertical dashed line represents the cut-off (15.57 a.u.) of mean PLCζ level (M) per patient's sperm, while the horizontal dash line represents the cut-off (71%) of the proportion of sperm containing PLCζ (P) per patient's sperm. "I, II, III, IV" represent the four categories associated with PLCζ parameters. (I) patients with  $M < 15.57$  a.u, but  $P > 71\%$ ; (II) patients with  $M > 15.57$  a.u and  $P > 71\%$ ; (III)  $M \leq 15.57$  a.u and  $P \leq 71\%$  (PLCζ deficiency, and likely favours AOA) and (IV)  $M > 15.57$  a.u, but  $P < 71\%$ . Patients with PLCζ deficiency are labelled in red; the red triangle represents patients who have had clinical pregnancies/live-birth with AOA, red dots represent those that did not receive AOA, and the red star represents those who received AOA and experienced chemical pregnancy. Green cubes represent patients who have had live-births without AOA, and black dots depict patients with unknown outcomes of subsequent IVF/ICSI treatment.

**Table 3.4 Information and IVF/ICSI outcomes of infertile patients whose sperm showed reduced/normal PLC $\zeta$  expression when compared to fertile controls (n=28).**

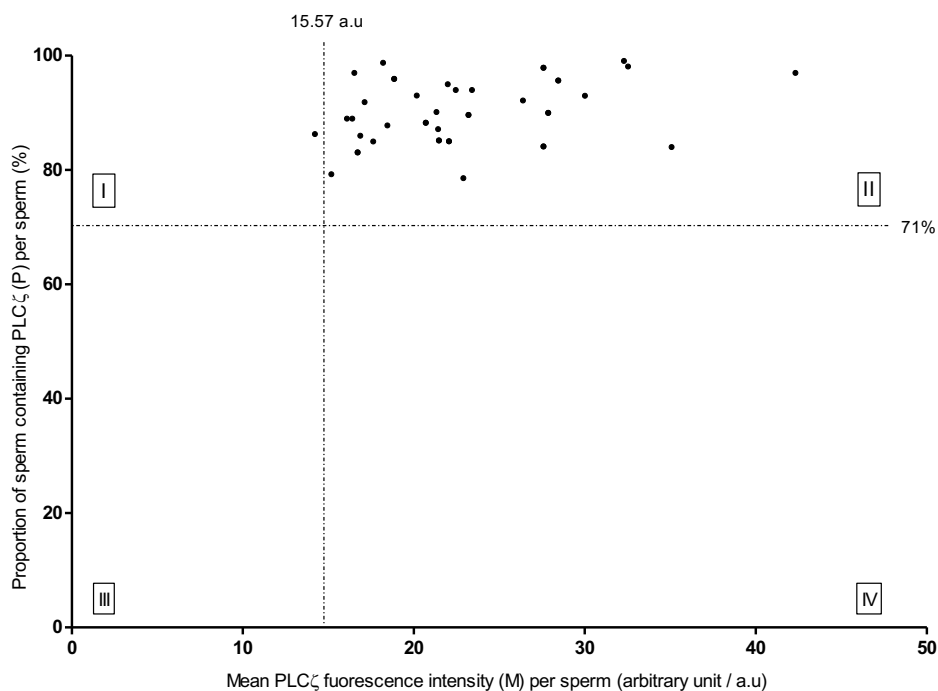
| Patient No. | Male age at date of sample | Female age at date of sample | IVF/ICSI history |                                   |               | Pregnancy outcome |
|-------------|----------------------------|------------------------------|------------------|-----------------------------------|---------------|-------------------|
|             |                            |                              | Cycles (n)       | Fertilisation rate % (2pn/n)      | No. of ET (n) |                   |
| 16          | 43                         | 32                           | 1                | 0.0(0/16) (IVF)                   |               | N. A              |
| 17          | 32                         | 37                           | 1                | 0.0 (0/12) (IVF)                  |               | N. A              |
| 18          | 36                         | 35                           | 2                | 0.0 (0/4) (ICSI)                  |               | N. A              |
|             |                            |                              |                  | 25.0 (2/8) (ICSI)                 | 2             | Live birth        |
| 19          | 33                         | 39                           | 1                | 2.6 (1/38) (IVF)                  | 1             | Negative          |
| 20          | 38                         | 35                           | 2                | 0.0 (0/12) (IVF)                  |               | N. A              |
|             |                            |                              |                  | 80.0 (4/5) (ICSI)                 | 1             | Live birth        |
| 21          | 43                         | 41                           | 1                | 0.0 (0/2) (ICSI)                  |               | N. A              |
| 22          | 33                         | 36                           | 2                | 10.0 (1/10) (IVF)                 | 1             | Negative          |
|             |                            |                              |                  | 45.5 (5/11) (ICSI)                | 2             | Negative          |
| 23          | 32                         | 30                           | 2                | 0.0 (0/7) (IVF)                   |               | N. A              |
|             |                            |                              |                  | 88.9 (8/9) (ICSI)                 | 1             | Live birth        |
| 24          | 37                         | 31                           | 1                | 0.0 (0/17) (IVF)                  |               | N. A              |
| 25          | 43                         | 42                           | 1                | 0.0 (0/8) (ICSI)                  |               | N. A              |
| 26          | 32                         | 27                           | 1                | 0.0 (0/16) (IVF)                  |               | N. A              |
| 27          | 43                         | 29                           | 2                | 9.1 (1/11) (IVF)                  | 1             | Negative          |
|             |                            |                              |                  | 83.3 (10/12) (ICSI)               | 2             | Live birth        |
| 28          | 41                         | 39                           | 1                | 15.4 (2/13) (IVF)                 | N. A          |                   |
| 29          | 38                         | 36                           | 2                | 0 (0/4) (IVF))                    |               |                   |
|             |                            |                              |                  | 0 (0/5) (ICSI)                    |               |                   |
| 30          | 34                         | 35                           | 1                | 0 (0/5) (ICSI)                    |               |                   |
| 31          | 38                         | 38                           | 3                | 0 (0/14) (IVF)                    |               |                   |
|             |                            |                              |                  | 27.8 (5/18) (ICSI)                | 1             | Negative          |
| 32          | 36                         | 33                           | 2                | 14.3 (2/14) (ICSI)                | 1x twice ET   | Negative          |
| 33          | 37                         | N. A                         | 0                | Family history, bother of patient |               |                   |

(Continued)

| Patient No.     | Male age at date of sample | Female age at date of sample | IVF/ICSI history |                              |               | Pregnancy outcome  |
|-----------------|----------------------------|------------------------------|------------------|------------------------------|---------------|--------------------|
|                 |                            |                              | Cycles (n)       | Fertilisation rate % (2pn/n) | No. of ET (n) |                    |
| 34              | 32                         | N. A                         | 2                | 0.0 (0/6) (IVF)              |               | N. A               |
|                 |                            |                              |                  | 0.0 (0/4) (ICSI)             |               | N. A               |
| 35              | 37                         | N. A                         | 4                | 12.5 (4/32) (ICSI)           | 1             | Negative           |
| 36 <sup>#</sup> | 33                         | 30                           | 1                | 66.6 (8/12) (ICSI)           | 1             | Live birth         |
| 37              | 44                         | 41                           | 1                | 0.0 (0/14) (IVF)             |               | N. A               |
| 38              | 33                         | 33                           | 2                | 25.0 (1/4) (IVF)             | 1             | N. A               |
|                 |                            |                              |                  | 0.0 (0/7) (ICSI)             |               | N. A               |
| 39              | 31                         | 31                           | 2                | 15.4 (2/13) (IVF)            | 2             | Negative           |
|                 |                            |                              |                  | 21.4 (3/14) (ICSI)           | 1             | Chemical pregnancy |
| 40              | 33                         | 30                           | 1                | 0.0 (0/3) (IVF)              |               | N. A               |
| 41              | 36                         | 35                           | 1                | 0 (0/11) (ICSI)              |               | N. A               |
| 42              | 35                         | 33                           |                  | 0 (0/5) (IVF)                |               |                    |
| 43              | 36                         | 34                           | 1                | 22.2 (2/9) (ICSI)            | 1             | Negative           |

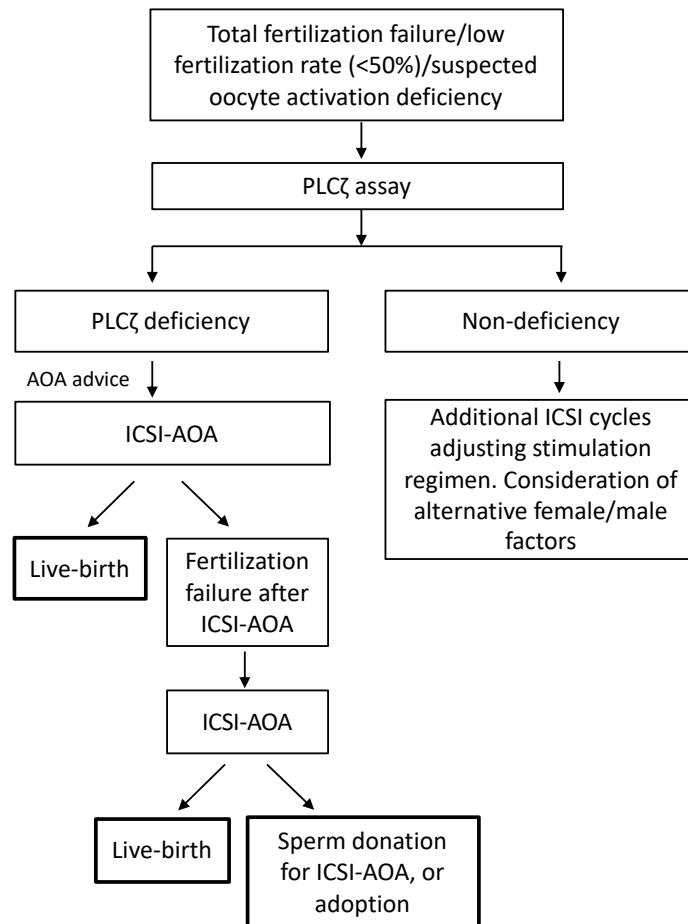
IVF/ICSI history may be displayed as mixed cycles performed by different IVF clinics. Abbreviations: N. A: not applicable; IVF: *in-vitro* fertilization; ICSI: intracytoplasmic sperm injection; ET: embryo transfer; FET: frozen embryo transfer. Chemical pregnancy: human chorionic gonadotropin was detected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

PLC $\zeta$  categorisation also showed that all fertile controls were classified into category II and one was assigned to category I (**Figure 3.8 and 3.9**); thus indicating the accuracy of this assay in identifying PLC $\zeta$ -deficient patients. Based on these results, I therefore proposed an algorithm relating to PLC $\zeta$  deficiency, to help identify infertile patients who may benefit from AOA (**Figure 3.10**). By using the algorithm, our laboratory-based in-house PLC $\zeta$  assay can evaluate PLC $\zeta$  expression in infertile males, therefore identifying whether the state of infertility is male-related. This allows the development of an individually targeted strategy for the management of AOA, which can save effort, time and cost for both IVF clinics and patients.



**Figure 3.9 PLC $\zeta$  categorisation of fertile controls by representation of mean PLC $\zeta$  level in sperm (X-axis) against proportion of sperm containing PLC $\zeta$  (Y-axis).** Vertical dashed line represents the cut-off 15.57 a.u. of mean PLC $\zeta$  level (M) per person's sperm, horizontal dashed line represents the cut-off 71% of proportion of sperm containing PLC $\zeta$  (P) per person's sperm. "I, II, III, IV" represent the four categories associated with PLC $\zeta$

parameters. All 13 fertile controls were assigned to category II and one was from category I.



**Figure 3.10 A suggested clinical algorithm for assessing the eligibility of patients for artificial oocyte activation (AOA).** Laboratory-based PLCζ analysis represents an individual targeted approach, which can save effort, time and cost for both IVF clinics and patients.

### 3.4 Discussion

This part of my thesis aimed to quantify PLC $\zeta$  protein levels in the sperm of infertile males and to investigate the applicability of a PLC $\zeta$  assay as a diagnostic tool in the assessment of patient eligibility for AOA. My results, comparing relative fluorescent PLC $\zeta$  levels in the sperm of infertile and fertile males, were in line with our previous findings (Kashir *et al.* 2012c; Kashir *et al.* 2013b; Yelumalai *et al.* 2015; Yeste *et al.* 2016b). Infertile patients had PLC $\zeta$  deficiency in their sperm, indicating that any abnormalities in the PLC $\zeta$  protein are likely to play a key role in IVF/ICSI failure (Heytens *et al.* 2009; Kashir *et al.* 2011c). Indeed, the crucial role of PLC $\zeta$  in oocyte activation is highlighted by evidence showing that PLC $\zeta$  levels, localisation patterns, and the proportion of human sperm exhibiting PLC $\zeta$  significantly correlate with ICSI fertilisation rates (Yelumalai *et al.* 2015; Nazarian *et al.* 2019). In addition, PLC $\zeta$  has been associated with abnormal sperm morphology. Sperm from teratozoospermic males showed significantly reduced PLC $\zeta$  levels compared to fertile males (Azad *et al.* 2018), and sperm from globozoospermic males exhibited significantly lower PLC $\zeta$  mRNA and protein levels, and abnormal localisation patterns compared to fertile males (Tavalaee and Nasr-Esfahani 2016). Collectively, these results suggest that PLC $\zeta$  is a promising biomarker for diagnosing OAD and predicting fertilisation outcomes in ICSI.

AOA is a potential option for low fertilisation or TFF and is the only option with which to rescue OAD at present (Ebner *et al.* 2012; Nikiforaki *et al.* 2016). However, there is no consensus guidance for the use of AOA in clinical practice. Clinics undertake AOA mainly based on patient history such as recurrent fertilisation failure, low fertilisation rates in ICSI, or suspected OAD. Unsurprisingly, bias may exist when choosing AOA treatment for patients; indeed, the effect of

patient choice to undergo self-funded treatment and clinician bias cannot be excluded. Furthermore, AOA is not beneficial in all infertile conditions (Borges Jr *et al.* 2009a; Aydinuraz *et al.* 2016), which suggests that a consensus guidance in clinical practice for the use of AOA is needed (Vanden Meerschaut *et al.* 2014b; Santella and Dale 2015). In the absence of objective diagnostic criteria to support the use of AOA, our data demonstrate that PLC $\zeta$  analysis has significant potential in accurately informing clinicians about patient eligibility to undergo AOA as well as treatment prognosis. In line with this, the HFEA suggests the use of AOA only on selected patients with specific indications, such as PLC $\zeta$  deficiency (HFEA 2018b).

Previous attempts have been made to predict oocyte activation capacity and successful fertilisation by assessing PLC $\zeta$  mRNA, RNA, or protein levels in human sperm (Aghajani *et al.* 2011; Kashir *et al.* 2013b; Abadi *et al.* 2016). Here we report, for the first time, that quantitative immuno-analysis of PLC $\zeta$  can help with the clinical decision-making process in AOA treatment. In addition, we demonstrate the clear association of PLC $\zeta$  deficiency and OAD (**Figure 3.6**), which was in line with previous findings (Yelumalai *et al.* 2015). The success of 4 ICSI-AOA cases in achieving clinical pregnancy (patient 3, 5, 6 and 7) indicates that PLC $\zeta$  analysis is able to elucidate, at least partially, the mechanisms underlying fertilisation failure in such patients, and supports the use of AOA according to our suggested algorithm (**Figure 3.10**).

Interestingly, two of ICSI-AOA patients had normal semen parameters upon undergoing ICSI-AOA, suggesting that PLC $\zeta$  analysis may complement routine semen analysis and target AOA-eligible patients with normozoospermia, not only teratozoospermia (Azad *et al.* 2018). The present study did not correlate semen parameters (**Appendix Table 3**) with PLC $\zeta$  expression, as previous evidence has

shown that PLC $\zeta$  characteristics significantly correlate with semen quality (sperm concentration, motility and morphology). Mean PLC $\zeta$  levels in sperm correlated significantly with sperm motility (Yelumalai *et al.* 2015), and the proportion of sperm exhibiting PLC $\zeta$  was significantly correlated with the percentage of progressively forward motile sperm, sperm concentration and morphology (Yelumalai *et al.* 2015; Tavalae *et al.* 2017). However, Ferrer-Vaquer *et al.* suggested that PLC $\zeta$  localisations did not correlate with sperm motility and concentrations (Ferrer-Vaquer *et al.* 2016). Furthermore, routine semen analysis does not always correlate with the functional quality of sperm and cannot be used to predict all cases of pregnancy outcomes (Wang and Swerdloff 2014). The present data support the use of immunofluorescent labelling of PLC $\zeta$  as an adjuvant to routine basic semen analysis in accurately evaluating AOA applicability (**Figure 3.7**).

The immunochemistry method used in our PLC $\zeta$  assay was the same as that previously published by our research group (Kashir *et al.* 2012c; Kashir *et al.* 2013b; Yelumalai *et al.* 2015; Yeste *et al.* 2016b). Accuracy analysis proved that the results of in-house PLC $\zeta$  analysis are reliable (**Figure 3.7**). It should be noted that fertile males exhibit variable levels of PLC $\zeta$  in sperm (Kashir *et al.* 2011a; Kashir *et al.* 2013b; Yeste *et al.* 2016b), and for this reason, fertile controls recruited in the current study were first tested to ensure they exhibited similar parameters of PLC $\zeta$  to previous controls.

As discussed, 5 patients (patients 18, 20, 23, 27, and 36) achieved live births following subsequent IVF/ICSI treatment without AOA (**Table 3.4**). Individual PLC $\zeta$  analysis revealed that these patients either had normal PLC $\zeta$  protein levels or reduced PLC $\zeta$  (i.e., categories I and II, **Figure 3.8**), which further supports the use of our suggested algorithm in the selection of AOA-eligible patients (**Figure 3.10**).



It is worth mentioning that patient 7 (from category IV, **Figure 3.8**) had AOA treatment following the diagnosis of PLC $\zeta$  deficiency. As a result, the couple involved experienced biochemical pregnancy only (**Table 3.3**). This suggests that PLC $\zeta$  analysis may be helpful in predicting the chances of fertilisation success, and that combining PLC $\zeta$  analysis results and PLC $\zeta$  categorisation may better predict clinical pregnancy success.

Importantly, we expect that AOA success, as well as the accuracy of our in-house quantitative assay, should be more related to the chances of fertilisation success rather than to clinical pregnancy outcomes. Indeed, AOA is targeted to rectify oocyte activation problems, while pregnancy success remains largely dependent upon other factors such as abnormal maternal hormone levels, poor endometrium receptivity and embryo aneuploidy (Shahine *et al.* 2016). In addition, data relating to IVF/ICSI outcomes among infertile patients with normal PLC $\zeta$  (category II, **Figure 3.8**) were not available, precluding matched comparisons between participants, specifically with regards to clinical pregnancy and live birth rates. Some of the infertile patients in our cohort with normal PLC $\zeta$  experienced IVF failure, reiterating that OAD is not exclusively the result of PLC $\zeta$  abnormalities and likely relates to additional unknown male and female factors (Yeste *et al.* 2015).

The PLC $\zeta$  assay and PLC $\zeta$  categorisation revealed different results for one patient; Patient 8 was classified in category III, whereas the PLC $\zeta$  assay revealed that he exhibited PLC $\zeta$  deficiency. Clinical ICSI/AOA outcomes of the patient would have provided useful information with which to examine the disparity between the two assays and the accuracy of the two. However, the patient has not yet undergone ICSI/AOA. Interestingly, patient 8 exhibited round sperm with small acrosomes, and

studies have shown that globozoospermic males can benefit from AOA treatment (Heindryckx *et al.* 2005; Kochhar and Ghosh 2018).

Undergoing ICSI treatment after fertilisation failure in IVF is a standard clinical approach in ART. In this study, I also analysed PLC $\zeta$  levels in males who had experienced IVF failure, to investigate the extent of PLC $\zeta$  analysis in predicting AOA outcomes. Indeed, patient 3 and 5 experienced total fertilisation failure in their previous IVF cycles, and PLC $\zeta$  analysis showed they both had PLC $\zeta$  deficiency. Subsequent ICSI with AOA resulted in both of patients achieving live-births. More evidence could be acquired if future studies involve split cycles in which oocytes from female individuals are equally distributed to ICSI and ICSI/AOA treatment.

The functional release of Ca<sup>2+</sup> at the time of oocyte activation requires specific periodic frequency, appropriate amplitude and an adequate number of oscillations (Ducibella *et al.* 2006). Accordingly, insufficient Ca<sup>2+</sup> oscillations in a human oocyte leads to a failure to activate (Ferrer-Buitrago *et al.* 2018c). Admittedly, the use of AOA generates some concerns. One such concern is that it may lead to either insufficient or excessive oscillations in the oocyte by altering physiological Ca<sup>2+</sup> oscillatory patterns (Ebner and Montag 2016). In turn, this may impair embryogenesis in both the pre- and post-implantation stages (Ozil *et al.* 2006). Another concern is that AOA protocols vary between IVF units, with significant differences in reagents (ionomycin, SrCl<sub>2</sub>, or calcimycin combined with SrCl<sub>2</sub>), incubation times and the use of supplements. Furthermore, patient responses using the same protocol may be discrepant due to individual factors (Fawzy *et al.* 2018). There is a paucity of data to support the universal recommendation of AOA in clinical practice, and the HFEA states that randomized controlled trials and follow-up studies are required (HFEA 2019).

Commercially-available GM508 remains, thus far, the most commonly used reagent for OAD rescue in a clinical setting. One study showed that GM508 is efficient in promoting zygote development to the 2-cell stage (Darwish and Magdi 2015), and in improving implantation, fertilisation and pregnancy rates (Ebner *et al.* 2012; Ebner *et al.* 2015a). Furthermore, safety data from AOA-born children using GM508 have been investigated (Vanden Meerschaut *et al.* 2014a; Miller *et al.* 2016). These studies specifically focused on congenital abnormalities, birth weight and neurodevelopmental outcomes of AOA-born children, and the overall results have been reassuring so far. Long-term follow-up data would be paramount in informing clinicians about the immediate and delayed risks associated with GM508. A recombinant form of human PLC $\zeta$  protein (rhPLC $\zeta$ ) has been proposed as a suitable candidate given its ability to trigger Ca<sup>2+</sup> oscillation in a mouse model (Kashir *et al.* 2011b; Sanusi *et al.* 2015). Producing a pure, functional rhPLC $\zeta$  molecule using mammalian cells, better mimicking its endogenous equivalent, has thus far proven a challenge that our group endeavors to solve and is addressed further in Chapter 5.

Fertilisation is a complex process dependent on factors not exclusive to PLC $\zeta$  deficiency. Other male factors that may also lead to fertilisation failure include sperm structural and morphological defects, sperm DNA damage, or sperm flagellar abnormalities. Sperm DNA damage has been associated with fertilisation failures in IVF/ICSI cycles and is commonly tackled by different techniques: alkaline and neutral Comet assay, sperm chromatin dispersion test, TUNEL assay and sperm chromatin structure assay (Benchaib *et al.* 2003; Ribas-Maynou *et al.* 2013). In addition, cation channel of sperm (CatSper) in the sperm tail is important in the sperm's ability to penetrate the oocyte (Avidan *et al.* 2003), while the K<sup>+</sup> ion

channels-associated factor, IKSper, is expressed in the sperm flagellum and plays a key role in the acrosome reaction (Brenker *et al.* 2014). Other forms of dysfunction can also lead to OAD, such as DNA fragmentation in the sperm head, inadequate oocyte maturation, low oocyte numbers, or even technical problems encountered during ART (Vanden Meerschaut *et al.* 2012). As mentioned earlier, there are several other methods that have been experimentally applied to evaluate sperm ability for oocyte activation, such as the MOAT (Heindryckx *et al.* 2008) or HOAT (Ferrer-Buitrago *et al.* 2018c). Sperm DNA integrity, in particular, has been associated with successful pregnancy in IVF/ICSI, although its applicability as a diagnostic tool requires further research (Collins *et al.* 2008; López *et al.* 2013).

In the absence of a gold standard test to diagnose OAD, as well as the small numbers of AOA cases in this study, I am unable to fully validate the sensitivity, specificity and overall accuracy of PLC $\zeta$  analysis as a diagnostic tool. However, the PLC $\zeta$  categorisation of fertile controls at least elucidates, to some extent, that our PLC $\zeta$  assay is able to accurately identify PLC $\zeta$  deficient patients. Further study is needed to include more ICSI/AOA cases. Another limitation of this study is that our assay requires fixed sperm and is thus not necessarily reflective of the PLC $\zeta$  levels and activity within live sperm used in IVF/ICSI. Further research is now required to investigate whether patients with normal PLC $\zeta$  parameters would benefit from receiving AOA.

### **3.5 Conclusions**

In conclusion, this study, for the first time, demonstrated the efficacy and accuracy of our in-house assay to identify PLC $\zeta$  abnormalities and inform clinical decisions in the treatment of couples with male-factor infertility. These findings

provide further evidence of the association between abnormalities in PLC $\zeta$  and AOA and support the use of a PLC $\zeta$  assay to identify patients who would benefit from AOA while undergoing ICSI. Crucially, we show the success rate of AOA treatment in this cohort, as measured by a fertilisation rate of 56.8% and a pregnancy rate of 60.0% per initiated cycle. An algorithm is also proposed for the diagnosis and management of patients in whom AOA is likely to be beneficial, allowing for a targeted and individualised approach. This study has limited case numbers of patients receiving AOA treatment, as well as small numbers of infertile males. Further research should increase patient numbers and focus on the potential short- and long-term complications of AOA. In addition, further studies should focus on the development of an effective form of rhPLC $\zeta$  in order to best mimic its endogenous counterpart and increase therapeutic success, and this provides an alternative option to replace AOAs.

### **3.6 Key findings**

- Our in-house assay showed efficacy and accuracy in detecting PLC $\zeta$  abnormalities.
- The in-house PLC $\zeta$  assay supports the clinical decision-making process in the AOA treatment of couples with male-factor infertility, and helped to improve the fertilisation rate from 18.6% to 56.8% ( $p < 0.001$ ) in ICSI cycles and achieve the first series of cases of AOA-born live-births in Oxford (60% pregnancy rate and 40% live birth rate in ICSI-AOA per initiated cycle).
- The cut-offs for PLC $\zeta$  deficiency was determined; mean PLC $\zeta$  levels in sperm was 15.57 [a.u.], and the proportion of sperm exhibiting PLC $\zeta$  was 71%). This was further applied for categorisation of PLC $\zeta$  main characteristics.

- A decision procedure was proposed to support the management of AOA, thus allowing individualised targeted strategy
- Further evidence was presented to suggest the association between PLC $\zeta$  expression in infertile males and an infertile status.

## Chapter 4. An investigation of the genetic mechanisms linking PLCζ and male infertility

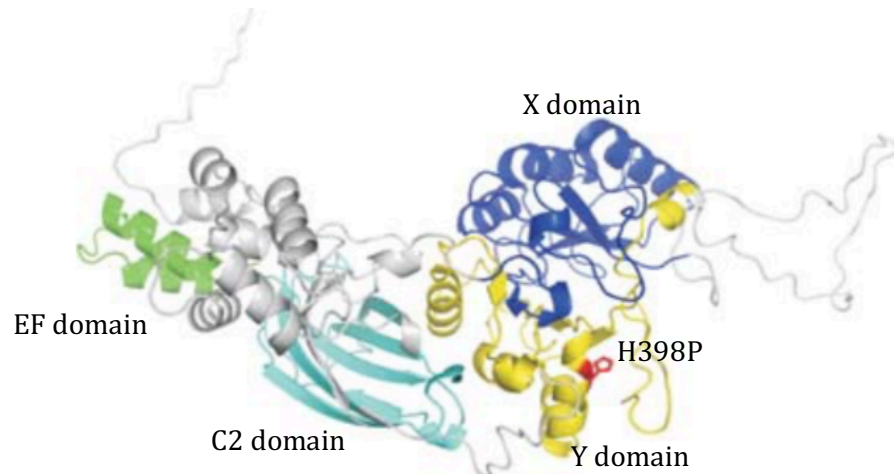
### 4.1 Introduction

Chapters 2 and 3 investigated PLCζ protein expression and its association with male infertility; this Chapter attempts to link the *PLCζ* gene with male infertility. As introduced in Chapter 1 (Section 1.3.4), the *PLCζ* gene is located on chromosome 12 and consists of 15 exons. Correspondingly, the PLCζ protein structure is composed of EF, X, Y, and C2 domains. An XY-linker connects the X and Y domains. Therefore, it is very likely that *PLCζ* genomic variations may result in protein dysfunction and impact upon oocyte activation. To date, eleven mutations in human *PLCζ* genes have been reported; ten were reported to impair protein function (**Figure 1.9**) (Heytens *et al.* 2009; Kashir *et al.* 2011c; Escoffier *et al.* 2015; Ferrer-Vaquer *et al.* 2016; Torra-Massana *et al.* 2019).

Heytens *et al.* were the first to identify a normozoospermic male with defects in oocyte activation that exhibited a substitution mutation in *PLCζ* exon 11; the corresponding protein contained a histidine to proline (PLCζ<sup>H398P</sup>) substitution in the Y domain (**Figure 4.1**) (Heytens *et al.* 2009). 3D structural modelling of PLCζ<sup>H398P</sup> indicated that this mutation could impair enzymatic activity because histidine at residue 398 (H398) is preserved in the alpha-helix structure; this is a secondary structure vulnerable to disruption by proline. The mutation could therefore result in helical destabilisation in the protein structure. Nomikos *et al.* (2017b) further demonstrated that mouse PLCζ<sup>H435P</sup>, which is equivalent to human PLCζ<sup>H398P</sup>, abolished the hydrolytic activity of PLCζ and was unable to trigger Ca<sup>2+</sup> oscillations. These authors further replaced histidine with a non-helix-destabilising

amino acid, alanine, thus generating PLC $\zeta$ <sup>H435A</sup>. Microinjection of PLC $\zeta$ <sup>H435A</sup> cRNA resulted in Ca<sup>2+</sup> oscillations similar to those of wild-type PLC $\zeta$ , which indicated that the inability of mutant PLC $\zeta$  to trigger oocyte activation may be due to disruption of the protein secondary structure (Nomikos *et al.* 2017b).

Kashir *et al.* subsequently identified a second mutation in PLC $\zeta$  exon 6 from the same patient, in which the corresponding histidine was replaced with leucine in the X domain (PLC $\zeta$ <sup>H233L</sup>) (Kashir *et al.* 2011c). Interestingly, PLC $\zeta$ <sup>H398P</sup> and PLC $\zeta$ <sup>H233L</sup> were both heterozygous, indicating that PLC $\zeta$ -linked infertility can be inherited via the maternal lineage (**Figure 4.2**).

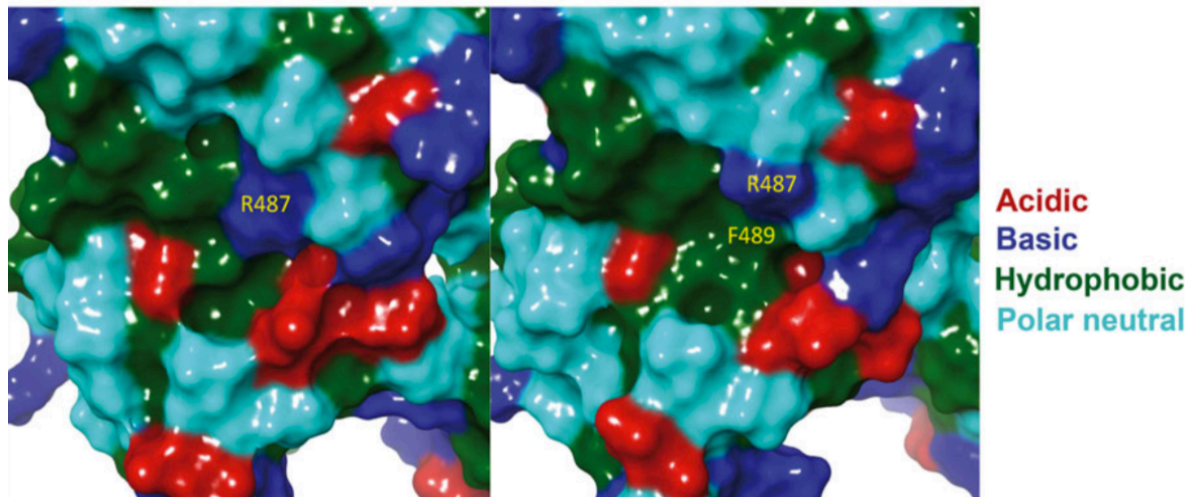


**Figure 4.1 Model of human PLC $\zeta$  showing domain structure and the H398P mutation.** Domains are coloured as follows: green—EF domain (residues 35–70), blue—X catalytic domain (residues 156–300), yellow—Y catalytic domain (residues 348–465), cyan—C2 (residues 484–572). Histidine to proline (H398P) is shown in red. Figure modified from Heytens *et al.* (2009).





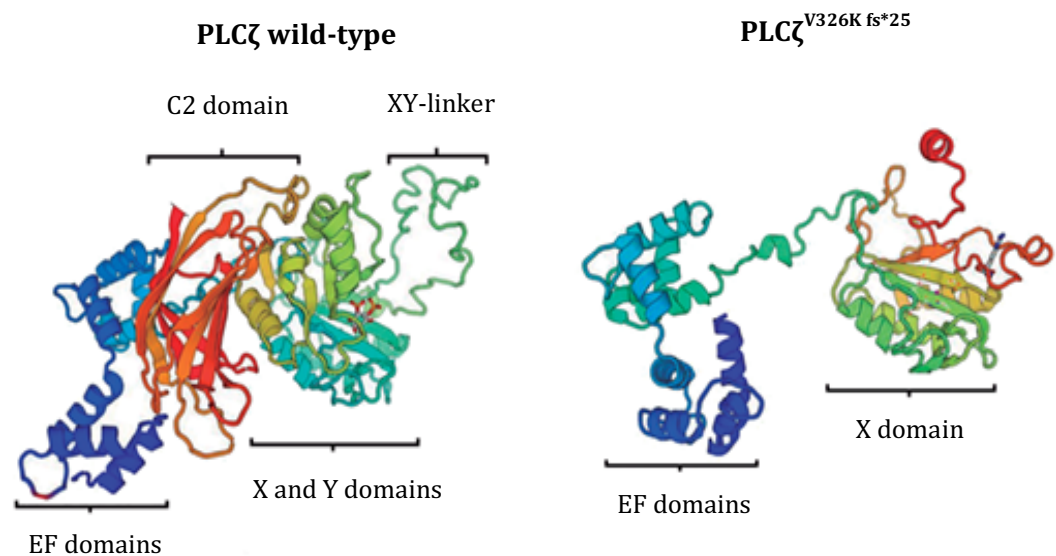
2016). However, functional analysis is now needed to investigate whether this mutation can impair protein function.



**Figure 4.3** Three-dimensional modelling of mutant *PLCζ* in which isoleucine at residue 489 is substituted with phenylalanine (*PLCζ*<sup>I489F</sup>). The molecular surfaces of WT and mutant *PLCζ* in the vicinity of residue 489 are shown, demonstrating the formation of a novel hydrophobic sub-site at the C2/EF-hand junction. Basic, acidic, hydrophobic, and polar neutral residues are shown in blue, red, green, and cyan, respectively. Reproduced from Escoffier *et al.* (2015).

Recently, Torra-Massana *et al.* reported three novel *PLCζ* mutations and confirmed the previously identified mutations (R197H, H233L and S500L) from infertile males. The three newly-identified mutations are I120M mutation (isoleucine to methionine) from exon 4, L224P mutation (leucine to proline) from exon 6, and V326K fs\*25 (a frame shift, valine to lysine) from exon 9 (**Figure 1.9**) (Torra-Massana *et al.* 2019). Further bioinformatics analysis (by Phyre2) suggested that the L224P and V326K fs\*25 mutations exert detrimental effects on *PLCζ* protein function (Torra-Massana *et al.* 2019). These authors subsequently microinjected cRNA of *PLCζ*<sup>L224P</sup>, *PLCζ*<sup>S500L</sup>, *PLCζ*<sup>H233L</sup> and *PLCζ*<sup>R197H</sup> into *in-vitro* matured human oocytes and found that R197H and H233L mutations caused low

oocyte activation rates (30% and 30.77%, respectively), whereas L224P and S500L mutations had no influence on oocyte activation rate (64.29% and 60%, respectively) (Torra-Massana *et al.* 2019). These findings indicate that not all genetic variation is detrimental to PLC $\zeta$  protein function. Notably, the V326K fs\*25 mutation is located in the *XY-linker* region and is caused by the deletion of two nucleotides, A and G, resulting in a frame shift and a stop codon downstream of the mutation (Torra-Massana *et al.* 2019). This is the first PLC $\zeta$  mutation identified in the *XY-linker* region, and further bioinformatics analysis suggested that the V326K fs\*25 mutation could lead PLC $\zeta$  to form a truncated XY-linker and lack both Y and C2 domains (Figure 4.4). In line with this, the microinjection of PLC $\zeta^{V326K\text{ fs*25}}$  cRNA into human *in-vitro* matured oocytes caused TFF (0/10), thus demonstrating the deteriorate effect of this mutation on protein function (Torra-Massana *et al.* 2019).

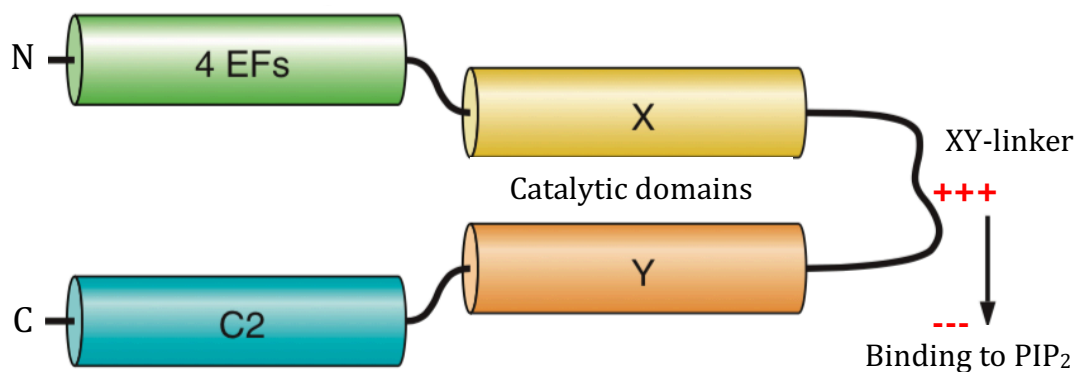


**Figure 4.4 Three-dimensional modelling of the protein structures of PLC $\zeta$  wild type and PLC $\zeta^{V326K\text{ fs*25}}$ .** Frame shift of mutation PLC $\zeta^{V326K\text{ fs*25}}$  resulting in the absence of the Y and C2 domains. Reproduced from Torra-Massana *et al.* (2019).

More recently, Dai *et al.* identified a novel nonsense mutation in *PLCζ* exon 6 (C196X, cysteine to an undetermined amino acid), and two novel missense mutations from *PLCζ* exons 7 and 10 (S350P, serine to proline, and L246F, leucine to phenylalanine) (Dai *et al.* 2019). These mutations were identified from three infertile males who experienced low fertilisation rates (0 – 7.7%). Further 3D modelling suggested that the C196X mutation created a premature termination codon, that may impair protein function. In line with this, the infertile patient who exhibited the C196X mutation showed no *PLCζ* expression in his sperm. The other two mutations, S350P and L246F, also showed hydrogenic changes in the secondary structure of *PLCζ* protein. Interestingly, *PLCζ* was found to be expressed in sperm from patients exhibiting these two mutations. Unfortunately, it is not known whether *PLCζ* expression levels were reduced in these mutant sperm. Nevertheless, all three patients received AOA treatment in subsequent ICSI cycles and achieved significantly improved fertilisation rates (from 4.4 – 56%), resulting in two successful on-going pregnancies.

Collectively, these data show that eleven mutations have been identified in human *PLCζ* genes; ten of these are suggested to have deleterious effect on protein function. These ten mutations are situated in six exons and impair the functions of the X domain (C196X, R197H, L224P, H233L and S350P), Y domain (L246F and H398P), C2 domain (I489F and S500L) and XY-linker (V326K fs\*25). Indeed, the biological roles of the four domains of *PLCζ* have been extensively studied, while the XY-linker region of *PLCζ*, which is a highly conserved structure among PLC isoforms, has received far less attention. Thus far, only one mutation was found in the XY-linker region, which forms a truncated XY-linker and exerts a deleterious effect on oocyte activation (Torra-Massana *et al.* 2019).

As mentioned in Chapter 1 (Section 1.3.3), the XY-linker that connects the X and Y domains is positively charged in PLC $\zeta$  but negatively charged in all other PLC isoforms, thus indicating that a different regulatory role may be present for PLC $\zeta$ ; the linker is thought to bind to a negatively charged substrate (**Figure 4.5**). Accordingly, dysfunctions in the XY-linker impair PLC $\zeta$  enzymatic activity (Nomikos *et al.* 2011a; Nomikos *et al.* 2011b; Swann and Lai 2015). Therefore, I aimed to investigate whether the XY-linker of PLC $\zeta$  plays an important role at the molecular level. My working hypothesis was that genetic variations in the XY-linker may impair the targeting and hydrolytic activity of the translated PLC $\zeta$  protein.



**Figure 4.5 Schematic diagram of the primary structure of PLC $\zeta$ .** Each of the PLC $\zeta$  domains (4 EF, X, Y, and C2 domains) is represented from the N terminus to the C terminus. The XY-linker is represented as a curved line and may play a role in binding to PIP<sub>2</sub>. Reproduced from Swan and Lai (2015).

Apart from mutations, the impaired expression/function of PLC $\zeta$  could be due to the presence of SNPs in the PLC $\zeta$  gene or other genetic variations. Previous sequencing of the PLC $\zeta$  gene in infertile males identified several SNPs in introns 1, 3, 5, 12, and 13 (Yoon *et al.* 2008; Durban *et al.* 2015; Ferrer-Vaquero *et al.* 2016).

However, these SNPs have been identified previously. In addition, four novel SNPs were reported only in *PLCζ* introns, of which two were located in intron 3 and two in intron 12 (Yoon *et al.* 2008; Ferrer-Vaquer *et al.* 2016).

Introns are non-coding DNA sequences and were once considered to be ‘functionless junk DNA’ in the eukaryotic genome. However, emerging evidence shows that introns are functional, to some extent, in some eukaryotes. For example, introns can encode RNAs, such as mRNA, microRNA and long non-coding RNAs (Engreitz *et al.* 2016; Wong *et al.* 2016; Hubé *et al.* 2017; Steiman-Shimony *et al.* 2018). Introns can also contribute to the formation of different protein isoforms, predominately via alternative splicing (Carazo *et al.* 2018). Furthermore, introns can regulate gene expression through a number of different mechanisms which are referred to as intron-mediated enhancements (IMEs). One of the best examples of IME is the fact that introns have been shown to improve the expression levels of the growth hormone gene in transgenic mice (Palmiter *et al.* 1991). Indeed, mammalian intron-bearing genes have higher and broader expression levels in tissue than intronless genes (Shabalina *et al.* 2010). In addition, intron-bearing genes in yeast produce more mRNA and protein than intronless genes (Juneau *et al.* 2006). At the DNA level, introns are involved in the initiation and termination of transcription, and in genome organisation (Carmel and Chorev 2012).

Previous *PLCζ* research has focused extensively on exons and have mostly ignored introns. This chapter aimed to investigate potential defects in both *PLCζ* exons and introns, particularly in the areas corresponding to the XY-linker region.

As mentioned earlier, *PLCζ* shares a bidirectional promoter with *CAPZA3*, which is essential in the stabilisation of sperm structure. Genome-wide sequencing previously suggested that bidirectional promoters are present in over 10% of

human genes, and that these genes may be associated with variable expression (Trinklein *et al.* 2004). It has not yet been determined whether variations in the shared promoter have dual effects on downstream transcription on both *PLCζ* and *CAPZA3*. One study reported two variations in the bull *PLCζ* promoter region that may alter the transcriptional process; these variations led to reduced semen quality (Pan *et al.* 2013). In addition, a cytosine (C) to thymine (T) substitution was found in the human *CAPZA3* promoter, which may influence the downstream transcriptional factor regulatory factor X (RFX4), a dimeric DNA-binding protein (Javadian-Elyaderani *et al.* 2016). However, this substitution was predicted to lay outside of the *PLCζ* promoter region (Javadian-Elyaderani *et al.* 2016). Nevertheless, no variations has yet been identified in the regulatory region of human *PLCζ* (i.e., promoter and 5' untranslated region (5'UTR)). As such, it is worth investigating the *PLCζ* promoter region to understand how abnormality in this region might affect *PLCζ* function.

*CAPZA3* is a type of actin-capping protein involved in actin polymerisation during spermiogenesis, formation of the actin cytoskeleton and acrosome, and the acrosome reaction (Hurst *et al.* 1998; Miyagawa *et al.* 2002; Sosnik *et al.* 2010). An immunofluorescence study showed that *CAPZA3* protein is expressed in mature mouse sperm and its localisation was redistributed during capacitation and acrosome reaction (Sosnik *et al.* 2010). However, few studies have linked *CAPZA3* with human infertility.

The human *CAPZA3* gene is intronless; it resides on the reverse strand of chromosome 12 and encodes a 298-amino acid protein (Tanaka *et al.* 2003). One mutagenesis study, using random N-ethyl-N-nitrosourea (ENU), produced a sterile phenotype in mice that harboured a missense mutation in *CAPZA3* (methionine to

lysine, M44K) (Geyer *et al.* 2009). The M44K mutation was proven to disrupt mice spermiogenesis procedures including the organisation of filament actin and midpiece integrity. Furthermore, mutant mice had low sperm motility, a low sperm count, and abnormal morphology. Since the CAPZA3 amino-acid sequence is highly conserved in mammalian species (96% similarity), the M44K mutation may also impair human spermiogenesis (Geyer *et al.* 2009). However, no study has detected such a mutation in the human *CAPZA3* gene thus far. This study aimed to investigate the association between *CAPZA3* and male infertility and identify whether abnormality in the bidirectional promoter can impair the expression of the genes downstream.

Next-generation sequencing (NGS) has been applied extensively in clinical research to identify the genetic causes of a disease. Compared to Sanger sequencing, which produces one forward and one reverse read, NGS generates a huge amount of paralleled DNA sequencing data that provide more sensitive results and are highly suitable for genotyping and discovering new variants (Behjati and Tarpey 2013). The main procedures involved in NGS include template preparation, sequencing, imaging and analysis (Metzker 2010). Thus far, the high-throughput NGS method has not been applied in any study relating to *PLCζ*, and I aimed to utilise NGS for *PLCζ* and *CAPZA3* sequencing, hoping to expand our knowledge on male infertility relating to *PLCζ* with fine detail.

The general aim of this chapter was to investigate the genetic mechanisms relating to *PLCζ* that may contribute to male infertility. More precisely, this chapter used Sanger sequencing to screen all *PLCζ* 15 exons (particular exons 6, 11 and 13 as most mutations were identified in these regions), and used NGS to investigate variations in (1) *PLCζ* regulatory regions (i.e., promoter, *PLCζ* exons 1 and 2), (2) the



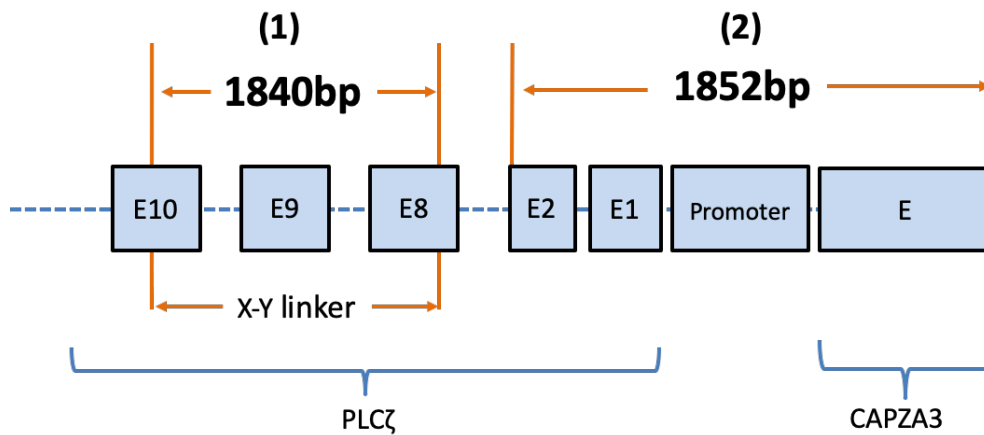
*PLCζ* regions corresponding to the XY-linker (i.e., *PLCζ* exons 8-10) and (3) the *CAPZA3* gene.

## 4.2 Materials and methods

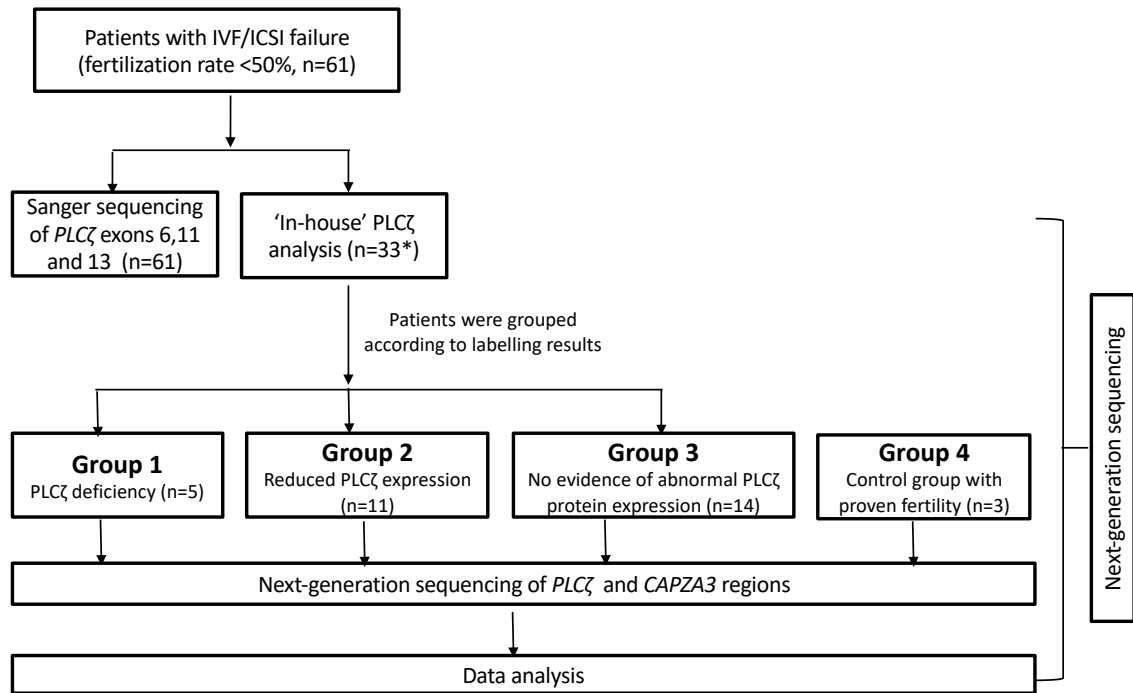
### 4.2.1 Patients and study design

Thus far, my project investigated a total of 61 infertile patients and their genomic DNA had undergone Sanger sequencing. Patients were recruited from Oxford Fertility, UK or Ninewells Hospital, Dundee, UK. The research study was approved by the National Research Ethics Service (Reference number: 10/H0606/65) and the East of Scotland Research Ethics Service (Reference number: 13/ES/0091). Patient selection criteria are described in Chapter 3 (Section 3.2.1). The methods used for genomic screening in this chapter included Sanger sequencing and NGS. Of the 61 patients, 16 received whole *PLCζ* exon sequencing and 45 received sequencing of only exons 6, 11 and 13. Screening of all *PLCζ* exons is costly, and previous work has identified mutations mostly in exons 6, 11 and 13. Meanwhile, NGS was used to study 33 of the 61 males with particular regards to (1) the *PLCζ* promoter region together with *CAPZA3* exon (1840 bp), and (2) *PLCζ* exons and introns corresponding to the XY-linker structure (1852 bp) (**Figure 4.6**). The 33 males that were used for NGS were grouped according to their *PLCζ* protein expression in sperm, as previously described in Chapter 3, Section 3.2.1. Grouping information on 33 males in the present chapter are shown in **Figure 4.7 and Table 4.3**. To be precise, group 1 (n=5) included patients whose sperm exhibited *PLCζ* deficiency (i.e., significantly lower *PLCζ* levels and a significantly lower proportion expressing *PLCζ*, when compared to matched fertile controls). Group 2 (n=11) included patients whose sperm exhibited significantly lower *PLCζ* levels, or a

significantly lower proportion expressing PLC $\zeta$ , when compared to matched fertile controls. Group 3 (n=14) included patients with no statistical evidence of abnormal PLC $\zeta$  protein expression. Group 4 (n=3) included fertile controls who had either fathered children, naturally or via IVF treatment.



**Figure 4.6 Schematic representation of the regions subject to next-generation sequencing (NGS).** NGS screening focused on two regions of *PLC $\zeta$*  and *CAPZA3*: (1) *PLC $\zeta$*  exons 8-10 and the connecting introns, corresponding to the XY-linker structure (1840 bp); (2) regions of the promoter, *PLC $\zeta$*  exons 1 and 2, and *CAPZA3* exon (1852 bp).



**Figure 4.7 Flow chart showing procedures involved in the patient selection for Sanger sequencing and next-generation sequencing.** \*: 33 of 61 patients were sequenced by both methods, i.e., Sanger sequencing and Next-generation sequencing.

#### 4.2.2 DNA extraction

Genomic DNA was provided by 61 patients from Oxford Fertility and extracted using saliva collected with the Oragene Saliva Self Collection Kit (DNA Genotek, Canada). The PrepIT•L2P kit (DNA Genotek, Canada) was used for DNA extraction, in accordance with the manufacturer's instructions. In brief, 1:25 (v/v) of PT-L2P solution was added to each saliva sample, which had been preheated at 50°C for 1 hour. After incubation for 10 min on ice, the mixture was centrifuged at 15,000 g for 5 min. The DNA-containing supernatant was then collected, leaving cell debris at the bottom of the tube. Subsequently, 100% ethanol and 70% (v/v) ethanol were applied to precipitate the DNA, followed by 10 min of incubation and centrifugation (15,000 g for 2 min) after each precipitation. DNA was then eluted

with 50 µl of nuclease-free water (Invitrogen, UK). DNA concentration was measured spectrophotometrically using a Qubit Fluorometric Quantitation kit (ThermoFisher Scientific, UK). DNA was stored at 4°C to await further analysis.

#### 4.2.3 Polymerase chain reaction (PCR) and purification

For PCR, I used 300-500 ng of DNA and primers (forward (F) and reverse (R)), designed against target exons of *PLCζ*. Oligonucleotides were produced commercially (Invitrogen, UK) and diluted to 500 µmol as a stock concentration. The nucleotide sequences for each primer pair are shown in **Table 4.1**.

**Table 4.1 Primer pair sequences for screening the 15 *PLCζ* exons.**

| Exons    | Forward                     | Reverse                     | Expected sizes (bp) |
|----------|-----------------------------|-----------------------------|---------------------|
| Exon 1   | TCAAGGTGTTAACATTCAAGCTG     | GCAAAGTGCTGTCACTCTTGAAAC    | 333                 |
| Exon 2   | GTGTGTTCTCTGCTTTCTCCCTA     | GCAGAAACAAGCTATCAATGGAT     | 329                 |
| Exon 3   | CAGTAATCAAATGTTTCTTTAGGAAT  | CTAACCTTTATATGACAATTACTGAC  | 286                 |
| Exon 4   | CGTCACTCCATGCAGCTTTGTAT     | CGGTAATTTGATAACCACAATTCAT   | 509                 |
| Exon 5   | GAGTAAGTTTACAAGTGAGATGTAG   | CCTACTGAGGTAGACACTGCCA      | 361                 |
| Exon 6   | TTTAATGCAAATTTCTAGAGACTCAAA | TATCATCTAAAGTAAGGCTAAGCA    | 421                 |
| Exon 7   | AGGTACTAAACGTTAGAAGTAAGT    | CACAGGCCAATATAACAGTGACA     | 335                 |
| Exon 8-9 | GCTAATTGGAAATATAGTGAACAC    | AAGTAAATTGTAAATGATAGACTAGAG | 508                 |
| Exon 10  | TCAAATGGTGTGTTGAGGAGATAAT   | GAGCAATCTTAACACCCTAGCAG     | 365                 |
| Exon 11  | TTCCTTGAATGATCAGTGACATA     | TACTTCTGCAAACAACCTCAATATC   | 413                 |
| Exon 12  | TCAGTAAGTGGTTTGTGTTCTT      | GAAATTGAAGCAAATCAGTGGAAT    | 339                 |
| Exon 13  | GCTGACTTACAGTCCATCAACTT     | CAGGTACATTTCTGTATTGATAATC   | 458                 |
| Exon 14  | AAAGTTTGGAGAACAAATGTAATCTAT | CTTTATGATAGAGCTATTCCTATGT   | 330                 |
| Exon 15  | TTCTCAGCAAGGCTATTGGAGAC     | GAATGGGCTCAATATTTCTAGTTT    | 290                 |

bp: base pair

Other reagents used in PCR, except DNA and primers, were from the Fidelity PCR Master Kit (Roche Bioscience, Germany). PCR was carried out in a G-Storm GS4 thermal cycler (Genetic Research Instrumentation, UK) following the procedure outlined in **Table 4.2**.

**Table 4.2 Procedure for performing polymerase chain reaction (PCR).**

| Step          | Temperature (°C) | Time (min.sec) |
|---------------|------------------|----------------|
| Start cycle   |                  |                |
| Denaturation  | 94               | 4.00           |
| Cycle 1 (x10) |                  |                |
| Denaturation  | 94               | 0.10           |
| Annealing     | 60-62            | 1.10           |
| Elongation    | 72               | 2.00           |
| Cycle 2 (x20) |                  |                |
| Denaturation  | 94               | 0.15           |
| Annealing     | 60-62            | 0.30           |
| Elongation    | 72               | 2.00           |
| End cycle 2   |                  |                |
| Elongation    | 72               | 7.00           |
| Storage       | 4                | Infinity       |

PCR products were then analysed by electrophoresis using 2% agarose gels (ThermoFisher Scientific, UK). To prepare an agarose gel, 4 g of agarose (Promega, UK) was added to 200 ml of tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer (40mM Tris, 20mM acetic acid, and 1mM ethylenediaminetetraacetic acid (EDTA)), ThermoFisher Scientific, UK), with adequate heating to dissolve thoroughly. After the gel had cooled down, ethidium bromide (Amresco, USA) was added to reach a final concentration of 0.5 µg/ml. PCR products, together with 1x Orange DNA loading dye (ThermoFisher Scientific, UK), were then loaded into the wells of the gel. By applying a voltage of 120 V, DNA was then allowed to migrate for a period of 40 min. An ultraviolet-transilluminator was then used to visualise DNA bands, which were then compared with a 50 bp DNA ladder (ThermoFisher Scientific, UK).

Successfully amplified PCR products were then purified with the QIAquick PCR Purification Kit (QIAGEN, UK), in accordance with the manufacturer's

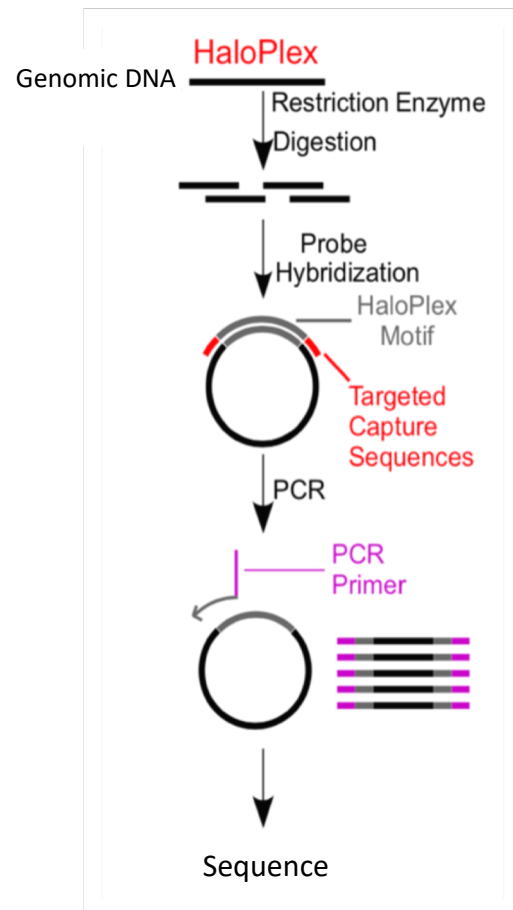
instructions. Then, appropriate amounts of purified PCR products were sent for Sanger sequencing (Source Bioscience, UK). DNA sequences were finally aligned with wild-type sequences (via database, <https://genome.ucsc.edu/>) to detect variants using the sequencing editor, Bioedit (accessible via <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>).

#### 4.2.4 Next-generation sequencing (NGS, Illumina)

NGS was performed by a commercial company (Source Bioscience, UK). The company also processed the data from FASTQ format to unmapped BAM and vcf formats. I then analysed data using Integrative Genomics Viewer (IGV, 2.0 software, <https://software.broadinstitute.org/software/igv/>). Prior to NGS, the coordinates for the two regions under investigation were identified using the UCSC web tool (<https://genome-euro.ucsc.edu/index.html>). Probes were designed according to specific coordinates for *PLCζ* and *CAPZA3* (18,890,284-18,892,124 and 18,854,710-18,852,858, respectively, accession number NM\_033123). As required by the company, the human hg19 assembly was used as this includes comprehensive allele information. The Illumina sequencing flow was composed of four main procedures: (1) library preparation, (2) sequencing, (3) imaging and (4) data analysis. Library preparation was performed with the Haloplex HS custom-designed kit, which requires a concentration of DNA  $\geq 50$  ng. DNA was digested with 16 restriction enzymes, which was followed by probe hybridisation and PCR amplification (**Figure 4.8**). The Herculase II Fusion DNA Polymerase kit (Agilent Technologies, USA) was used for PCR amplification, with the following conditions: (1) denaturation at 98°C for 2 min, (2) 30 cycles of denaturation at 98°C for 30s, annealing at 60°C for 30s, and elongation at 72°C for 1 min, and (3) final elongation at 72°C for 10 min. The

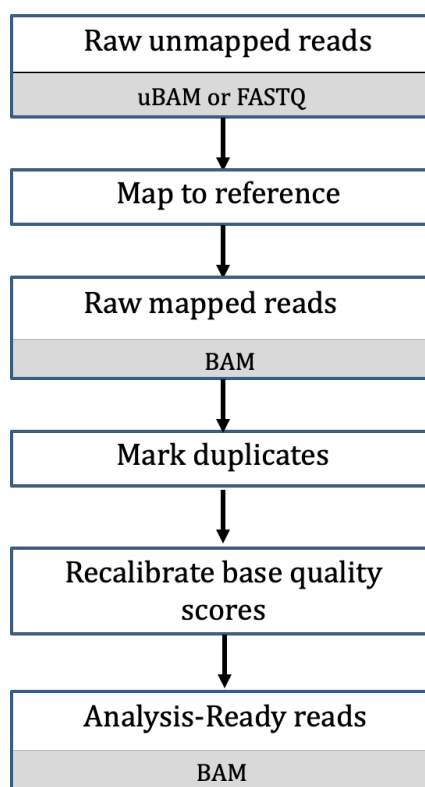
PCR products were then purified with AMPure XP beads. The quality of the library from each patient's DNA was validated with 2100 Bioanalyser Software (Agilent Technologies, USA) prior to sequencing.

The Illumina MiSeq System was used for sequencing, which integrates cluster amplification, sequencing, imaging, and data analysis, into one tool. The read length was 150 bp in this study, which allowed for maximum coverage. The data were further processed and converted from FASTQ to bam format (**Figure. 4.9**). Following this, I then used IGV software to analyse individual patient sequences. Variant results were considered valid if the total count at one nucleotide was read more than 500 times.



**Figure. 4.8** Flow chart showing the procedure from DNA fragment to complementary sequences prior to next-generation sequencing. Reproduced from Samorodnitsky *et al.* (2015).



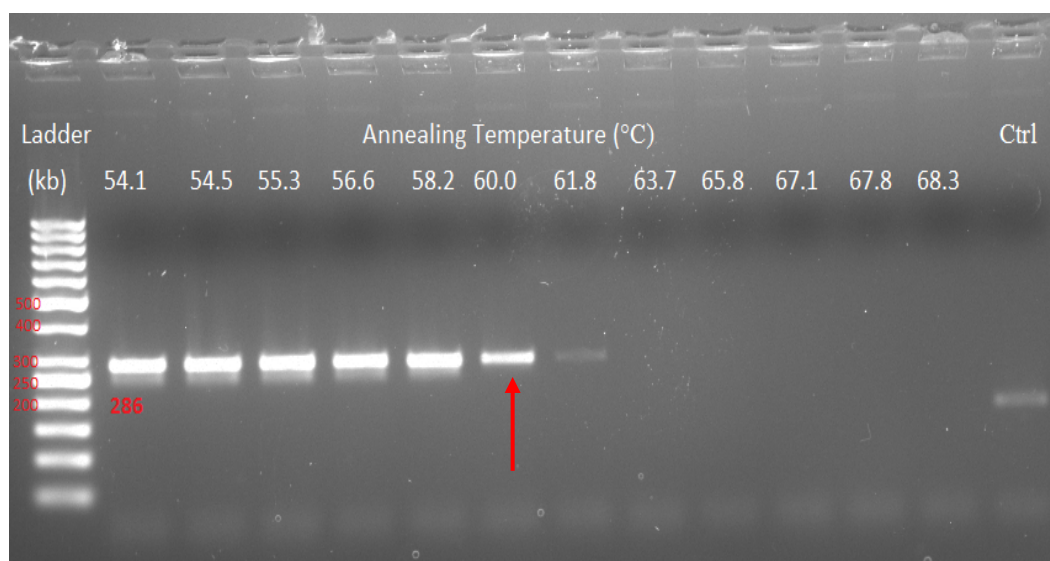


**Figure 4.9 Flow chart showing the workflow of processing raw data from FASTQ to BAM format.** Reproduced from the Broad Institute (<https://software.broadinstitute.org/gatk/best-practices/workflow?id=11146>).

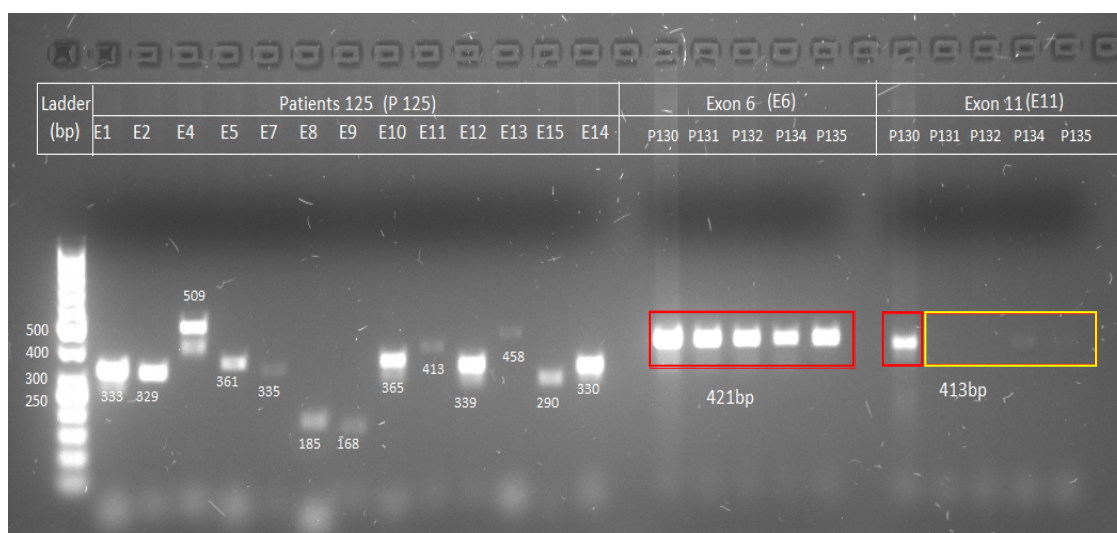
## 4.3 Results

### 4.3.1 Sanger sequencing of *PLCζ* exons in infertile males

The annealing temperatures for the primer pairs were first checked by gradient PCR (**Figure 4.10**). After PCR amplification, electrophoresis was used to confirm target regions before sending PCR products for sequencing (**Figure. 4.11**). Then, the Sanger sequencing reads were analysed and compared to fertile males, using Bioedit software (**Figure 4.12**). No *PLCζ* mutation was found in any of the 61 infertile patients.



**Figure 4.10 Annealing temperature of *PLCζ* exon 3 primers (286 kb) as determined by gradient PCR.** The red arrow indicates the best annealing temperature for the amplification of *PLCζ* exon 3.



**Figure 4.11 Representative PCR products as determined by electrophoresis (2% agarose gels).** The sizes of the amplified products from the 15 exons were labelled and checked against the expected sizes. PCR products that gave visible bands were purified and sequenced (highlighted by red box), whereas weak bands underwent a second round of PCR (highlighted by yellow box).

Sequence logo for the 150-220 bp region. The top track shows nucleotide conservation with a scale from 0 to 1. The sequence below is: 5'-GCCCTTGTGAAGGATGCCGTTGTTTGGAGATTGACTGCTGGGATGGAGCACAAATGAACCTGTTGTATATCATGGCTA-3' (top strand) and 3'-GCCCTTGTGAAGGATGCCGTTGTTTGGAGATTGACTGCTGGGATGGAGCACAAATGAACCTGTTGTATATCATGGCTA-5' (bottom strand). The bottom strand is the reverse complement of the top strand. Asterisks mark positions 150-220.

### 4.3.2 NGS of *PLCζ*

Aside from Sanger sequencing, which focused exclusively on the exonic regions of *PLCζ*, the NGS experiment was carried out to extend our analysis of the *PLCζ* promoter and introns. Individual *PLCζ* assay results and genomic DNA concentrations are summarised in **Table 4.3**.

**Table 4.3 Information relating to genomic DNA concentrations and *PLCζ* immunofluorescence assay results for the subjects used for NGS analysis (n=33).**

| Patient number                                                 | Genomic DNA                                 | PLCζ immunofluorescence assay |                                        |
|----------------------------------------------------------------|---------------------------------------------|-------------------------------|----------------------------------------|
|                                                                | concentration (ng/μl)<br>by Qubit Fluro 2.0 | Mean total PLCζ levels        | Proportion of sperm<br>exhibiting PLCζ |
| Group 1 PLCζ deficiency (n=5)                                  |                                             |                               |                                        |
| 59                                                             | 122.7                                       | Reduced                       | Reduced                                |
| 114                                                            | 37.4                                        | Reduced                       | Reduced                                |
| 126                                                            | 51                                          | Reduced                       | Reduced                                |
| 130                                                            | 52                                          | Reduced                       | Reduced                                |
| 147                                                            | 57.2                                        | Reduced                       | Reduced                                |
| Group 2 Reduced PLCζ expression (n=11)                         |                                             |                               |                                        |
| 116                                                            | 147                                         | Reduced                       | No significant differences             |
| 119                                                            | 46.5                                        | Reduced                       | No significant differences             |
| 125                                                            | 53                                          | Reduced                       | No significant differences             |
| 132                                                            | 120                                         | Reduced                       | No significant differences             |
| 135                                                            | 8.77                                        | Reduced                       | Increased                              |
| 136                                                            | 19.2                                        | Reduced                       | No significant differences             |
| 137                                                            | 17.5                                        | Reduced                       | No significant differences             |
| 138                                                            | 74.8                                        | Reduced                       | No significant differences             |
| 139                                                            | 29.6                                        | Reduced                       | No significant differences             |
| 144                                                            | 57                                          | Reduced                       | No significant differences             |
| 146                                                            | 56                                          | No significant differences    | Reduced                                |
| Group 3 No evidence of abnormal PLCζ protein expression (n=14) |                                             |                               |                                        |
| 54                                                             | 160.5                                       | No significant differences    | No significant differences             |
| 60                                                             | 101.4                                       | No significant differences    | No significant differences             |
| 117                                                            | 85.8                                        | No significant differences    | No significant differences             |
| 118                                                            | 120                                         | No significant differences    | No significant differences             |
| 120                                                            | 35.2                                        | No significant differences    | No significant differences             |
| 121                                                            | 23.7                                        | Increased                     | No significant differences             |
| 122                                                            | 110                                         | Increased                     | Increased                              |
| 123                                                            | 89                                          | Increased                     | No significant differences             |
| 124                                                            | 50                                          | No significant differences    | No significant differences             |
| 127                                                            | 135                                         | No significant differences    | No significant differences             |
| 131                                                            | 86.2                                        | No significant differences    | No significant differences             |
| 134                                                            | 13.8                                        | No significant differences    | Increased                              |
| 140                                                            | 41.4                                        | No significant differences    | No significant differences             |
| 141                                                            | 114                                         | No significant differences    | No significant differences             |
| Group 4 Fertile controls (n=3)                                 |                                             |                               |                                        |
| Ctrl 1                                                         | 52                                          |                               |                                        |
| Ctrl 2                                                         | 85.2                                        |                               |                                        |
| Ctrl 3                                                         | 180.3                                       |                               |                                        |

#### 4.3.2.2 Genetic variations in the intronic regions of *PLCζ* in infertile males

Compared to the human genome database (NCBI, NM\_033123), the NGS experiment did not detect mutations, or SNPs, in the exonic regions of *PLCζ* (i.e., exons 1, 2, and 8-10). Instead, ten SNPs were identified in the *PLCζ* introns of fertile and/or infertile males; and 6 of these variants were present only in infertile males (**Table 4.4**). The SNPs identified in this study were searched in SNP databases in order to determine whether they were novel (NCBI, <https://www.ncbi.nlm.nih.gov/snp/>, UCSC, <https://genome.ucsc.edu/> and Ensembl <https://www.ensembl.org/index.html>). The ten SNPs were situated in introns 1, 8 and 9, and have been previously reported: rs7133682, rs529457993, rs1428813971, rs1470348899, rs563105487, rs148182265, rs17488186, rs7302811, rs748476303 and rs1476908253 (**Table 4.4**).

Although the database is continuously updated with new SNPs, the *PLCζ* SNPs in the database are not yet associated with clinical data. As such, I provided more information on *PLCζ* SNPs that are associated with individual fertility. Infertile patients were retrospectively assigned to groups, according to the level of protein expression in sperm based on immunofluorescence analysis (**Table 4.3**). One deletion from AC to A in intron 9 (rs1470348899) was found only in an infertile male (patient 59) who showed *PLCζ* deficiency (**Table 4.4**). Two SNPs, rs7133682 and rs148182265, were identified only in infertile males from group 3, and one SNP (rs563105487) was found only in a patient from group 2 (patient 144). In contrast, two SNPs were present in *PLCζ*-reduced, *PLCζ* deficiency, and/or normal groups (rs529457993 and rs1428813971, **Table 4.4**).

**Table 4.4 List of SNPs identified in *PLCζ* introns 1 and 9 of infertile patients.**

NM\_033123, GRCh37/hg19, chr12:18836110-18890993.

| SNP No. | SNP position on Chr12 | No. of patients processing SNP | Patients No.          | Intron No. | He/Ho?   | SNP       | Frequency   | Variant No.  |
|---------|-----------------------|--------------------------------|-----------------------|------------|----------|-----------|-------------|--------------|
| 1       | 18890787              | 2                              | 54, 60                | 1          | He       | T --> C   | 0.03-0.06   | rs7133682    |
| 2       | 18890472              | 2                              | 59, 120               | 1          | Deletion | GT --> G  | 0.00001     | rs529457993  |
| 3       | 18853446              | 5                              | 54, 59, 60, 116, 120, | 9          | Deletion | CAA --> C | N/A         | rs1428813971 |
| 4       | 18853445              | 1                              | 59                    | 9          | Deletion | AC --> A  | 0.00001     | rs1470348899 |
| 5       | 18853188              | 1                              | 144                   | 9          | He       | G --> A   | 0.001-0.002 | rs563105487  |
| 6       | 18852922              | 2                              | 117, 124              | 9          | He       | A --> G   | 0.01-0.02   | rs148182265  |

Six SNPs were found in eight infertile males. Patient number in red represents a patient from group 1 (*PLCζ* deficiency); patient number in blue represents a patient from group 2 (reduced *PLCζ* levels in sperm or low proportions of sperm expressing *PLCζ*), and patient number in black represents a patient from group 3 (no evidence of abnormal *PLCζ* expression). The phenotypes of these SNPs were investigated; 'Ho' represents a SNP that is homozygous, while 'He' represents a SNP that is heterozygous. Frequencies of SNPs were obtained via NCBI and Ensembl database; 'N/A' represents the frequency of a SNP is not applicable. 'No.' represents number.

#### 4.3.2.3 Mutations in the *CAPZA3* exon

Two heterozygous mutations were found in *CAPZA3*, after matching with NCBI, UCSC, Ensembl and HGMD (<http://www.hgmd.org>) databases. Infertile patient 138 (**Table 4.3**) showed a C/T substitution, resulting in an arginine to cysteine substitution at position 39 (R39C, rs61912355, **Figure 4.13**). The second mutation (a C/T substitution) was identified in patient 118 (**Table 4.3**), causing an alanine to valine change at position 153 (A153V, rs199684920, **Figure 4.14**).

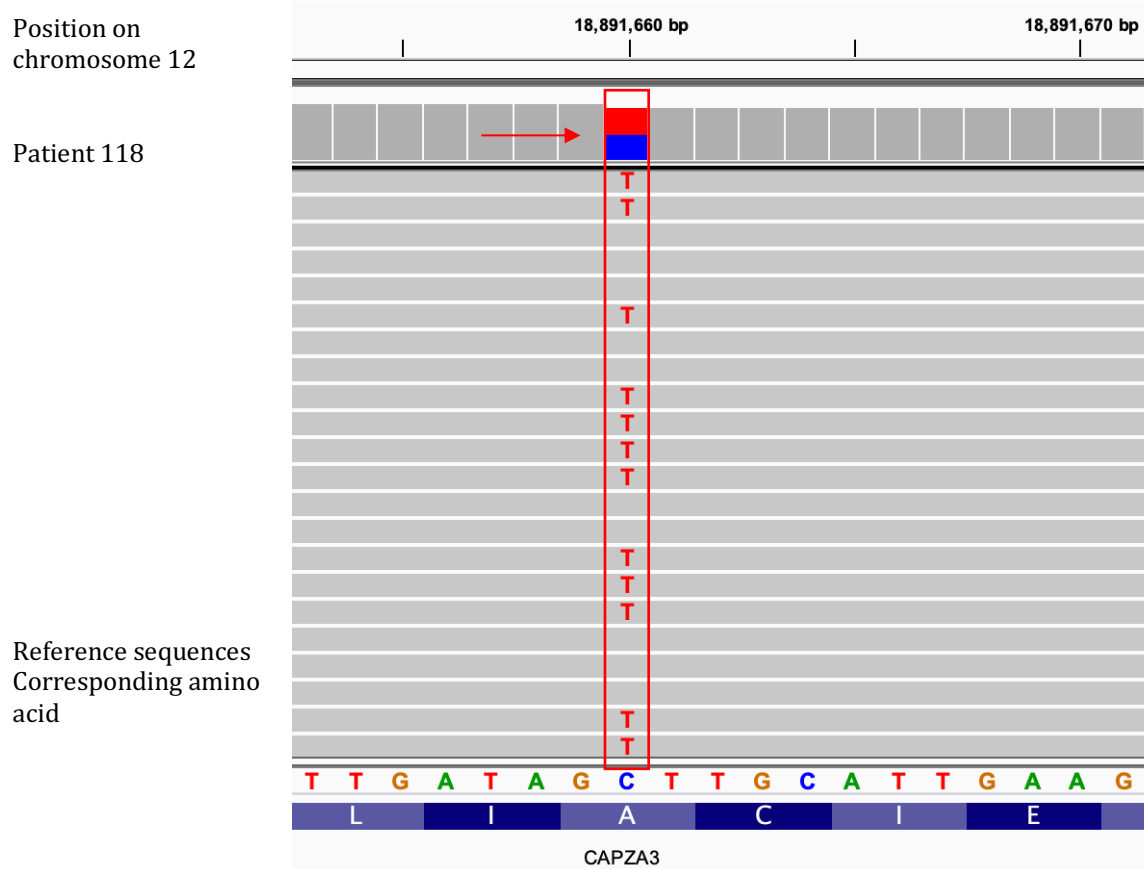
It is worth mentioning that two patients (119 and 138) showed another homozygous G/T alteration, in which valine was changed to phenylalanine at

position 145 (V145F, novel). However, the frequency of this allele change was low (1%). Interestingly, patient 138 exhibited two mutations (R39C and V145F); **Figure 4.15** summarises the amino acid changes in these patients compared to the reference human genome. The R39C and A153V mutations are suggested to be 'probably damaging' to protein function, as predicted by Netgene2 (Adzhubei *et al.* 2010); the V145F mutation is predicted to be benign.

Further structural analysis, and 3D modelling, using a webtool (<https://swissmodel.expasy.org/>) indicated that the two mutations cause alterations in the properties of the residue (**Figure 4.16**). The R39C mutation gave a more positive charge to the SH domain, thus making the cysteine smaller. In turn, this mutation may influence the corresponding surface ligand binding properties (**Figure 4.17**, predicted by Phyre2, <http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>). The A153V mutation causes an expansion of the carbon branched chain in the strand of the CAPZA3 secondary structure. However, this mutation may not influence the protein surface ligand. Although NGS revealed that the V145F mutation had low reliability (1% frequency of allele change), and is predicted to be benign, the mutation increased the sizes of the hydrophobic sites (**Figure 4.18**, produced by Phyre2 webtool, <http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>).



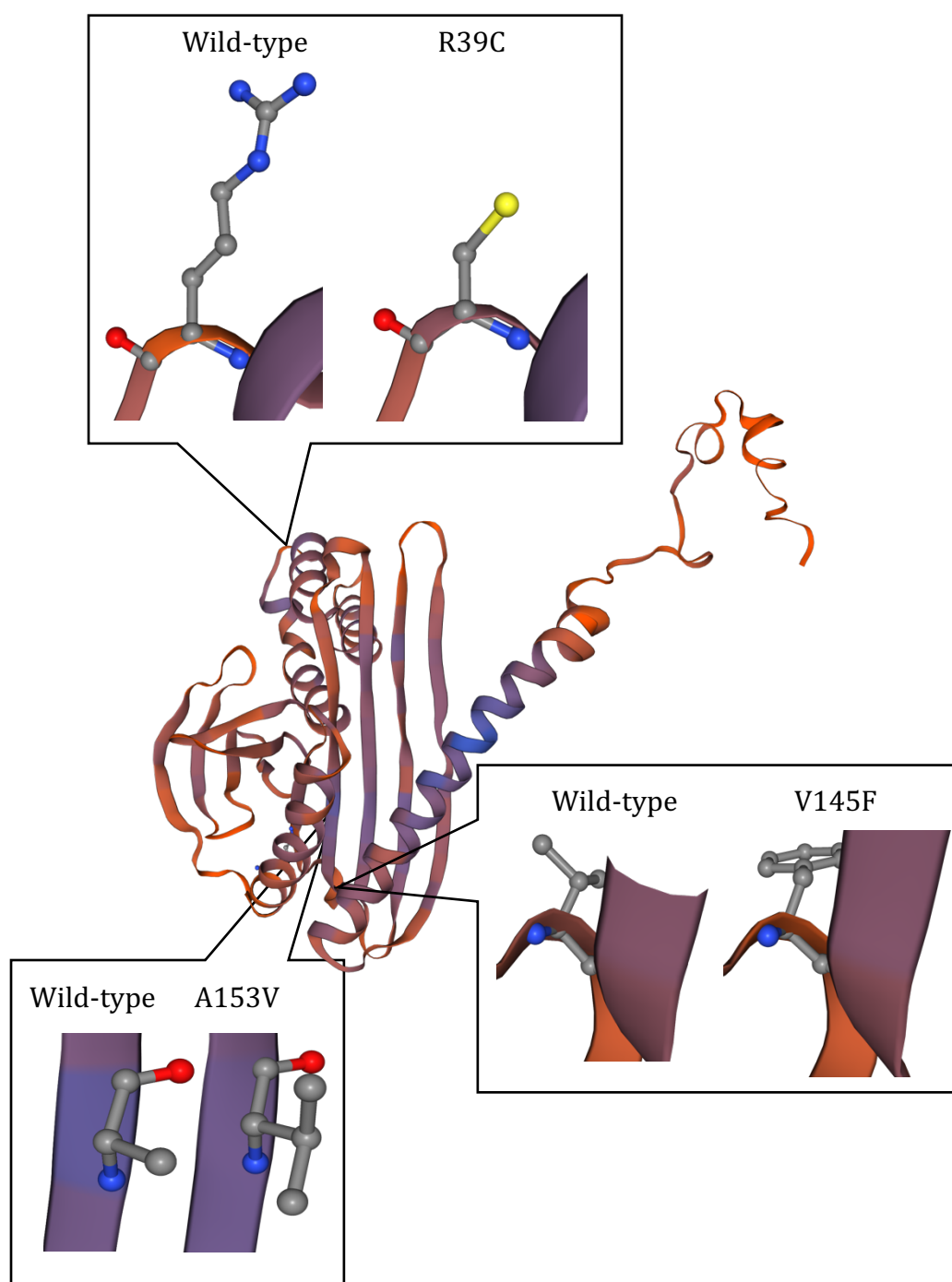




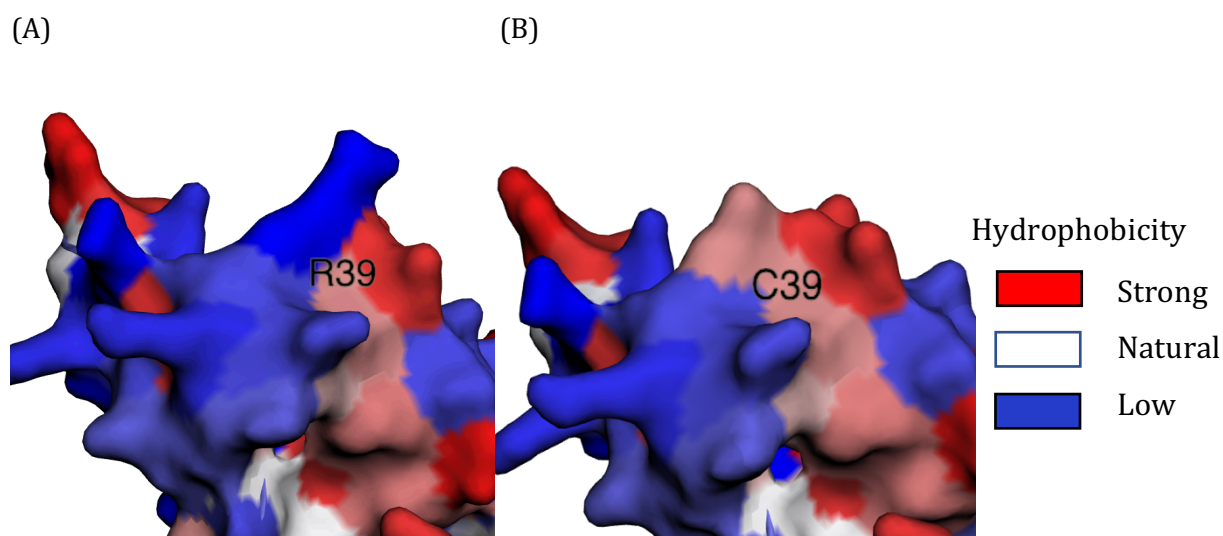
**Figure 4.14 Integrative Genomics Viewer (IGV) viewer showing a heterozygous mutation (A153V) in infertile patient 118.** The red box indicates a cytosine (C) to thymine (T) change, resulting in the substitution of alanine with valine. The red arrow indicates that the mutation is heterozygous. NM\_033328, chr12:18891045-18892122.

|             |           |           |        |                 |          |          |       |
|-------------|-----------|-----------|--------|-----------------|----------|----------|-------|
|             | 31        |           | 50     | 130             |          | 160      |       |
| Wild-type   | -FDDLCLLI | RDEKLMHHQ | GEC--  | NDHYPKGNCNMLRKT | VKSKEYLI | ACIEDHNY |       |
| Patient 138 | -FDDLCLLI | CDEKLMHHQ | GEC--- | NDHYPKGNCNMLRKT | FKSKEYLI | ACIEDHNY |       |
| Patient 118 | -FDDLCLLI | RDEKLMHHQ | GEC--  | NDHYPKGNCNMLRKT | VKSKEYLI | VCI      | EDHNY |
| Patient 119 | -FDDLCLLI | RDEKLMHHQ | GEC--  | NDHYPKGNCNMLRKT | FKSKEYLI | ACIEDHNY |       |

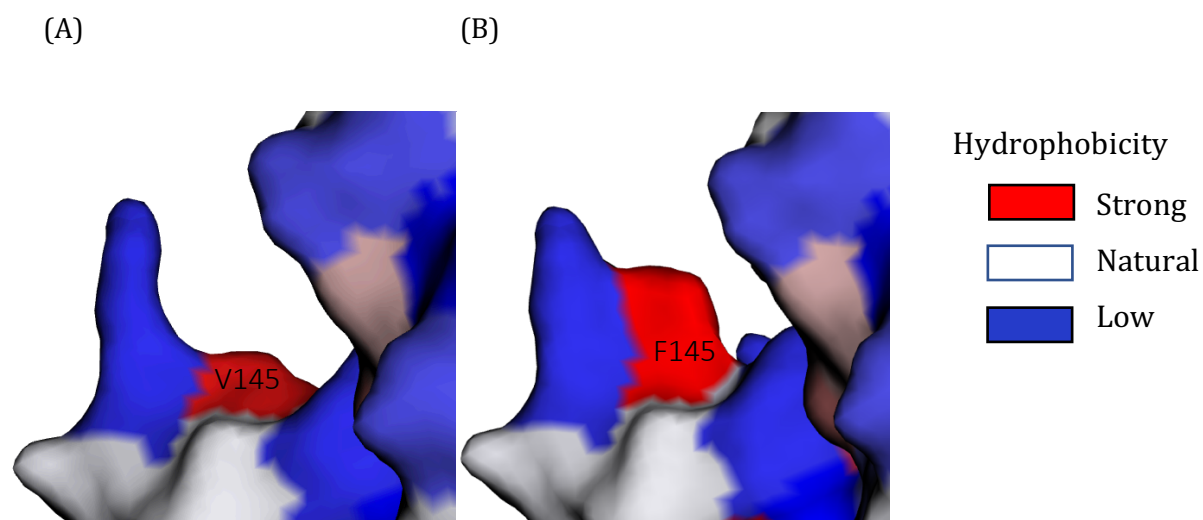
**Figure 4.15 Three infertile patients exhibiting mutation(s) in *CAPZA3*.** Red boxes indicate the position of mutations in the amino acid sequences. Three mutations were found in patient 138 (R39C and V145F), patient 118 (A153V) and patient 119 (V145F).



**Figure 4.16 Three-dimensional modelling of the wild-type CAPZA3 protein and the three mutations.** Three mutations are as follows; arginine to cysteine at position 39 (R39C), alanine to valine at position 153 (A153V) and valine to phenylalanine at position 145 (V145F).



**Figure 4.17 Three-dimensional modelling of CAPZA3 protein and the R39C mutation.** Arginine was replaced by cysteine at position 39, creating a more positive charge in the SH domain, thus making the cysteine smaller and increasing hydrophobicity.



**Figure 4.18 Three-dimensional modelling of CAPZA3 protein and the V145F mutation.** The substitution of valine with phenylalanine led to an increase in the residue size and hydrophobicity.

#### 4.4 Discussion

In this chapter, I investigated *PLCζ* exons, the *PLCζ* XY-linker structure, *CAPZA3*, and the bidirectional promoter, using traditional Sanger sequencing and NGS. Genetic screening results of *PLCζ* exons and promoter regions in infertile patients did not identify any mutations. However, NGS identified ten intronic variants in fertile and/or infertile males, and three *CAPZA3* mutations were identified in three infertile patients; R39C, A153V and V145F. Furthermore, 3D modelling predicted that R39C and V145F altered the hydrophobicity of *CAPZA3* residues, which may impair normal sperm function.

Although eleven mutations have previously been reported in *PLCζ* exons and shown to abolish *PLCζ* activity in terms of its ability to trigger  $\text{Ca}^{2+}$  oscillations and oocyte activation (Heytens *et al.* 2009; Kashir *et al.* 2011c; Escoffier *et al.* 2015; Ferrer-Vaquer *et al.* 2016; Dai *et al.* 2019; Torra-Massana *et al.* 2019), genetic defects within *PLCζ* exons remain very rare. Sanger sequencing technology, as a traditional sequencing method, reads four different fluorophores representing A, T, C, and G, respectively. Despite its merits, however, Sanger sequencing is limited in terms of reading larger sequences, the amplification of targets with natural variants, and its sensitivity in detecting low-frequency mutant alleles (Kircher and Kelso 2010; Fakruddin and Chowdhury 2012).

My data shows that infertile patients clearly expressed lower levels of *PLCζ* protein than fertile controls, but exon sequencing showed that their genomic sequences were the same as those of controls. In other words, the lower expression level was not due to mutation in the *PLCζ* exons. We currently do not know why this is the case, although it is tempting to suspect that there are several mechanisms that can affect gene expression at the transcriptional and/or translational levels. In my

study using Sanger sequencing, genetic analyses focused upon the exonic regions of *PLCζ* and largely ignored the regulatory regions that might exist in the promoter or intronic regions. The NGS experiment aimed to address these shortfalls in further detail. In addition, the NGS experiment also screened intron-exon boundaries corresponding to the XY-linker region, to investigate whether intronic SNPs may exert influence upon alternative splicing at the intron-exon boundary.

NGS experiments were performed to investigate the *PLCζ-CAPZA3* shared promoter sequences. *CAPZA3* is an actin-capping protein and is important in determining the final shape of sperm heads. Furthermore, *CAPZA3* has been associated with sperm count, motility and fertilisation rate (Javadian-Elyaderani *et al.* 2016). *CAPZA3* mRNA expression has also been associated *PLCζ* mRNA and protein levels (Tavalaee and Nasr-Esfahani 2016). Taken together, the abnormal expression of *PLCζ* and *CAPZA3* in infertile male sperm may be due to defects in their shared bidirectional promoter.

However, the study described in this chapter did not find any mutation nor SNP in these regions, indicating that the promoter is not the reason for infertility in these males, and the problems causing infertility may exist in the expression of downstream transcripts (i.e., mRNA) or upstream regulatory elements, such as enhancers, activators, transcription factors (TF, TFIIF TFIIE TFIIH), and RNA polymerase II, etc (Fischer *et al.* 2019). In addition, variations in chromatin, including chromatin decondensation and DNA methylation (Klemm *et al.* 2019), can also impair initiation of transcription, resulting in *PLCζ* dysfunction. Abnormalities in the genome, for example, DNA rearrangements and gene deletion/amplification have been proven to result in sperm DNA fragmentation (García-Peiró *et al.* 2011) and asthenozoospermia (abnormal defects in sperm tail) (Baccetti *et al.* 2005).

However, it is not known whether such genome can affect *PLCζ* function; this further investigate.

In the past, introns were considered to represent junk DNA. However, novel roles for introns are currently being explored. Among other functions, introns have been shown to be involved in transcript processing, such as the initiation and termination of transcription or genome organisation (Carmel and Chorev 2012). DNA elements that regulate transcription initiation have been found in intron-containing DNA sequences (Schohy *et al.* 2000; Gaunitz *et al.* 2005; Bianchi *et al.* 2009; Javadian-Elyaderani *et al.* 2016). Interestingly, DNA elements such as enhancers and silencers are also located in the 5'-first introns and the 5'UTR (Schohy *et al.* 2000; Gaunitz *et al.* 2005; Bianchi *et al.* 2009; Li *et al.* 2012), which suggests that 5'UTR in some eukaryotes are longer than downstream introns, because the 5'UTR host DNA elements responsible for transcription initiation (Bradnam and Korf 2008).

In this NGS experiment, nearly 50% of the males (14 infertile and 1 fertile) exhibited a SNP in the 5'UTR of *CAPZA3* (data not shown, T to C alteration, rs1075421). Further analysis using the Genomatix program (<http://www.genomatix.de>.) indicated that this SNP influences the binding sites of two putative regulators: the POZ domain zinc finger in the forward direction and the GHF-1 pituitary-specific POU domain transcription factor in the reverse direction. The POZ domain is a motif responsible for interactions between proteins, and is involved in biological processes such as apoptosis and transcription repression (Lee *et al.* 2002). However, further experimental studies are now required to validate whether disruptions in these regulators can impair target gene function.

Spliceosomal introns are formed in all eukaryotic genomes analysed thus far, and include core components that are critical for the splicing reaction, such as an

‘AG’ at the 3’ splice site boundary, a short 5’ splice site, and a catalytic adenosine (Irimia and Roy 2014). Abnormalities in these splicing sites may influence intron-exon architecture (Tilgner *et al.* 2009). In line with this, Schrimpf *et al.* identified two variants in the alternative splice site of horse *PLCζ* introns; as a result of these variants, exon 4 can be extended by three base pairs (Schrimpf *et al.* 2014).

The NGS experiment described herein identified a total of ten SNPs in human *PLCζ* introns 1, 8 and 9, which adds more information to the current database. Further categorisation of these SNPs revealed that fertile males possessed four of these SNPs, indicating that these four SNPs may not be the cause of impaired fertility. The other SNPs were found only in infertile males. However, none of these SNPs were located in spliceosomal introns predicated by NetGene2 (Hebsgaard *et al.* 1996), which indicates that no splicing problem was found in the regions investigated. This finding also correlates with the fact that no mutation was found in the neighbouring exons (exons 9 a and 10).

The first intron is generally more important than the other introns because it may contain regulatory elements that drive transcriptional initiation (Schrimpf *et al.* 2014). A genome-wide study sequenced the first *PLCζ* intron of the horse and found that this region contained two variants that cause downstream transcripts to contain a premature termination codon. As a result, this may cause the mature mRNA to vary in sizes (Schrimpf *et al.* 2014). In the present study, two SNPs (one deletion) were identified in four infertile patients’ *PLCζ* intron 1 (patient 54, 59, 60 and 120), but these SNPs seem not to affect any transcriptional factors, as suggested by bioinformatic analysis (Genomatix, <https://www.genomatix.de/>).

Collectively, the present NGS study investigated *XY-linker* regions and showed no evidence of abnormality. This provides us with an insight into the other



factors that may impair *PLCζ* gene function. Indeed, multiple regulatory mechanisms are involved in gene expression at the transcriptional and translational levels. Any abnormalities in regulatory molecules, including transcription factors and coding/non-coding RNAs, can lead to gene dysfunction. For example, one third of miRNAs derive from the introns of a host gene (Wessels *et al.* 2019), suggesting that problems in introns can hinder the generation of miRNAs and subsequent translation. Indeed, Schrimpf *et al.* suggested that intronic SNPs could disrupt miRNA function, thus resulting in variable *PLCζ* levels in horse sperm (Schrimpf *et al.* 2014). Therefore, it is essential for future studies to investigate the role(s) of these factors in *PLCζ*-related infertility.

This NGS study also identified three patients (118, 119, and 138) who possessed mutations in their *CAPZA3* exon, which may relate to their fertilisation failures in IVF treatment. *CAPZA3*, a known actin-capping protein, is involved in sperm capacitation and the acrosome reaction via the regulation of actin polymerisation (Sosnik *et al.* 2010). Previous immunofluorescence labelling showed that mouse sperm first expresses *CAPZA3* in the anterior acrosome, after which it is redistributed to the whole sperm head and post-acrosomal region following capacitation and the acrosome reaction, respectively (Tokuhiro *et al.* 2008; Sosnik *et al.* 2010). This suggests that any defects in the structure of *CAPZA3* protein could have an impact on these biological processes. Accordingly, in this experiment, two of the three patients who showed nonsense *CAPZA3* mutations received IVF treatment and experienced total fertilisation failure (patient 119, 0/16 fertilised oocytes) or low fertilisation (patient 138, 1/10 fertilised oocytes), which suggests that sperm–oocyte fusion problems potentially exist in these patients.

Interestingly, patients 119 and 138 exhibited normal sperm parameters (sperm count 55-118 mill/ml, sperm motility 55-60%, and morphology 4-6%), indicating that *CAPZA3* mutations in these patients may not alter the acrosomal formation of sperm. Indeed, globozoospermia has a heterogenetic aetiology, which can be driven by several different genes, including *Zpbp1*, *Spaca1*, and *Dpy19L2* (De Boer *et al.* 2015). Additionally, 52% of sperm produced in a sample by patient 138 exhibited DNA fragmentation. DNA damage can exert influence over the success of ART by virtue of chromatin abnormalities resulting from chromosomal abnormalities, gene mutations, or abnormal apoptotic degeneration during spermatogenesis (Evenson *et al.* 2002). Thus far, no study has suggested an association between DNA damage and *CAPZA3*; this needs to be investigated further.

Testis-specific CAPAZ3 is a subunit of the actin-capping protein that was originally found in the skeletal muscle Z-line (Casella *et al.* 1987). The capping protein in somatic cells has been considered to play a role in preventing actin depolymerisation by binding to the end of actin filaments and connecting with other Z-line proteins (Cooper and Sept 2008). The previously identified mutation M44K in *CAPZA3* has been proven to influence the development of mouse spermatids in terms of condensation and elongation, possibly by disrupting filamentous actin structures in spermatids. As a result, sperm in the cauda epididymis was low in concentration, had poor motility, and exhibited abnormal morphology, including the presence of cytoplasmic droplets (Geyer *et al.* 2009). In addition, Howes *et al.* suggested that actin-capping proteins may be involved in membrane remodelling during sperm maturation (Howes *et al.* 2001).

The role of *CAPZA3* in sperm–oocyte fusion remains unclear, although evidence suggests that *CAPZA3* is needed for sperm capacitation and the acrosome

reaction (Sosnik *et al.* 2010), and that actin polymerisation may play a role in acrosomal exocytosis (Howes *et al.* 2001). In addition, it is suggested that phosphoinositides, such as PIP<sub>2</sub>, bind to actin-capping protein and inhibit capping activity in organisms such as yeast, mammals, and plants (Heiss and Cooper 1991; Huang *et al.* 2006). In addition, PIP<sub>2</sub> is suggested to be involved in the acrosome reaction mainly through its hydrolysis (Breitbart and Finkelstein 2015). Taking all evidence together, one hypothesis is that mutations may alter the hydrophobicity of CAPZA3 residue and therefore hinder PIP<sub>2</sub> hydrolytic activity. As a result, this may influence the acrosome reaction at fertilisation. In line with this, a point mutation in the actin-capping protein at the C terminus was previously shown to hinder PIP<sub>2</sub> binding activity (Kim *et al.* 2007). In addition, Geyer *et al.* suggested that a CAPZA3 mutation resulting in the substitution of lysine to methionine increased residue hydrophobicity, which may disrupt the CAPZA3 tertiary structure and result in an abnormal sperm phenotype and mouse infertility (Geyer *et al.* 2009). This finding is in line with the present study. In the present study, three mutations were identified in CAPZA3; two of these related to a protein surface ligand, as predicted by Phyer2 (Kelley *et al.* 2015). The R39C mutation causes a change from a positively charged arginine to a hydrophobic cysteine, while the V145F mutation causes a change from valine to phenylalanine, which increases residue hydrophobicity. However, further structure/function analysis and cellular experiments will be needed to validate whether the newly-found CAPZA3 mutations influence PIP<sub>2</sub> hydrolysis activity at fertilisation.

Regardless of the mechanisms underlying reproductive events, the CAPZA3 mutations in the present study were found only in infertile males, which confirmed

the correlation between *CAPZA3* and human infertility. Accordingly, in a recent study, *CAPZA3* mRNA expression was significantly lower in infertile males than in fertile controls (Javadian-Elyaderani *et al.* 2016).

One limitation that should be noted is that the numbers of patients in the current study was relatively small. Also, only two fertile males were included in the NGS study. Future studies should increase the numbers of infertile and fertile male candidates. Although the SNPs in intron 9 identified by the current study are unlikely to be involved in the splicing of intron-exon boundaries, bioinformatic and experimental studies can be performed in future to further investigate the specific functions of intron 9, as many SNPs from different infertile patients were found in intron 9. Mutagenesis could be used to produce truncated *PLCζ* genes lacking intron 9; the functional ability of the translated protein could then be further investigated. In addition to splicing functions, the effects of the SNPs identified by this study can also be further investigated via enhancers. SNPs may be located in the binding sites of enhancers, which may further influence exon recognition (Fairbrother *et al.* 2004).

Furthermore, other approaches can be used to investigate *PLCζ*-related mechanisms of male infertility. For example, genome-wide sequencing of the whole mouse oocyte after injected with infertile male sperm could detect whether  $\text{Ca}^{2+}$  signalling mediators, such as  $\text{PIP}_2$  and  $\text{InsP}_3$ , exhibit abnormal activities upon microinjection. Because oocyte factor can also account for oocyte activation failure via downstream signalling cascade triggered by sperm fusion (Yeste *et al.* 2016a). In addition, screening of oocytes from the partner of infertile males could be performed to investigate possible genetic or molecular abnormalities in the oocyte during activation.

## 4.5 Conclusions

In conclusion, this chapter aimed to carry out a preliminary study to investigate the genomic mechanisms underlying human infertility that may be associated with *PLCζ*. I applied two sequencing approaches, Sanger sequencing and NGS, to investigate genetic variations in the *PLC-CAPZA3* promoter, *PLCζ* exon regions, the *XY-linker* regions, and *CAPZA3*. I identified a number of infertility-related SNPs in *PLCζ* intronic regions, which provides more information for the current database. Furthermore, three *CAPZA3* mutations were found in infertile males, which is the first link to the male infertility. Further structural modelling revealed that the R39C and V145F mutations altered residue hydrophobicity, which may influence *CAPZA3* function at fertilisation.

## 4.6 Key findings

- This preliminary study did not find mutations in the sequenced *PLCζ* exons (mainly exons 6, 11 and 13), the promoter, or regions encoding the *PLCζ* XY-linker structure, in 61 patients. This highlights the fact that there may be other genetic mechanisms impairing male fertility.
- Six SNPs were identified in *PLCζ* introns 1 and 9, in infertile males only.
- Three *CAPZA3* mutations were found in three infertile males; R39C, A153V and V145F (novel).
- In-silico analysis suggested that R39C and A153V may affect *CAPZA3* function

- 3D modelling of *CAPZA3* mutations indicated that R39C and V145F mutations may change the hydrophobicity of residues involved, thus may impair the sperm function at fertilisation (possibly via acrosome reaction).

## Chapter 5. Artificial oocyte activation (AOA) with ionomycin, calcimycin, and recombinant PLC $\zeta$ protein

### 5.1 Introduction

The aim of AOA in clinics is to rescue OAD in patients undergoing IVF/ICSI treatment. ICSI is carried out on couples mostly due to male-related infertile/subfertile factor(s) and provides the best opportunity for couples to have biologically related children. However, fertilisation failure in ICSI, especially TFF, still occurs, and the cause of such failure is still unclear, although evidence suggest that OAD is the main cause of TFF in ICSI and is responsible for 15% of TFF causes in IVF (Rawe *et al.* 2000). In addition, OAD has been linked with recurrent TFF and low fertilisation rates in IVF/ICSI (Kashir *et al.* 2018). At present, AOA represents the only option to alleviate OAD and allows patients to have their own children.

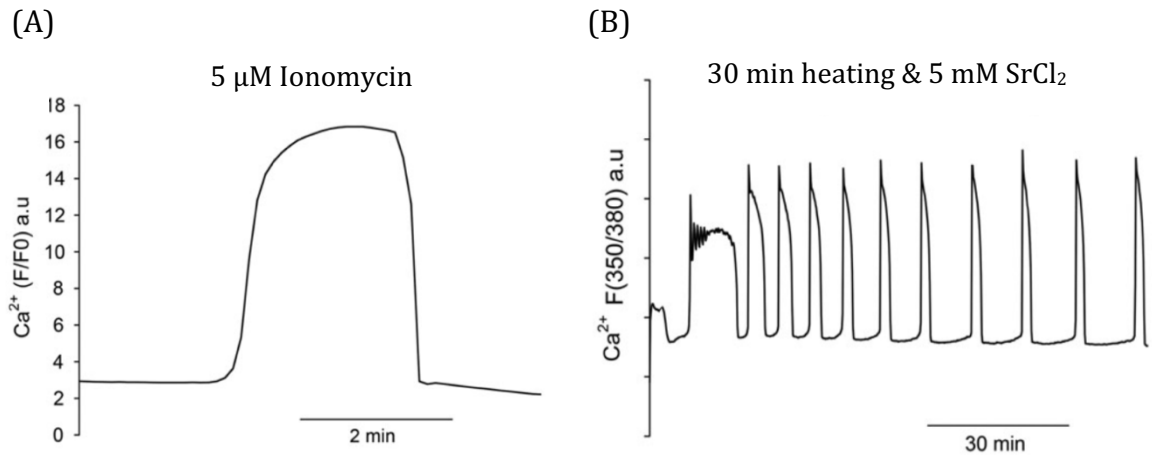
AOA involves raising intracellular Ca<sup>2+</sup> levels in an artificial manner. This has been studied extensively in animal models and applied as a complementary treatment in ART. Chemical AOA is the most commonly used method for AOA, and involves the incubation or microinjection of oocytes with chemicals including ionomycin, calcimycin, puromycin, SrCl<sub>2</sub>, ethanol, TPEN, or a commercialised chemical incorporating calcimycin, referred to as GM508. As introduced in Chapter 1 (Section 1.4), the application of chemical AOAs has been shown to improve fertilisation rate and pregnancy outcomes in human oocytes.

However, discrepancies exist in the AOA protocols utilised by different research groups, in terms of chemical amounts, incubation time and chemicals for post-treatment after activation (**Table 1.1**). In addition, different strategy for AOA

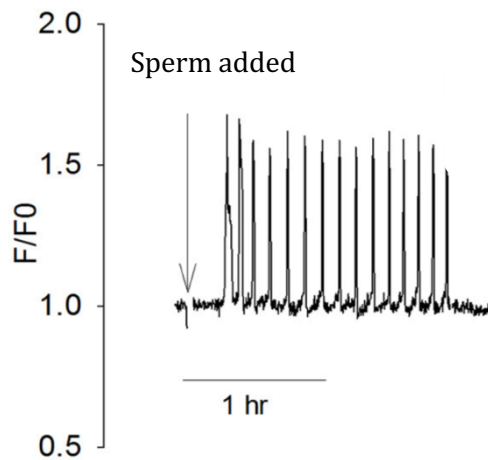
treatment may also depend on the background of infertility of each couple. All of these factors make it very difficult to evaluate the efficiency of AOA protocols and select the best one for clinical practice. Only one study compared the efficiency of ionomycin and GM508 in human and mouse oocytes. Nikiforaki *et al.* microinjected with sperm from a wobbler mouse model (i.e., round-shape sperm), and sperm from a globozoospermic male, into mouse oocytes and human oocytes matured *in-vivo*, respectively (Nikiforaki *et al.* 2016). GM508 and ionomycin were applied alongside with the microinjection. Then, the authors compared  $\text{Ca}^{2+}$  oscillations in the injected oocytes and suggested that ionomycin is more efficient for both human and mouse oocytes (Nikiforaki *et al.* 2016). However, sperm from globozoospermia patients may be able to fertilise oocytes without assistance with AOA (Kashir *et al.* 2012c). Therefore, it is difficult to identify whether the globozoospermic sperm contributed to oocyte activation in the study by Nikiforaki *et al.* In contrast, Heytens *et al.* simply applied ionomycin onto mouse oocytes and achieved oocyte activation parthenogenetically; this indicated that AOAs have the ability to activate oocytes without the presence of sperm (Heytens *et al.* 2008). However, Heytens *et al.* only examined the activation effects of ionomycin, without evaluating other types of AOA (Heytens *et al.* 2008). No other studies have further compared activation efficiency between different reagents and protocols. However, the efficiency of different AOAs on oocytes, in the absence of sperm, needs to be further investigated, as this may provide valuable information on AOA outcomes without the influence of a sperm factor. Therefore, the first aim of this chapter was to address this problem by evaluating the parthenogenetic activation efficiency of ionomycin, GM508, and rhPLC $\zeta$  on mouse oocytes.



Although AOA has achieved success in improving fertility outcomes clinically, several studies have reported that patients with particular medical conditions do not benefit from AOA treatment, such as males with teratozoospermia (Nasr-Esfahani *et al.* 2008; Aydinuraz *et al.* 2016), azoospermia (Borges Jr *et al.* 2009a), abnormal sperm morphology (i.e., amorphous head, tapered head and bent neck) (Moaz *et al.* 2006), or females with diminished ovarian reserve (Aytac *et al.* 2015). Several AOA studies have reported that infants born as a result of calcimycin treatment possessed malformations, such as knee dislocation, unilateral hip, imperforate anus, an anal atresia (Chapter 1, Section 1.4.3) (Ebner *et al.* 2015a; Van Blerkom *et al.* 2015; Mateizel *et al.* 2018), although Deemeh *et al.* suggested that there was no significant difference in rates of major birth defects between infants born through ICSI-AOA and ICSI (Deemeh *et al.* 2015). In addition, mouse studies further support the fact that ionomycin causes a single  $\text{Ca}^{2+}$  transient,  $\text{SrCl}_2$  leads to more frequent oscillations, and that ethanol fails to trigger  $\text{Ca}^{2+}$  release (Rogers *et al.* 2006; Suzuki *et al.* 2010; Sanusi *et al.* 2015; Nikiforaki *et al.* 2016)(**Figure 5.1**). In contrast, at normal fertilisation,  $\text{Ca}^{2+}$  pattern is released in a series of oscillations occurring every 30-60 min in human oocytes, and every 10 min in mouse oocytes (**Figure 5.2**)(Swann 2018a).



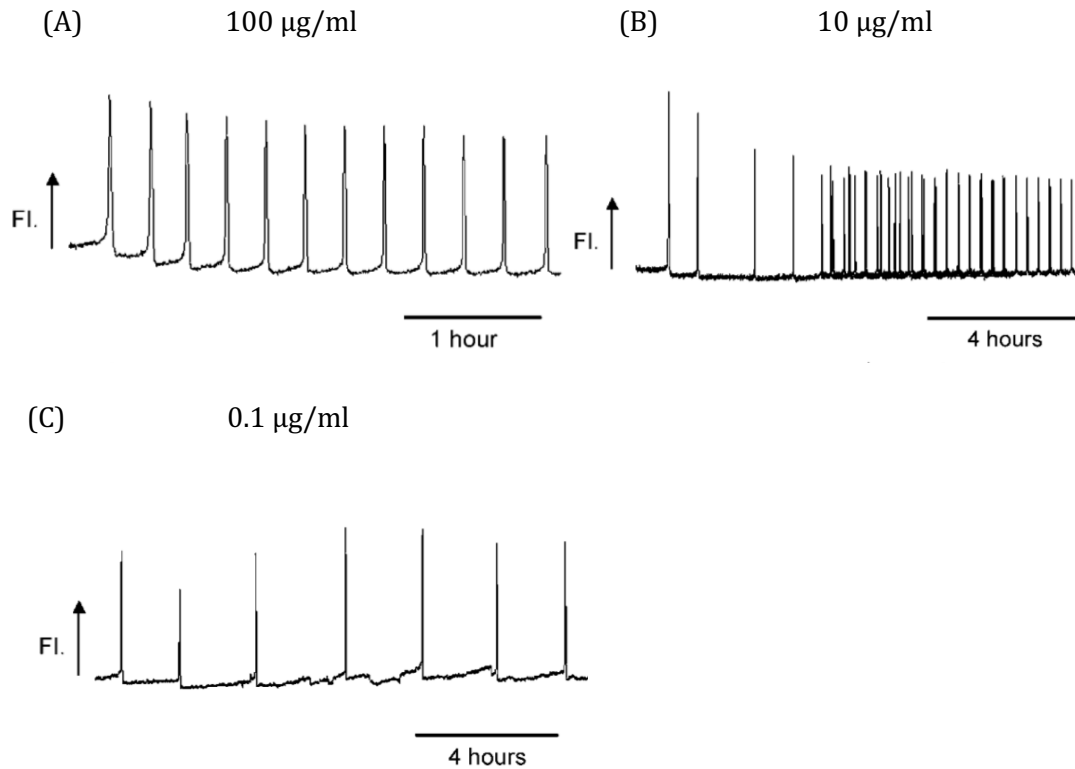
**Figure 5.1 Patterns of Ca<sup>2+</sup> release in mouse oocytes induced by artificial oocyte activators (AOAs).** (A) Ionomycin causes a single prolonged Ca<sup>2+</sup> peak. (B) High frequency oscillations induced by SrCl<sub>2</sub> with 30 min heating treatment. Figures reproduced from Sanusi *et al.* (2015) and Rogers *et al.* (2006).



**Figure 5.2 *In-vitro* fertilisation (IVF)-induced Ca<sup>2+</sup> oscillations in mouse oocytes.** Figure reproduced from Sanders *et al.* (2018).

The specific pattern of  $\text{Ca}^{2+}$  oscillations is crucial for successful mammalian oocyte activation. At oocyte activation, one of the most important biological developments is that the oocyte is released from meiotic arrest; this involves the  $\text{Ca}^{2+}$  dependent down-regulation of cyclin B and cyclin dependent kinase (CDK1) (Jones 2005). In other words, oscillatory  $\text{Ca}^{2+}$  changes digest cyclin B and suppress CDK1 activity; these activities promote oocyte development and PN formation. In contrast, a single  $\text{Ca}^{2+}$  increase is insufficient to maintain such suppression and can lead to the regeneration of cyclin B and CDK1 activity, thus resulting in the failure of oocytes to develop (Swann 2018a).

Therefore, considering the potential risks with regards to the safety of AOAs, the second aim of this chapter was to develop a safer endogenous reagent as an alternative option for OAD. As introduced in Chapter 1 (Sections 1.1.2 and 1.3), PLC $\zeta$  has been proposed as the dominant SOAF and the physiological trigger of oocyte activation (Amdani *et al.* 2016; Dai *et al.* 2019; Torra-Massana *et al.* 2019). It is therefore reasonable to investigate the therapeutic role of PLC $\zeta$  to overcome OAD. Indeed, the microinjection of human PLC $\zeta$  cRNA has been shown to trigger fertilisation-like  $\text{Ca}^{2+}$  oscillations in MII-arrested mammalian oocytes (Cox *et al.* 2002; Saunders *et al.* 2002). Furthermore, the microinjection of human PLC $\zeta$  cRNA into human oocytes that had failed during ICSI (i.e., aged oocytes) also triggered robust  $\text{Ca}^{2+}$  oscillations, although injection with different amounts of PLC $\zeta$  cRNA led to variable  $\text{Ca}^{2+}$  oscillatory patterns (**Figure 5.3**) (Rogers *et al.* 2004). These authors also found that a small amount of PLC $\zeta$  RNA (0.1  $\mu\text{g}/\text{ml}$ ) could effectively activate human oocytes to develop into blastocysts, while higher amounts of PLC $\zeta$  RNA prevented oocytes from reaching the 2-cell stage (Rogers *et al.* 2004).



**Figure 5.3  $\text{Ca}^{2+}$  oscillatory patterns in unfertilised human oocytes triggered by different amounts of human PLC $\zeta$  cRNA.** Figures reproduced from Rogers *et al.* (2004). FI: fluorescence intensity of  $\text{Ca}^{2+}$ .

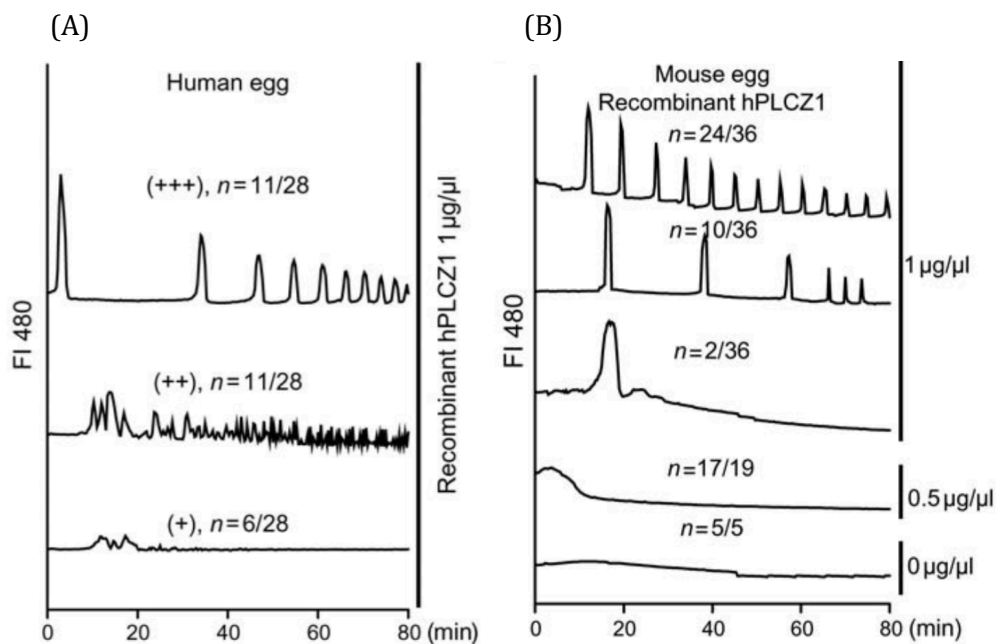
However, the injection of RNA may be problematic for the clinic as oocytes contain reverse transcriptase or alternative promoter. Consequently, exogenous forms of RNA may become reverse transcribed into DNA. In addition, RNA is unstable *in-vivo* (degradable), potentially immunogenic, and difficult to keep efficiency in a consistent manner in the process of protein translation (Burnett and Rossi 2012). These factors represent obstacles for the use of PLC $\zeta$  RNA as a therapeutic reagent in clinic. A better option for therapy is to use recombinant protein. Kouchi *et al.* were the first synthesise recombinant mouse PLC $\zeta$  (rmPLC $\zeta$ ) and recombinant mouse PLC $\delta$ 1 (rmPLC $\delta$ 1) from an insect cell expression system, baculovirus/Sf9 (Kouchi *et al.* 2004). After microinjection into mouse oocytes,

rmPLC $\zeta$  successfully triggered fertilisation-like Ca<sup>2+</sup> oscillations and showed better ability than rmPLC $\delta$ 1 in terms of sensitivity to Ca<sup>2+</sup> and hydrolytic activity (Kouchi *et al.* 2004). Following this, efforts were made to synthesise functional recombinant human PLC $\zeta$  (rhPLC $\zeta$ ) protein from bacteria, insect, mammalian and viral vectors.

The first major step forward was Kashir *et al.* (2011b). These authors were the first to synthesise rhPLC $\zeta$  from the lysates of a mammalian cell line, human embryonic kidney 293 cells (HEK293T), and microinjected these rhPLC $\zeta$ -containing lysates into mouse oocytes, which successfully triggered fertilisation-like Ca<sup>2+</sup> oscillations in 79% of injected oocytes. (Kashir *et al.* 2011b). However, this protein could not be purified from cell lysates, thus preventing this protein from being developed further for clinical purpose. Since then, no further achievement has been made in synthesising rhPLC $\zeta$  using a mammalian expression system, possibly because the efficiency of the mammalian expression system in producing protein is low, and the rhPLC $\zeta$  needs to be functional and active to trigger Ca<sup>2+</sup> release (Kashir *et al.* 2011b). All subsequent publications referred to rhPLC $\zeta$  were made from *E. coli*, which I believe was inappropriate in terms of both functionality and clinical application. Ideally, producing rhPLC $\zeta$  protein from a mammalian cell line would be more beneficial, as mammalian cells contains a nucleus and ER that are essential structures in processing protein post-translational modification; bacteria lack these key organelles (Muir *et al.* 2017). In addition, PLC $\zeta$  enzymatic activity is intimately related to PLC $\zeta$  domains that are required to undergo functional processing during protein production.

Yoon *et al.* were the first to generate a His-tagged rhPLC $\zeta$  protein using *E. coli* and investigated its activation ability in mouse oocytes and *in-vitro* matured human oocytes from patients who had failed ICSI (Yoon *et al.* 2012). Microinjection of

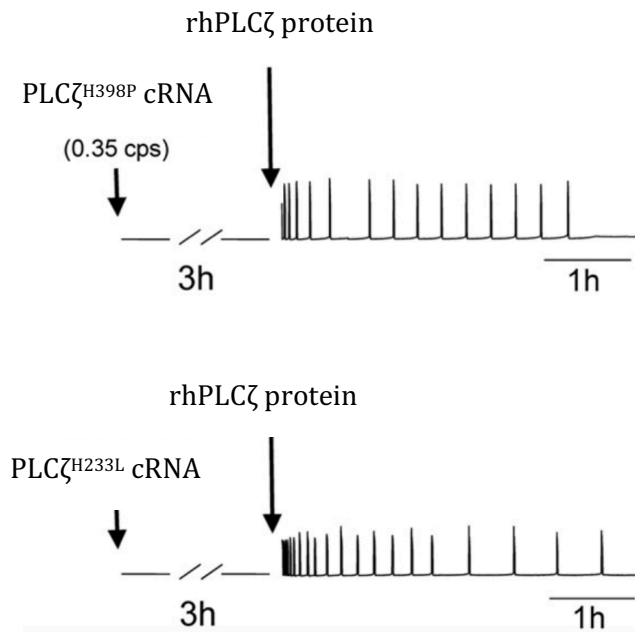
rhPLC $\zeta$  into human oocytes triggered PN but with variable patterns of Ca<sup>2+</sup> oscillations that were dose-dependent. Similarly, rhPLC $\zeta$  activated mouse oocytes, as judged by the second polar body and PN, but with a delayed response to Ca<sup>2+</sup> (Yoon *et al.* 2012)(**Figure 5.4**). However, this rhPLC $\zeta$  protein gradually showed a reduction in enzymatic activity, and the *E. coli* system yielded low protein amounts (Yoon *et al.* 2012). Of most concern, however, concentrations of this protein required to trigger oocyte activation was high (Yoon *et al.* 2012). Furthermore, some of the Ca<sup>2+</sup> oscillation patterns produced were abnormal in terms of their amplitude and frequency.



**Figure 5.4** The rhPLC $\zeta$  protein produced by Yoon *et al.* (2012) triggered dose-dependent Ca<sup>2+</sup> oscillatory patterns in (A) human oocytes matured *in-vitro* and (B) mouse oocytes. Gradual reduced Ca<sup>2+</sup> activity was observed in both human and mouse oocytes. FI: Ca<sup>2+</sup> fluorochrome intensity. Figures reproduced from Yoon *et al.* (2012).

In a subsequent study, Nomikos *et al.* produced a rhPLC $\zeta$  protein using the NusA-hexahistidine fusion system and, for the first time, investigated the potential therapeutic role of rhPLC $\zeta$  protein in rescuing OAD (Nomikos *et al.* 2013). As mentioned earlier in Chapter 1 (Section 1.3.4) and Chapter 4 (Section 4.1), PLC $\zeta^{\text{H398P}}$  and PLC $\zeta^{\text{H233L}}$  are two known PLC $\zeta$  mutations that lead to male infertility. The NusA-tagged rhPLC $\zeta$  protein triggered fertilisation-like Ca<sup>2+</sup> oscillations in mouse oocytes that were previously injected with PLC $\zeta^{\text{H398P}}$  and PLC $\zeta^{\text{H233L}}$  cRNA, which indicated that rhPLC $\zeta$  could rescue PLC $\zeta$  deficiency-related infertility in ART (**Figure 5.5**) (Nomikos *et al.* 2013). Furthermore, the rhPLC $\zeta$  activity was lost once the tag was removed (Nomikos *et al.* 2013). However, the size of the NusA tag is ~60 kd, which is similar to the molecular weight of PLC $\zeta$  (~ 70 kd). The biosafety of introducing such a large piece of exogenous protein into an oocyte has yet to be determined.

More recently, a Chinese group synthesised rhPLC $\zeta$  protein from a baculovirus/Sf9 cell expression system using the pFastBac-HTA plasmid. The rhPLC $\zeta$  protein was then purified by Ni<sup>2+</sup> and showed high enzymatic activity (326.8 U/mL, as measured by time-of-flight mass spectrometry). However, no further tests were provided in terms of protein functionality, efficiency, and the biosafety of this protein in terms of oocyte activation (Chen *et al.* 2019).



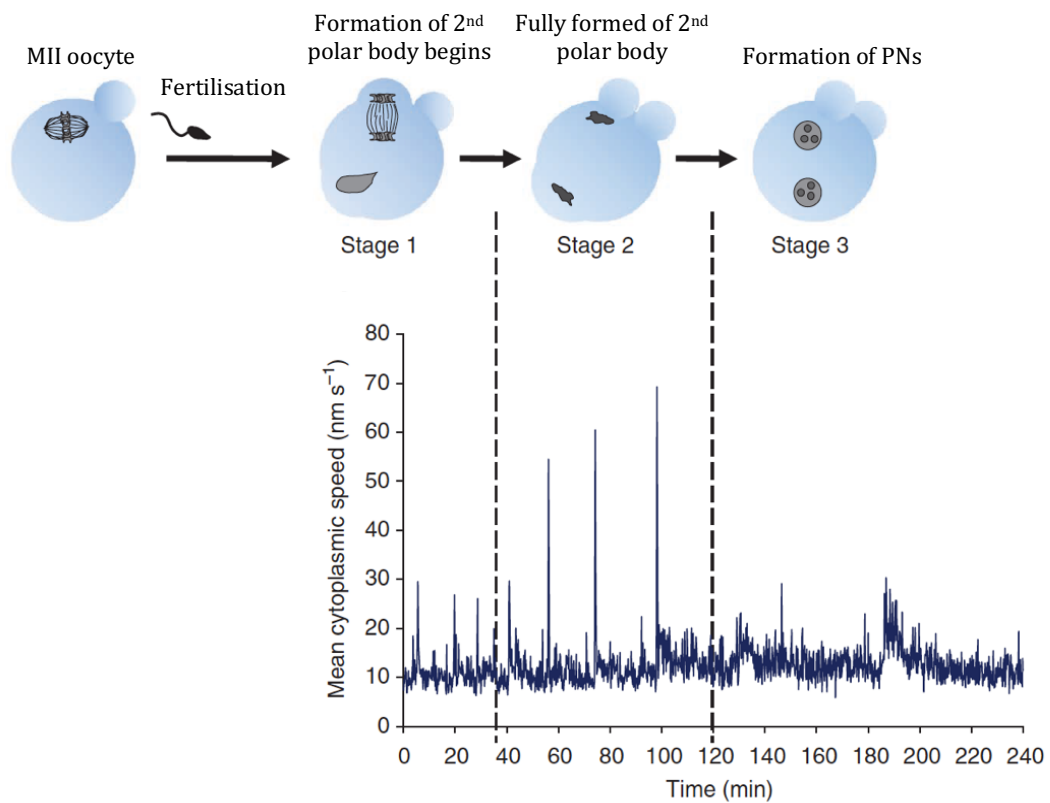
**Figure 5.5 The rhPLC $\zeta$  protein produced by Nomikos *et al.* (2013) rescued PLC $\zeta$ -associated infertility.** PLC $\zeta^{\text{H398P}}$  or PLC $\zeta^{\text{H233L}}$  cRNA were first microinjected into mouse oocytes, followed by 3 h culture to allow the expression of mutant protein. The rhPLC $\zeta$  was then microinjected into the same oocytes, which successfully generated Ca<sup>2+</sup> oscillations. Figures reproduced from Nomikos *et al.* (2013).

In this chapter, I prepared a rhPLC $\zeta$  protein using a vector, pHLsec, which was designed based on pLEXm (Aricescu *et al.* 2006). Previously, Kashir *et al.*, used the pTracer-EF/Bsd vector and failed to purify the rhPLC $\zeta$  from the cell lysates. (Kashir *et al.* 2011b). Unfortunately, mammalian cell lysates that contain cell debris and exogenous genomes are inappropriate for clinical use. To produce pure recombinant protein in the present study, the vector pHLsec (~4.5 kb) was engineered to contain a secretion signal sequence (SSS), a Fc-fusion site, and a 3C protease cleavage site. Accordingly, the target full-length PLC $\zeta$  cDNA could be incorporated into the vector via the Fc-fusion site. Then, the SSS site would allow the target protein, Fc-tagged PLC $\zeta$ , to be secreted into the culture medium, thus making the purification easier. The Fc-tag could then be removed via 3C protease,

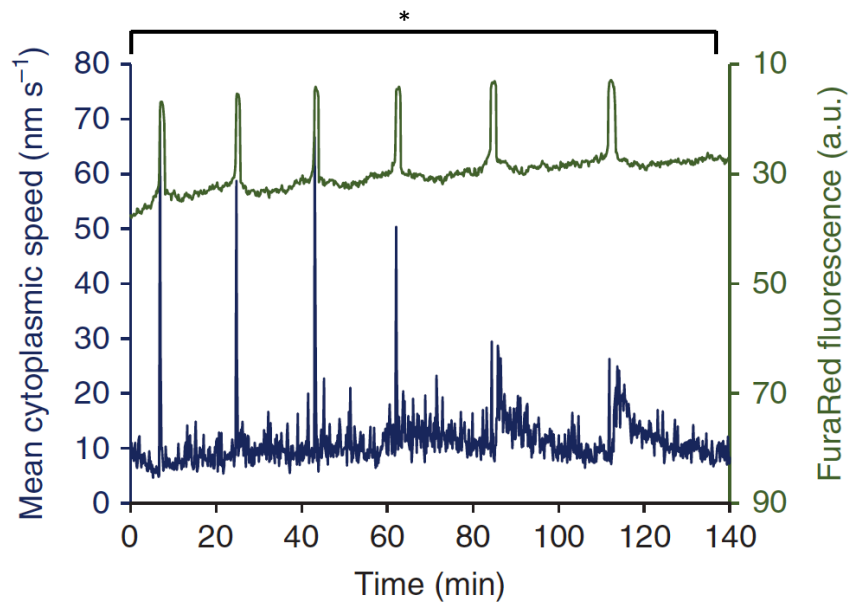


which generates untagged PLC $\zeta$ . Via these procedures, I hypothesised that rhPLC $\zeta$  protein could be generated and purified from Fc-tagged rhPLC $\zeta$  to untagged rhPLC $\zeta$ .

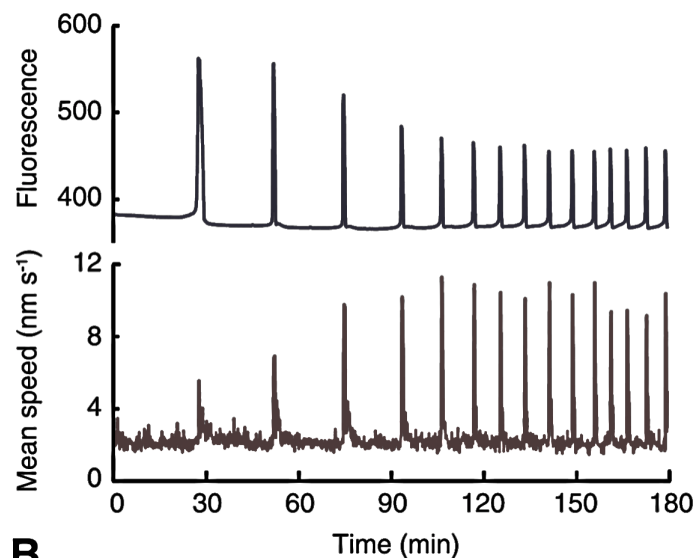
After the synthesis of Fc-tagged PLC $\zeta$  protein, I tested protein functionality by analysing the cytoplasmic movements of oocytes; this represents an indirect indicator of Ca<sup>2+</sup> oscillations. Cytoplasmic movements can therefore help us to understand the associations between oocyte movement at the 1-cell stage and the blastocyst stage, and therefore identify the potentials for oocytes to become embryos (Ajduk *et al.* 2011). Ajduk *et al.* analysed the cytoplasmic dynamics of mouse oocytes by performing particle image velocimetry (PIV), and found that mammalian oocytes displayed rhythmical cytoplasmic movements which were coordinated with the formation of the 2<sup>nd</sup> polar body that were triggered by sperm fusion (**Figure 5.6**)(Ajduk *et al.* 2011). These authors further identified that changes in cytoplasmic movements of mouse oocytes were in synchrony with Ca<sup>2+</sup> oscillations (**Figure 5.7**). In line with this, Swann *et al.* further showed that the microinjection of PLC $\zeta$  cRNA into human oocytes caused delayed Ca<sup>2+</sup> oscillations, and each Ca<sup>2+</sup> peak was in synchrony with cytoplasmic movements in the oocyte (**Figure 5.8**)(Swann *et al.* 2012). These authors further proposed that using PIV analysis to observe cell movement can provide a non-invasive approach with which to evaluate oocyte activation (Swann *et al.* 2012). Therefore, this chapter used PIV analysis as a non-invasive indicator of Ca<sup>2+</sup> oscillations to evaluate the activation efficiency of rhPLC $\zeta$  in mouse oocytes.



**Figure 5.6 Cytoplasmic movements and morphological changes in a representative mouse oocyte after fertilisation.** After sperm fusion, the mean cytoplasmic velocity of a representative mouse oocyte exhibits oscillatory patterns that are coordinated with the formation of the 2<sup>nd</sup> polar body (Stage 2). Figures reproduced from Ajduk *et al.* (2011).



**Figure 5.7 Oscillatory patterns of cytoplasmic movements and the  $\text{Ca}^{2+}$  release of a representative mouse oocyte after fertilisation.** \*: peaks in mean cytoplasmic velocity of a representative mouse oocyte (blue) were coordinated with  $\text{Ca}^{2+}$  oscillations (green) after normal fertilisation. Figure reproduced from Ajduk *et al.* (2011).



**Figure 5.8 Cytoplasmic movements and  $\text{Ca}^{2+}$  oscillations in a representative mouse oocyte after PLC $\zeta$  cRNA injection.** After the microinjection of PLC $\zeta$  cRNA into a mouse oocyte, the oscillation pattern of  $\text{Ca}^{2+}$  release (upper panel) coincided with cytoplasmic speed peaks (lower panel). Figure reproduced from Swann *et al.* (2012).

In terms of the general mechanisms underlying  $\text{Ca}^{2+}$  signalling pathways, previous studies have shown that most  $\text{PIP}_2$  resides in the plasma membrane of somatic cells and that this is responsible for the generation of  $\text{InsP}_3$  and  $\text{Ca}^{2+}$  release, following stimulation with an agonist (Gamper and Shapiro 2007). Furthermore, other isoforms of PLC, apart from  $\text{PLC}\zeta$ , have all been found to target phospholipase-based residues in the plasma membrane, in response to stimulation with an agonist (Watt *et al.* 2002; Gamper and Shapiro 2007; Yu *et al.* 2012).

However,  $\text{PLC}\zeta$  does not appear to target  $\text{PIP}_2$  in the plasma membrane (Yu *et al.* 2012). Previous immunofluorescence studies expressed  $\text{PLC}\zeta$  in somatic cells, or microinjected  $\text{PLC}\zeta$  cRNA into mouse oocytes, but failed to detect any fluorescence labelling of  $\text{PLC}\zeta$  in the plasma membrane (Yoda *et al.* 2004; Ito *et al.* 2008a; Phillips *et al.* 2011). In line with these observations, the depletion of plasma  $\text{PIP}_2$  did not interfere with  $\text{PLC}\zeta$ -induced  $\text{Ca}^{2+}$  oscillations (Yu *et al.* 2012). Yu *et al.* further suggested that there might be only small amounts of  $\text{PLC}\zeta$  localised to the plasma membrane, which is insufficient for the immunological detection (Yu *et al.* 2012). Alternatively, another form of  $\text{PIP}_2$  may exist for  $\text{PLC}\zeta$  hydrolysis. Indeed,  $\text{PIP}_2$  has been identified in the intracellular vesicles of mouse oocytes, and the microinjection of  $\text{PLC}\zeta$  cRNA has been shown to synchronise with the reduction of  $\text{PIP}_2$  levels in these vesicles (Yu *et al.* 2012). Sanders *et al.* further suggested that  $\text{PIP}_2$  was distributed throughout the ooplasm and was associated with the generation of  $\text{InsP}_3$  and  $\text{Ca}^{2+}$  oscillations (Sanders *et al.* 2018). These findings suggest that cytoplasmic  $\text{PIP}_2$  may be the substrate for  $\text{PLC}\zeta$  during oocyte activation. However, the true function of the  $\text{PIP}_2$ -containing vesicles in the cytoplasm of mammalian oocytes remains unknown.

In addition, it is not known how PLC $\zeta$  translocates into the oocyte cytoplasm and targets PIP $_2$  in the cytoplasm at normal fertilisation. Previous studies exclusively microinjected PLC $\zeta$  cRNA/protein into mouse oocytes, which inevitably disrupts the cellular structure and homeostasis of the oocytes (Yoda *et al.* 2004; Kashir *et al.* 2011b; Yoon *et al.* 2012; Yu *et al.* 2012; Nomikos *et al.* 2013; Sanusi *et al.* 2015; Sanders *et al.* 2018). The failure to detect PIP $_2$  in the plasma membrane of mammalian oocytes could be due to the disruption of the plasma membrane upon microinjection. Interestingly, previous work by our laboratory (unpublished) suggested that, instead of microinjection, the simple incubation of rhPLC $\zeta$  protein with oocytes could cause oocyte activation. This indicated that a possible alternative mechanism might exist that involves the plasma membrane. Indeed, *in-vivo* mating of fertile female mice with KO mice lacking the PLC $\zeta$  gene still generated viable pups, thus supporting the fact that a complementary mechanism may exist that operate when the classical Ca $^{2+}$  cascade is disabled or partly dysfunctional (Nozawa *et al.* 2018). Based on these observations and evidence, this chapter includes a pilot study, which, for the first time, investigated whether rhPLC $\zeta$  protein could activate mouse oocytes by simple incubation and to investigate the possibility of a mechanism involving the plasma membrane.

In this chapter, I aimed to evaluate the activation efficiency of current AOAs (GM508 and ionomycin), synthesise 'in-house' rhPLC $\zeta$  protein using a novel pHLsec vector, examine the functionality of rhPLC $\zeta$ , and, eventually, compare whether rhPLC $\zeta$  is more effective than current AOAs in terms of activation efficiency. I chose the three AOA protocols that are most commonly used by different research/clinical groups and have been proven to be effective. GM508 comes with a standard protocol that has been used clinically in humans (Ebner *et al.* 2012; Ebner *et al.* 2015a;

Nazarian *et al.* 2019). AOA treatments featuring 10  $\mu$ M ionomycin for both mouse and human oocytes were recommended by both Heytens *et al.* (2008) and Nikiforaki *et al.* (2016). AOA with 3  $\mu$ M ionomycin was derived from a nuclear transfer method (Yamada *et al.* 2014), and has been further optimised for the activation of human oocytes by another group member, Professor Chris Graham. I then aimed to synthesise a rhPLC $\zeta$  protein using the novel pHLsec vector, incubate the protein with mouse oocytes, and investigate its activation efficiency by various methods (i.e., morphology observation, cytoplasmic labelling, and fluorescence labelling). My working hypothesis was that an 'in-house' rhPLC $\zeta$  could be produced from a mammalian cell line, using the pHLsec vector, and that this protein would be functional and be able to serve as a therapeutic reagent for activating mouse oocytes with better efficiency than current AOAs.

## **5.2 Methods and materials**

### **5.2.1 Preparation of mouse oocytes**

Mouse oocytes at metaphase II were obtained from super-ovulated female mice ovaries (strain C57BL/6). Ovulation was stimulated by the injection of 5 IU of pregnant mare's serum gonadotrophin, followed by the injection of HCG after 48 hours. Oocytes were collected 15-16 h after HCG injection. Cumulus cells were removed with 0.3 mg/ml of hyaluronidase from bovine testes (Sigma-Aldrich, UK), followed by 2-3 min of incubation and gentle pipetting until the cumulus mass was dissociated. Denuded oocytes were then washed in M2 medium (Sigma, UK) and incubated in KSOM medium (Merck Millipore, UK) at 37.5°C with 5% CO<sub>2</sub> until ready for oocyte activation tests.

### 5.2.2 AOA using GM508

GM508 CultActive (GYNEMED, Germany) was used for oocyte activation and was carried out using a protocol described elsewhere (Ebner *et al.* 2012). In brief, GM508 was first equilibrated at 37.5°C with 5% CO<sub>2</sub> for 4 h before use. Then, mouse oocytes were exposed to GM508 for 15 min followed by two washes in M2 medium. Activated oocytes were then cultured with pre-equilibrated KSOM for further development. Mouse oocytes receiving no AOA treatment were used as negative controls, as mouse oocytes can be self-activated without any treatment. Evaluation of parthenogenetic development was carried out after 4, 24, 48 and 96 h of post-activation.

### 5.2.3 AOA using ionomycin and ethanol

In this part of my study, two ionomycin protocols were applied based on published activation protocols (Heytens *et al.* 2008; Yamada *et al.* 2014). I compared two activation protocols for the use of ionomycin, as procedures varied in different literature (Heytens *et al.* 2008; Yamada *et al.* 2014). For AOA using 3 µM ionomycin (Sigma, UK) (Yamada *et al.* 2014), 3 µM of ionomycin and 2 µg/ml of Trichostatin (Sigma, UK) were separately dissolved in aliquots of KSOM medium 30 min before activation. Then, oocytes were first exposed to ionomycin for 5 min and then to Trichostatin for 16 h. Alternatively, for AOA with 10 µM ionomycin, (Heytens *et al.* 2008), 10 µM of ionomycin and 2 µg/ml of Cytochalasin D (Sigma, UK) was dissolved separately in KSOM medium 30 min before activation. Then, oocytes were exposed to ionomycin twice for 10 min with a 30 min interval, followed by incubation with Cytochalasin D for 3 h. After either AOA method, mouse oocytes were then cultured in KSOM, and the activation rate was assessed at the same time points as with the

GM508 experiment. For AOA experiments with 10  $\mu$ M ionomycin, I used 7% ethanol as a positive control to activate mouse oocytes. The protocol for AOA with ethanol was described previously by Rogers *et al.* (2006). **Table 5.1** describes the AOA protocols that I used in the AOA experiment for mouse oocytes.

**Table 5.1 Summary of artificial oocyte activation (AOA) protocols for mouse oocytes**

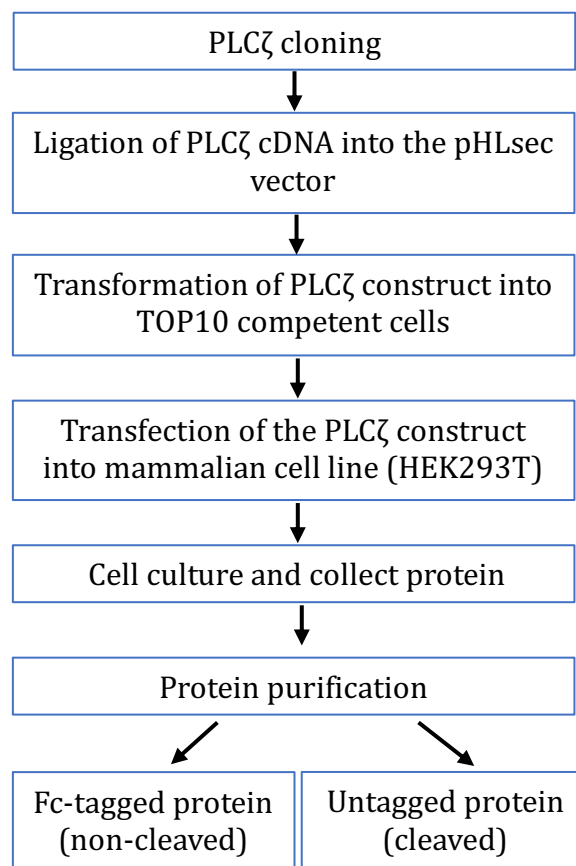
| Activation reagent         | Protocol                                                                     |                                    |
|----------------------------|------------------------------------------------------------------------------|------------------------------------|
|                            | Step 1                                                                       | Step 2                             |
| GM508                      | <i>Ready-to-use</i> for 15 min                                               | None                               |
| Ionomycin                  | 3 $\mu$ M for 5 min                                                          | 2 $\mu$ g/ml trichostatin for 16 h |
| Ionomycin                  | 10 $\mu$ M for 10 min;<br>30 min in M2 medium;<br>then 10 $\mu$ M for 10 min | 2 $\mu$ g/ml cyto D for 3 h        |
| Ethanol (positive control) | 7% for 7 min                                                                 | 2 $\mu$ g/ml cyto D for 2 h        |
| Negative control           | KSOM medium                                                                  | KSOM medium                        |

Mouse oocytes arrested at meiosis II received AOA following four different protocols. GM508: commercial-available chemicals incorporating calcimycin; cyto D: cytochalasin D.

#### 5.2.4 Synthesis of recombinant human PLC $\zeta$ (rhPLC $\zeta$ ) protein

**Figure 5.9** shows a schematic flow chart of how I generated rhPLC $\zeta$  from a mammalian cell line, HEK293T, with the protocol optimised from Kashir *et al.* (2011b).

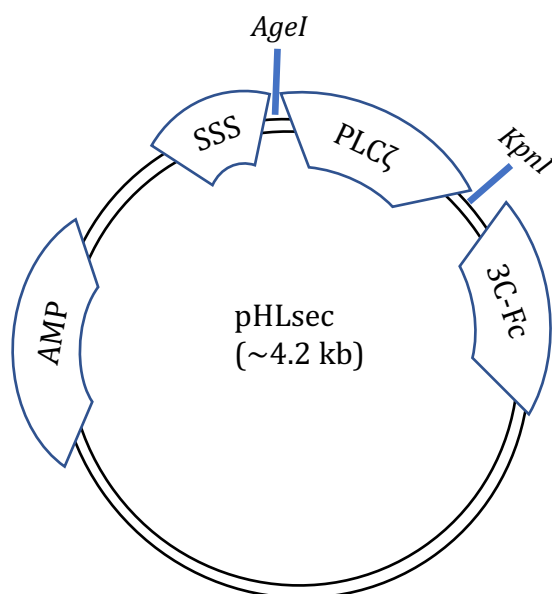




**Figure 5.9** Flow chart showing rhPLC $\zeta$  synthesis from a mammalian cell line, human embryonic kidney 293 cells (HEK293T).

#### 5.2.4.1 PLC $\zeta$ cloning and ligation of PLC $\zeta$ cDNA into the pHL vector

A map of the pHLsec vector is shown in **Figure 5.10**. The DNA sequence encoding the full-length human PLC $\zeta$  was amplified and inserted into the pHLsec vector. The pHLsec vector and the PLC $\zeta$  construct used in this chapter are generous gifts from Dr. A. Radu Aricescu and our laboratory manager, Mrs Johns. The PLC $\zeta$  construct has been fully verified with sequencing (Source Bioscience, Oxford). The synthesis of the PLC $\zeta$  plasmid is described in **Appendix 5**.



**Figure 5.10 The protein expression construct used to make recombinant PLC $\zeta$ .** The full-length human PLC $\zeta$  cDNA was inserted into the pHlsec vector using *AgeI* and *KpnI* restriction enzymes. AMP: ampicillin, SSS: secretion signal sequence, 3C-Fc: 3C cleavage site followed by Fc-tag. The Fc tag can therefore be cleaved with human rhinovirus protease.

#### 5.2.4.2 Plasmid transformation

The PLC $\zeta$  construct was then transformed into TOP10 competent *E. coli* cells (Thermo Fisher Scientific, UK). In brief, the construct, and the TOP10 competent cells, were incubated on ice for 30 min and then incubated at 42°C for 45s, followed by incubation on ice for 2 min. The transformation mixture was then cultured in 600  $\mu$ l of super optimal broth with catabolite repression medium (SOC medium, Thermo Fisher Scientific, UK) for 1h at 37°C, with rotation at 220 rpm. The mixture was then spread onto an agar plate containing 100  $\mu$ g/ml ampicillin (Sigma-Aldrich, UK) and

35 g/L luria broth with agar (Sigma-Aldrich, UK), followed by overnight incubation at 37°C to allow transformation. The next day, a single colony was selected and cultured in 100 ml of culture medium containing 25 g/L luria broth (Sigma-Aldrich, UK) for 16 h at 37°C.

The plasmid DNA (450-500 ng/μl) was then extracted from the culture medium with the 'HiSpeed plasmid Midi Kit (Qiagen, UK), following the manufacturer's protocol. The concentration of plasmid DNA was then measured with a spectrophotometer and determined by the following formula:

*plasmid DNA concentration (ng/μl) =*

*absorbance value at 260 nm wave length × optical density of one unit of DNA*

#### **5.2.4.3 Cell transfection**

Cell transfection was performed with the jetPEI® *in vitro* DNA transfection kit (Polyplus, France) in accordance with the manufacturer's protocol. HEK293T cells were cultured at 37.5°C in a 10 cm x 75 cm<sup>2</sup> flask with Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, UK) containing 10% Fetal Bovine Serum (FBS), 4mM glutamine and 100U/ml penicillin (Thermo Fisher Scientific, UK). For each flask, 30 μg of plasmid DNA was prepared with 150 nM NaCl (total volume was 500 μl), and 60 μl of jetPEI® was prepared with 150 nM NaCl (total volume was 500 μl). The jetPEI®-NaCl mixture was then added to the DNA-NaCl mixture and incubated for 30 min at room temperature. A total of 1 ml of jetPEI®-DNA mixture was added dropwise to one flask, following by incubation overnight at 37°C. The culture medium was then changed to DMEM without FBS 24 h after post-

transfection. Transfected cells were cultured for 5 days to allow secretion of the recombinant protein. After this, the culture media was collected and purified.

#### **5.2.4.4 Removal of the Fc tag and protein purification**

Complete™ Protease Inhibitor Cocktail (0.2 mM EDTA, Merck, UK) was added to the collected media to prevent protein degradation. This was followed by centrifugation at 1500 g for 30 min. The supernatant was then collected and added to a Poly-Prep® chromatography column (Bio-Rad, USA) which contained a polyethylene membrane (30 µm of frit pore size) to retain the target protein. Then, 600 µl of protein-A-sepharose (GE Healthcare, UK) and 10 ml of PBS were added to the column; this helped to capture the target protein (1 ml captures 20 mg of protein) and to remove residue ethanol. To obtain the Fc-tagged protein (non-cleaved), 600 µl of glycine buffer (0.1 M, pH=3.0) was added to the column to elute the protein, and a microcentrifuge tube containing 60 µl of 1 M tris was used to collect and neutralise the eluates. To harvest the untagged protein (i.e., cleaved), 1x human rhinovirus (HRV) 3C reaction buffer and HRV protease enzyme were added. After an overnight incubation, the eluates were then collected.

#### **5.2.4.5 Protein identification**

The chemical reagents used in this part of the study were all from Thermo Fisher Scientific, UK, unless otherwise stated. SDS-NuPAGE electrophoresis and silver staining were used to visualise the target protein. To prepare a 30 µl sample for electrophoresis, 1x NuPAGE reducing agent and 1x NuPAGE loading buffer were mixed with the protein sample. The mixture, together with a Novex™ Sharp Pre-stained protein ladder, were incubated at 101°C for 5 min and were loaded onto a

NuPAGE™ 10% Bis-Tris protein gel. Covered with 1x NuPAGE MESS SDS running buffer, the sample and ladder were run at 160 V for 1 h. Silver staining was then performed with the Pierce™ Silver Stain Kit, following the manufacturer's protocol, in order to visualise the target protein.

#### **5.2.4.6 Transfer of rhPLCζ into Ca<sup>2+</sup>-free buffer**

Ca<sup>2+</sup>-free buffer was used to store the rhPLCζ protein, prior to functional testing. This avoids Ca<sup>2+</sup>-induced oocyte activation in subsequent experiments. In brief, Ca<sup>2+</sup>-free solution was prepared with 120 mM KCl and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in ultraclean water, with a pH of 7.4. The sartorius VIVASPIN 20 (Appleton Woods, UK), which contained an ultrafiltration membrane, was used as a filter to capture the protein and to exchange the buffer. Ca<sup>2+</sup>-free buffer was first loaded into the filter and centrifuged at 1000 g for 5 min. After disposal of the flow-through, the protein mixture, and 3 ml of Ca<sup>2+</sup>-free buffer, was added to the filter, followed by centrifugation at 1000 g for 10 min. The protein was then retained on the filter. Centrifugation at 1000 g for 10 min was performed twice after discarding the flow-through and 2 ml of Ca<sup>2+</sup>-free buffer was added each time. The concentrations of Fc-tagged and untagged proteins in Ca<sup>2+</sup>-free buffer were then measured with a spectrophotometer.

#### **5.2.5 AOA using rhPLCζ**

The protocol for oocyte activation using rhPLCζ was derived from our previous method (Kashir *et al.* 2011b), with some modifications. In brief, mouse oocytes were incubated with either 47-63 µg/ml of untagged rhPLCζ or 147 µg/ml of Fc-tagged rhPLCζ at 37.5°C for 15 or 30 min. Then, oocytes were washed twice

with M2 medium, followed by culture in KSOM medium. Activation rate was then assessed at the same time points as with the other AOA experiments (i.e., GM508, Section 5.2.2 and ionomycin, Section 5.2.3). Along with the rhPLC $\zeta$  group, two negative control groups were included; a buffer control using mouse oocytes that were treated with only Ca<sup>2+</sup>-free buffer (i.e., HEPES and KCl), and a control group using mouse oocytes with no AOA treatment.

### **5.2.6 Nuclear staining of mouse oocytes**

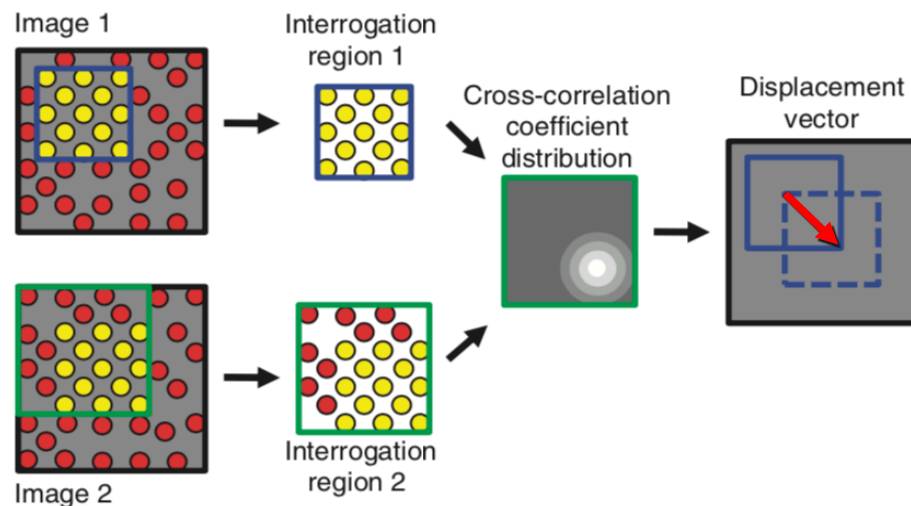
Nuclear staining was used to check PN formation. The protocol was optimised previously in Chapter 2 (Section 2.2.4). Fixation and permeabilisation were the same as described in Chapter 2 (Section 2.2.4). Mouse oocytes were then incubated with 5% BSA for 30 min and washed with PBS, followed by incubation with 4  $\mu$ l of DAPI for 5 min. The oocytes were then evaluated under a fluorescence microscope for PN formation.

### **5.2.7 Cytoplasmic movements of mouse oocytes activated by rhPLC $\zeta$**

The cytoplasmic movement study was carried out with the assistance of Professor Chris Graham. Following oocyte activation, mouse oocytes were cultured in 300  $\mu$ l of KSOM covered with 150  $\mu$ l of oil at 37.5°C with 5% CO<sub>2</sub>. After incubation with rhPLC $\zeta$ , oocytes were imaged with a standard microscope (Zeiss Axiovert) under  $\times 20$  and  $\times 40$  magnification, with transmitted light at 635 nm. For the first 2 h of recording, images of oocyte movements were taken every 10s (720 frames in total), followed by 21.5 h of recording with 30 min intervals (42 frames in total).

The cytoplasmic movements of mouse oocyte were analysed with filmed images, based on the PIV method described by Ajduk *et al.* (2011). This method is

based on fluid dynamics (Willert and Gharib 1991; Westerweel 1997) and involves the cross-linking of sequential cell images and calculation of the coefficient distribution according to interrogation regions (**Figure 5.11**). Then, the displacement vector over time was calculated based on the changes in location between the current image and the previous image (**Figure 5.11**). The mean velocity of cell movement was then calculated by the magnitude of vectors in each region.



**Figure 5.11 The algorithm used for particle image velocimetry (PIV) analysis.** Cross-correlating cell images were taken (Image 1 and 2) and divided into square interrogation regions (Interrogation region 1 and 2). Then, the cross-correlation coefficient distribution was calculated by placing Interrogation region 1 in Interrogation region 2. Following this, the displacement vector was calculated by the patterns of contrast of one pixel over many pixels. Accordingly, the movement of cytoplasmic granules (i.e., speed of cytoplasmic movement) could be measured. Figures reproduced from Ajduk *et al.* (2011).

### 5.2.8 Statistical analysis

The statistical analysis of oocyte development after activation was carried out using the Fisher's exact test or Chi-square test where appropriate, using Prism

software 5.0 (Graph-Pad, CA, USA). Statistical significance was defined when the p value was <0.05.

## 5.3 Results

### 5.3.1 AOA using GM508

The development and morphology of oocytes/embryos were checked each day for four days. Two replicates, including a total of 56 oocytes, showed that only 5 oocytes developed to the 2-cell stage when assessed 24 h after AOA, but none of these oocytes developed to the blastocyst stage when assessed 96 h after AOA (**Table 5.2**). According to the manufacturer's protocol, oocytes were incubated with GM508 for 15 min; however, few mouse oocytes developed into the 2-cell stage (27.7% of the total number of treated oocytes). Considering that different species may response to drugs in different ways, the incubation time was increased to 30 min in the second round to investigate whether a longer incubation period could improve activation rate. Unfortunately, this did not result in oocyte activation (0%).

**Table 5.2 Effects of GM508 on mouse oocyte activation and development.**

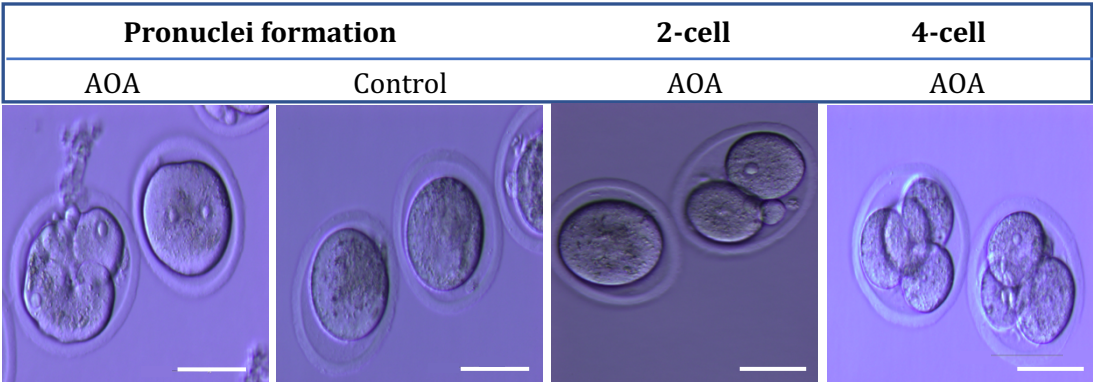
| Group numbers | Incubation time | Tested oocytes, n | 2-cell embryos at 24 h, n (% of treated) | 4-cell embryos at 96 h, n (% of treated) |
|---------------|-----------------|-------------------|------------------------------------------|------------------------------------------|
| 1             | GM508           | 11                | 3 (36)                                   | 0 (0)                                    |
|               | Control         | 24                | 7 (29.1)                                 | 3 (12.5)                                 |
| 2             | GM508           | 7                 | 2 (28.5)                                 | 1 (14.2)                                 |
|               | GM508           | 7                 | 0 (0)                                    | 0 (0)                                    |
|               | Control         | 7                 | 0 (0)                                    | 0 (0)                                    |

Mouse oocytes arrested at meiosis II were incubated with GM508 for 15 or 30 min, respectively. Activation efficiency was evaluated based on 2-cell and 4-cell formations at 24 h and 96 h after activation.



### 5.3.2 AOA using ionomycin

In contrast to my previous activation study (Section 5.3.1), I additionally evaluated PN formation in oocytes 4 h after AOA in this next experiment, as it is debateable whether the morphological sign of oocyte activation is based on PN formation (4 h after AOA), or the 2-cell stage (24 h after AOA). Therefore, I recorded both parameters in this experiment. For AOA with 3  $\mu$ M ionomycin, four replicate experiments were performed, and a total of 74 mouse oocytes were selected randomly for activation (AOA group); 77 oocytes served as controls. **Figure 5.12** shows representative images of mouse oocyte development after treatment with ionomycin, when compared to the control group. Judged by PN formation, the rate of oocyte activation was significantly higher in the AOA group than the controls (10.8% vs. 1.2%,  $p < 0.05$  by the Fisher's exact test, **Table 5.3**). While more oocytes developed to the 2-cell stage in the AOA group, this was not significantly different to the controls (21.6% vs. 11.6%,  $p = 0.12$  by the Fisher's exact test). There was no significant difference in terms of the other stages of embryo development when compared between the two methods.



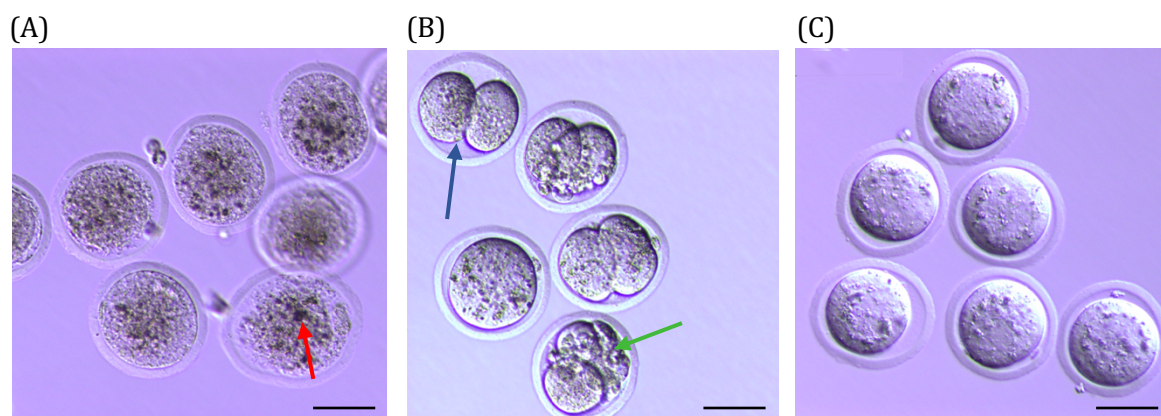
**Figure 5.12 Representative images of oocyte development with/without 3  $\mu$ M ionomycin.** Images were taken at 4 h (pronuclei formation, red arrow), 24 h (2-cell, white asterisk), 48 h (4-cell, orange colour) after artificial oocyte activation (AOA), under x40 magnification, scale bar=50  $\mu$ m.

**Table 5.3 Effects of ionomycin (3μM) on mouse oocyte activation and development.**

| Group numbers |         | Treated oocytes, n | PN n (% of treated)   | 2-cell embryos, n (% of treated) | 4-cell embryos, n (% of treated) | Fragmented /dead n (% of treated) |
|---------------|---------|--------------------|-----------------------|----------------------------------|----------------------------------|-----------------------------------|
| 1             | AOA     | 12                 | 5 (22.7)              | 4 (57.1)                         | 0 (0)                            | 15 (68.1)                         |
|               | Control | 18                 | 0 (0)                 | 3 (17.6)                         | 2 (11.8)                         | 1 (5.6)                           |
| 2             | AOA     | 18                 | 0 (0)                 | 3 (16.7)                         | 0 (0)                            | 0 (0)                             |
|               | Control | 11                 | 0 (0)                 | 1 (9.1)                          | 0 (0)                            | 3 (27.2)                          |
| 3             | AOA     | 9                  | 1 (11.1)              | 5 (55.5)                         | 0 (0)                            | 2 (22.2)                          |
|               | Control | 14                 | 1 (7.1)               | 2 (16.7)                         | 1 (8.3)                          | 0 (0)                             |
| 4             | AOA     | 25                 | 2 (8)                 | 4 (20.0)                         | 4 (20)                           | 0 (0)                             |
|               | Control | 34                 | 0 (0)                 | 3 (9.1)                          | 0 (0)                            | 0 (0)                             |
| Total (n=4)   | AOA     | 74                 | 8 (10.8) <sup>a</sup> | 16 (21.6)                        | 4 (5.4)                          | 17 (22.9)                         |
|               | Control | 77                 | 1 (1.2)               | 9 (11.6)                         | 3 (3.8)                          | 4 (5.1)                           |

Mouse oocytes were incubated with 3μM ionomycin for 5 min, followed with post-treatment with 2 μg/ml trichostatin for 16 h. A total of four replicates were performed. Activation efficiency was evaluated via morphological observations; PN (4 h), 2-cell embryos (24 h) and 4-cell embryos (48 h) formations after AOA. AOA: artificial oocyte activation; PN: pronuclei; Conc.: concentrations; <sup>a</sup> denotes a significant difference when compared with the corresponding control group according to the Fisher's exact test (p<0.05).

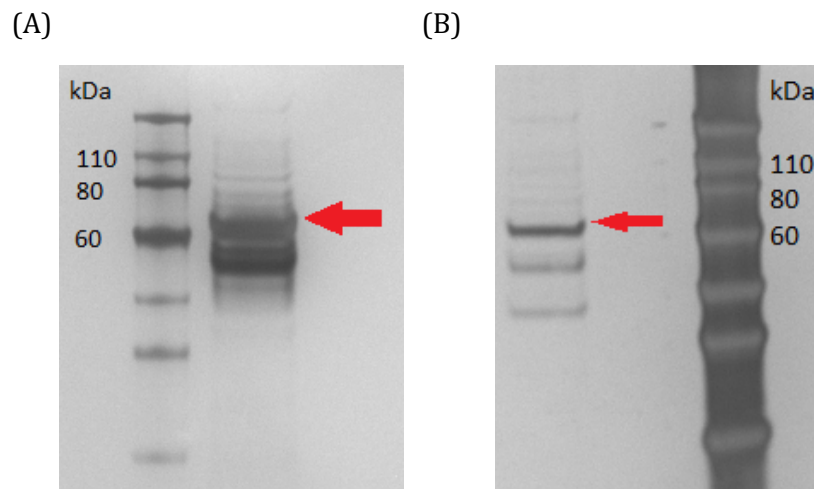
For AOA with a double exposure to 10 μM ionomycin, I followed the protocol described by Heytens *et al.* (2008) and Nikiforaki *et al.* (2016); I also performed three replicate experiments. However, oocytes that were treated with 10 μM ionomycin started to show signs of necrosis; as a result, a total of 52 oocytes (52 in total) died 24 h after ionomycin exposure. Oocytes from the ethanol group (positive control, 6 out of 9 activated, 66.6%), and control groups, remained alive 24 h after AOA (**Figure 5.13**).



**Figure 5.13 Morphology of mouse oocytes treated with different artificial oocyte activators (AOAs).** Images were taken 24 h after treatment with (A) 10  $\mu$ M ionomycin (B) 7% ethanol, and (C) no AOs. Necrosis (red arrow) can be seen in oocytes treated with 10  $\mu$ M ionomycin, which indicates necrosis. Blue arrow shows 2-cell formation, and green arrow shows 2-cell embryo with fragmentation. Scale bar = 50  $\mu$ m.

### 5.3.3 AOA using rhPLC $\zeta$

Both untagged (cleaved) and Fc-tagged (non-cleaved) rhPLC $\zeta$  protein were tested using electrophoresis and silver staining (**Figure 5.14 A and B**, respectively). The target size of rhPLC $\zeta$  protein is  $\sim$ 70 kDa, while the bands of Fc-tagged and untagged rhPLC $\zeta$  protein on the gel were  $\sim$ 65 kDa and  $\sim$ 61 kDa. The concentrations of untagged rhPLC $\zeta$  protein and Fc-tagged rhPLC $\zeta$  protein were 147  $\mu$ g/ml and 47-63  $\mu$ g/ml, respectively.



**Figure 5.14 Recombinant human PLC $\zeta$  (rhPLC $\zeta$ ) protein on a NuPAGE™ 10% Bis-Tris protein gel. (A) Fc-tagged rhPLC $\zeta$ , indicated by red arrow. (B) Untagged rhPLC $\zeta$  indicated by blue arrow.**

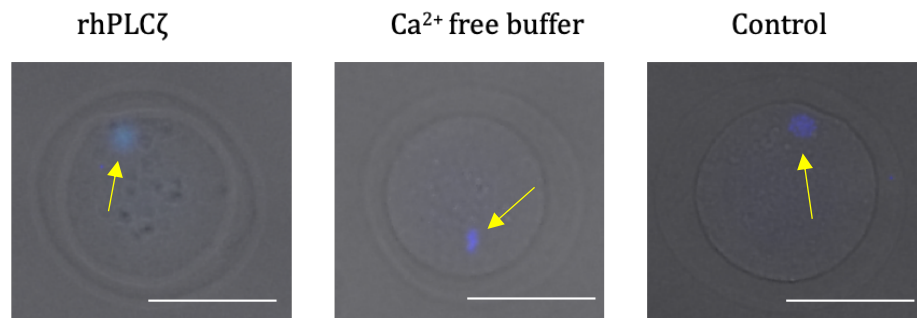
After removing the Fc tag, untagged rhPLC $\zeta$  was used for mouse oocyte activation. However, only 4 out of 90 oocytes tested reached the 2-cell stage, after incubation with rhPLC $\zeta$  for 15 min (**Table 5.3**). A subsequent experiment, using Fc-tagged rhPLC $\zeta$ , showed that the activation rate was low (11.9%, **Table 5.3**). In the next experiment, the incubation time was increased to 30 min, to investigate whether a longer incubation period could improve activation outcome. The rationale of doing this was because simple incubation allowed rhPLC $\zeta$  to only interact with the oocyte membrane, and rhPLC $\zeta$  may take longer for the reactions from cell membrane to the cytoplasm. A 30 min incubation period resulted in an activation rate that was significantly higher than the buffer control and negative controls (42.3% vs. 8.1% and 18.75%,  $p < 0.001$ , **Table 5.4**). In addition, 2 of the 22 embryos at the 2-

cell stage developed further to the 4-cell stage. Additionally, PN formation was not observed in rhPLC $\zeta$ -treated oocytes 4 h after activation. To further examine the activation effect, nuclear staining was performed 4 h after AOA. However, no PN was observed in oocytes from all three groups; only single nucleus was observed (Figure 5.15).

**Table 5.4 Effects of recombinant human PLC $\zeta$  (rhPLC $\zeta$ ) on the development of mouse oocyte**

| Groups                  | Incubation time | Treated oocytes, n | 2-cell embryos, n (% of treated) | Fragmentation n (% of treated) |
|-------------------------|-----------------|--------------------|----------------------------------|--------------------------------|
| Untagged rhPLC $\zeta$  | 15 min          | 90                 | 4 (4.4)                          | 0 (0)                          |
| Buffer Control          |                 | 75                 | 4 (5.3)                          | 6 (8.0)                        |
| Control                 |                 | 105                | 5 (4.8)                          | 1 (0.9)                        |
| Fc-tagged rhPLC $\zeta$ | 15 min          | 42                 | 5 (11.9)                         | 4 (9.5)                        |
| Buffer Control          |                 | 29                 | 4 (13.8)                         | 0 (0)                          |
| Control                 |                 | 31                 | 1 (3.2)                          | 3 (9.7)                        |
| Fc-tagged rhPLC $\zeta$ | 30 min          | 52                 | 22 (42.3)***                     | 3 (5.8)                        |
| Buffer Control          |                 | 37                 | 3 (8.1)                          | 1 (2.7)                        |
| Control                 |                 | 48                 | 9 (18.75)                        | 13 (27.1)                      |

The concentrations of untagged rhPLC $\zeta$  protein and Fc-tagged rhPLC $\zeta$  protein used to activate mouse oocytes were 147  $\mu$ g/ml and 47-63  $\mu$ g/ml, respectively. Embryos at the 2-cell stage and fragmentation were observed 24 h after AOA. PN: pronuclei formation; Conc.: concentrations; \*\*\* denotes a significant difference from the corresponding control groups, according to the Chi-square test ( $p < 0.001$ ).



**Figure 5.15 Representative immunofluorescence images of mouse oocytes 4 h after artificial oocyte activation (AOA).** Mouse oocytes were treated with recombinant human PLC $\zeta$  (rhPLC $\zeta$ ) or Ca<sup>2+</sup> free buffer (buffer control) and compared to a control group (no AOA treatment). Yellow arrows indicate the presence of a single nucleus. Images of nuclear staining were captured at 400ms exposure. Scale bar = 50  $\mu$ m

#### 5.3.4 Cytoplasmic movements of mouse oocytes activated by rhPLC $\zeta$

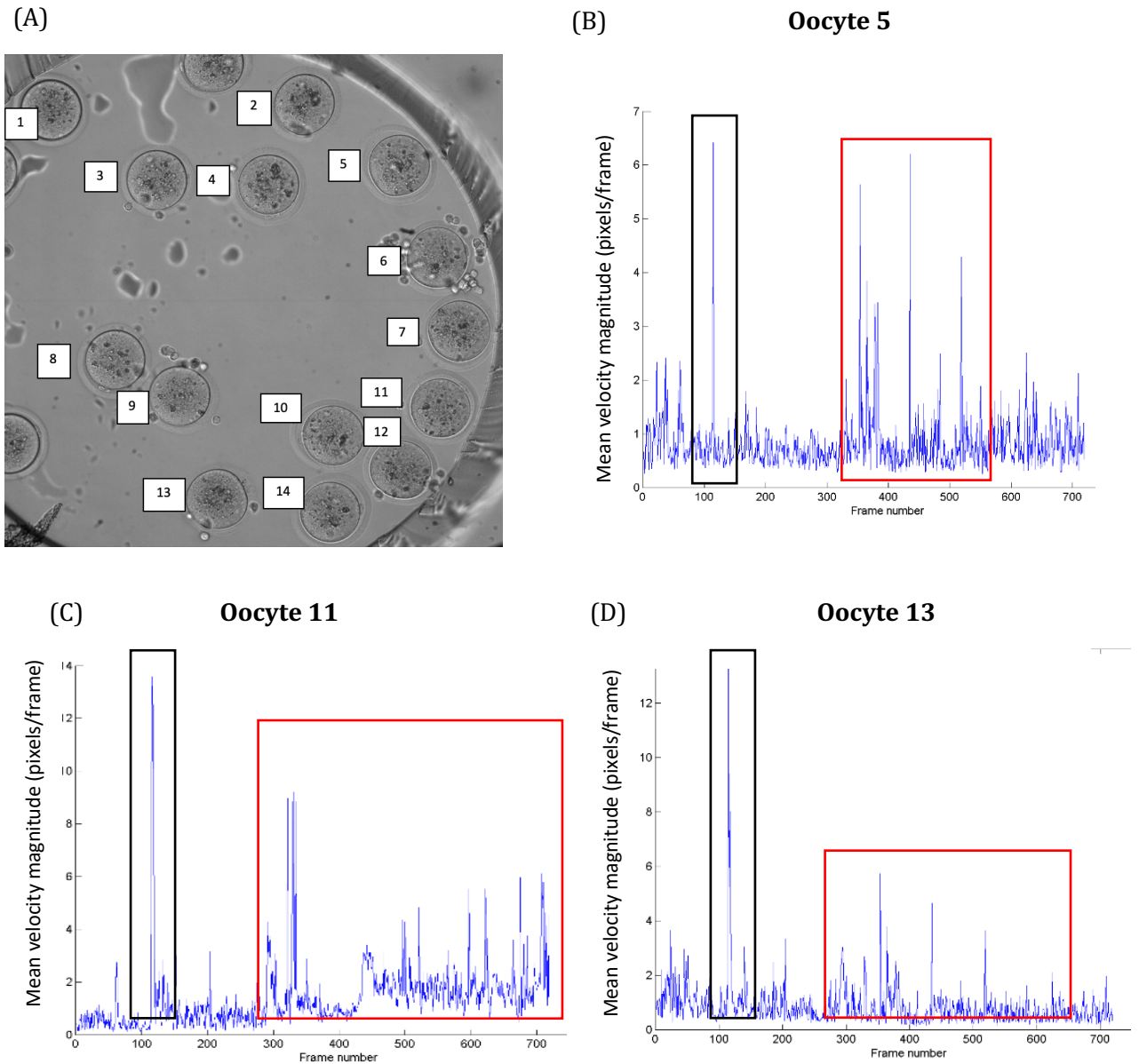
Eighteen MII mouse oocytes received AOA with rhPLC $\zeta$ ; one of these formed pronuclei and a 2<sup>nd</sup> polar body 3 h after activation. This oocyte developed further to the 2-cell stage 15 h after AOA. Fourteen MII mouse were incubated in Ca<sup>2+</sup>-free buffer for 10 min as a negative control; three of these underwent lysis. The others did not show any features of activation (i.e., no activation-related movement).

**Figure 5.16 (A)** shows a recording of 14/18 oocytes treated with rhPLC $\zeta$  after 2 h. Of these, oocytes 5, 11 and 13 showed rhythmic cytoplasmic movement (**Figure 5.16, B-E**); this is a sign relating to the formation of 2<sup>nd</sup> polar body at oocyte activation that represents a sign of Ca<sup>2+</sup> oscillations, as described by Ajduk *et al.* (2011). Oocytes 5, 11 and 13 showed a high mean velocity around frame 400~500 (in red box, **Figure 5.16, B-E**), indicating that there might be formation of the 2<sup>nd</sup>

polar body in these oocytes. However, there was no evidence to indicate that the other mouse oocytes exhibited activation-related movement after rhPLC $\zeta$ -AOA (**Figure 5.17**).

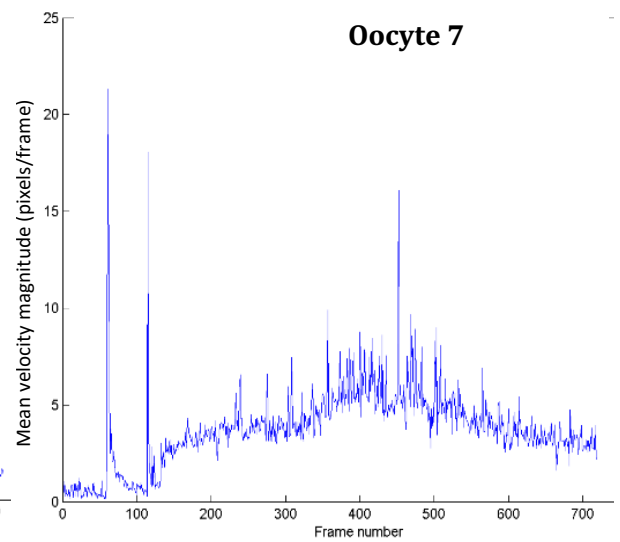
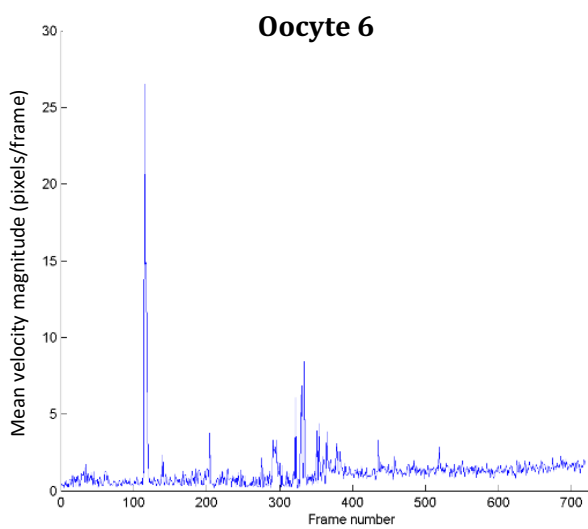
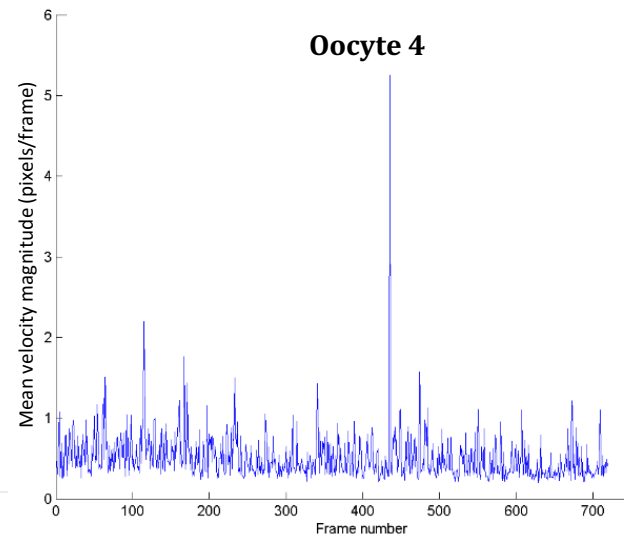
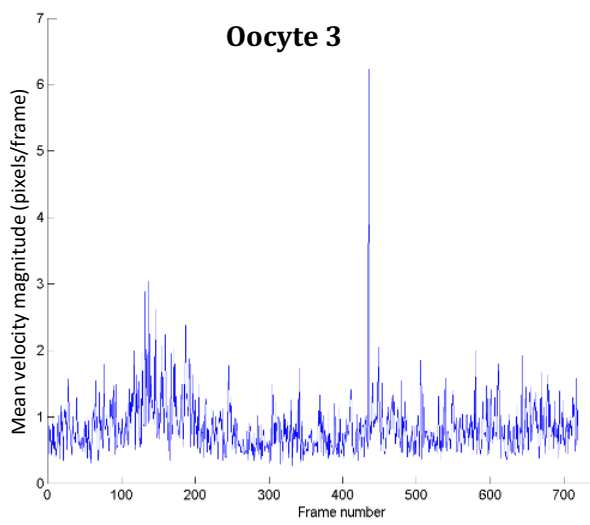
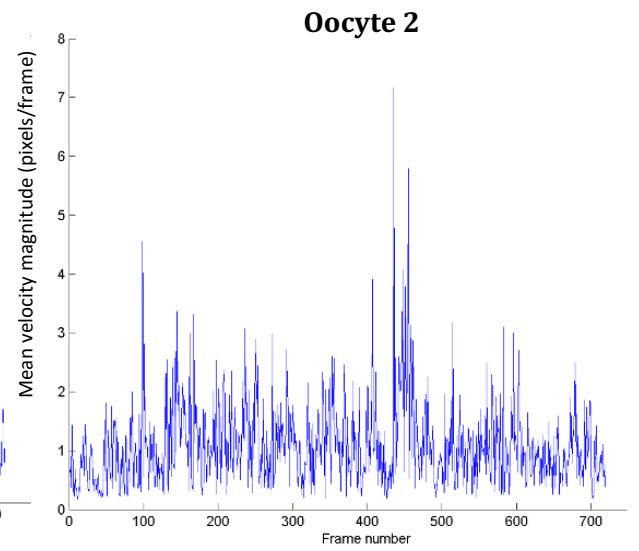
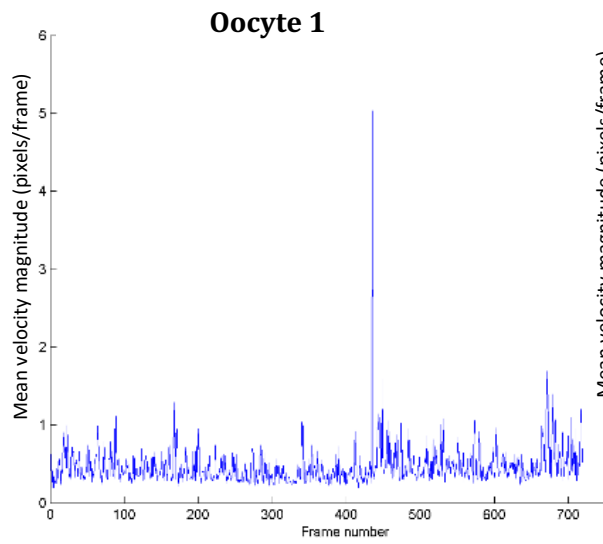
Because air bubbles interfered with focusing and recording, it was necessary to remove bubbles and restart the recording; this resulted in the oocytes being renumbered (**Figure 5.18**). In an attempt to discover further evidence of movement associated with polar body and pronuclei formation, a longer duration of filming was carried out (21.5 h in total, with 30 min intervals). Overall, a total of 15 rhPLC $\zeta$ -treated oocytes were filmed in the second experiment; one oocyte (number 3, **Figure 5.18**) formed a 2<sup>nd</sup> polar body and developed into the 2-cell stage, 3 h and 12 h after rhPLC $\zeta$ -AOA, respectively (**Figure 5.19**). Four oocytes (number 4, 10, 11 and 13 **Figure 5.18**) showed a high velocity of cytoplasmic movements which indicated the formation of the 2<sup>nd</sup> polar body and successful activation (**Figure 5.20**). However, no further morphological division was observed in these oocytes. The other 10 oocytes showed variable cytoplasmic movements but no convincing evidence for successful activation (**Figure 5.21**).

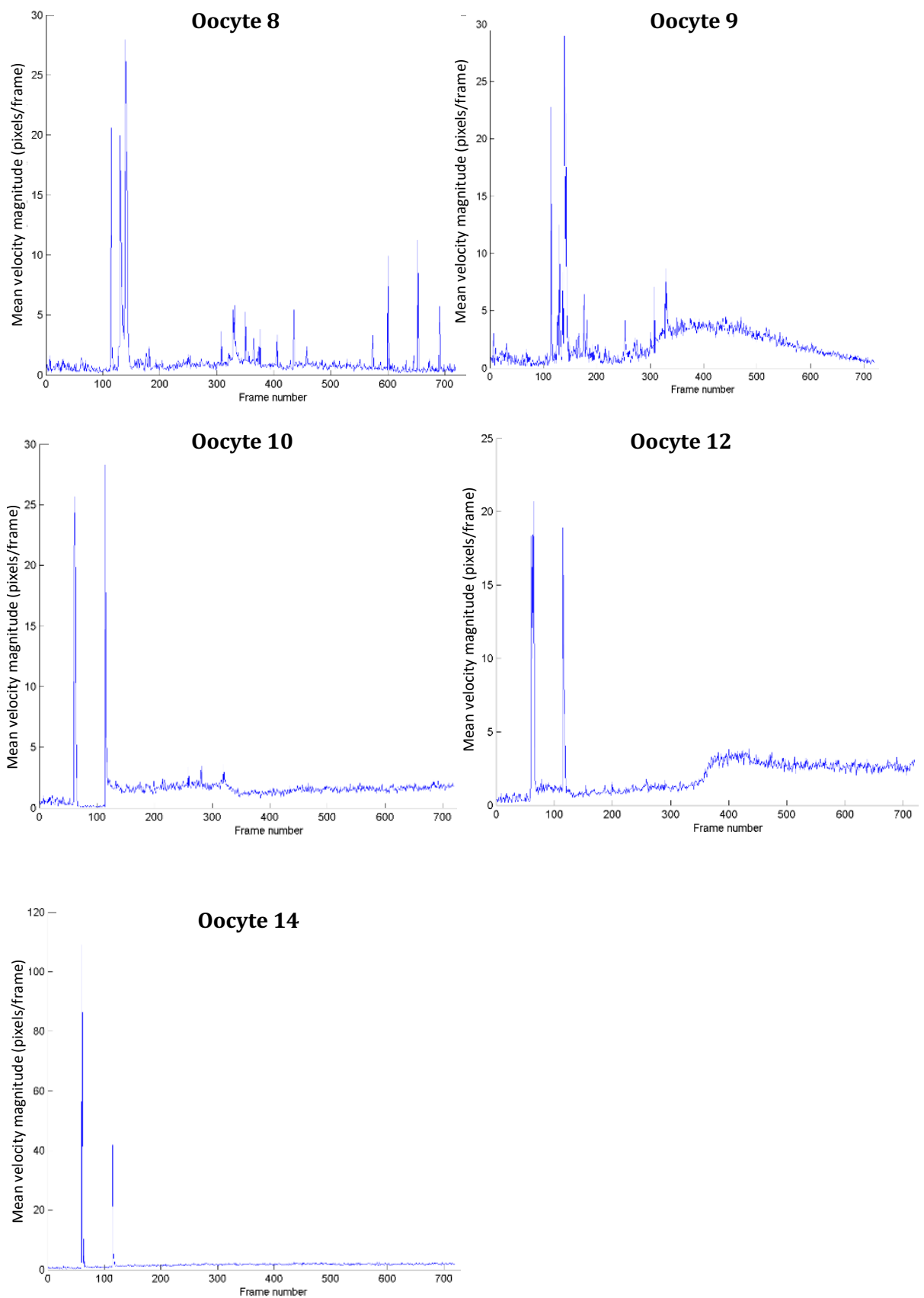




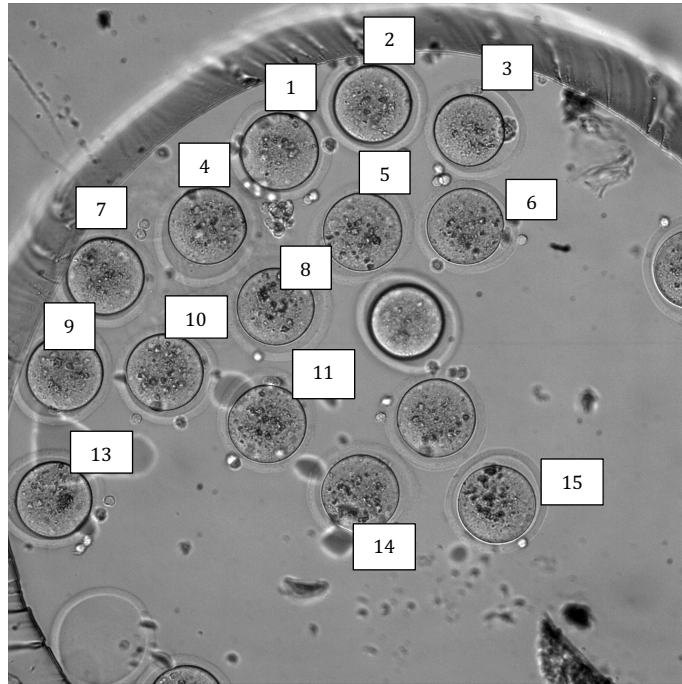
**Figure 5.16 Cytoplasmic movements in mouse oocytes treated with recombinant human PLC $\zeta$  during the first 2 h. (A): a total of 14 oocytes were filmed with a 10s interval. (B-D): oocytes 5, 11 and 13 showed patterns of activation-related movements (in red box). A starting high peak appeared in all graphs at the same time, which could be non-biological and resulted from external noise**



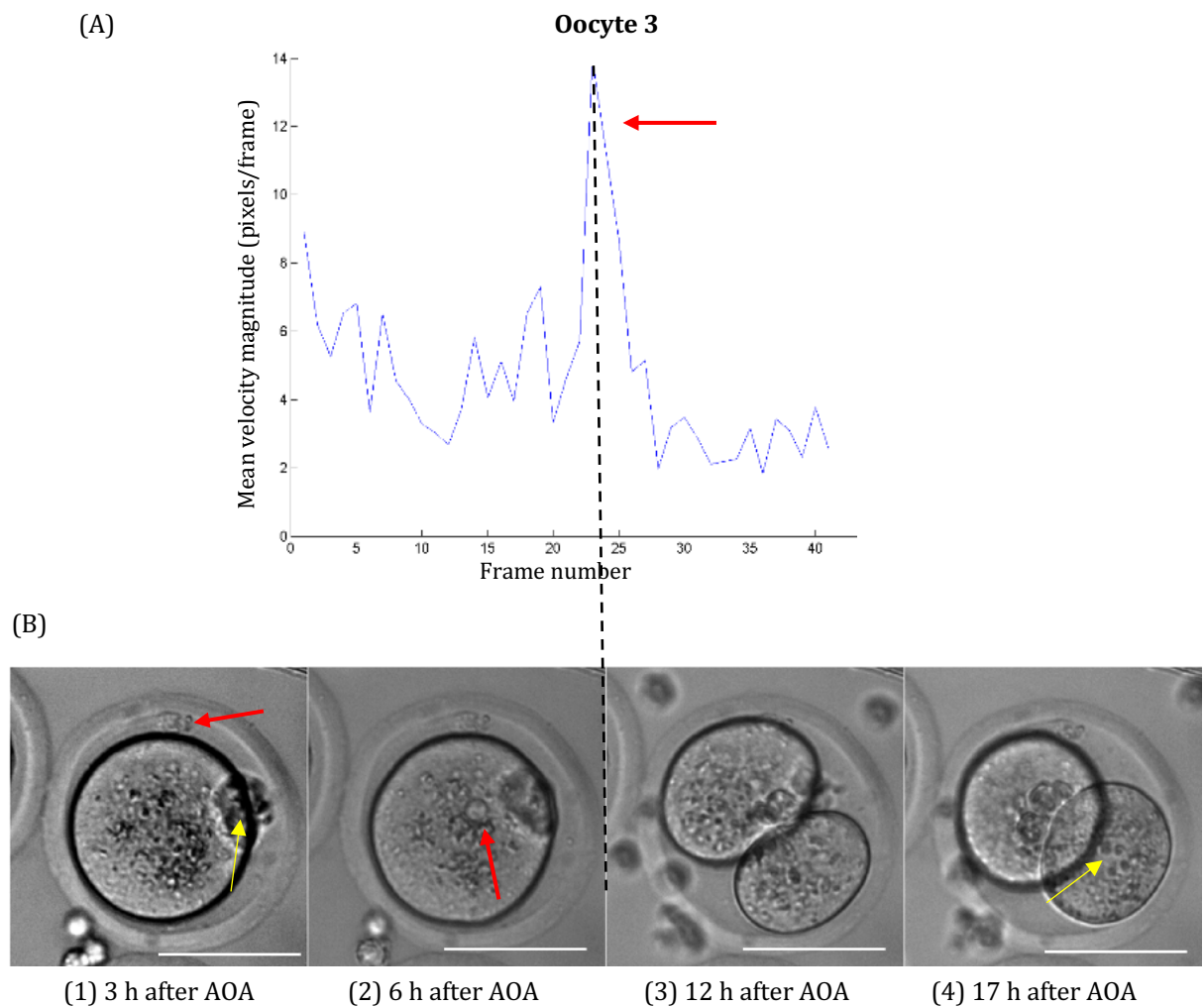




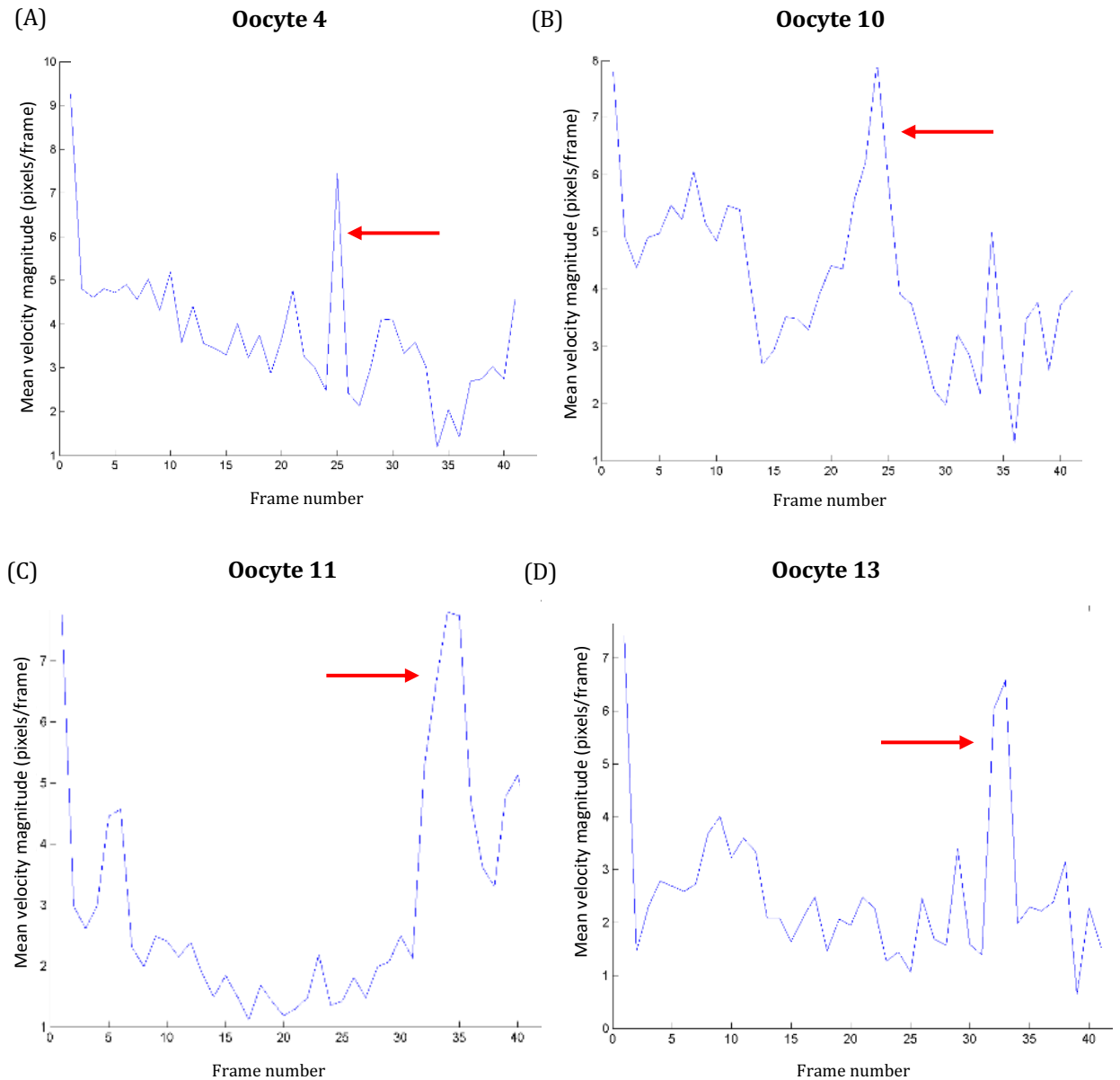
**Figure 5.17** The cytoplasmic movement of rhPLC $\zeta$ -treated mouse oocytes that did not exhibit activation-related movements (11 out of 14).



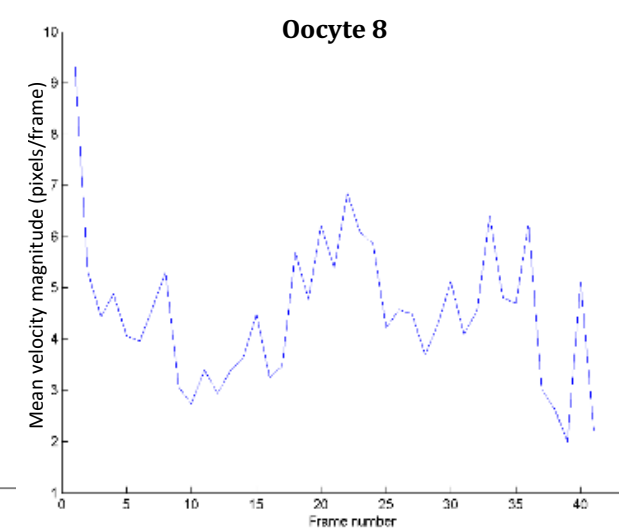
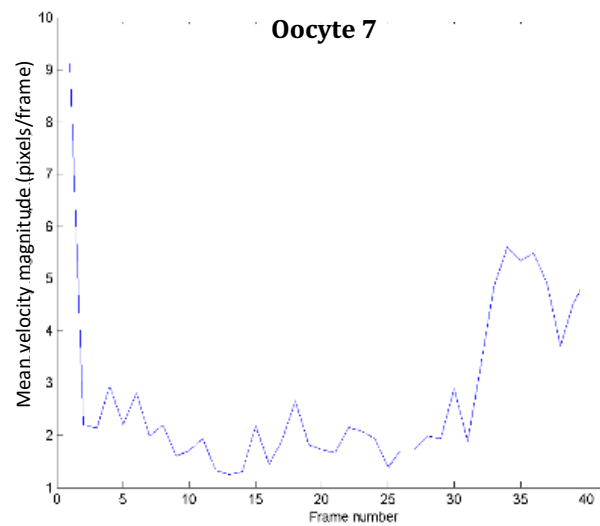
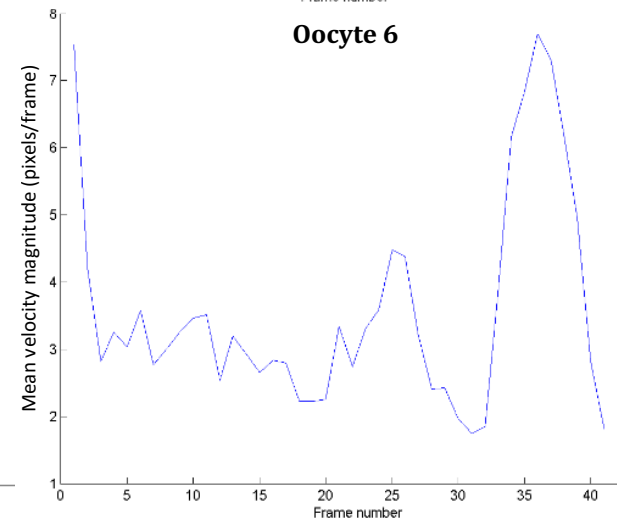
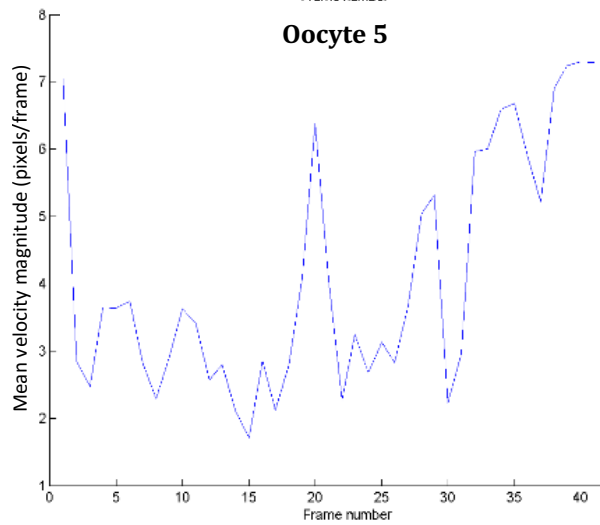
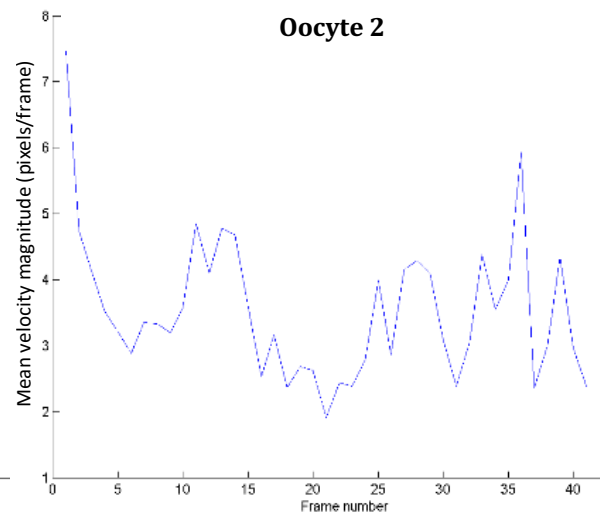
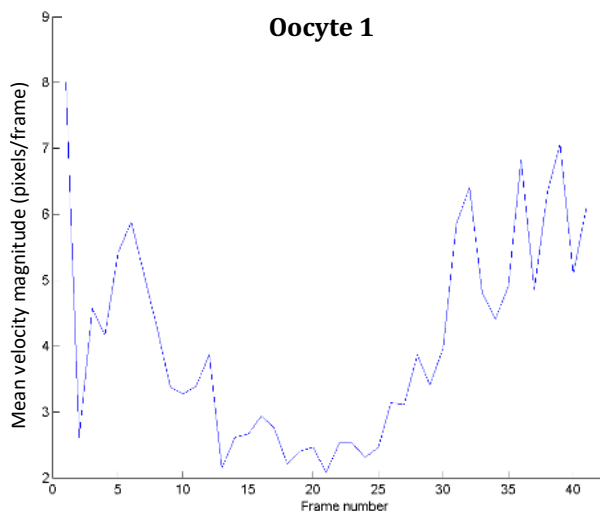
**Figure 5.18 Images of mouse oocytes treated with recombinant human PLC $\zeta$  were recorded from 2 h to 23.5 h. A total of 15 oocytes received a second period of filming with a longer duration (21.5 h in total, with a 30 m interval). Oocytes were labelled by numbers.**

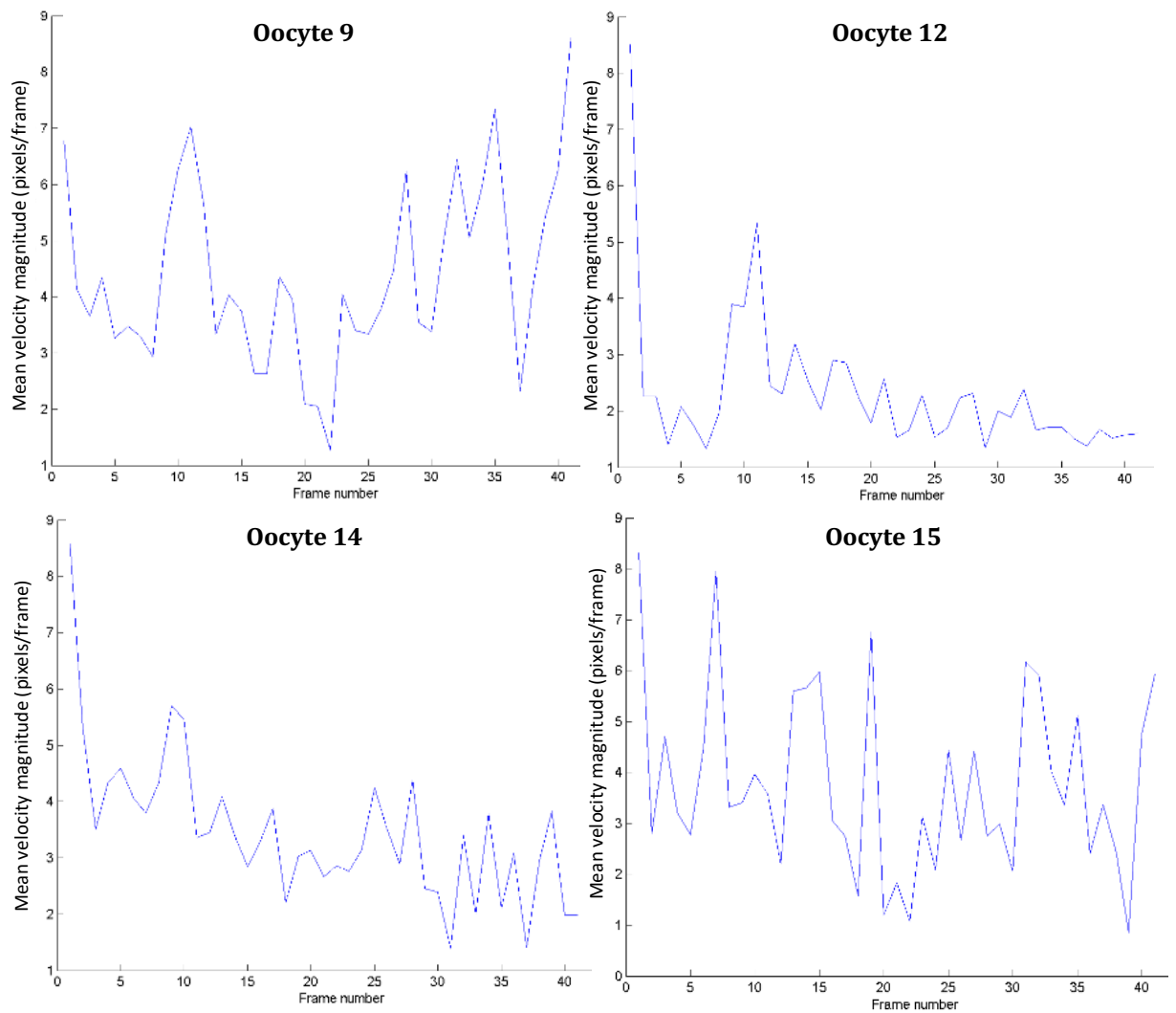


**Figure 5.19 The cytoplasmic movement of a recombinant human PLC $\zeta$  (rhPLC $\zeta$ )-treated mouse oocyte and its corresponding morphological changes. (A)** High velocity magnitude of the cytoplasmic movement in an oocyte was observed at frame 24 (red arrow), corresponding to the stage of the 2-cell stage (black dash line). **(B)** Representative images of morphological development in oocyte 3 after incubation with rhPLC $\zeta$ . The oocyte formed a second polar body 3 h after AOA and divided into a 2-cell 12 h after AOA. (1) A lysed polar body (red arrow) and second polar body (yellow arrow) were seen 3 h after AOA. (2) A pronucleus observed 6 h after AOA (red arrow). (3) Oocyte cleaved 12 h after AOA. (4) A nucleus was seen in the right-hand cell 17 h after AOA (yellow arrow). Scale bar = 50  $\mu$ m



**Figure 5.20 Cytoplasmic movements in mouse oocytes showing signs of 2<sup>nd</sup> polar body formation after treated with recombinant human PLC $\zeta$  (rhPLC $\zeta$ ).** The cytoplasmic velocity of mouse oocytes was recorded from 2 h to 23.5 h every 30 min, after incubation with rhPLC $\zeta$ . Four oocytes showed a peak in the velocity of cytoplasmic movement that was associated with successful activation (red arrow). **(A-D)**: Oocytes were labelled as number 4, 10, 11 and 13, respectively in the **Figure 5.18**.





**Figure 5.21 Ten out of fifteen mouse oocytes did not exhibit activation-related cytoplasmic movements.** The cytoplasmic velocities of mouse oocytes were recorded from 2 h to 23.5 h every 30 min, after incubation with recombinant human PLC $\zeta$ . The peaks representing cytoplasmic velocities could be non-biological and may be due to external interference. Oocytes were labelled as numbers corresponding to those in the Figure 5.18.

## 5.4 Discussion

This chapter evaluated three AOA protocols (GM508, 3  $\mu$ M and 10  $\mu$ M ionomycin) that have been shown to be effective in the activation of human/mouse oocytes. I planned to compare current AOAs on mouse oocytes, without involvement of the sperm factor. If the experiments worked, this would be the first comparative study which parthenogenetically activated mouse oocytes by using the most common AOA protocols designed for humans. Parthenotes that only pass on maternal genetic information can represent as an effective model with which to study maternally-derived effects, for example, imprinting of the maternal genome or the reprogramming of maternal cytoplasmic factors (Sepulveda-Rincon *et al.* 2016). However, this comparison was not performed because one AOA protocol with 10  $\mu$ M ionomycin caused oocytes to undergo necrosis, while the other AOA protocols (GM508 and 3  $\mu$ M ionomycin) both exhibited low efficiency in activation.

GM508 is a “ready-to-use” solution designed specifically for clinical use. Only one study has applied GM508 to mouse oocytes, but obtained only few activated oocytes (Nikiforaki *et al.* 2016); more evidence is needed to examine the applicability of GM508 on mouse oocytes. Therefore, I first examined the effect of GM508 on mouse oocytes; if GM508 is efficient for mouse oocytes, I could then use GM508 for subsequent comparison. However, AOA with GM508 was unsuccessful as none of oocytes developed to the blastocyst stage and few developed to the 2-cell stage (27.7%). This may be due to the fact that different species exhibit different responses to AOAs (Heindryckx *et al.* 2008). In line with this, Nikiforaki *et al.* previously applied GM508 on a mouse model and found that only 15.1% of oocytes parthenogenetically developed to the 2-cell stage (Nikiforaki *et al.* 2016). These authors also applied GM508 to oocytes injected with mouse wobbler sperm; none



of these oocytes were activated (Nikiforaki *et al.* 2016). The low activation rate of GM508 in mouse oocytes in the present study shows that the mouse model is not ideal for investigating AOA protocols designed for human oocytes. Although my finding was in line with Nikiforaki *et al.*, it should be noted that Nikiforaki *et al.* also reported that 43.7% of oocytes developed to the blastocyst stage. The poor quality of the mouse oocytes used in my study could be a potential reason for the failure of oocytes to reach the blastocyst stage.

Nevertheless, the present data observed from mouse models further indicates that different species may respond to the same activator in different way. Indeed, although mouse oocytes showed poor responses to GM508, clinical studies using GM508 for human oocytes have shown to be successful, with fertilisation rates ranging from 52.6 to 99%, as justified by 2-cell formation (Ebner *et al.* 2015a; Nicopoullos *et al.* 2015). Furthermore, accumulating evidence shows that GM508 has successfully helped infertile couples with low fertilisation rate, or total fertilisation failure, to achieve live-births (Bonte *et al.* 2019; Li *et al.* 2019b). To best evaluate the activation effects of GM508, future studies should use human oocytes instead of mouse oocytes.

I then applied ionomycin to activate mouse oocytes, in order to select an effective AOA protocol for subsequent comparison with rhPLC $\zeta$  protein. I used two established protocols; one created by Professor Chris Graham and designed for human oocytes (3  $\mu$ M, unpublished)(Yamada *et al.* 2014) and another from Heytens *et al.*, and designed for mouse oocytes (10  $\mu$ M)(Heytens *et al.* 2008). The use of 3  $\mu$ M ionomycin activated 21.6% oocytes underwent cleavage; however, none of these oocytes developed to blastocysts. I then increased the concentration of ionomycin to 10  $\mu$ M as suggested by Heytens *et al.*, as mammalian oocytes have a wider

response to calcium stimulation (Ducibella *et al.* 2006; Heytens *et al.* 2008). However, oocytes showed evidence of necrosis after incubation with higher concentrations of ionomycin. This is the first evidence suggesting that 10  $\mu$ M ionomycin can exert detrimental effects on oocyte viability; this is not indicated by any of the published literature.

However, the use of 10  $\mu$ M ionomycin for AOA is more often applied to human and mouse oocytes, when compared to other concentrations (de Fried *et al.* 2008; Heytens *et al.* 2008; Nikiforaki *et al.* 2016). Several studies have demonstrated the successful use of 10  $\mu$ M ionomycin for the activation of mouse oocytes (Heytens *et al.* 2008; Nicopoullos *et al.* 2015; Karabulut *et al.* 2018). In addition, Nikiforaki *et al.* suggested that ionomycin was better than calcimycin in triggering both human and mouse activation (Nikiforaki *et al.* 2016). In contrast, Vaden Meerschaut *et al.* reported that ionomycin cannot rescue all cases of fertilisation failure in human oocytes (Vanden Meerschaut *et al.* 2012). Thus far, no study has investigated the toxicity of ionomycin for oocytes. In contrast, the current data indicates that higher concentrations of ionomycin can be lethal to mouse oocytes. Future studies should involve the further investigation of oocyte apoptosis, with the use of the TUNEL assay, or other cellular assays associated with cell viability, cell metabolism and DNA degradation.

As well as using ionomycin for AOA, I also used 7% ethanol as a positive control to examine my AOA technique; six out of nine mouse oocytes successfully developed into the 2-cell stage (66.6%), thus indicating that the failure of activation in these mouse oocytes could be due to the use of AOAs and not my techniques.

Taking all this evidence together, the current study showed that ionophore-based AOAs were not effective in initiating mouse oocyte development to the 2-cell

stage; this finding differed from previous studies (Heytens *et al.* 2008; Nicopoulos *et al.* 2015; Karabulut *et al.* 2018). The AOA protocols I tested are designed for either human or mouse oocytes; however, all yielded very few activated oocytes. Furthermore, my experiment suggest that 10  $\mu$ M ionomycin can cause necrosis in mouse oocytes, indicating that AOAs should be applied with careful justification in a clinical setting.

In terms of the possible reasons for the failure of oocytes to develop to the blastocyst stage; firstly, the quality of the harvested mouse oocytes collected from Biomedical Services varied widely. I cannot exclude that the weak response of some mouse oocytes to AOAs was because of poor quality. Secondly, some oocyte activation protocols used in this study were designed for humans; this study only utilised mouse oocytes. However, differences between species is a factor that cannot be neglected.

Considering the scarcity of human oocytes, and the ethical issues involved in procuring human oocytes for research, investigations in a mouse model are more realistic, although inter-species differences may limit the extent of research investigation. Indeed, AOA strategies have been optimised differently between human and mouse oocytes. For example, Nikiforaki *et al.* applied 10  $\mu$ M ionomycin twice with human oocytes and incubated for 10 min each time, whilst de Fried *et al.* applied 10  $\mu$ M ionomycin to human oocytes once only and incubated for 6 min (de Fried *et al.* 2008; Nikiforaki *et al.* 2016). Discrepancies in AOA protocols can bring difficulties in deciding upon an appropriate regime to study; this is the reason that I repeated several published AOA protocols and aimed to determine the most effective one.

Furthermore, the use of further treatments after AOA varies in terms of chemicals (cytochalasin B, D or 6-dimethylaminopurine), amounts (0.5-5 µg/ml) and incubation time (1.5-5 h) (Rogers *et al.* 2004; Suzuki *et al.* 2010; Nomikos *et al.* 2013; Liu *et al.* 2014). However, these differences should not influence oocyte activation because cytochalasin and 6-dimethylaminopurine are protein inhibitors, and the aim of using them is to promote oocyte development from the stage of arrest (Szollosi *et al.* 1993; Yi and Park 2005). Therefore, the current study only focused on the differences between AOAs and not in the reagents for treatments after AOA.

Indeed, calcium oscillations with appropriate frequency, amplitude and duration are critical to mammalian embryo development. In contrast, exogenous reagents, such as ionomycin and GM508, may activate oocytes, but there are uncertainties for the long-term safety of infants born from AOA treatment, largely due to the pattern of Ca<sup>2+</sup> release. In line with this, evidence shows that Ca<sup>2+</sup> release patterns differ when compared between oocytes fertilised normally and AOA-treated oocytes (Rogers *et al.* 2006). Abnormal calcium patterns were also shown to lead to lethal developmental problems during organogenesis in rabbits (Ozil and Huneau 2001). These findings, together with data from the present study, indicate the essential need of producing a safer endogenous reagent as a replacement for current AOAs.

Therefore, I synthesised untagged and Fc-tagged rhPLCζ with the use of a novel vector, pHLsec, and examined the functionality of the rhPLCζ by observing morphological changes and cytoplasmic movements in oocytes. Electrophoresis and silver staining showed that the molecular weight bands of rhPLCζ were lower than expected 75 kDa (~65 kDa). Similarly, Yoon *et al.* observed that rhPLCζ produced from pGEM-T vector in *E. coli* was ~68 kDa and successfully activated

mouse oocytes after microinjection (Yoon *et al.* 2012). Notably, the PLC $\zeta$  plasmid used in the present study has been previously verified via sequencing. Discrepancies of molecular weights of rhPLC $\zeta$  protein in my study and the native PLC $\zeta$  in sperm may be due to the biological differences in protein post-translational modification between the mammalian cell line and human sperm (Yoon *et al.* 2012). In terms of the purity of protein, lower sizes of molecular bands on the Bis-Tris protein gel were also observed (**Figure 5.14 A and B**); thus may partial explain why the activated mouse oocytes failed to develop to the blastocyst stage.

After purification, Fc-tagged and untagged rhPLC $\zeta$  proteins were successfully generated and purified (~147  $\mu\text{g/ml}$  of Fc-tagged and 47-63  $\mu\text{g/ml}$  of untagged rhPLC $\zeta$ ). These were higher concentrations than previously published work (Kashir *et al.* 2011b; Nomikos *et al.* 2013). However, no one has incubated protein with oocytes, as the proteins used in previous studies were exclusively microinjected into mouse oocytes. Therefore, I did not know the appropriate concentrations for activating oocytes by simple incubation. Saunders *et al.* showed that the amount of rhPLC $\zeta$  triggering mouse oocyte activation via microinjection was ~44–75 femtograms (Saunders *et al.* 2002). In my study, a higher concentration of protein may be needed; however, this is the highest concentration from the stock that I can obtained thus far.

The design of the pHLsec vector not only features a secretion site; it also features a small Fc tag (30 bp). Therefore, the molecular weights of untagged and Fc-tagged rhPLC $\zeta$  were similar. In contrast, the latest work of Nomikos *et al.* used a ~60 kDa Nus-A tag for synthesising rhPLC $\zeta$ , although the large size of this tag is likely to reduce clinical applicability (Nomikos *et al.* 2013). I then attempted to remove the tag; unfortunately, the rhPLC $\zeta$  failed to activate oocytes after tag

removal. This failure could possibly be due to protein degradation during the cleavage procedure, a reduction in protein stability, or the low yields of the expression system. An alternative possibility is that the rhPLC $\zeta$  needs the Fc-tag to maintain protein stability, because the Fc-tag may have been incorporated into rhPLC $\zeta$  during the post-translational modification. Microinjection of the untagged protein into mouse oocytes would provide more informative evidence relating to whether my rhPLC $\zeta$  can retain its functionality after tag removal.

The most exciting result from the present experiment is that the Fc-tagged rhPLC $\zeta$  protein successfully activated mouse oocytes. This represents the success of synthesising a functional Fc-tagged rhPLC $\zeta$  protein from the mammalian cell line, with a novel approach (the pHLsec vector), although the safety profile needs to be further investigated. More importantly, the fact that simple incubation with Fc-tagged rhPLC $\zeta$  triggered oocyte activation provides us with a new insight into the understanding that a plasma-membrane bound mechanism may be relevant to oocyte activation. We do not know the exact underlying mechanism; however, this could be related to the non-specific binding of molecules in the plasma membrane, an alternative pathway related to classic G-protein-coupled receptor (GPCR) pathway (Hannan *et al.* 2018), or tyrosine kinases receptor (Piiper and Zeuzem 2004), which leads to the activation of a transmembrane domain in the plasma membrane and the subsequent release of Ca<sup>2+</sup>.

Interestingly, incubation for 30 min triggered oocyte activation, but not 15 min, indicating that this unknown mechanism of oocyte activation may be time-dependent. However, the current study is at the preliminary stage and needs more evidence to confirm my findings. To verify this plasma membrane-bound mechanism, further studies could use rhPLC $\zeta$  made by Kashir *et al.* and incubate

with oocytes, as the function of this protein has been verified via microinjection (Kashir *et al.* 2011b).

Nevertheless, it is acknowledged that more than one mechanism is involved in  $\text{Ca}^{2+}$  oscillations, and that the role of PLC $\zeta$  at oocyte activation is more complex than expected. Hachem *et al.* mutated two exons of the mouse PLC $\zeta$  gene and produced a mutant KO mouse. These authors subsequently naturally mated these KO mice with fertile female mice, but produced viable pups (Hachem *et al.* 2017a). Notably, no  $\text{Ca}^{2+}$  response was observed in mouse oocytes after fertilisation with sperm from KO mice, neither by IVF nor ICSI. These lines of evidence indicate that an alternative mechanism may be involved in oocyte activation. Hachem *et al.* hypothesised the existence of an atypical  $\text{Ca}^{2+}$  signal, which was not observed during  $\text{Ca}^{2+}$  monitoring (Hachem *et al.* 2017a). Indeed, in my experiment, the cytoplasmic movement study did not show fertilisation-like cytoplasmic movements in oocytes after incubation with my rhPLC $\zeta$  protein. However, we cannot exclude the potential existence of an atypical pattern of  $\text{Ca}^{2+}$  release which differs from that seen at natural fertilisation. Nevertheless, this is the first time that this type of assay has been used to evaluate rhPLC $\zeta$  functionality in oocyte activation. This assay allows us to indirectly correlate the pattern of cytoplasmic movement with  $\text{Ca}^{2+}$  oscillations and thus serves as an indirect indicator to predict oocyte activation. Apart from studying patterns of cytoplasmic movement and  $\text{Ca}^{2+}$  patterns, it would be imperative to investigate the possibility of whether other sperm factors (i.e., PAWP) are involved in oocyte activation when PLC $\zeta$  is dysfunctional (Hachem *et al.* 2017a; Nozawa *et al.* 2018).

Indeed, oocyte activation can be triggered in a manner different from  $\text{Ca}^{2+}$ -dependent pathway. For example, MPF is responsible for oocyte arrestment, and

reduction in MPF can trigger oocyte activation without applying  $\text{Ca}^{2+}$  stimuli (Phillips *et al.* 2002). In addition, Jones suggested that there may be a sperm cryptic factor(s) responsible for  $\text{Ca}^{2+}$  oscillations when PLC $\zeta$  is absent (Jones 2018). Furthermore, successful oocyte activation in oocytes incubated with Fc-tagged rhPLC $\zeta$  provides us with new insights into the mechanism underlying PLC $\zeta$  function; there might be two forms of PLC $\zeta$ , one responsible for targeting cytoplasmic PIP $_2$ ; the other acting as an alternative regulator and triggers oocyte activation possibly via plasma membrane-based mediator(s). This is speculative, however, and needs further investigation.

The present study has some limitations that need to be considered. Only small numbers of mouse oocytes were used for AOA (especially for activation using GM508). Future studies should aim to increase sample size. In addition, to test the function of my rhPLC $\zeta$  protein, microinjection should be performed to allow comparison with previous studies. However, microinjection was not performed in the present study as this requires an experienced embryologist with appropriate microinjection experience. No embryologist was available during the time these experiments were carried out. Alternatively, other approaches could also be considered, such as  $\text{Ca}^{2+}$  measurement, rhPLC $\zeta$ -combined ICSI using sperm from infertile males, enzymatic hydrolysis assays, or changes in the regulators that are relevant to the  $\text{Ca}^{2+}$  cascade (i.e., PIP $_2$ , InsP $_3$  and MPF). Due to time constraints and the need for  $\text{Ca}^{2+}$  imaging equipment, I did not perform these experiments.

## 5.5 Conclusion

In this chapter, I evaluated the efficiency of current AOA protocols on mouse oocyte activation: calcimycin (GM508) and ionomycin (3  $\mu\text{M}$  and 10  $\mu\text{M}$ ), together



with ethanol (7%). I also synthesised Fc-tagged/untagged rhPLC $\zeta$  protein and examined the functionality of Fc-tagged/untagged rhPLC $\zeta$  by observing morphological changes and cytoplasmic movements in the mouse oocytes, aiming to develop a better and safer endogenous reagent for AOA. Only a small number of oocytes were activated by the use of GM508 and 3  $\mu$ M ionomycin (respective 27.7% and 21.6% at the 2-cell stage), which indicates that mouse models are not suitable for investigating AOA with these ionophores. In addition, the present study provides valuable information that 10  $\mu$ M ionomycin may induce oocytes to undergo necrosis, thus indicating that the use of AOAs should be given careful evaluation. In contrast, a 42.3% activation rate was observed in mouse oocytes incubated with Fc-tagged rhPLC $\zeta$  protein, which provides a break-through achievement in that functional Fc-tagged rhPLC $\zeta$  can be synthesised from a novel method involving the use of a pHLsec vector. In addition, these results indicate that a novel plasma membrane-dependent mechanism may be involved in oocyte activation. Unfortunately, no activation was observed when oocytes were incubated with untagged rhPLC $\zeta$ , indicating that the rhPLC $\zeta$  protein may need the Fc tag to retain functionality and that the Fc tag may have been incorporated into the rhPLC $\zeta$  structure during post-translational modifications.

## 5.6 Key findings

- The use of GM508 and ionomycin was not effective in triggering mouse oocyte activation, despite the fact that Heytens *et al.* (2008) and Nikiforaki *et al.* (2016) suggested that 10  $\mu$ M ionomycin was efficient in triggering oocyte activation in the mouse. However, the present study showed that 10  $\mu$ M ionomycin can be detrimental to oocytes

- An Fc-tagged rhPLC $\zeta$  protein was successfully synthesised using a novel method involving the use of pHLsec vector and a mammalian cell expression system
- Untagged rhPLC $\zeta$  protein failed to activate mouse oocytes, indicating that the protein needs the Fc tag to remain functional and that the tag may be incorporated into rhPLC $\zeta$  during post-translational modification
- Cytoplasmic movements study was used as a non-invasive indicator to examine the functionality of 'in-house' rhPLC $\zeta$  in triggering oocyte activation, via correlating the patterns of cytoplasmic movements with Ca<sup>2+</sup> oscillations. This study did not detect fertilisation-like patterns of cytoplasmic movement, thus dictating that an atypical Ca<sup>2+</sup> release might occur upon rhPLC $\zeta$  stimulation
- A 42.3% activation rate (i.e., 2-cell formation) was achieved via simple incubation of the Fc-tagged rhPLC $\zeta$ , thus suggesting that a novel plasma membrane-bound mechanism of oocyte activation may exist; this needs to be investigated further

## Chapter 6 General discussion and summary

### 6.1 Summary

Infertility affects an estimated 8 – 12% of couples in the world (Inhorn and Patrizio 2015). In particular, 20 - 30% of infertile cases are attributed to male-related factors (ESHRE 2018). Helping infertile couples to achieve successful fertilisation and pregnancy are the ultimate aims of applying IVF and ICSI. However, fertilisation failure occurs in ~10-15% of IVF cycles, and ~30% ICSI cycles (Kuczyński *et al.* 2002). The precise reasons for fertilisation failure are not yet fully understood. However, the failure of oocyte activation, referred to as OAD, is considered as a principle reason leading to fertilisation failure in IVF/ICSI (Yeste *et al.* 2016a).

It has been identified that the release of intracellular  $\text{Ca}^{2+}$  is the universal trigger of oocyte activation (Stricker 1999). During mammalian oocyte activation,  $\text{Ca}^{2+}$  is released in a manner that exhibits repetitive peaks with a particular amplitude and frequency, referred to as  $\text{Ca}^{2+}$  oscillations (Stewart and Davis 2019). Accordingly,  $\text{Ca}^{2+}$  oscillations in mammalian oocytes promote a series of biological events, including PN formation, the prevention of polyspermy, the recruitment of maternal RNA, the exocytosis of cortical granules, and the synthesis of DNA and protein. Numerous studies have demonstrated that PLC $\zeta$  is the principal sperm factor contributing to oocyte activation, a key stage of development representing oocyte-embryo transition (Saunders *et al.* 2002; Kashir *et al.* 2013a). At mammalian fertilisation, PLC $\zeta$  is released from sperm to the oocyte and triggers  $\text{Ca}^{2+}$  oscillations via the inositol phosphate pathway. Numerous studies have shown that the failure of oocyte activation leads to fertilisation failure and has been attributed to

abnormalities in PLC $\zeta$  (Heytens *et al.* 2009; Ferrer-Vaquer *et al.* 2016; Yeste *et al.* 2016a; Nazarian *et al.* 2019; Torra-Massana *et al.* 2019). However, little is known with regards to the precise mechanism of how PLC $\zeta$  targets its substrate on PIP<sub>2</sub>, the factors that create a dysfunctional PLC $\zeta$  gene/protein, or the involvement of other mechanisms contributing to oocyte activation.

Currently, the only available option in clinics with which to alleviate OAD is to use AOA. This involves the use of physical, mechanical, or chemical methods to artificially increase the release of Ca<sup>2+</sup> in oocytes and therefore promote oocyte activation. Chemical AOAs mainly include ionomycin and calcimycin. However, the use of chemical AOAs for clinical patients is the cause for some concern, mainly because chemical AOAs cause abnormal patterns of Ca<sup>2+</sup> release in oocytes, and may cause subsequent epigenetic issues (Rogers *et al.* 2006). Currently, there is no assay available for clinics to assist with AOA justification. Most IVF clinics apply AOAs based mainly on whether patients have experience with (repeated) fertilisation failure. Therefore, it is imperative that we create a diagnostic assay with which to assist the clinical decision-making process in AOA treatment. Furthermore, it is important to produce a rhPLC $\zeta$  protein to replace current AOAs, as PLC $\zeta$  is the endogenous reagent and is naturally responsible for oocyte activation.

Collectively, the main objective of this thesis was to address the aforementioned questions and to investigate the diagnostic and therapeutic applicability of PLC $\zeta$  for clinical purpose. I also aimed to gain a better understanding of the mechanisms underlying PLC $\zeta$ -related male infertility and oocyte activation deficiency. **Chapter 2 and 3** focused on the diagnostic role of PLC $\zeta$ . **Chapter 2** optimised a standard (in-house) protocol to visualise PLC $\zeta$  expression in male sperm and compared these with a recent published assay (AUM) involving antigen

unmasking, in order to determine the best immunofluorescence assay. **Chapter 3** further investigated the possibility of applying this assay for clinical diagnosis with which to allow individualised strategy for AOA treatment. **Chapter 4** was a preliminary study which aimed to investigate the genetic mechanisms related to *PLCζ* that may cause male infertility. A PCR-based approach was used to screen *PLCζ* exons (mainly 6, 11 and 13, where the majority of mutations have been found). In addition, NGS was applied to screen gDNA from infertile males with which to detect genetic variations in the *PLCζ X-Y linker*, *CAPZA3* and the shared bidirectional promoter. **Chapter 5** evaluated current AOA protocols (GM508 and ionomycin) and further explored the therapeutic role of *PLCζ* in oocyte activation. In this chapter, I synthesised a recombinant protein using a novel approach that featured the use of a pHLsec vector with a secretion signal and the mammalian HEK293T expression system. **Chapter 5** also yielded new evidence related to a potential novel mechanism for *PLCζ* which acts via the plasma membrane of oocytes.

## **6.2 The ‘in-house’ laboratory assay demonstrated the best visualisation efficiency for *PLCζ* compared to another recently published assay**

Following the ground-breaking discovery of *PLCζ*, substantial evidence has accumulated to show that *PLCζ* expression and localisation in human sperm is associated with male infertility. For example, infertile males exhibit lower *PLCζ* levels in sperm than fertile males (Kashir *et al.* 2013b), and higher *PLCζ* levels in male sperm is correlated with higher fertilisation rates in IVF/ICSI (Yelumalai *et al.* 2015; Nazarian *et al.* 2019). In addition, *PLCζ* was found to be predominantly expressed in the equatorial region of mammalian sperm. Alternatively, *PLCζ* expression has been demonstrated in the the post-acrosomal/acrosome regions.

In most studies of PLCζ, immunofluorescence labelling is used (Yoon *et al.* 2008; Kashir *et al.* 2011a; Kashir *et al.* 2012c; Kashir *et al.* 2013b; Yelumalai *et al.* 2015; Yeste *et al.* 2016b; Azad *et al.* 2018; Moreau *et al.* 2019; Nazarian *et al.* 2019). However, Kashir *et al.* proposed that the antibodies used in all previous studies of human PLCζ were not completely specific to PLCζ antigens; consequently, these authors believe that earlier expression and localisation data might not be entirely accurate (Kashir *et al.* 2017). Kashir *et al.* applied an AUM method (HCl, AT, or heating) with sperm from porcine, mouse and human sperm, and reported that the AUM method provides highly consistent results in visualising PLCζ expression in human sperm. However, these authors only used sperm from fertile donors and proposed that their AUM method could be used to evaluate PLCζ expression in sperm from infertile males in a clinical setting.

Chapter 2 therefore investigated the efficiency of visualising PLCζ expression in infertile and fertile male sperm, by comparing two immunofluorescence methods; our in-house laboratory assay, and the AUM method. The in-house assay, which was developed by the Coward group, uses a polyclonal antibody synthesised from the same sequences as the AUM method. In previous studies, our in-house assay was shown to be highly specific to native PLCζ sperm (Yoon *et al.* 2008; Heytens *et al.* 2009). In this study, I used sperm from infertile males who have shown significantly reduced/absent PLCζ expression, as identified by our in-house assay. The use of acidic reagents in the AUM method increased the level of PLCζ in sperm from some fertile males, but failed to improve levels in sperm from infertile males. This indicated that the AUM method cannot serve as a diagnostic assay for clinics. In addition, different acid treatment (i.e., HCl and AT) caused fluctuating PLCζ levels in sperm from the same male; inconsistency of these AUM treatments will be a

problem for a clinical purpose. In contrast, the in-house assay consistently showed the best visualisation efficiency of PLC $\zeta$  in sperm from both fertile and infertile males, when compared with the AUM method. This indicates, that although both methods identified poor PLC $\zeta$  expression, the in-house assay provided much better visualisation of PLC $\zeta$  and therefore more accurate quantification. Therefore, the present study demonstrated that the in-house assay is more reliable and consistent than AUM method.

I additionally evaluated the specificity of the antibody used in this study and found that the in-house assay showed good specificity, while the AUM method still showed fluorescence signals after peptide blocking; this indicated that the immunofluorescence signals from sperm stained with the AUM method may not accurately represent PLC $\zeta$  expression. This may partially explain why the AUM method resulted in higher levels of PLC $\zeta$  expression in sperm, when compared to those without acid treatment. It is possible that acid solutions may disrupt the cell membrane and result in non-specific binding. These findings further support the inapplicability of the AUM method for clinical purposes, as the rationale for performing the PLC $\zeta$  assay is to assess PLC $\zeta$  levels in human sperm as close to native status as possible. To achieve this, immunolabelling procedures can be optimised. For example, changing to another fixative which may not cause crosslinking between antigens and antibody, therefore allowing better immunofluorescence reactivity.

Overall, Chapter 2 compared the in-house assay with the AUM method in terms of the quantification of PLC $\zeta$  expression in sperm from fertile and infertile males and demonstrated that the in-house assay showed the best and most consistent outcomes of immunofluorescence labelling. The in-house assay should

therefore remain as the main method for scientific and clinical studies investigating PLC $\zeta$ . However, future studies should aim to improve efficiency and accuracy further. For example, a better option would be to develop a monoclonal antibody, as all immunofluorescence studies relating to PLC $\zeta$  are currently using polyclonal antibodies; a monoclonal antibody will be more specific to antigens and thus eliminate the chances of non-specific binding.

### **6.3 PLC $\zeta$ analysis identified candidates for artificial oocyte activation (AOA), resulting in a more evidence-based approach for the application of AOA**

Ionophore-based chemicals, such as calcimycin (i.e., GM508) and ionomycin, are considered to represent an appropriate solution for OAD. However, the use of these chemicals has been considered to be experimental for many years. Several studies reported that AOAs can improve fertilisation, pregnancy and live-birth rates in IVF/ICSI (Vanden Meerschaut *et al.* 2013; Ebner *et al.* 2015a; Economou *et al.* 2017; Ferrer-Buitrago *et al.* 2018c; Karabulut *et al.* 2018; Bonte *et al.* 2019). However, there is no standard criteria for the justification of AOA use, particularly in terms of the selection of eligible patients, the types of AOA to be used and the specific AOA protocol to be applied. Most IVF clinics apply AOAs based on patient treatment history in previous IVF/ICSI cycles, usually a poor fertilisation rate. However, the cut-off thresholds for low fertilisation rate (the number of fertilised oocytes/total oocytes) in different IVF clinics varies between 33.3% and 50% (Ebner *et al.* 2012; Ebner *et al.* 2015a), thus causing extreme difficulty when evaluating the efficacy of AOA treatment.

It is important to bear in mind that AOA treatment aims to rescue severe infertility conditions in which the sperm is incapable of triggering oocyte activation.



Sanatella *et al.* were concerned that the use of AOAs might become a routine approach in IVF clinics, as clinicians may use AOAs to help guarantee at least successful fertilisation, but without appropriate justification for the use of AOAs (Sanatella and Dale 2015). In addition, AOA treatment does not solve all infertile problems; chemical AOAs did not improve the fertilisation outcomes of cases involving sperm with bent necks or azoospermic sperm aspirated from testicles (Moaz *et al.* 2006; Borges Jr *et al.* 2009a).

It is imperative to define a specific diagnostic method as a guidance to identify patients who may benefit from AOA. It is also important to generate a safer and more endogenous therapeutic reagent as an alternative option for OAD; however, until such time, the justification of AOA treatment must be made logistically and based on evidence. Chapter 3 therefore attempted to use our in-house PLC $\zeta$  assay as a diagnostic assay to translate PLC $\zeta$  characteristics from the laboratory to clinical purpose; as a result, the current results provide us with new insight into the use of PLC $\zeta$  as a predictive indicator to select patients who may be eligible for AOA treatment.

In Chapter 3, I compared two key parameters of PLC $\zeta$  (mean PLC $\zeta$  level and the proportion of sperm expressing PLC $\zeta$ ) between fertile and infertile individuals and created the term 'PLC $\zeta$  deficiency'. This is the first demonstration of the applicability of a PLC $\zeta$  assay as a predictive indicator for AOA treatment by assessing two parameters associated with PLC $\zeta$  in male sperm on an individual basis. The expression of PLC $\zeta$  in sperm has been compared previously between infertile and fertile groups, and have been associated with fertilisation outcomes (Yelumalai *et al.* 2015; Ferrer-Vaquer *et al.* 2016). In addition, Kashir *et al.* suggested that fertile males exhibited significant variability in the mean PLC $\zeta$  levels in sperm, which may

limit the application of quantitative immunofluorescence PLC $\zeta$  for diagnostic purpose (Kashir *et al.* 2013b). In addition, these authors found that the proportion of sperm exhibiting PLC $\zeta$  was significantly higher in a fertile control group than an infertile group (Kashir *et al.* 2013b). Therefore, in the present study, I evaluated both parameters, as these two parameters provide information from different aspects. However, a recent randomised clinical trial used the ‘proportion of sperm expressing PLC $\zeta$ ’ as the only standard for grouping patients and compared fertilisation outcomes between groups with and without AOA treatment (Nazarian *et al.* 2019). Although a significantly improved fertilisation rate was observed in the PLC $\zeta$ -deficient group (proportion of PLC $\zeta$  < 60%) after AOA treatment, scientific evidence is needed to support the rationale behind this practice.

In the present study, appropriate cut-off thresholds of these two key parameters were calculated and were used to examine the accuracy of the PLC $\zeta$  assay. The Youden’s index test reflects the overall capacity of the assay performance and indicates a number(s) that can best differentiate the two populations. Therefore, this test was used to indicate the cut-off thresholds by analysing data from all 56 fertile and infertile males. A cut-off reference can therefore overcome the problem created by individual fertile males exhibiting variable PLC $\zeta$  levels in sperm. Accordingly, the cut-offs divided infertile patients into 4 types; deficient PLC $\zeta$  (category III), partial deficient PLC $\zeta$  (category I and IV) and non-deficient PLC $\zeta$  (category II). Strikingly, four healthy AOA-assisted live-births were achieved by four couples in which the male was categorised into category III and showed PLC $\zeta$  deficiency. This represented the first few cases of infants born after AOA treatment in Oxford. To the best of our knowledge, this is the first demonstration that a threshold for PLC $\zeta$  parameters can be determined empirically. These cut-offs can

therefore provide information for the future assessment of PLC $\zeta$  expression clinically. Furthermore, this shows the excellent applicability of our in-house PLC $\zeta$  assay as a diagnostic assay.

Five couples in which the male showed PLC $\zeta$  deficiency received AOA treatment; one achieved fertilised oocytes but experienced a miscarriage at week 6 (Patient 4, Table 3.2 and Figure 3.8). Interestingly, our in-house assay indicated that this patient was PLC $\zeta$  deficient, while PLC $\zeta$  categorisation assigned the patient to category IV (i.e., partial deficient PLC $\zeta$ ). This indicates that the best solution at the moment might be a combination of two results, in-house assay and PLC $\zeta$  categorisation, and analyse each patient on a case-by-case basis, which is in line with the principle of precision medicine. Nevertheless, the definition of successful oocyte activation should be based on the rates of fertilised oocytes, not the subsequent maintenance of pregnancies. Pregnancy failure after AOA may also be due to maternal-related abnormalities, such as hormonal disorders. It would be very interesting if future studies correlate outcomes of our in-house PLC $\zeta$  assay with live-birth and miscarriage rates from AOA cycles.

Patient 8 (Table 3.2 and Figure 3.8) showed PLC $\zeta$  deficiency but was assigned to category II. Notably, this patient was globozoospermic. This indicates that a PLC $\zeta$  assay for globozoospermic patients should be established separately from normozoospermic males and should be compared with globozoospermic controls who have fathered a child without AOA treatment. Thus far, I have received only one male with globozoospermia; therefore, I did not separate his PLC $\zeta$  data from the others.

In addition, in the present study, I also identified significant associations between PLC $\zeta$  expression levels and male infertility, which was in line with previous

studies (Yelumalai *et al.* 2015; Nazarian *et al.* 2019). To better understand PLC $\zeta$  expression at the physiological level, it would be very interesting if future studies investigate the expression of PLC $\zeta$  in sperm between different ejaculations from the same male, or the expression of PLC $\zeta$  between ejaculated sperm and surgical retrieved sperm.

Collectively, the findings of this chapter provide significant evidence on the applicability of our in-house PLC $\zeta$  assay as a diagnostic assay which allows for an individualised strategy for AOA treatment. The PLC $\zeta$  assay also meets the requirements of the HFEA in that AOA should only be applied in selected patients with proven evidence, such as PLC $\zeta$  deficiency (HFEA 2018b). However, no such assay had been established prior to this thesis. Therefore, our in-house PLC $\zeta$  assay represents the first assay with the potentials to provide such information. More importantly, this PLC $\zeta$  assay would be performed within IVF laboratories and should be therefore popularised, as performing the assay does not require special equipment, such as Ca<sup>2+</sup> monitor, although a fluorescence microscope, an imaging software, and a trained laboratory staff are needed.

There are some limitations in the present study, however. Due to small patient numbers in the present study, the sensitivity and specificity of the current PLC $\zeta$  assay could not be calculated; it would be imperative to increase patient numbers to examine the accuracy of this assay further and to enlarge the current database relating to PLC $\zeta$  parameters. In addition, as required by the HFEA, those patients who showed normal PLC $\zeta$  parameters are not eligible for AOA treatment in the U.K, which also limits the evaluation of PLC $\zeta$  accuracy. Future studies could include sperm samples from collaborating IVF laboratories in Europe, on the condition that ethical issues of transporting sperm samples can be approved.

Nevertheless, I agree with the HFEA guidance with states AOA treatment should be confined to patients with deficient PLC $\zeta$ , as not all infertile males gain benefit from AOA treatment. More importantly, AOA treatment may cause risks in terms of birth defects and epigenetic issues; producing an endogenous reagent to replace current AOA is therefore of importance. Another limitation of the PLC $\zeta$  assay is that the assay requires fixed sperm and three days for immunolabelling. This means that the assay cannot analyse the sperm chosen for actual fertilisation.

Future studies should also consider other assays when evaluating OAD, such as sperm DNA fragmentation (Pabuccu *et al.* 2017), sperm cation channel dysfunction (Stewart and Davis 2019), MOAT (Vanden Meerschaut *et al.* 2013) or oocyte factors such as CaMKII and MAPK (Yeste *et al.* 2016a).

#### **6.4 An investigation of the genetic mechanisms linking PLC $\zeta$ and male infertility**

Heytens *et al.* provided the first link between male infertility and genetic defects in PLC $\zeta$  exons and suggested that reduced/absent PLC $\zeta$  levels in sperm may be due to such defects (Heytens *et al.* 2009). Following this groundbreaking finding, more studies have been performed to screen PLC $\zeta$  exons (Heytens *et al.* 2009; Kashir *et al.* 2011c; Escoffier *et al.* 2015; Ferrer-Vaquer *et al.* 2016; Torra-Massana *et al.* 2019). However, thus far, only eleven mutations have been identified (**Figure 1.9**); five were found to affect the PLC $\zeta$  X domain (PLC $\zeta$ <sup>C196X</sup>, PLC $\zeta$ <sup>R197H</sup>, PLC $\zeta$ <sup>L224P</sup>, PLC $\zeta$ <sup>H233L</sup> and PLC $\zeta$ <sup>S350P</sup>), and four were found to impair the Y domain (PLC $\zeta$ <sup>L246F</sup> and PLC $\zeta$ <sup>H398P</sup>) and the C2 domain (PLC $\zeta$ <sup>I489F</sup> and PLC $\zeta$ <sup>S500L</sup>). Notably, these studies all involved a PCR-based method. However, the PCR approach is limited by the reading length of DNA sequences, and the performance of PCR can be affected by

contamination (Hyung-Suk *et al.* 1991). Nevertheless, PCR is a widely used approach which provides accurate data with good specificity. Therefore, the present study employed two approaches for screening genetic variations; PCR and high-throughput NGS.

In Chapter 4, a PCR-based approach was applied to screen *PLCζ* 15 exons from 61 infertile males. Due to limited research funding, I screened *PLCζ* entire exons for the first 16 patients, then I only screened exons 6, 11 and 13 for the rest. Exons 6, 11 and 13 received particular screening, as these regions are where the majority of mutations have been identified. PCR successfully sequenced patient DNA, which matched perfectly to the reference database. This suggested that no mutation was identified in the patients screened since I began working on this project. However, the current study only focused on a few selected exons. It would be helpful if future studies screened *PLCζ* all exons. In addition, future studies should recruit patients across different populations, as the current study only recruited patients from U.K. Of the 11 mutations that have been identified, the first two mutations were found in the same globozoospermic male from a Belgian hospital (Ghent, Belgium), the others were found in patients from France, Spain, and China. It is not known whether the distribution of infertile males carrying *PLCζ* mutations are related to population distribution. Therefore, it would be very useful if future studies recruit patients from Africa, Asia, America, Europe, and other parts of U.K.

In Chapter 4, NGS was employed to screen the *PLCζ* promoter, *XY-linker*, and *CAPZA3*, with an aim to detect variations in these regions and to investigate the alternative *PLCζ*-related reasons leading to infertility. The *XY-linker* received particular screening, as this may play a regulatory role for *PLCζ* activity; the

positively charged XY-linker helps PLC $\zeta$  to target on PIP<sub>2</sub> (Swann and Lai 2015; Amdani *et al.* 2016). I screened the entire *XY-linker* but did not identify any mutations. Instead, 4 SNPs were identified in *XY-linker* intron 9 and were only exhibited in infertile males. Furthermore, I attempted to correlate these SNPs with PLC $\zeta$  expression levels in patient sperm. Unfortunately, these infertile patients, who exhibited the same SNPs (rs1428813971), showed variable levels of PLC $\zeta$  expression (i.e., reduced and normal PLC $\zeta$  levels). In addition, the other three SNPs (rs1470348899, rs563105487, and rs148182265) were found in few infertile males only, thus limiting the further correlation analysis of SNPs and PLC $\zeta$  expression. Nevertheless, this is the first attempt to associate NGS data with male infertility. In addition, the present data showed that 4 SNPs were also found from fertile males, thus indicating that these SNPs are unlikely to impair fertility. The correlation of SNPs with male infertility in the present study also provided more detailed information for future studies.

I further investigated the possibility of the effect of these SNPs on gene function with regards to alternative splicing, to verify whether these SNPs would be situated in the intron-exon boundary and thus affecting intron-exon architecture. However, none of SNPs were found to be situated in the intron-exon boundary. Nevertheless, the present data suggests that *PLC $\zeta$*  intron 9 in infertile males is more likely to possess SNPs, although the *XY-linker* region showed no evidence of abnormality. It would be helpful if future studies investigate whether these SNPs affect the generation of miRNAs and thus protein expression, as introns are one source of miRNA production.

Interestingly, although I did not discover variations in the *XY-linker* region, a very recent study identified a mutation, PLC $\zeta$ <sup>V326K fs\*25</sup> that was situated in the exon

9 and represents the first mutation identified in the *XY-linker* region (Torra-Massana *et al.* 2019). Further bioinformatics suggested that the mutation could cause deleterious impact on protein structure and function; a truncated XY-linker, and loss of oocyte activation capability (Torra-Massana *et al.* 2019). The discovery of PLCζ<sup>V326K fs\*25</sup> confirmed the hypothesis of the present study, that a mutant XY-linker can impair PLCζ protein expression and function.

Thus far, few studies have investigated the bidirectional promoter of *PLCζ*; only one variant has been identified in the *CAPZA3* promoter but only in the vicinity of *PLCζ*, which may not affect the transcription of *PLCζ* (Javadian-Elyaderani *et al.* 2016). In the current study, I screened the promoter region, together with *PLCζ* exons 1 and 2; two SNPs were found in intron 1, which is unlikely to have a role. The overall data showed no evidence of any abnormality that could affect subsequent transcription and translation of *PLCζ*. The present study used NGS to investigate *PLCζ*-related genetic variations in infertile males, and this represents the first study to correlate PLCζ protein expression with genetic variations. Furthermore, the present data highlights the fact that there might be other regulatory factors that lead to a dysfunctional PLCζ at transcriptional and translational levels.

Indeed, multiple molecules are involved in gene expression at the transcriptional and translational levels, including RNA polymerase, enhancers, silencers, and RNA molecules, including coding and non-coding RNAs (Khalil *et al.* 2009; Behzadi and Issakhanian 2019). It would be therefore very interesting if future studies investigate whether abnormalities in these factors can impair *PLCζ* expression, which eventually contributes to the dysfunction of the PLCζ protein and male infertility. In addition, I found no evidence that an abnormality of *PLCζ* can impair protein activity, indicating that future studies should investigate factors



upstream of the initiation of transcription, for example, abnormalities in chromatin structure (i.e., chromatin decondensation, DNA methylation or histone modifications) (Klemm *et al.* 2019), DNA rearrangements (García-Peiró *et al.* 2011) and gene deletion/amplification (Baccetti *et al.* 2005). Therefore, future studies should also investigate the associations between these factors and *PLCζ* expression. Alternatively, nuclear position and nuclear neighbourhoods (such as nuclear pore complex, nuclear speckles, inner nuclear membrane, and nuclear lamina) are also suggested to be involved in gene regulation (Kumaran *et al.* 2008; Zhao *et al.* 2009). Therefore, it would be very helpful if future studies verify whether *PLCζ*-related infertility is associated with abnormalities in these factors.

In addition to investigating *PLCζ* genes, the sequencing of *CAPZA3* using NGS identified three mutations (R39C, V145F and A153V) in three infertile males whose sperm exhibited normal morphology. Although mutation R39C and A153V have been identified, this is the first time of correlation of *CAPZA3* expression with male infertility. *In-silico* analysis identified that R39C and V145F were detrimental to protein structures. Further 3D modelling of R39C and V145F mutations showed that the hydrophobicity of this amino acid was increased. These findings were further supported by the fact that, two patients, who possessed these two mutations, experienced zero or very low fertilisation rate in IVF (0% and 10%). This provides us with new insights into the possibility that IVF failure occurred in these two patients because of these *CAPZA3* mutations. Indeed, *CAPZA3* mutations can impair mouse fertility. A mutant mouse, possessing a *CAPZA3* mutation, M44K, exhibited low sperm motility and disorganisation in the mid-piece of the sperm, leading to subfertility or infertility (Geyer *et al.* 2009). It is therefore important that future studies attempt to investigate the effect of the *CAPZA3* mutations on spermiogenesis,

the organisation of the filament actin, sperm function and, eventually, fertility. An appropriate approach with which to investigate these subjects would involve using the gene editing tool (CRISPER-cas9) to knock out *CAPZA3* gene/mutations and *in-vivo* mouse models for studying biological effects. Alternatively, future studies could perform immunofluorescence labelling to investigate whether CAPZA3 expression in sperm is affected by these mutations.

Apart from the small number of patients/controls recruited in the current genetic study, another limitation was that the NGS study only focused on particular regions of the *PLCζ* gene, not the entire *PLCζ* gene. Consequently, it is possible that sequencing missed key abnormalities. However, the cost of screening the entire *PLCζ* entire gene is very high. Nevertheless, this is the first high throughput genetic investigation of *PLCζ*-related mechanisms causing infertility, which not only provides exonic information, but also information relating to the intronic, promoter, and untranslated regions. This study also attempted to associate SNPs with patient fertilisation states and *PLCζ* expression in sperm. Collectively, these provide valuable evidence for the possibility of the existence of other mechanisms causing male infertility.

## **6.5 Artificial oocyte activation (AOA) with ionomycin, calcimycin, and recombinant PLCζ protein**

The use of AOAs has been the source of much debates in the recent years. Although Ebner *et al.* were the first to report that GM508 successfully rescued OAD and achieved live-births for infertile couples (Ebner *et al.* 2012), the fact that ionophore-based AOAs cause abnormal  $\text{Ca}^{2+}$  patterns during oocyte activation and may exert epigenetic influences on infants after AOA treatment cannot be neglected

(Ozil *et al.* 2006; Van Blerkom *et al.* 2015; Norozi-Hafshejani *et al.* 2018). Indeed, birth defects have been observed in infants born via AOA treatment, including anal atresia (Ebner *et al.* 2015a), knee dislocation, unilateral hip, and imperforate anus (Mateizel *et al.* 2018). Although Deemeh *et al.* suggested that the major birth defects rate was not significantly different between infants born through ICSI-AOA and ICSI (Deemeh *et al.* 2015), the safety profile of AOAs should be carefully evaluated. Thus far, only one study compared the activation outcomes between GM508 and ionomycin in mouse and human oocytes, by monitoring intracellular  $\text{Ca}^{2+}$  changes upon microinjection with either wobbler mouse sperm or globozoospermic human sperm (Nikiforaki *et al.* 2016). However, the contributions of the sperm factor to this study cannot be excluded; this may exaggerate the efficacy of AOAs. Furthermore, only one previous study suggested that ionomycin can trigger mouse oocytes to undergo parthenogenetic development, which can serve as a useful option for AOA studies involving mouse/human oocytes (Heytens *et al.* 2008). However, these authors failed to indicate whether ionomycin is the best and safest form of AOA.

In addition, it is also imperative to determine a safer approach to replace current AOAs. Indeed, several attempts have been made to investigate the applicability of PLC $\zeta$  as an alternative option for OAD (Kouchi *et al.* 2004; Rogers *et al.* 2004; Kashir *et al.* 2011b; Yoon *et al.* 2012; Nomikos *et al.* 2013; Chen *et al.* 2019). These studies made a ground-breaking contribution to the understanding that the synthesis of a rhPLC $\zeta$  protein is more appropriate for clinics. Unfortunately, these studies also have shortfalls; mainly because the use of vectors prevented further protein purification, or enzymatic activity maintenance, and thus limited the

activation efficiency in oocytes (details see Chapter 1, Section 1.3.4 and Chapter 5, Section 5.1).

Collectively, in Chapter 5, my main aims were to address these questions by evaluating the activation efficiency of GM508 and ionomycin on mouse oocytes, to synthesise an 'in-house' rhPLC $\zeta$  protein, and by examining the functionality of rhPLC $\zeta$  protein to determine whether rhPLC $\zeta$  protein can serve as a therapeutic reagent for the treatment of OAD. In addition, I aimed to investigate whether simple incubation of rhPLC $\zeta$  protein with mouse oocytes could trigger oocyte activation. This decision was based on previous findings by our group (unpublished): that PLC $\zeta$  does not have to be injected into oocyte to cause Ca<sup>2+</sup> release.

Overall, GM508 and 3  $\mu$ M ionomycin did not effectively stimulate mouse oocytes to activate and develop (27.7% and 21.6%, respectively), while the positive control (7% ethanol) did successfully activate mouse oocytes (66.6%). The success of development in the ethanol-treated oocytes confirms my technique for activating oocytes, while the low activation rates for AOA-treated oocytes indicates that the activation efficiency of ionophore-based chemicals varies. Therefore, the present study highlighted that the mouse model is not the best for assessing activation efficiency of AOA protocols designed for humans. Indeed, although the mouse model helps us to understand biochemical regulations, and the physiological processes of an oocyte during maturation, activation, fertilisation and development, mouse oocytes are inherently different from humans. For example, mRNA profiles coding for enzymes in the last stage of oocyte maturation were found to be different between mouse and human (El Mouatassim *et al.* 1999). In addition, compared to human MII oocytes, mouse MII oocytes are smaller in size (90-100  $\mu$ m vs. 150-180  $\mu$ m), quicker to reach the 2-cell stage (12-16 h vs. 30 h) and the blastocyst stage (120

h vs. 70 h) (Ménézo and Hérubel 2002). Furthermore, embryo metabolism, in terms of ATP consumption and the ability to convert glucose, differs between the two species (Leese 2012). These factors may serve as potential factors that might be responsible for human and mouse oocytes responding to AOAs in different ways. Therefore, using human oocytes, or human oocytes matured *in-vitro*, would be a better approach with which to investigate the efficacy of AOA protocols, subsequent cellular development, and the safety profiles in AOA-treated oocytes.

In addition, the present study provides important evidence that 10  $\mu$ M ionomycin can cause oocyte degradation and necrosis; this was not indicated by any published literature previously (Heytens *et al.* 2008; Nikiforaki *et al.* 2016). In fact, I observed degradation in the mouse oocytes just 10 min after incubation with 10  $\mu$ M ionomycin. This reveals that 10  $\mu$ M ionomycin is very toxic to oocytes, thus highlighting that the use of such chemicals in the clinic should receive full caution. It should be noted that the mouse strain, and the brand of ionomycin used in my experiment, were the same as those used by Heytens *et al.* (2008). I also ensured that the procedures for preparing ionomycin from stock was correct, and that the KSOM and M2 medium for oocyte culture were both in good condition. One possible reason is that the mouse oocytes I used were very poor in quality and thus could not receive such concentrated stimulation. To better verify this, it is very important for future studies to apply ionomycin with gradually increased concentrations (i.e., 3  $\mu$ M, 6  $\mu$ M, 8  $\mu$ M and 10  $\mu$ M) onto mouse oocytes, in order to determine a safer concentration for activating mouse oocytes. Furthermore, it is also important that future studies should apply the TUNEL assay to evaluate DNA degradation and apoptosis in ionomycin-treated oocytes.

Considering the potential risks associated with several chemical AOA's, I attempted to synthesise a rhPLC $\zeta$  protein using a novel approach, the pHLsec vector, and attempted to characterise functionality. As mentioned earlier, previous studies failed to purify protein or retain enzymatic activation. For the first time, the pHLsec vector was used in the present study to generate rhPLC $\zeta$  protein. The pHLsec vector features a secretion signal, which makes purification possible and easier. The Fc-fusion site, and 3C cleavage site, of the vector allow the full length PLC $\zeta$  sequence to be inserted and allows the Fc tag to be removed. In addition, I attempted to generate rhPLC $\zeta$  from a mammalian cell line, instead of a bacterial expression system, in order to ensure my protein was able to reach an active state via post-translational modification.

Gel electrophoresis confirmed that the size of the target protein was appropriate (~65 kDa), although this was slightly lower than expected (70 kDa). Interestingly, Yoon *et al.* used a pET-6His vector and obtained rhPLC $\zeta$  at lower molecular sizes (~68kDa) than expected, but this protein successfully triggered mouse activation (Yoon *et al.* 2012). Differences between the molecular sizes of my rhPLC $\zeta$  protein and the native form of PLC $\zeta$  in male sperm are likely to be due to differences in post-translational modification between the mammalian cell expression system and biological spermiogenesis. Nevertheless, the pHL-PLC $\zeta$  plasmid was verified via sequencing before undergoing transformation and transfection, which ensured that the PLC $\zeta$  sequences have been successfully inserted.

Crucially, Chapter 5 described the successful synthesis of Fc-tagged rhPLC $\zeta$  from a mammalian cell line using the pHL vector that was able to trigger activation in mouse oocytes. This represents a crucial milestone in the establishment of a novel

protocol for rhPLC $\zeta$  synthesis and represents major progress in translating PLC $\zeta$  to the clinics. I also attempted to remove the Fc-tag and produce untagged rhPLC $\zeta$ , thus creating the most endogenous form of PLC $\zeta$  protein. Unfortunately, untagged rhPLC $\zeta$  may fail to maintain its enzymatic activity after tag removal. As a result, none of the mouse oocytes were activated. This could be due to the fact that the Fc tag had been incorporated into the protein structure during post-translational modifications. To better investigate the functionality of the Fc-tag and the untagged rhPLC $\zeta$ , it is essential for future studies to microinject the protein into the oocytes, as this is a direct and standard approach with which to examine the activation effects of the protein. Microinjection was not performed in the current study, as embryologists were not available during the time of this experiment. In addition, it is essential that future studies increase the numbers of mouse oocytes, to better examine the characteristics of rhPLC $\zeta$ . It would be especially useful for clinics, if future studies evaluate activation efficiency of rhPLC $\zeta$  on human oocytes and compared these to other AOA, including GM508 and ionomycin.

Another crucial finding in the present study is that simple incubation with Fc-tagged rhPLC $\zeta$  was able to activate mouse oocytes (42.3% of oocytes reaching the 2-cell stage). Although this study is at a preliminary stage, the current results represent a significant advance in broadening our knowledge of oocyte activation; it is possible that a plasma-membrane bound mechanism may exist. In line with this, the evidence that KO mice, lacking the entire or partial *PLC $\zeta$*  gene, both produced viable pups also supports the possibility of the existence of an alternative activation factor/mechanism (Hachem *et al.* 2017a; Nozawa *et al.* 2018). However, it remains unclear what this exact mechanism actually is. One possible explanation is that there an atypical form of Ca<sup>2+</sup> release might occur when PLC $\zeta$  is partially or

completely dysfunctional (Hachem *et al.* 2017a). Interestingly, the current study utilised the PIV assay as a non-invasive indicator to study the patterns of cytoplasmic movement, and found that the patterns were different to those at fertilisation. It cannot be excluded that an atypical form of  $\text{Ca}^{2+}$  release was induced via incubation with rhPLC $\zeta$  protein, and was not detected by the PIV assay. This is because the PIV assay only characterises the patterns of cytoplasmic movement at normal fertilisation (i.e., oscillatory patterns) (Swann *et al.* 2012).

Interestingly, the present study showed that incubation with rhPLC $\zeta$  for 15 min did not activate oocytes, which indicates that this mechanism could be time-dependent. However, the exact mechanism underlying PLC $\zeta$ -induced oocyte activation via the membrane needs to be further examined. This could be related to a receptor in the plasma membrane, for example, the tyrosine kinase receptor, (Piiper and Zeuzem 2004), non-specific binding molecules, regulators related to the GPCR or CaMKII pathways (Hannan *et al.* 2018), or some other oocyte unusual factor. Therefore, in order to further investigate this plasma membrane-bound mechanism, it is crucial for future studies to incubate mouse oocytes with a rhPLC $\zeta$  with proven functionality, for example, to use the rhPLC $\zeta$  as described in Kashir *et al.* (2011b), Yoon *et al.* (2012), or Nomikos *et al.* (2013). In addition, immunofluorescence labelling and enzymatic assays would also provide valuable information to help clarify whether these potential candidates play a role in oocyte activation. It would also be interesting if future studies investigate the activities of downstream regulators in the  $\text{Ca}^{2+}$  pathway (i.e.,  $\text{InsP}_3$ , DAG, PKC and MAPK), at oocyte activation induced by rhPLC $\zeta$  incubation. In addition,  $\text{Ca}^{2+}$  imaging could be performed to confirm the precise pattern of  $\text{Ca}^{2+}$  release. Such studies are essential



to further clarify the relative importance of the plasma membrane-related mechanisms induced via rhPLC $\zeta$  incubation.

## **6.6 Is PLC $\zeta$ the sole factor at oocyte activation?**

Since the discovery of PLC $\zeta$  (Saunders *et al.* 2002), an increasing body of evidence supports the significant role of PLC $\zeta$  in mammalian oocyte activation (Yoda *et al.* 2004; Knott *et al.* 2005; Nomikos *et al.* 2005; Yoon and Fissore 2007; Grasa *et al.* 2008; Heytens *et al.* 2009; Kashir *et al.* 2011b; Kashir *et al.* 2011c; Pan *et al.* 2013; Nomikos *et al.* 2014b; Ferrer-Vaquer *et al.* 2016; Yeste *et al.* 2016b; Sanders *et al.* 2018; Dai *et al.* 2019). However, recent studies have demonstrated that PLC $\zeta$ -deficient sperm from a KO mouse model was able to produce viable pups (Hachem *et al.* 2017a; Nozawa *et al.* 2018). These findings indicate that other sperm factors, or complementary mechanisms, may exist or co-exist with PLC $\zeta$ .

Indeed, other sperm factors have been proposed as SOAF candidates, including PAWP, tr-kit, and citrate synthase. A recent study evaluated PAWP expression in infertile male sperm and globozoospermic sperm, and found a positive correlation between PAWP expression levels and fertilisation rates in ICSI (Tanhaei *et al.* 2019). These authors further proposed that PAWP may play a functional role in fertilisation aside from oocyte activation, or assist PLC $\zeta$  in triggering oocyte activation (Tanhaei *et al.* 2019).

However, none of these SOAF candidates are associated with direct evidence relating to the independent ability to trigger Ca<sup>2+</sup> oscillations. Indeed, Ca<sup>2+</sup> oscillations represent a universal act in oocyte activation across species (Kashir *et al.* 2013a), and has always been associated with a sign of successful oocyte activation. However, as mentioned earlier, other mechanisms can trigger oocyte

activation bypassing  $\text{Ca}^{2+}$  signalling pathway, for example, MPF deactivation (Fujinami *et al.* 2004) or a reduction in  $\text{Zn}^{2+}$  levels (Uh *et al.* 2019). Therefore, it is unknown whether other sperm-borne factor(s)/molecules play a biological role in oocyte activation, when PLC $\zeta$  is completely/partially dysfunctional. Nevertheless, it is confirmed that PLC $\zeta$  remains as the dominant SOAF contributing to oocyte activation, at least, for now.

In addition to SOAF candidates, other molecules on the perinuclear theca, such as acrosin, calicin, cyclin I and II are also associated with sperm function at fertilisation, because they are involved in the skeletal formation and morphogenesis of sperm (Hess *et al.* 1995). Currently, there is no evidence to indicate that these factors directly regulate oocyte activation, which needs future studies to investigate further.

The fact, that I did not identify any variations in *PLC $\zeta$*  coding regions, or the promoters in the present study, indicates that the cause of male infertility in our patients might not be only related to *PLC $\zeta$*  dysfunction. Other male factors also lead to fertilisation failure, for example, sperm flagellar abnormalities, sperm structure defects (abnormal sperm head vacuole)(Perdrix *et al.* 2012), dysfunctional  $\text{Ca}^{2+}$  channel function, premature chromosomal condensation, and sperm DNA damage (Jeve *et al.* 2018). These were not evaluated in the present study, and future studies can investigate these aspects.

In addition, the results described in the present genetic study potentially indicate that the function of PLC $\zeta$  might be related to epididymosome-sperm interactions, although it is a very speculative hypothesis. A very recent study proposed that epididymosomes serve as a cargo to deliver protein, enzymes and cytokines to the post-acrosomal region of the sperm head, assisted by the

mechanoenzyme (Zhou *et al.* 2019). Such epididymosome-sperm interactions therefore assist sperm to mature and acquire functional capability. It is not known whether PLC $\zeta$  is required to undergo such processing to become functional, or whether PLC $\zeta$  is processed by mechanoenzyme or epididymosomes in the microenvironment of the epididymis. It would be therefore very interesting for future studies to investigate such aspects.

Apart from sperm factors, oocyte-related factors should be considered. Borges *et al.* applied AOA onto oocytes from women at different age, after injection with sperm from respective male partner (Borges Jr *et al.* 2009b). These authors did not find that AOA significantly improved the fertilisation and implantation rates in these couples, however, a statistical significance was found when evaluating these parameters only in women younger than 36 years. This indicates that successful oocyte activation also relates to the quality of oocyte. Indeed, sperm factor(s) plays a crucial role in triggering oocyte activation, while the successful development of an oocyte into an embryo involves multiple important mechanisms and oocyte-related factors. For example, abnormal formations of polar body and spindle structures have also been suggested to result in OAD (Combelles *et al.* 2010). In addition, mitochondrial activities, that supply energy to pronuclei, may also relate to successful oocyte activation (Ebner *et al.* 2004). All oocyte-related factors can serve as potential agents contributing to successful oocyte activation, which needs further investigation.

## **6.7 General conclusions**

In summary, this thesis investigated the diagnostic and therapeutic role of PLC $\zeta$  in male-related infertility and the mechanisms underlying oocyte activation. A

comparative immunofluorescence study between our in-house assay and a published AUM method demonstrated that our in-house assay provides the best options to evaluate PLC $\zeta$  expression in frozen/fresh-fixed sperm from fertile and infertile males. This supports the future use of our assay as a reliable and standard approach for evaluating PLC $\zeta$  parameters; mean PLC $\zeta$  levels in sperm and proportion of sperm exhibiting PLC $\zeta$ . Although the visualisation of PLC $\zeta$  was improved in fertile frozen sperm stained with an AUM method compared with a non-AUM method, our in-house assay showed the best efficiency and reliability in terms of visualising PLC $\zeta$  in sperm with/without cryopreservation. Furthermore, our in-house assay showed better specificity than the AUM method, showing that the improved PLC $\zeta$  visualisation shown by the AUM method might be related to non-specific binding. Collectively, these results show that the AUM method may not be needed and supports the continued use of our in-house assay for clinical screening.

Crucially, the present study demonstrates, for the first time, the applicability of our in-house assay as a diagnostic indicator to inform clinical decision-making for the treatment of couples suffering from male-factor infertility. This is the first demonstration of the efficacy and accuracy of our in-house assay in the provision of PLC $\zeta$  expression data which was successfully used to inform clinical decision making with regards to AOA. In addition, cut-offs for PLC $\zeta$  deficiency, and categorisation of PLC $\zeta$  parameters in patients, were defined. These can now be used as reference standards for evaluating PLC $\zeta$  deficiency in the clinic. These results represent a crucial step towards translating our in-house PLC $\zeta$  assay to a diagnostic indicator for clinical purpose. More importantly, guided by the PLC $\zeta$  assay, clinical outcomes further confirmed such application; the fertilisation rate in patients with PLC $\zeta$  deficiency improved from 18.6% to 56.8%, resulting in the birth of four

healthy infants following ICSI-AOA (pregnancy rate 60% and live-birth rate 40% per initiating cycle). These represent the first series of AOA cases in Oxford. In addition, an algorithm was created for the management of patients, for whom AOA is likely to be beneficial.

This thesis also investigated genetic variations in the *PLCζ*, and *CAPZA3* genes, in infertile males, using a PCR-based approach and NGS. The PCR-based approach did not find any mutations in *PLCζ* exons, indicating that PCR technology might not be sufficient for mutation screening, especially when large numbers of patient samples are involved. Therefore, a high throughput approach is needed. Subsequently, the NGS study identified 6 SNPs in *PLCζ* introns of infertile males. However, these SNPs did not appear to interfere with gene expression, indicating that screening of the *PLCζ* entire gene would provide more information. In addition, the infertile males who possessed SNPs were found to exhibit variable levels of *PLCζ* expression in sperm, thus suggesting that these SNPs may not be the reason underlying variable *PLCζ* expression.

In addition, three mutations were identified in the *CAPZA3* gene in infertile males (R39C, V145F and A153V); two of these (R39C and V145F) were suggested to impair protein structure. A review of the patients possessing these two mutations showed that the patients experienced a zero or a very low fertilisation rate in IVF. Consequently, these *CAPZA3* mutations can represent a causative factor for IVF failure in these patients. However, this needs to be investigated further. 3D modelling in the present study further indicated that the R39C and V145F mutations increased the hydrophobicity of *CAPZA3* residues.

Collectively, my overall data represent the first preliminary study to use NGS technology to investigate *PLCζ*-related mechanisms causing infertility. Screening of

*PLCζ* exons, the *XY-linker* and the promoter, showed no evidence of any abnormality that may affect subsequent transcription and translation in infertile males. These results indicate that other mechanisms are likely to be involved in the abnormal expression and function of *PLCζ*.

This thesis further evaluated the activation efficiency of ionophore-based AOAs and successfully synthesised purified rh*PLCζ* protein with a novel approach involving the use of the pHLsec vector. GM508- and ionomycin-activated oocytes both showed low activation rates (27.7% and 21.6%, respectively), thus indicating that the mouse model is inappropriate for evaluating AOA protocols designed for humans. Furthermore, the present study also provides important evidence to suggest that 10  $\mu$ M ionomycin can be harmful to mouse oocytes. In a subsequent study, I synthesised rh*PLCζ* protein and examined its function in two forms: Fc-tagged and untagged. Crucially, Fc-tagged rh*PLCζ* partially activated mouse oocytes to develop to the 2-cell stage (42.3%). However, untagged rh*PLCζ* protein did not trigger oocyte activation. This implies that my rh*PLCζ* protein requires a Fc tag to retain its enzymatic activity and the tag may have been incorporated into the protein structure during post-translational modification.

Another essential finding in this thesis is the finding that simple incubation with Fc-tagged rh*PLCζ* triggered oocyte activation in mouse. This implies that a plasma-membrane-bound mechanism might be involved in oocyte activation. Interestingly, the patterns of cytoplasmic movement in mouse oocytes incubated with Fc-tagged rh*PLCζ* were different to those observed at normal fertilisation. This also supports the possibility of the existence of an alternative factor/mechanism induced via *PLCζ* incubation.

Collectively, results described in this thesis support the crucial role of PLC $\zeta$  in mammalian oocyte activation, translate PLC $\zeta$  as a diagnostic indicator to clinical application, and demonstrate the potentials for rhPLC $\zeta$  as an endogenous reagent for therapeutic purpose. The present results also dictate the complexity of PLC $\zeta$ -induced mechanism of oocyte activation. Therefore, this thesis improved our understanding with regards to the complex biological role of PLC $\zeta$ , translated PLC $\zeta$  characteristics to clinical diagnosis and, hopefully, will help future studies in the progress of translating PLC $\zeta$  to a clinical-available AOA.

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**Appendix Table 1. Reports of clinical case(s) studies of artificial oocyte activation (AOA) on patients with fertilisation failure**

| References                      | AOA type                                | Patient selection criteria                                                                                             | Fertilisation rate % (PN/total injected oocytes)                |                                         |                           |          | Pregnancy rate % (number of pregnancy/ET) |              | Number of live births after AOA |
|---------------------------------|-----------------------------------------|------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------|-----------------------------------------|---------------------------|----------|-------------------------------------------|--------------|---------------------------------|
|                                 |                                         |                                                                                                                        | ICSI                                                            | AOA-ICSI                                | ICSI                      | AOA-ICSI | ICSI                                      | AOA-ICSI     |                                 |
| Darwish <i>et al.</i> (2015)    | Calcimycin                              | 4 couples whose embryos arrested at PN stage in ICSI                                                                   | (1 with ICSI and 1 with ICSI/AOA)                               | 55 (22/40)                              | 67.6 (25/37)              |          | 0                                         | 25 (1/4)     | 2                               |
|                                 |                                         |                                                                                                                        |                                                                 | Cleavage rate: 0 (0/22)                 | Cleavage rate: 44 (11/25) |          |                                           |              |                                 |
| Kim <i>et al.</i> (2015)        | Calcimycin                              | A couple with LF failure in ICSI of <i>in-vitro</i> maturation                                                         | (1 with ICSI and 2 with ICSI/AOA)                               | 18.2 (2/11)                             | 83.3 (20/24)              |          | 0 (0/2)                                   | 33 (2/6)     | 2                               |
| Nicopoulos <i>et al.</i> (2015) | GM508 or Mechanical                     | 2 couples with repeated TFF in ICSI and IMSI (9 with ICSI, 2 split cycles with IMSI and IMSI/AOA, and 1 with IMSI/AOA) | GM508 (IMSI)                                                    | ICSI: 2.6 (7/277)*<br>IMSI: 17.6 (3/17) | 52.6 (10/19)#             |          | 0 (0/2)                                   | 75 (3/4)     | on-going by the time of writing |
|                                 |                                         |                                                                                                                        | Mechanical AOA (IMSI)                                           |                                         | 0 (0/16)                  |          |                                           |              |                                 |
| Kim <i>et al.</i> (2014)        | Calcium ionophores or SrCl <sub>2</sub> | Cases report of 4 couples experienced TFF or LF in ICSI                                                                | No AOA (n=9)                                                    | 20 (14/70)                              |                           |          | 0 (0/15)                                  |              |                                 |
|                                 |                                         |                                                                                                                        | Calcium ionophore (n=15)                                        |                                         | 25.3 (40/158)             |          | 0 (0/9)                                   |              |                                 |
|                                 |                                         |                                                                                                                        | SrCl <sub>2</sub> (n=12)                                        |                                         | 61.7 (66/107)             |          | 41.7 (5/12)                               |              | 8                               |
| Isachenko <i>et al.</i> (2010)  | Calcium ionophores                      | A couple of which male partner is asthenoteratozoospermia                                                              | (1 with ICSI and 1 split cycle for ICSI and ICSI/AOA)           | 57 (4/7)                                | 83 (5/6)                  |          | 0 (0/2)                                   | 100 (2/2)    |                                 |
| Terada <i>et al.</i> (2009)     | Ionomycin                               | A couple with LF in ICSI (n=7)                                                                                         | (7 with ICSI 1 with ICSI/AOA)                                   | 17.8 (8/45)                             | 97 (23/33)                |          | 33.3 (1/3)                                |              | 1                               |
| Ahmady and Michael (2007)       | Calcimycin                              | A couple with suspected azoospermia (n=1)                                                                              | nd                                                              |                                         | 100 (1/1)                 |          | 0                                         | 100 (1/1)    | 1                               |
| Yanagida <i>et al.</i> (2006)   | SrCl <sub>2</sub>                       | A couple with LF in ICSI                                                                                               | (8 with ICSI and 1 with ICSI/AOA)                               | 17 (8/58)                               | 100 (6/6)                 |          | 0                                         | 50 (1/2)     | 1                               |
| Chi <i>et al.</i> (2004)        | Calcimycin                              | A couple with repeated fertilization failure in IVF and ICSI                                                           | (1 with ICSI and 1 with ICSI/AOA)                               | 25 (4/16)                               | 80 (12/15)                |          | 0                                         | 12.5 (1/8)   | on-going at the time of writing |
| Murase <i>et al.</i> (2004)     | Calcimycin + puromycin                  | A couple with repeated fertilization failure in IVF and ICSI                                                           | (1 with IVF, 1 with IVF/ICSI, 3 with ICSI, and 1 with ICSI/AOA) | 1.4 (1/71)                              | 66.7 (8/12)               |          | nd                                        | FET 20 (1/5) | 3                               |
| Eldar-Gev <i>et al.</i> (2003)  | Calcimycin                              | A couple with TFF in IVF and ICSI                                                                                      | (1 with IVF and 1 with ICSI, and 3 with ICSI/AOA)               | 0 (0/9)                                 | 37 (17/57)                |          | 0                                         | 40 (4/10)    | 3                               |

AOA: artificial oocyte activation; ET: embryo transfer; LF, low fertilisation after previous conventional ICSI; IMSI: introytoplasmic morphological selected sperm; nd: no data indicated; PN: pronuclei formation; TFF: total fertilisation failure; \*: estimated, some date in a cycle was not indicated; # data included a donor's oocytes

**Appendix Table 2. Summary of artificial oocyte activators (AOAs) on clinical outcomes**

| References                                                         | AOA type                        | Selection criteria and patient group information<br>(n=number of participants otherwise stated)                                             | Fertilisation rate % (PN/total injected oocytes ) |                   | Pregnancy rate per ET otherwise stated |               | Live-birth rate or No. of live-birth after AOA |
|--------------------------------------------------------------------|---------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------|-------------------|----------------------------------------|---------------|------------------------------------------------|
|                                                                    |                                 |                                                                                                                                             | ICSI                                              | AOA-ICSI          | ICSI                                   | AOA-ICSI      |                                                |
| Studies suggested that AOA is beneficial to investigated subjects: |                                 |                                                                                                                                             |                                                   |                   |                                        |               |                                                |
| Bonte <i>et al.</i> (2019)                                         | Ionomycin                       | Couples with either TFF (0-10%), or LF (≤33.3%) (n=122)                                                                                     | 9.7 (18/185)                                      | 70.1 (330/471)    | 0 (0/9)                                | 49.0 (25/51)  | 41.2 (21/51)                                   |
|                                                                    |                                 | Sperm-related OAD (MOAT 1) (n=19)                                                                                                           |                                                   |                   |                                        |               |                                                |
|                                                                    |                                 | Diminished oocyte activating capacity (MOAT 2) (n=56)                                                                                       | 14.8 (163/1,104)                                  | 63.0 (500/794)    | 7.0 (5/71)                             | 34.9 (37/106) | 22.6 (24/106)                                  |
|                                                                    |                                 | Presumably oocyte-related OAD (MOAT 3) (n=47)                                                                                               | 17.7 (105/595)                                    | 57.3 (274/478)    | 7.0 (3/43)                             | 29.4 (20/68)  | 22.1 (15/68)                                   |
| Li <i>et al.</i> (2019b)                                           | Ionomycin                       | Patients with severe teratozoospermia and couples with TFF (0%) or LF (<20%) (n=50)                                                         | 33.9 (85/251)                                     | 50.4 (132/262)    | 25 (6/24)                              | 38.5 (10/26)  | 38.5 (10/26)                                   |
| Nazarian, <i>et al.</i> (2019)                                     | Calcimycin                      | Couples referred to ICSI treatment (n=26), subgroup as percentage of sperm expressing PLCζ (<60%: PLCζ deficiency; >85%: control)           | 41.9 (106 cycles)                                 | 69.5 (114 cycles) | nd                                     | nd            |                                                |
|                                                                    |                                 | control (113 cycles)                                                                                                                        | 78.1                                              |                   | nd                                     |               |                                                |
| Ferrer-Buitrago, <i>et al.</i> (2018b)                             | Ionomycin                       | Males with LF in ICSI (<33.3) (n=16)                                                                                                        | 10.2 (28/273)                                     | 47.3 (61/129)     | nd                                     | nd            | 4 + 1 on-going by the time                     |
| Karabulut, <i>et al.</i> (2018)                                    | GM508                           | Couples with either TFF, no history of previous IVF, at least once ICSI failure or at least once successful ICSI, which were subclassified: |                                                   |                   |                                        |               |                                                |
|                                                                    |                                 | TFF in ICSI (n= 19 cycles):                                                                                                                 | nd                                                | 74.1              | nd                                     | 28.6          |                                                |
|                                                                    |                                 | Low oocyte count (n=17)                                                                                                                     | 66.7 (n=20)                                       | 80.2              | 12.5 (n=20)                            | 27.2          |                                                |
|                                                                    |                                 | Oligoasthenoteratozoospermia (n=23)                                                                                                         | 65.5 (n=20)                                       | 69                | 20 (n=20)                              | 66.6          |                                                |
|                                                                    |                                 | ICSI with frozen sperm (n=19)                                                                                                               | 59.2 (n=20)                                       | 61.2              | 40 (n=20)                              | 100           |                                                |
| Fawzy, <i>et al.</i> (2018)                                        | Calcimycin or SrCl <sub>2</sub> | TFF or LF (<30%) in previous two ICSI cycles                                                                                                |                                                   |                   |                                        |               |                                                |
|                                                                    |                                 | No AOA (n=115)                                                                                                                              | 61 (720/1180)                                     |                   | 27 (31/115)                            |               | 21                                             |
|                                                                    |                                 | Calcimycin (n=113)                                                                                                                          |                                                   | 67 (874/1310)     |                                        | 42 (48/113)   | 37                                             |
|                                                                    |                                 | SrCl <sub>2</sub> (n=115)                                                                                                                   |                                                   | 85 (1168/1377)    |                                        | 49 (56/115)   | 46                                             |

(Continued)

| References                              | AOA type                           | Selection criteria and patient group information<br>(n=number of participants otherwise stated)                                                                                                                                       | Fertilisation rate % (PN/total injected oocytes )                |                                                |                                           | Pregnancy rate per ET otherwise stated                       |                                          | Live-birth rate or No. of live-birth after birth after AOA |
|-----------------------------------------|------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------|------------------------------------------------|-------------------------------------------|--------------------------------------------------------------|------------------------------------------|------------------------------------------------------------|
|                                         |                                    |                                                                                                                                                                                                                                       | ICSI                                                             | AOA-ICSI                                       | ICSI                                      | AOA-ICSI                                                     |                                          |                                                            |
| Norozi-Hafshejani, <i>et al.</i> (2018) | Ionomycin and/or SrCl <sub>2</sub> | Infertile males (n=105) with 99-100% abnormal sperm morphology<br><br>Half of oocytes per patient received Ionomycin and the other half received SrCl <sub>2</sub> (n=66)<br><br>ICSI with Ionomycin (n=39)                           | oocytes received Ionomycin<br>oocytes received SrCl <sub>2</sub> | 65.23<br>49.65<br>68.58                        | nd                                        | nd                                                           | 25 (4/16)<br>23.8 (5/21)<br>23.52 (8/34) | 6<br>4<br>7                                                |
| Capalbo, <i>et al.</i> (2016)           | Calcimycin                         | Endometriosis, male factor, tubal or idiopathic etiologies (n=12)                                                                                                                                                                     | nd                                                               | 79.6 (39/49)                                   | nd                                        | nd                                                           | nd                                       | 9                                                          |
| Ebner, <i>et al.</i> (2015a)            | GM508                              | Fertilization abnormalities (≤ 50% fertilized oocytes in ICSI) (n=101 cycles)                                                                                                                                                         | nd                                                               | 99 (100/101)                                   | nd                                        | 37 (37/100)                                                  | 28                                       | 28                                                         |
| Ebner, <i>et al.</i> (2015b)            | GM508                              | Couples whose embryo failed to reach blastocyst stage (n=57)                                                                                                                                                                          | 73.2 (358/489)                                                   | 75.3 (386/512)                                 | 12.8 (6/39)                               | 45.1 (28/51)                                                 | 23                                       | 23                                                         |
| Vanden Meerschaut, <i>et al.</i> (2013) | Ionomycin                          | TFF or LF (≤33.3%) after ICSI<br><br>MOAT ≤ 20% (n=7)<br><br>MOAT 21 - 84% (n=10)<br><br>MOAT = 94% (n=9)                                                                                                                             | 18.7 (23/123)<br>13.4 (31/230)<br>24 (52/216)                    | 63.5 (75/118)<br>70.5 (91/129)<br>53.7 (51/95) | nd                                        | nd                                                           | 4<br>4 (2 twins)<br>4                    | 4                                                          |
| Yoon, <i>et al.</i> (2013)              | Calcimycin+ SrCl <sub>2</sub>      | TFF (n=98) or LF (<50%, n=72) in ICSI with subclassied as sperm source:<br><br>Ejaculated-normal (n=57)<br>Ejaculated-oligo-astheno-terato (n=69)<br>Testicular-extracted (n=44)<br><br>TFF or LF (<50%) in ICSI with subclassied as: | 29.9<br>38.2<br>37.4<br>0<br>34.8                                | 71.5<br>74.6<br>76.3<br>75.7<br>68.2           | 4.5 (1/22)<br>7.9 (3/38)<br>0<br>0<br>5.6 | 26.3 (15/57)<br>26.1 (18/69)<br>36.4 (16/44)<br>32.7<br>23.6 | 38 babies from AOA treatment             | 38                                                         |
| Ebner, <i>et al.</i> (2012)             | Calcimycin                         | Azoo- or cryotozoospermia (n=66)                                                                                                                                                                                                      | 34.7 (244/706)                                                   | 56.9 (379/666)                                 | 6.3 (6/79)                                | 29.7 (34/73)                                                 | 32                                       | 32                                                         |

(Continued)

| References                              | AOA type                        | Selection criteria and patient group information<br>(n=number of participants otherwise stated) | Fertilisation rate % (PN/total injected oocytes ) |                | Pregnancy rate per ET otherwise stated |              | Live-birth rate or No. of live-birth after AOA |
|-----------------------------------------|---------------------------------|-------------------------------------------------------------------------------------------------|---------------------------------------------------|----------------|----------------------------------------|--------------|------------------------------------------------|
|                                         |                                 |                                                                                                 | ICSI                                              | AOA-ICSI       | ICSI                                   | AOA-ICSI     |                                                |
| Kyono, <i>et al.</i> (2012)             | Calcimycin or SrCl <sub>2</sub> | TFF or LF (≤33.3%) after ICSI (n=85) with subclassified:                                        |                                                   |                |                                        |              |                                                |
|                                         |                                 | LF group with calcimycin (n=50)                                                                 | 20.9 (50/239)                                     | 62.4 (131/210) | 0 (0/20)                               | 24.3 (9/37)  | 10                                             |
| Montag, <i>et al.</i> (2012)            | Calcimycin                      | LF group with SrCl <sub>2</sub> (n=35)                                                          | 24.7 (48/194)                                     | 54.5 (97/178)  | 0 (0/15)                               | 27.3 (6/22)  | 12 (including 1 twin)                          |
|                                         |                                 | ICSI with previous (n=89):                                                                      |                                                   |                | in initial cycle /ET                   |              |                                                |
| Vanden Meerschaut, <i>et al.</i> (2012) | Ionomycin                       | TFF (n = 27)                                                                                    | 0 (0/114)                                         | 41.6 (96/231)  | 0                                      | 18.8 (6/32)  | 5                                              |
|                                         |                                 | LF between 0-29% (n = 38)                                                                       | 19.3 (72/373)                                     | 44.4 (161/363) | 12.8 (6/47)                            | 31.4 (16/51) | 14                                             |
|                                         |                                 | LF between 30-50% (n = 24)                                                                      | 36.8 (64/174)                                     | 56.1 (124/221) | 16 (4/25)                              | 27.6 (8/29)  | 6                                              |
|                                         |                                 | TFF or LF (≤33.3%) after ICSI                                                                   |                                                   |                | per cycle                              |              |                                                |
| Borges, <i>et al.</i> (2009b)           | Calcimycin                      | Couples attending clinics (n=314) with subclassified:                                           |                                                   |                |                                        |              |                                                |
|                                         |                                 | Ejaculated (n = 92)                                                                             | 43.5 (40/92)                                      | 74.2 (75/101)  | 0 (0/28)                               | 35.7 (5/14)  | 2                                              |
|                                         |                                 | Epididymal (n = 82)                                                                             | 76.2 (259/340)                                    | 69.4 (220/317) | 21.7 (10/46)                           | 23.9 (11/46) |                                                |
|                                         |                                 | Testicular (n = 140)                                                                            | 66.6 (399/599)                                    | 48.9 (254/519) | 31.7 (13/41)                           | 26.8 (11/41) |                                                |
| Heindryckx, <i>et al.</i> (2008)        | Ionomycin                       | LF (n=30) after ICSI, with subclassified:                                                       |                                                   |                |                                        |              |                                                |
|                                         |                                 | MOAT 0-20% (n=16)                                                                               | 5.9 (11/186)                                      | 75 (307/407)   | 0 (0/19)                               | 34 (12/35)   | 17                                             |
|                                         |                                 | MOAT 20-85% (n=7)                                                                               | 22 (36/164)                                       | 73 (81/111)    | 0 (0/18)                               | 43 (6/14)    | (13 singleton and 4 twins)                     |
|                                         |                                 | MOAT 85-100% (n=7)                                                                              | 14 (19/138)                                       | 75 (78/104)    | 0 (0/13)                               | 17 (20/61)   |                                                |

**(Continued)**

| References                                               | AOA type                                       | Selection criteria and patient group information<br>(n=number of participants otherwise stated) | Fertilisation rate % (PN/total injected oocytes ) |                | Pregnancy rate per ET otherwise stated |             | Live-birth rate or No. of live-birth after AOA |
|----------------------------------------------------------|------------------------------------------------|-------------------------------------------------------------------------------------------------|---------------------------------------------------|----------------|----------------------------------------|-------------|------------------------------------------------|
|                                                          |                                                |                                                                                                 | ICSI                                              | AOA-ICSI       | ICSI                                   | AOA-ICSI    |                                                |
| Heindryckx, <i>et al.</i> (2005)                         | Calcimycin                                     | TFF or LF (n=10) after ICSI, with subclassified:                                                |                                                   |                |                                        |             |                                                |
|                                                          |                                                | TFF (n=4):                                                                                      | nd                                                | 78 (25/32)     | nd                                     | 0           |                                                |
|                                                          |                                                | MOAT 0-9% (n=2)                                                                                 |                                                   |                |                                        |             |                                                |
|                                                          |                                                | MOAT 9-94% (n=1)                                                                                | nd                                                | 74 (14/19)     | nd                                     | 0           |                                                |
|                                                          |                                                | MOAT >94% (n=1)                                                                                 | 0 (0/5)                                           | 66.6 (6/9)     | 0 (0/1)                                | nd          |                                                |
| Tesarik, <i>et al.</i> (2002)                            | Calcimycin                                     | LF (n=4)                                                                                        | 9 (11/118)                                        | 79 (77/97)     | nd                                     | nd          |                                                |
|                                                          |                                                | LF + embryo arrest (n=2)                                                                        | 50 (6/12)                                         | 64.7 (11/17)   | nd                                     | 0 (0/5)     |                                                |
|                                                          |                                                | Fertilization failure in ICSI with female factor-related OAD (n=3)                              | 25 (18/72)                                        | 78.5 (11/14)   | 30 (3/10)                              | 33.3 (1/3)  | 1                                              |
| Hoshi, <i>et al.</i> (1995)                              | Calcimycin, electroporation, or mechanical AOA | Failed fertilization in IVF with oligozoospermia or asthenozoospermia (n=109):                  |                                                   |                |                                        |             |                                                |
|                                                          |                                                | Calcimycin (n=24)                                                                               |                                                   | 48.4 (107/221) |                                        | 5.9 (2/34)  | 1                                              |
|                                                          |                                                | Electroporation (n=13)                                                                          | nd                                                | 51.9 (27/52)   | nd                                     | 0 (0/10)    |                                                |
|                                                          |                                                | Mechanical AOA with immobilized sperm (n=11)                                                    |                                                   | 45.3 (29/64)   |                                        | 0 (0/6)     |                                                |
| Studies suggested that AOA did not improve ICSI outcomes |                                                | Mechanical AOA with mobilized sperm (n=61)                                                      |                                                   | 49.9 (205/411) |                                        | 12 (6/50)   | 1                                              |
|                                                          |                                                |                                                                                                 |                                                   |                |                                        |             |                                                |
| Aydinuraz, <i>et al.</i> (2016)                          | GM508                                          | Teratozoospermia and LF (<50%) (n=21)                                                           | 73.2 (71/97)                                      | 82.5 (80/97)   | 40 (4/10)                              | 33.3 (2/6)  | 2                                              |
| Aytac, <i>et al.</i> (2015)                              | GM508                                          | Female with diminished ovarian reserve (n=296)                                                  | 55.4 (79/142)                                     | 60.7 (89/147)  | 12.8 (19/148)                          | 21 (31/148) |                                                |



**(Continued)**

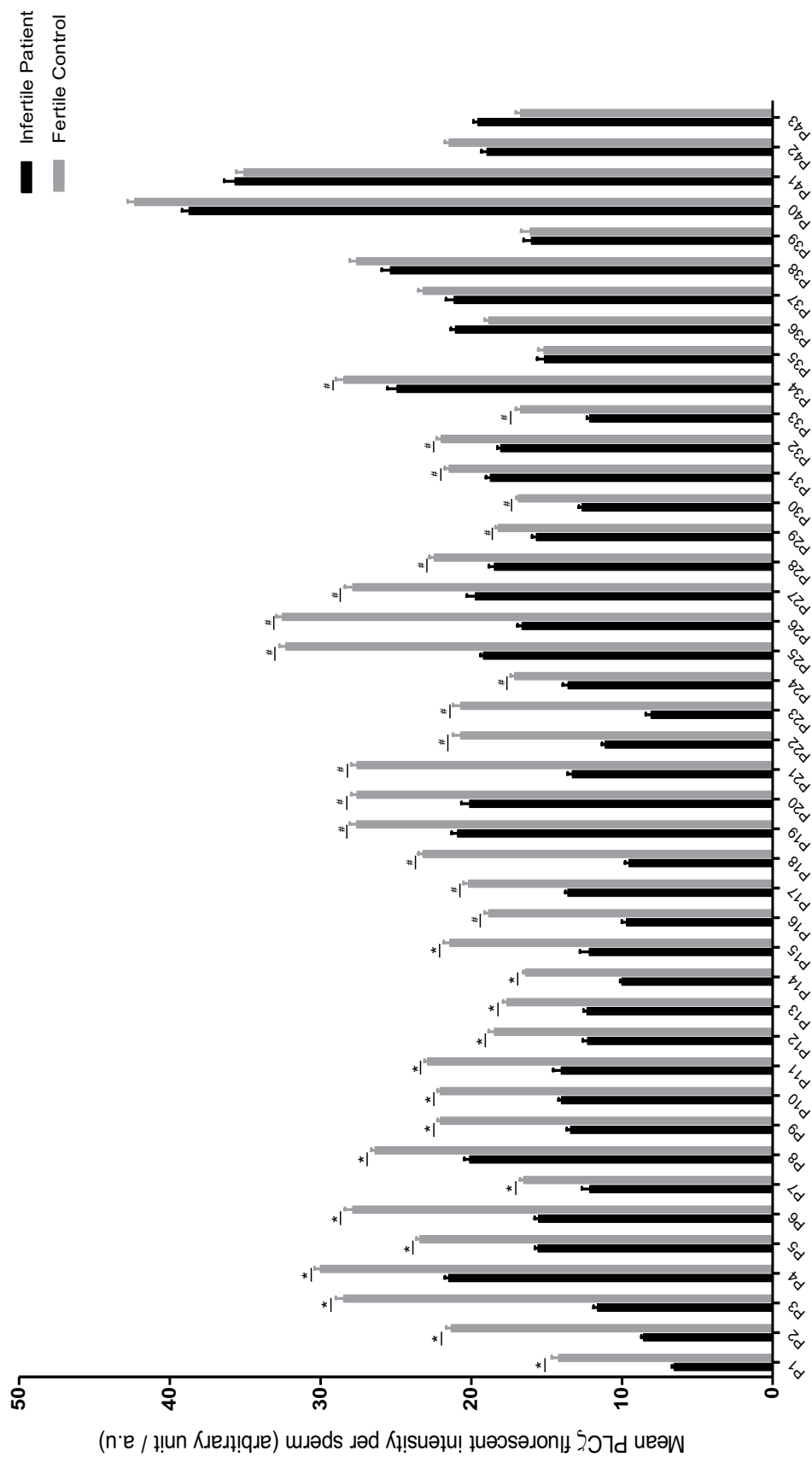
| References                                               | AOA type   | Selection criteria and patient group information<br>(n=number of participants otherwise stated) | Fertilisation rate % (PN/total injected oocytes ) |                | Pregnancy rate per ET otherwise stated |              | Live-birth rate or No. of live-birth after AOA |
|----------------------------------------------------------|------------|-------------------------------------------------------------------------------------------------|---------------------------------------------------|----------------|----------------------------------------|--------------|------------------------------------------------|
|                                                          |            |                                                                                                 | ICSI                                              | AOA-ICSI       | ICSI                                   | AOA-ICSI     |                                                |
| Studies suggested that AOA did not improve ICSI outcomes |            |                                                                                                 |                                                   |                |                                        |              |                                                |
| Borges, <i>et al.</i> (2009a)                            | Calcimycin | Azoospermia undergoing sperm retrieval (n=204) with subclassied:                                | ET                                                |                |                                        |              |                                                |
|                                                          |            | TESA in nonobstructive patients (n = 58)                                                        | 44 (13/29)                                        | 44.7 (14/29)   | 20.8 (6/29)                            | 18.5 (5/29)  |                                                |
|                                                          |            | TESA in obstructive patients (n = 48)                                                           | 65.2 (16/24)                                      | 55 (13/24)     | 29.2 (5/24)                            | 39.1 (9/24)  |                                                |
|                                                          |            | PESA in obstructive patients (n = 98)                                                           | 65.8 (32/29)                                      | 67 (33/49)     | 29.1 (14/49)                           | 35.9 (18/49) |                                                |
| Nasr-Esfahani, <i>et al.</i> (2008)                      | Ionomycin  | Severe teratozoospermia subclassied according to fertilization rate (n=87) :                    | per patient                                       |                |                                        |              |                                                |
|                                                          |            | 0                                                                                               | 0                                                 | 57.8           | 0                                      | 2            |                                                |
|                                                          |            | 1-33%                                                                                           | 14.3                                              | 58.3           | nd                                     | 44 (4/9)     |                                                |
|                                                          |            | 34-65%                                                                                          | 47                                                | 63.4           | nd                                     | 58 (11/19)   |                                                |
| Moaz, <i>et al.</i> (2006)                               | Ionomycin  | 66-100%                                                                                         | 85.8                                              | 77.9           | nd                                     | 40 (8/20)    |                                                |
|                                                          |            | TFF & LF (<40%) of patients with abnormal sperm morphology (n=56) with subclassied:             | 36.7 (33/90)                                      | 82.7 (81/98)   | nd                                     | nd           |                                                |
|                                                          |            | Amorphous head (n = 18)                                                                         | 39.3 (46/117)                                     | 81.7 (103/126) | nd                                     | nd           |                                                |
|                                                          |            | Tapered head (n = 23)                                                                           | 49.4 (39/79)                                      | 48.2 (40/83)   | nd                                     | nd           |                                                |

AOA, artificial oocyte activation; ET, embryo transfer; nd: no data indicated; GM-CSF: Granulocyte-macrophage colony-stimulating factor; LF, low fertilisation after previous conventional ICSI; MOAT: mouse oocyte activation test; OAD oocyte activation deficiency; PN, pronuclei; PESA, percutaneous epididymal sperm aspiration; SrCl<sub>2</sub>: Strontium Chloride; TESA, testicular sperm aspiration; TFF, total fertilisation failure after previous conventional ICSI.

**Appendix Table 3** Semen analysis of patient from Oxford Fertility (n=25).

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

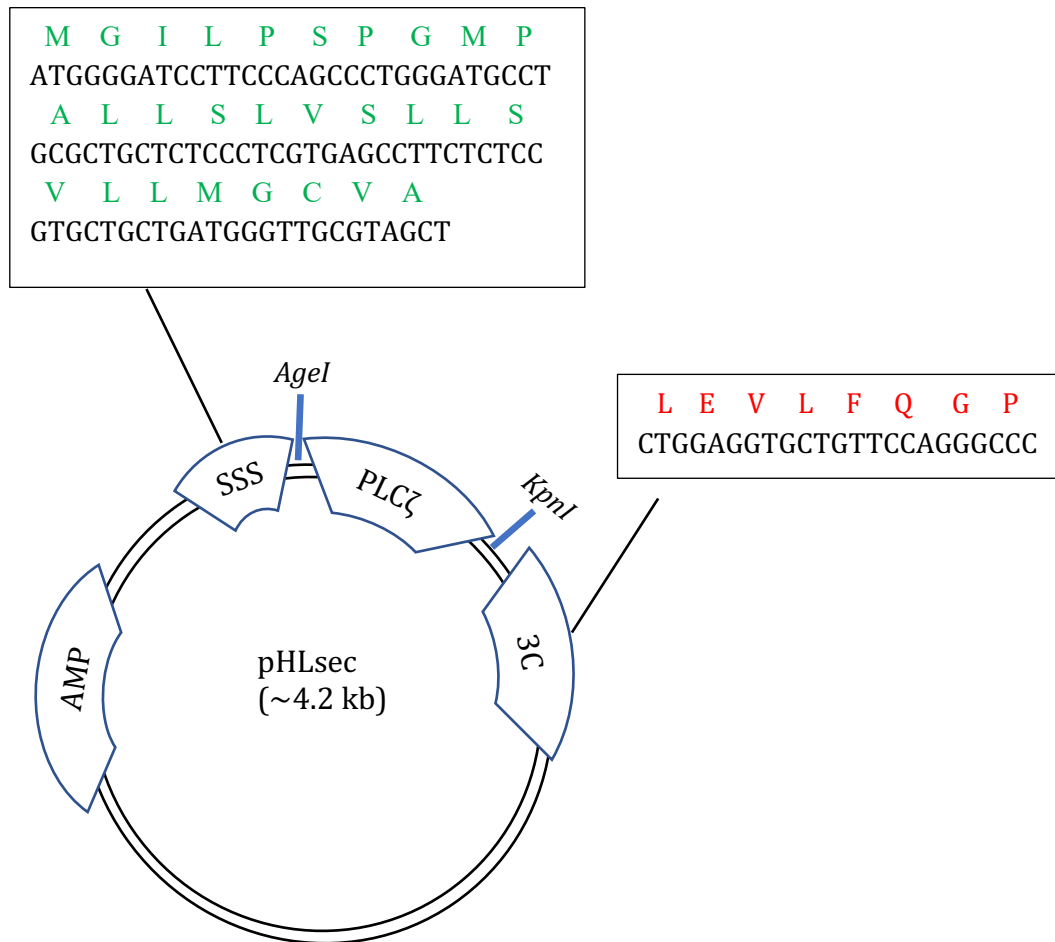
Appendix Figure 4. Larger version of the total levels of PLC $\zeta$  in sperm from 43 individual males, as compared to a matched fertile control.



## Appendix 5 PLC $\zeta$ cloning and ligation of PLC $\zeta$ cDNA into the pHL vector

The DNA sequence encoding full-length human PLC $\zeta$  was amplified (Forward primer: 5'-TTATCCAAGCTATACTCAAGTATGCATTCAT-3'; Reverse primer: 5'-ATGAATGCATACTTGAGTATAGCTTGGATAA-3') by PCR as follows: an initial denaturation at 94°C for 4 min, followed by 10 cycles of denaturation (94°C, 10s), annealing (58°C, 60s) and elongation (72°C, 2min). This was then followed by 20 cycles of denaturation (94°C, 15s), annealing (58°C, 30s) and elongation (72°C for 2 min). The final extension step was at 72°C for 7 min and the amplicons were stored at 4°C. The amplicons were then analysed and purified using methods described in Chapter 4 (Section 4.2.3).

The pHLsec vector was designed based on the pLEXm backbone (Aricescu *et al.* 2006) and contains a secretion signal sequence, a Fc fusion site and a 3C cleavage site. The PLC $\zeta$  amplicons were then inserted into the pHLsec vector via the restriction enzymes, *AgeI* and *KpnI*, with the 'Rapid DNA ligation kit', according to the manufacturer's protocol (Roche UK). The sequence of the forward primer, containing the *AgeI* restriction site (**in bold**) was: 5'-TAT**ACCGG**TATGGAAATGAGATGGTTTTTG-3', and the sequences of the reverse primer included the *KpnI* restriction site (**in bold**) are: 5'-TAT**GGTAC**CTCTGACGTACCAAACATA-3'. The final PLC $\zeta$  construct (pHLsec vector containing full-length PLC $\zeta$  cDNA) was verified by a genetic company (Source Bioscience, Oxford) and stored at -20°C for further validation and testing.



#### Appendix figure 4. The protein expression construct used to make recombinant PLCζ.

The full-length human PLCζ cDNA was inserted into the pHlsec vector by *AgeI* and *KpnI* restriction enzymes. AMP: ampicillin, SSS: secretion signal sequence (in green), 3C:3C cleavage site (in red) followed by Fc fusion site. Sequences for Fc fusion site are:

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AAATCTTGTGACAAACTCACACATGCCACCGTGCCAGCACCTGAACTCCTGGGGGGACCGT
CAGTCTTCTCTTCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACA
TGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCG
TGGAGGTGCATAATGCCAAGACAAAGCCGCGGAGGAGCAGTACAACAGCACGTACCGTGTGGT
CAGCGTCCTCACCCTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCC
AACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAAC
CACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTGAGCCTGACCTGC
CTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGA
ACAACTACAAGGCCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTC
ACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTC
TGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAA

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## Research output

### Publications

**Meng Xin**, Melo Pedro, Jones Celine, Ross Caroline, Ginny Mounce, Turner Karen, Child Tim, and Coward Kevin (2019). The Use of PLC zeta Analysis to Identify Candidates for Artificial Oocyte Activation (AOA): A Case Series of Clinical Pregnancies and Proposal of Management Algorithm. Fertility and Sterility (submitted, November 2019)

**Meng Xin**, Jones Celine, Ginny Mounce, Child Tim, and Coward Kevin (2019). Antigen Unmasking Method cannot Improve PLC zeta Visualization Efficiency in Infertile Males (in preparation)

**Meng Xin**, Alexandra Wilson, Jones Celine, Ginny Mounce, Child Tim and Coward Kevin (2019). An Investigation of PLC zeta Genomic Abnormalities in Infertile Males (in preparation)

### Presentations

#### Oral presentation

Annual meeting of the British Andrology Society in London, 06/2018

“Does PLC $\zeta$  Screening Provide an Efficient Tool to Assist the Clinical Decision-making Process when Considering Artificial Oocyte Activation (AOA) Treatment for Patients with Total Fertilization Failure (TFF)?”

#### Poster presentation

**Meng Xin**, Melo Pedro, Jones Celine, Ross Caroline, Ginny Mounce, Turner Karen, Child Tim, and Coward Kevin. “The Use of PLC zeta Analysis to Identify Candidates for Artificial Oocyte Activation (AOA): A Case Series of Clinical Pregnancies and Proposal of Management Algorithm” ESHRE’s 35th Annual meeting, Vienna, Austria, 06/2019

**Meng Xin**, Jones Celine, Amdani Siti, Ginny Mounce, Turner Karen, Child Tim, and Coward Kevin. “Clinical Screening of Phospholipase C zeta (PLC $\zeta$ ) Protein Levels and Localisation Patterns in Infertile Males” Medical Science Division’s DPhil symposium, University of Oxford, Oxford, U.K. 07/2018