



**THE OOCYTE ACTIVATION FACTOR,
PHOSPHOLIPASE C ZETA (PLC ζ): POTENTIAL
MECHANISMS OF ACTION AND SCOPE FOR
HUMAN INFERTILITY TREATMENT**

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A thesis submitted for the degree of Doctor of Philosophy

DEDICATION

This work is dedicated...

..to my Mother, Dr Wafaa Naguib, for all the sacrifices she made for her family,

for all the sacrifices she made for me...

..to my Father, Dr Mohamed Ramadan, for his support throughout the years,

for always believing in me...

This is also for my beloved Grandmother, who would have been so proud...

Acknowledgements

First and foremost, I give praise and thanks to God, for the innumerable favours that He has bestowed upon me. I thank Him for giving me the strength, patience and will to persevere and complete what I started despite the challenges and hurdles I encountered.

Secondly, I would like to express my deep, heartfelt and sincere gratitude to my supervisor and my mentor Dr Kevin Coward, for his invaluable support, patience, and guidance. I am especially grateful to Dr Coward for his understanding and immense support particularly when I was writing my thesis. In Dr Coward I see an excellent supervisor, who pushes his students to excel and to develop, and a mentor who is available, supportive and always ready to guide, build and advice. As well as Dr Coward, I am very grateful for Mrs Celine Jones, the Laboratory Manager who was also my day-to-day supervisor, for all the time, help, teaching and endless effort she offered me. Her efficiency is truly remarkable.

I am forever grateful to my parents, for all they have done, which is too numerous to mention. I am where I am because of them and to them I owe everything. I am also deeply grateful to my brothers Amr and Ahmed, and my sister-in-law Teresa, who have supported and believed in me over the years, even when I didn't. I am also indebted forever to my other family, the whole Altikriti family, whose unwavering support, love, companionship, encouragement and presence in my life is truly a priceless gift and a blessing I am ever so grateful for. I owe a special mention to Malath Shakir and Raghad Altikriti for their friendship, love, support and patience over the years. I owe an equally special mention to all the kids of the family who I love endlessly and consider my own (Aseel, Seba, Osama, Zaid, Yasir, Lena, Mais and baby Jude). It is truly a pleasure to watch them grow and be part of their lives. I owe a very very special mention to their wonderful beloved Grandmother, Mrs Wakar Altikriti, who took me under her wings and never stopped praying for me. Her love and heartfelt care means so much to me.

Other close people who I owe a mention for their support over the years include the Birawi family (with a special mention to Yaman Birawi), the lovely 'Tant' Amany and family, and my dear friends Amaan Al-Afandi and Zamzam Al-Basri.

From the University of Oxford, I owe my gratitude to Professor Katharine Carr, my College advisor, for her committed support, guidance and advice since I started my DPhil in October 2008. I also thank Professor Helen Mardon for providing me the opportunity to join the Department and I thank all the former members of the Mardon group, with special gratitude to Ms Janet Carver for all she has taught me and her invaluable help, even after I joined the Coward group. I am also grateful to Professor Ian Sargent, the Director of Graduate studies and Professor Stephen Kennedy, the head of Department, for their support since I joined the Department. I'm also grateful to my colleague Dr Junaid Kashir for all the teaching and advice he provided when I joined the Coward group. I owe thanks to Dr Rebecca Dragovic for her sincere assistance with the flow cytometry and her help with trouble-shooting. I am also very grateful for all the help Dr Alan Wainman (Dunn School of Pathology) provided, in training me with the confocal microscope and all his help thereafter.

I am also very grateful for the lovely company I had during my time at the NDOG, some of which have developed into strong friendships which I will cherish after I leave the University. I am particularly thankful to my lovely dear friend Pilar Vazquez who was my companion along this long DPhil journey. I would also like to especially thank Souraya Jaroudi, Muna El-Kasti, Leila Cheikh Ismail, Patricia Grasa-Molina, Samer Alfarawati, Katharina Späth, Maria Enciso and Jonas Sarasa for their great company, and for making the journey more pleasant.

There are many other people who have helped me over the years, through their support, friendship and prayers. I wish I could mention each one by name but space will not allow, but I thank each and every one who has supported me, encouraged me, prayed for me, or helped me in any way whatsoever.

Walaa M Ramadan,

September 2013

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Abbreviations

AOA	Artificial oocyte activation	HEK293T	Human embryonic kidney cells
ART	Assisted reproductive technology	HRP	Horse-radish peroxidase
BSA	Bovine serum albumin	ICSI	Intra-cytoplasmic sperm injection
Ca²⁺	Calcium ions	IP₃	Inositol triphosphate
CASA	Computer-assisted sperm analysis	IP₃R	IP ₃ receptor
CG	Cortical granule	IVF	<i>In-vitro</i> fertilisation
cRNA	Complementary RNA	mRNA	Messenger RNA
DAB	Diaminobenzidine	NLS	Nuclear localisation signal
DAPI	4',6-diamidino-2-phenylindole	NOA	Non-obstructive azoospermia
DGW	Density-Gradient Wash	NTR	Nuclear transport receptor
DMEM	Dulbecco's Modified Eagle Medium	OAD	Oocyte activation deficiency
DNA	Deoxyribonucleic acid	OD	Optical density
ER	Endoplasmic reticulum	OFU	Oxford Fertility Unit
EV	Empty vector	ORF	Open reading frame
FBS	Fetal bovine serum	PBS	Phosphate-buffered saline
FITC	Fluorescein isothiocyanate	PCR	Polymerase chain reaction
GST	Glutathione S-transferase	PFA	Paraformaldehyde

PH	Pleckstrin homology
PIP₂	Phosphatidylinositol 4,5-diphosphate
PLC	Phospholipase C
PM2	Protamine-2
PN	Pro-nucleus
PNA	Peanut agglutinin
PTM	Post-translational modifications
ROI	Region of interest
ROSI	Round spermatid injection
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SF	Sperm factor
SH	Src homology
TESE	Testicular sperm extraction
WHO	World Health Organisation
WT	Wild-type
ZP	Zona Pellucida

ABSTRACT |

Title | The oocyte activation factor, phospholipase C zeta (PLC ζ): potential mechanism of action and scope for human infertility treatment.

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Degree | D.Phil in Obstetrics and Gynaecology

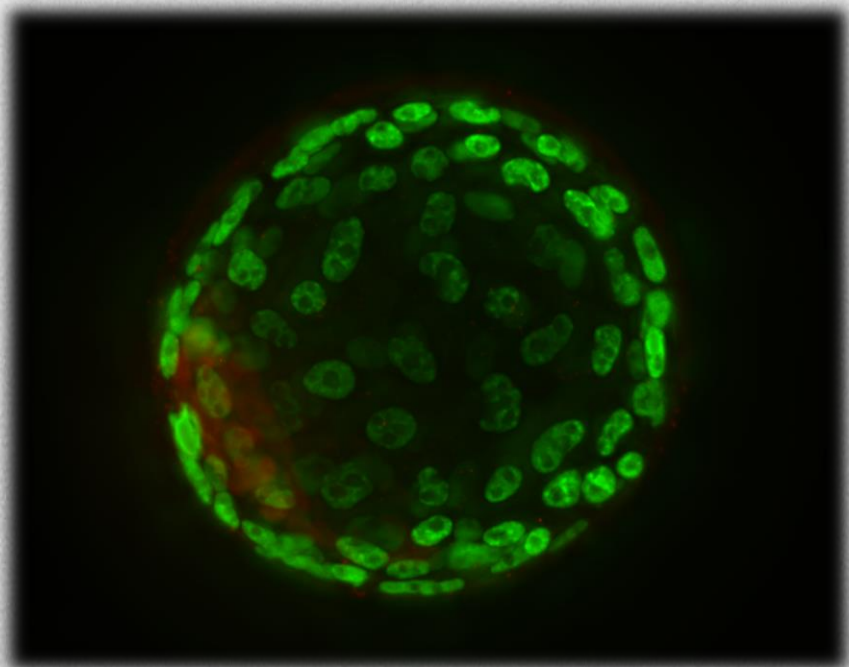
Department | Nuffield Department of Obstetrics and Gynaecology

College | St Catherine's College

Year/Term submitted | HT 2013

It is widely believed that the sperm-specific protein phospholipase C zeta (PLC ζ) is released into the oocyte upon fusion and initiates calcium (Ca²⁺) oscillations which regulate oocyte activation. Reduced amounts, abnormal localisation patterns, and aberrant forms of PLC ζ underlie certain types of male-factor infertility, prompting significant interest in the use of this fundamental protein as a clinical therapeutic agent or diagnostic marker. This thesis systematically addressed a range of outstanding questions relating to the expression, localisation, and therapeutic/diagnostic application of PLC ζ . Using immunohistochemical and quantitative immunofluorescence techniques, the current study investigated the effect of paternal age and epididymal maturation upon PLC ζ expression and localisation in mouse sperm. Neither age nor epididymal maturity exhibited significant effect upon the total level of PLC ζ in mouse sperm, although there was a significant trend towards post-acrosomal localisation as sperm matured during transit in the epididymis. Immunohistochemical analysis of mouse testis showed that the expression of PLC ζ first appeared at the round-spermatid stage of spermatogenesis. Paternal age did not influence total levels of PLC ζ expression or localisation pattern in human sperm samples (n=29), with PLC ζ being predominantly localised to the post-acrosomal region. A significant reduction (34%) was detected in the proportion of sperm exhibiting PLC ζ from an oocyte-activation deficient patient compared with fertile controls. Novel immunohistochemical analyses suggested, for the first time, that PLC ζ is first expressed from the round spermatid stage in humans, and that the analysis of PLC ζ expression in human testicular biopsies (n=29) may provide credible indication of the oocyte activation ability of surgically-retrieved sperm samples from infertile patients. Initial data also suggest that flow cytometry may serve as a useful prognostic/diagnostic tool for the quantitative analyses of PLC ζ expression in human sperm samples. Mammalian and bacterial expression systems were utilised to generate recombinant human PLC ζ protein as a therapeutic agent and for the generation of a novel monoclonal anti-PLC ζ antibody respectively. Expression studies using HEK293T cells demonstrated that human PLC ζ appeared to localise to the endoplasmic reticulum and that the EF hand domain appeared to be involved in regulating this pattern of localisation. Collectively, these studies extend our fundamental knowledge of how PLC ζ is expressed in developing sperm and assist in the translation of PLC ζ as a clinical therapeutic and diagnostic biomarker for oocyte activation ability.

Chapter 1 | INTRODUCTION



1.1 Sexual reproduction

Sexual reproduction is the process whereby two parents bequeath their genes to their progeny producing offspring with a mix of genes inherited from each gender. This occurs through a process known as fertilisation, brought about by the physical union of the male and female gametes, the sperm and the oocyte, resulting in a genetically unique cell with the extraordinary capacity to develop into a pluripotent zygote. The fusion of the sperm and oocyte is the culmination of gametogenesis and all the steps that precede fertilisation (Primakoff and Myles, 2002). Gametogenesis, or the formation of the male and female gametes, involves meiotic cell division, which occurs only in the germ cells of the gonads, the testis and ovary respectively, resulting in a haploid gamete comprising one set of the parental chromosomes. The unison of the haploid sperm and oocyte culminate in a diploid zygote upon their fusion at fertilisation, the latter comprising two sets of chromosomes, one from each parent. The work described in this thesis focuses upon a sperm-specific protein which plays a fundamental role in the process of fertilisation.

1.1.1 Spermatogenesis

The mature spermatozoon is a highly specialised cell and the process by which it is produced from diploid spermatogonial cells is known as spermatogenesis. Spermatogenesis is a complex, highly organised and regulated process occurring within the testes, with final maturation events undertaken in the epididymis. The entire spermatogenic cycle takes approximately 75 days in man and 35 days in mice (Clermont, 1963; Cooke and Saunders,

2002). Testis are composed of highly organised structures, namely seminiferous tubules, which contain sperm cells at differing stages of maturation, with the most mature cells lying closest to the tubule lumen [Figure 1.1].

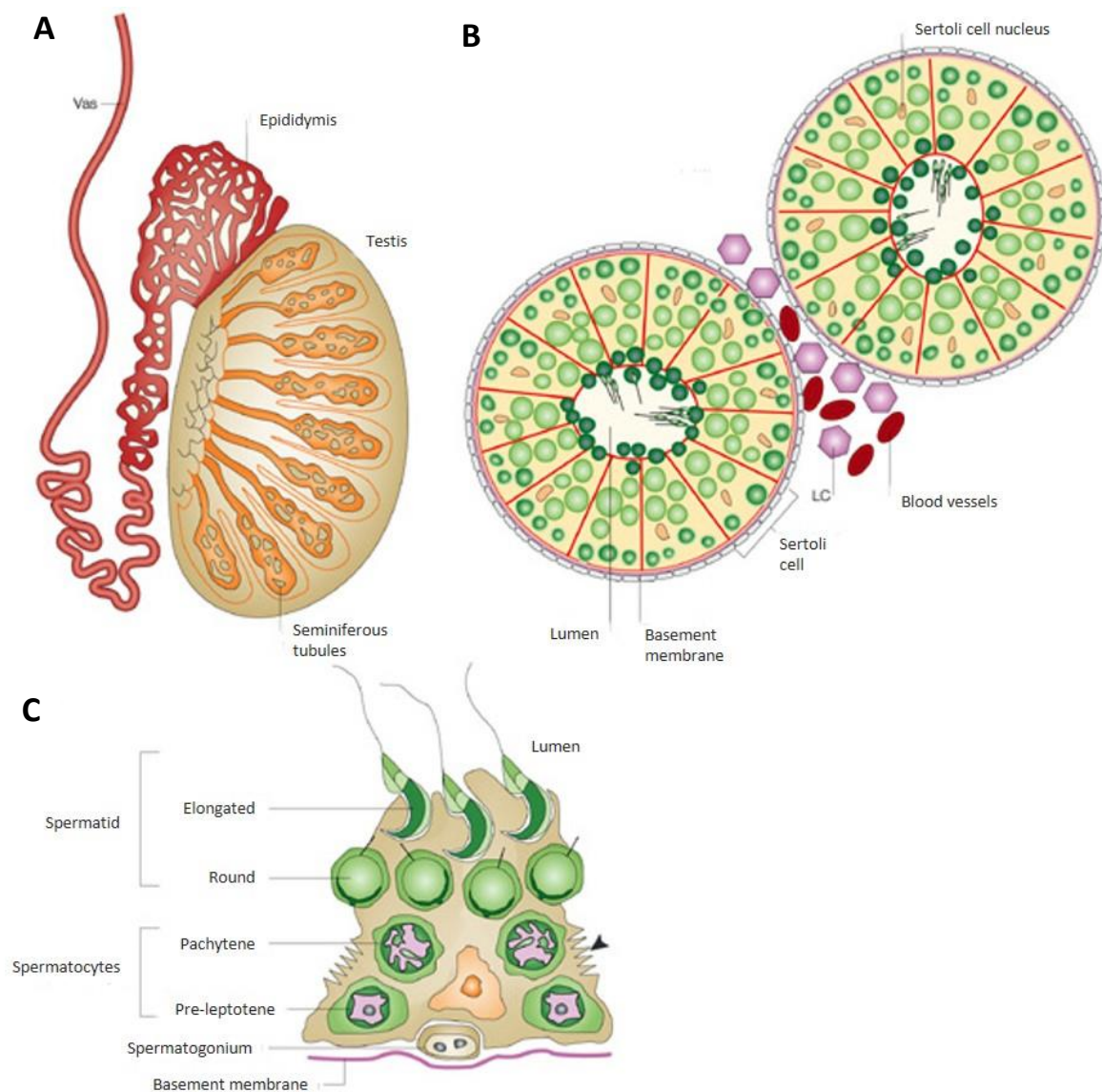


Figure 1.1 | Schematic showing the organisation of the mammalian testis. A| Cross-section through a testis showing the location of the seminiferous tubules, epididymis and vas deferens. **B|** Cross-section through a testicular seminiferous tubule showing germ cells (green) at various stages of development nurtured by Sertoli cells (red) which maintain integrity of the epithelium and support spermatogenesis by cell-cell contact with the germ cells. Leydig cells (LC), which produce testosterone, are present in the interstitium. Maturing sperm accumulate in the lumen of the tubules where they are released into the epididymis. **C|** A single Sertoli cell with its associated germ cells. Germ cells are organised in a strict order of maturation with the stem cells and pre-meiotic spermatogonia located towards the basement membrane, and the post-meiotic round and elongating spermatids towards the lumen.

Adapted from Cooke and Saunders (2002)

Spermatogenesis commences with the proliferative expansion of progenitor cells involving rounds of mitosis of the spermatogonial stem cells to repopulate the testicular stem cell population by producing a reservoir of self-generating cells, as well as producing progenitor cells that can undergo spermatogenesis [Figure 1.2]. Amidst this reservoir emerge spermatogonia which undergo a series of irreversible differentiation steps, first by going through further rounds of mitotic divisions before they commence meiosis as primary spermatocytes (de Rooij and Russell, 2000). The first meiotic division involves the separation of homologous chromosomes yielding secondary spermatocytes. Sister chromatids are subsequently separated into individual cells in the second meiotic division yielding haploid round spermatids, with four round spermatids resulting from each spermatocyte. Thereafter, round spermatids undergo dramatic morphological changes (spermiogenesis) during which the sperm nucleus is compacted and remodelled forming an elongated spermatid, and finally a spermatozoon. Immature immotile sperm are then released from the seminiferous epithelium into the tubule lumen and flow passively into the epididymis via the rete testis for further maturation.

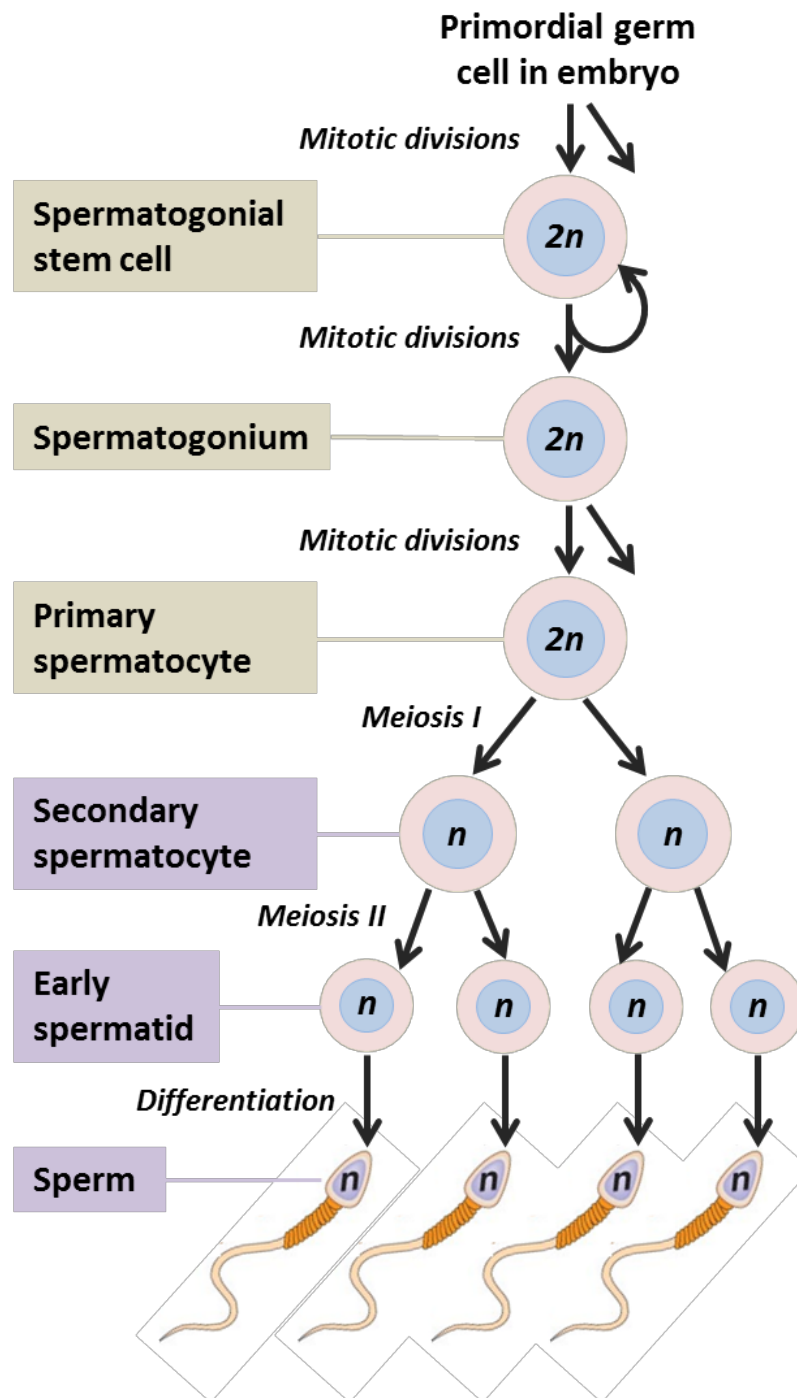


Figure 1.2 | **Schematic showing the process of spermatogenesis.** Germ cells near the outer wall of the seminiferous tubules of the testis differentiate into stem cells called spermatogonia. Spermatogonia divide by mitosis, and mature into primary spermatocytes. Each primary spermatocyte undergoes meiosis: meiosis I yields two haploid secondary spermatocytes; meiosis II yields four equal-sized spermatids. The spermatids migrate toward the lumen. Sertoli cells supply nutrients for the spermatids, which mature into motile sperm in the epididymis.

1.1.2 The epididymis

Once released in the lumen of the seminiferous tubule, spermatozoa are transported through the efferent ducts to begin their journey down the epididymis [Figure 1.3]. The average time for transit of human sperm through the epididymis is 12 days (Rowley *et al.*, 1970). Sperm entering the epididymis after exiting the testis are relatively immotile, and not yet mature functionally, morphologically or biochemically. During their passage through the epididymis, sperm become functionally mature, acquiring the ability to move forward when released from the epididymis, ascend the female genital tract, undergo the acrosome reaction, and finally, bind to and penetrate the egg (Robaire *et al.*, 2006). The epididymis is divided into the caput, corpus and cauda, and in all species examined thus far, during the passage through the different stages of the epididymis, a gradient of fertilising potential is observed as sperm traverse the epididymis.

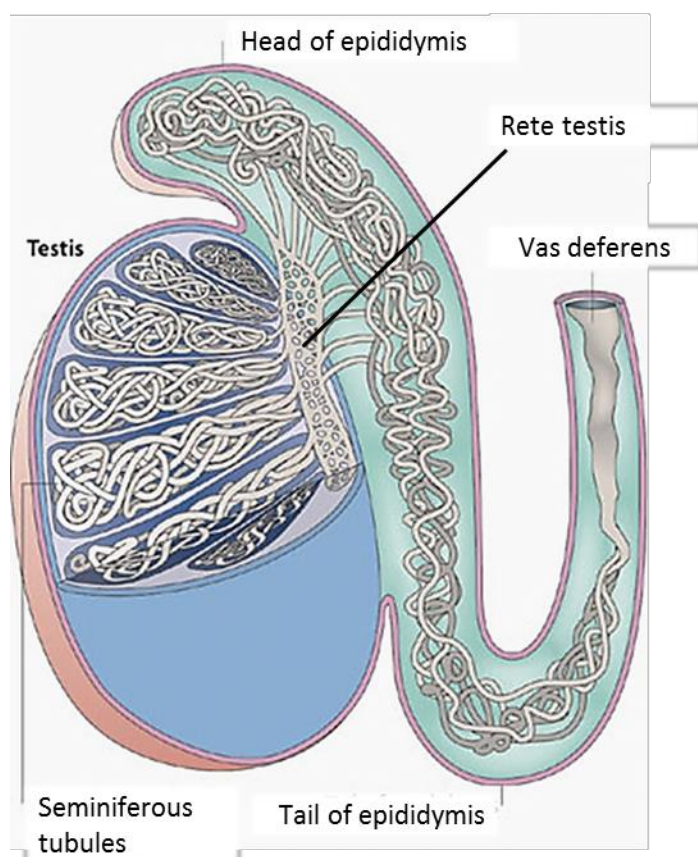


Figure 1.3 | Schematic of a human testis with the epididymis attached.

A cross-section through the epididymis and partial exposure of the interior of the testis shows the seminiferous tubules. The testis is oriented with respect to the head and tail of the epididymis. The lumen of each tubule provides a pathway for sperm to reach the epididymis and the vas deferens. The sections from the head to tail of the epididymis are: caput, corpus, and cauda.

Adpated from Arnheim and Calabrese (2009)

With this acquisition of fertilising ability, sperm undergo various maturational changes, the potential for motility being the first characteristic to be recognised (Tournade, 1913). In addition to the capacity for progressive motility, epididymal sperm also develop the capacity to undergo the acrosome reaction, recognise and bind to the zona pellucida, and fuse with the vitelline membrane of the oocyte. Concomitant with these functional changes, structural changes are also evident as sperm traverse the epididymis. This includes migration of the cytoplasmic droplet along the sperm flagellum, acrosomal reshaping, changes in the sperm nuclear chromatin, and changes in the sperm plasma membrane (Bedford, 2004). Collectively, these changes underpin the functional maturation of sperm for subsequent storage until ejaculation.

1.1.3 Capacitation and the acrosome reaction

Further to the functional maturation that the sperm acquired during epididymal transit, freshly ejaculated mammalian sperm is not yet ready to fertilise an oocyte and must undergo further structural and functional changes in order to become competent, known as capacitation (Yanagimachi, 1970; Publicover *et al.*, 2007; Suarez, 2008). These changes occur through the interaction with environmental stimuli in the female reproductive tract and allow the sperm the ability to penetrate and progress through the viscous and visco-elastic mucous and the gelatinous cumulus matrix to reach the fibrous glycoprotein zona pellucida (ZP) to which it must bind and penetrate. Concomitant with capacitation are complex changes in the biochemical, physiological and cellular properties of the sperm (Florman *et al.*, 2008). As a result of this process, sperm develop novel patterns of motility, including hyperactivation, which provides sperm with greater propulsive force to reach the

oocyte through mucous, cumulous cells and the ZP (Yanagimachi, 1970; Publicover *et al.*, 2007; Suarez, 2008; Florman *et al.*, 2008; Phillips *et al.*, 2008). This is achieved by the flagellum developing deep bends at greater amplitude producing a beat pattern that is highly unsymmetrical (Suarez, 2008). Furthermore, sperm acquire oriented motility towards chemoattractants from the female reproductive tract, as well as the ability to undergo a ZP-evoked acrosome reaction (Florman *et al.*, 2008).

The acquirement of the propulsive hyperactive force of the flagellum, as well as the ensuing biochemical interaction between the spermatozoon and the ZP, are both required in order that the sperm can cross the ZP. The latter include first, binding of specific receptors on the head of the spermatozoon with ZP3 ligands on the ZP (Primakoff and Myles, 2002), and second, the acrosome reaction, whereby enzymes are released from the sperm head, including hyaluronidase and acrosin, which locally digest and soften the ZP so that the spermatozoon can penetrate deeper (Primakoff and Myles, 2002). Ca^{2+} signalling plays a central regulatory role in all the key activities which allow sperm to attain fertilising ability, including capacitation, hyperactivation, chemotaxis and acrosome reaction (Costello *et al.*, 2009). Indeed, the acrosome reaction is caused in response to sustained Ca^{2+} influx which follows the sperm interaction with the ZP (Florman *et al.*, 2008). To prevent polyspermic entry, the ZP of the oocyte undergoes hardening at all points except the acrosome site, blocking the penetration of further sperm.

1.2 Oocyte activation

Prior to fertilisation, mature oocytes remain arrested in the metaphase stage of the second meiotic division (MII). The process leading up to this arrest, known as *oocyte maturation*, during which the oocyte extrudes the first polar body, renders the oocyte fully competent and receptive to any forthcoming signals during fertilisation (Jones *et al.*, 1995). Upon fusion with a sperm, the oocyte is released from arrest and meiosis resumes, converting the oocyte into a totipotent zygote, able to form all cells of the body, through a series of events known as *oocyte activation* (Dale *et al.*, 2010; Kashir *et al.*, 2010). These morphological and biochemical events include cortical granule (CG) exocytosis leading to the prevention of polyspermy, extrusion of the second polar body, maternal RNA recruitment, male and female pro-nuclear (PN) formation, and the initiation of embryonic gene expression (Kline and Kline, 1992; Stricker, 1999; Ducibella *et al.*, 2002, 2006; Malcuit *et al.*, 2006; Miyazaki and Ito, 2006; Liu, 2011).

It has long been established that fertilisation is universally and unconditionally regulated and triggered by intracellular calcium (Ca^{2+}), arising from intracellular stores in the endoplasmic reticulum (ER), and that the oocyte must produce a programmed species-specific pattern of intracellular Ca^{2+} release in order for development to proceed (Kline and Kline, 1992; Stricker, 1999; Miyazaki, 2006). These intracellular Ca^{2+} transients are the universal trigger for oocyte activation and development in all species studied thus far (Kline and Kline, 1992; Miyazaki and Ito, 2006; White *et al.*, 2010). However, the nature, amplitude, duration and frequency of this signal are largely species-specific (Jones, 1998; Stricker, 1999; Ducibella *et al.*, 2006). While oocytes of non-mammalian species such as medaka fish and sea urchins require only a single Ca^{2+} transient that lasts several minutes

(Whitaker, 2006), mammalian oocytes are activated by distinctive repetitive Ca^{2+} increases, or *oscillations* lasting several hours (Miyazaki and Ito, 2006) [Figure 1.4].

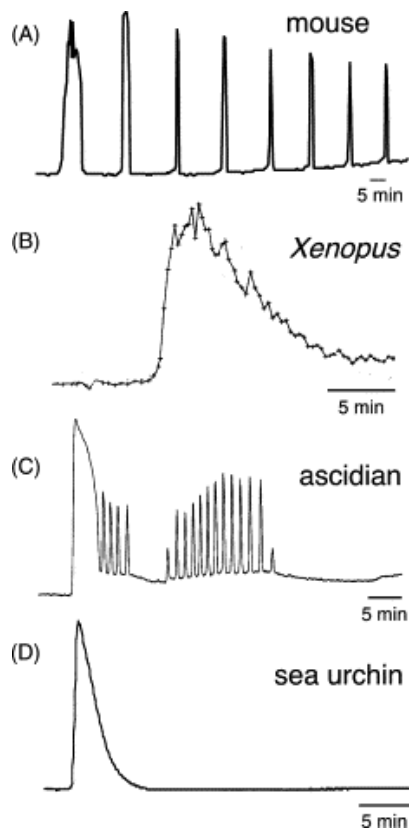


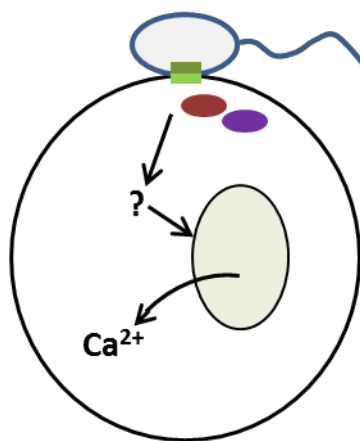
Figure 1.4 | **Ca^{2+} responses at fertilisation in oocytes of various species.** Ca^{2+} transients vary in frequency and amplitude between species.

Adapted from Miyazaki (2006)

The idea that intracellular Ca^{2+} signals are initiated by events that are closely linked to the fusion of the sperm and oocyte has existed for some time (Lawrence *et al.*, 1997) and scientists have theorised extensively as to how gamete fusion elicits such responses. Two main hypotheses endeavour to explain the mechanism by which sperm initiate Ca^{2+} oscillations in mammals (Oda, 2006; Swann *et al.*, 2006; Parrington *et al.*, 2007; White *et al.*, 2010) [Figure 1.5]. The first relates to potential interaction of a sperm ligand with a coupled receptor on the plasma membrane of the oocyte which in turn stimulates the release of intracellular Ca^{2+} , either through a G-protein linked cascade or via tyrosine kinase activity (Jaffe, 1990; Schultz and Kopf, 1995; Evans and Kopf, 1998). This membrane receptor model

was the dominant theory for many years since surface-mediated interactions were a normal cell-signalling feature in somatic cells (Jaffe, 1990; Parrington *et al.*, 2007). The second model proposed that a soluble sperm factor (SF) is released into the oocyte upon gamete fusion, and interacts with oocyte cytosolic components leading to Ca^{2+} release [Figure 1.5]. For many years this model was not accepted due to the failure to identify such a factor, but several lines of evidence now strongly support this theory. The mere fact that direct injection of sperm or sperm extract into an unfertilised oocyte initiates Ca^{2+} oscillations similar to those seen in fertilisation, and subsequent embryo development (Swann and Ozil, 1994; Tesarik *et al.*, 1994; Jones *et al.*, 1998), poses significant doubt over the receptor model since membrane fusion is bypassed altogether.

Membrane receptor model



Soluble sperm factor model

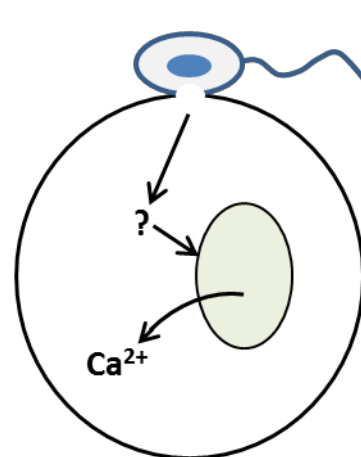


Figure 1.5 | The two main models of oocyte activation. The membrane receptor model suggests that upon fusion of the sperm with the oocyte, a receptor is activated on the surface of the oocyte relaying a signal which initiates Ca^{2+} release; The soluble sperm factor model suggests that sperm deliver a molecule upon fusion which initiates a Ca^{2+} release pathway.

Since fertilisation takes place at different stages of meiotic progression in different species, the frequency of this signal varies amongst different organisms (Miyazaki and Ito, 2006), and can range from a single Ca^{2+} increase every two minutes, to a single Ca^{2+} increase every hour in the mouse and bovine oocytes respectively (Fissore *et al.*, 1992; Kline and Kline, 1992; Miyazaki *et al.*, 1993; Ducibella *et al.*, 2002, 2006; Swann *et al.*, 2004, 2006). The initial increase in intracellular Ca^{2+} appears to be critical for the initiation of both early and late events of oocyte activation (Fissore *et al.*, 1992; Kline and Kline, 1992; Ducibella *et al.*, 2006) as well as much later consequences peri-implantation, specifically in the long-term effect on both gene expression and development to term (Ozil, 1998; Ozil *et al.*, 2006; Ducibella *et al.*, 2006; Jones, 2007).

Many early activation events are Ca^{2+} -dependent, but are not simultaneously instigated following the initial Ca^{2+} transient, but rather occur in a temporal order (Ducibella *et al.*, 2006; Ozil *et al.*, 2006) via spatial and temporal control of Ca^{2+} release (Boulware and Merchant, 2008). Individual events that occur during activation require different numbers of Ca^{2+} transients, hence total Ca^{2+} inputs (Kline and Kline, 1992). An example of this was demonstrated in a study by Ducibella *et al.* (2002) which controlled the number of Ca^{2+} oscillations in the mouse oocyte experimentally, and found that 20-50% CG exocytosis required 1-4 Ca^{2+} rises, whereas metaphase II exit required 4-8 rises (Ducibella *et al.*, 2002, 2006). Indeed, from a physiological point of view, CG exocytosis is initiated before cell cycle resumption, and its early initiation is important for the prevention of polyspermy. It is debatable however whether it is the Ca^{2+} oscillations that cause the membrane block to polyspermy by CG exocytosis since injecting a sperm directly into the oocyte does not cause a block to polyspermy (Wortzman-Show *et al.*, 2007). This suggests that events associated

with or downstream to fusion of the gametes may be an effector to the membrane blockade (Wortzman-Show *et al.*, 2007). Later, I will discuss how the Ca^{2+} -oscillatory patterns are coordinated in a cell-cycle dependent manner (Ducibella *et al.*, 2006).

It is commonly accepted that Ca^{2+} release at fertilisation and embryo development involve the activation of the phosphoinositide pathway (Malcuit *et al.*, 2006). Activation of this pathway in oocytes results in the production of 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG) via the hydrolysis of phosphatidylinositol 4,5-diphosphate (PIP_2). Both products are involved in shaping the intracellular Ca^{2+} responses. Increases in intracellular IP_3 are responsible for mediating Ca^{2+} release by binding to IP_3 -receptors (IP_3R) on the endoplasmic reticulum (ER) (Miyazaki *et al.*, 1992; Fissore *et al.*, 1999; Kurokawa *et al.*, 2004; Malcuit *et al.*, 2005; Lee *et al.*, 2006; Oda, 2006; Swann and Yu, 2008; Berridge, 2009). Injection of IP_3 parthenogenetically activates mammalian oocytes in multiple species (Kline and Kline, 1994; Oda *et al.*, 1999; Goud *et al.*, 2002), and blocking (Miyazaki *et al.*, 1993); down-regulating (Brind *et al.*, 2000; Jellerette *et al.*, 2000); or reducing the expression of IP_3 by injection of siRNA (Xu *et al.*, 2003) before fertilisation, inhibits Ca^{2+} oscillations in oocytes that are fertilised subsequently. IP_3 increase is measured by the down-regulation of IP_3Rs , and studies involving the injection of sperm extracts into mouse oocytes demonstrated down-regulation of IP_3R , signalling an increase in IP_3 production (Brind *et al.*, 2000; Jellerette *et al.*, 2000; Cooney *et al.*, 2010).

Numerous studies have proposed that IP_3Rs may contribute in shaping the pattern of sperm-initiated oscillations in the oocyte through regulatory changes during oocyte maturation and the first zygotic cycle (Xu *et al.*, 1994). Early studies demonstrate that during oocyte maturation, the concentration of IP_3R , its cellular distribution, and sensitivity,

are increased (Mehlmann and Kline, 1994; Jones *et al.*, 1995) for optimal Ca^{2+} responses in fertilisation. IP_3 Rs undergo degradation via persistently high levels of IP_3 and cell-cycle dependent regulation following sperm entry (Jellerette *et al.*, 2000, 2004; FitzHarris *et al.*, 2003; Lee *et al.*, 2006, 2009) modifying the pattern of Ca^{2+} oscillations which result from such degradation. Since sperm induce Ca^{2+} release from the ER via IP_3 R, the action of the proposed SF, once released into the ooplasm must be that of producing IP_3 . Therefore, the most likely candidate was considered to be a phospholipase C (PLC), which is the only enzyme known to generate IP_3 in living cells (Kurokawa *et al.*, 2004; Oda, 2006). The specific identity of this PLC isoform, however, remained unknown for some time.

1.3 Phospholipase C zeta (PLC ζ) as the putative oocyte activation factor

Recombinant protein synthesised against all known PLC isoforms failed to induce Ca^{2+} oscillations upon injection into mouse oocytes (Jones *et al.*, 2000), and it was not until 2002 that Saunders *et al.* proposed the existence of a novel, uncharacterised PLC isoform, which was a sperm-specific PLC and designated PLC zeta (PLC ζ). PLC ζ appears to have a universal role in triggering oocyte activation with its identification both in mammals – humans, mice, monkeys, boars, hamsters and bulls (Cox *et al.*, 2002; Saunders *et al.*, 2002; Yoneda *et al.*, 2006; Young *et al.*, 2009; Cooney *et al.*, 2010); and in non-mammals, such as the chicken, medaka fish and quail (Coward *et al.*, 2005; Ito *et al.*, 2008; Mizushima *et al.*, 2009).

Numerous studies since the discovery of PLC ζ provide compelling evidence that PLC ζ is indeed the physiological agent responsible for IP_3 -mediated Ca^{2+} release in activating

oocytes (Cox *et al.*, 2002; Saunders *et al.*, 2002; Malcuit *et al.*, 2006) [Figure 1.6]. Micro-injection of both PLC ζ cRNA and recombinant protein into mouse and bovine oocytes results in the initiation of oscillations and oocyte activation, similar to that seen during normal fertilisation [Figure 1.7], and stimulates embryonic development to the blastocyst stage (Cox *et al.*, 2002; Saunders *et al.*, 2002; Kouchi *et al.*, 2004; Campbell and Swann, 2006; Yu *et al.*, 2008; Ross *et al.*, 2008, 2009). Injection of human PLC ζ cRNA also causes Ca²⁺ oscillations and the activation of pre-implantation development in human oocytes (Rogers *et al.*, 2004). Consistent with its role as the putative SF, depletion of PLC ζ from sperm extracts (Saunders *et al.*, 2002), or the inhibition of PLC ζ expression in sperm using transgenic RNA interference (Knott *et al.*, 2005), significantly abolishes the ability to initiate Ca²⁺ release, or causes premature termination of Ca²⁺ oscillations and reduction in litter size.

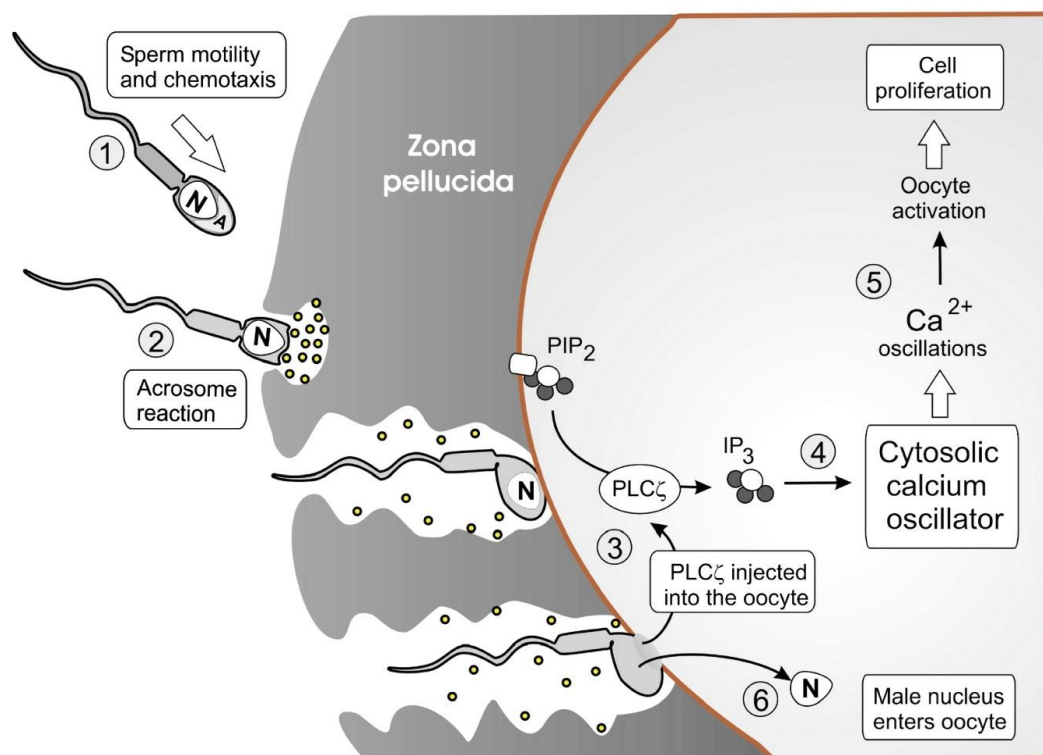


Figure 1.6 | Role of the IP₃/Ca²⁺ signalling pathway in the control of mammalian fertilization, illustrating the events that occur from sperm-oocyte fusion to the activation of the oocyte via the IP₃/Ca²⁺ signalling pathway. (N – sperm nucleus; A – acrosome; PIP₂ – phosphatidylinositol 4,5-diphosphate, IP₃ – 1,4,5-triphosphate)

Adapted from Berridge (2009)

A Fertilization

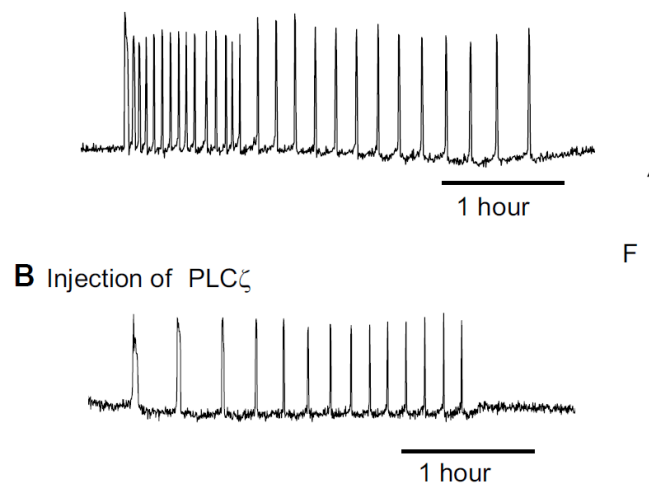


Figure 1.7 | Intracellular Ca^{2+} oscillations in mouse oocytes measured by the fluorescence of a Ca^{2+} -sensitive dye. A | Normal fertilisation. B | Following microinjection of mouse $\text{PLC}\zeta$ cRNA.

Adapted from Swann and Yu (2008)

Although much evidence supports $\text{PLC}\zeta$ as being the putative oocyte activation factor, other sperm proteins have been suggested as alternative candidates able to induce meiotic progression or Ca^{2+} release in various species. Three such molecules, truncated c-Kit (Sette *et al.*, 1997); citrate synthase (Harada *et al.*, 2007); and post-acrosomal sheath domain-binding protein (PAWP) (Wu *et al.*, 2007) are indeed potential candidates since their injection into mammalian and non-mammalian oocytes induces activation. However, the precise molecular mechanisms underlying the function of these proteins remain unknown, with no demonstration of such proteins triggering Ca^{2+} oscillations in mammalian oocytes, thereby casting doubt over their candidacy of being the oocyte activation factor (Wu *et al.*, 2007; Aarabi *et al.*, 2010). It is indeed possible that many factors may act collectively, especially within the egg, but future work needs to investigate this further.

$\text{PLC}\zeta$ exhibits a typical PLC domain structure (Saunders *et al.*, 2002) with characteristic X and Y catalytic domains which form the active site in all other known PLC isoforms (β , γ , δ , ϵ and η) (Williams, 1999; Rebecchi and Pentyla, 2000; Suh *et al.*, 2008), a

single C2 domain and four tandem EF hand domains [Figure 1.8]. PLC ζ exhibits closest homology with PLC δ , with 33% similarity (Saunders *et al.*, 2002). However, one major difference to other PLCs is the absence of pleckstrin homology (PH) and Src homology (SH) domains, making PLC ζ the smallest known mammalian PLC with a molecular mass of ~70kDa in humans and ~74kDa in mice (Cox *et al.*, 2002; Saunders *et al.*, 2002). Another distinctive feature of PLC ζ is its high sensitivity to Ca²⁺ (Kouchi *et al.*, 2004) and greater potency in humans than mouse, with the minimal amount of human PLC ζ cRNA required to cause Ca²⁺ oscillations in mouse oocytes (0.2–0.02 μ g/ml) nearly a hundred times less than the effective dose of mouse PLC ζ cRNA (2 μ g/ml) (Cox *et al.*, 2002).

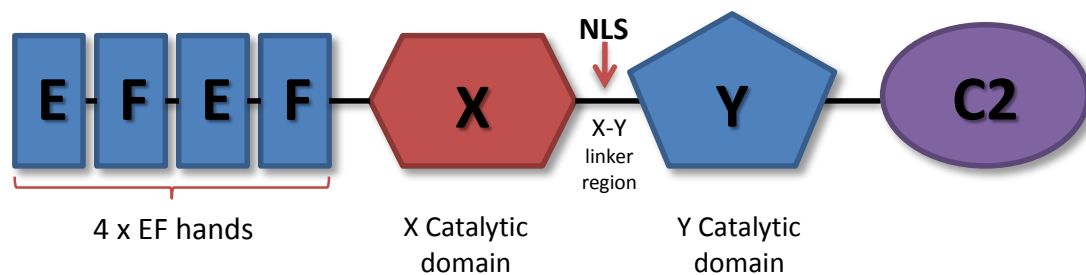


Figure 1.8 | **Schematic representation of PLC ζ domain distribution.**

The catalytic XY domain of PLC ζ is highly conserved showing close homology with PLC δ , and mutagenesis of just one active site residue leads to total loss of Ca²⁺-release ability (Swann *et al.*, 2006; Heytens *et al.*, 2009). However, the X-Y linker region is poorly conserved amongst PLC ζ isoforms except for the presence of positively charged amino acids, pre-empting speculation that differing motifs in this region may underlie the well-established species-specific patterns of Ca²⁺ oscillations amongst mammals (Swann *et al.*, 2006; Kurokawa *et al.*, 2007; Saunders *et al.*, 2007).

Deletion of the PLC ζ EF hand domains leads to a loss of *in-vitro* catalytic activity (Kouchi *et al.*, 2005; Bi *et al.*, 2009) while injection of high concentrations of cRNA encoding this truncated form failed to cause Ca²⁺ oscillations in mouse oocytes (Kouchi *et al.*, 2005; Nomikos *et al.*, 2005; Kurokawa *et al.*, 2007). Furthermore, injection of cRNA encoding PLC ζ without the C2 domain does not generate Ca²⁺ oscillations (Kouchi *et al.*, 2005; Kurokawa *et al.*, 2007), implying that both the EF hands and the C2 domain play an essential role in oocyte activation.

A sequence of amino acids predicting a nuclear localisation signal (NLS) was identified in the X-Y linker region of mouse PLC ζ (residues 374-381; KKRKRKMK) which is thought to be a binding site for the nuclear transport receptor (NTR) responsible for the nuclear translocation of mouse PLC ζ (Kuroda *et al.*, 2006). Studies have shown that the distribution of PLC ζ within the activating mouse oocyte changes during the first cell cycle, exhibiting PN localisation during the zygotic interphase (Marangos *et al.*, 2003; Larman *et al.*, 2004; Yoda *et al.*, 2004; Ito *et al.*, 2008) thereby restricting access of the enzyme to its substrate, PIP₂, resulting in termination of Ca²⁺ oscillations. Mutation of this putative NLS sequence in mouse PLC ζ prevented nuclear accumulation, as well as the termination of oscillations at interphase (Larman *et al.*, 2004; Swann *et al.*, 2006). Kuroda *et al.* (2006) hypothesised that PLC ζ is driven into the nucleus primarily by the NLS and the co-ordinated 3D structure, possibly formed by folding in the X-Y linker region (Kuroda *et al.*, 2006). Close contact between EF/C2 domains appears to be required not only for enzymatic activity but also for nuclear translocation ability (Kouchi *et al.*, 2004, 2005; Yoda *et al.*, 2004; Nakanishi *et al.*, 2008) [Figure 1.9]. However, when injected into mouse oocytes, human, bovine, rat or medaka fish PLC ζ do not translocate to the PN (Ito *et al.*, 2008ab; Cooney *et al.*, 2010). It

has yet to be verified as to whether PLC ζ in humans or other species also have a distinctive NLS sequence, although nuclear localisation may prove to be a result of a conformational change or modification arising from interaction with other adaptors.

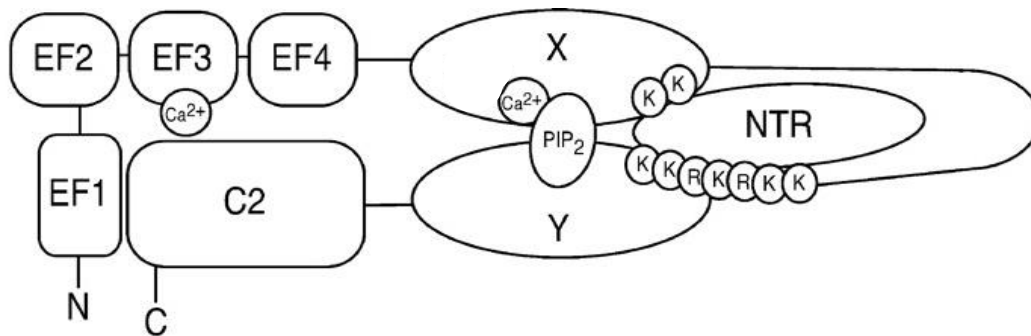


Figure 1.9 | **Schematic drawing of a model for three-dimensional functional structure of mouse PLC ζ** having Ca²⁺ oscillation-inducing ability and nuclear translocation ability through the folding of the X-Y linker region and the close contact between the EF hands and the C2 domain. (NTR – nuclear transport receptor; EF1-4 – EF hands)

Adapted from Kuroda et al. (2006)

Upon PN envelope breakdown during entry into mitosis, Ca²⁺ oscillations return (Jones *et al.*, 1995; Marangos *et al.*, 2003; Larman *et al.*, 2004; Yoda *et al.*, 2004; Yoon and Fissore, 2007) [Figure 1.10] and may act in conjunction with modifications to the IP₃R to terminate Ca²⁺ signals at fertilisation (Larman *et al.*, 2004; Lee *et al.*, 2006). Sone *et al.* (2005) have shown that this pattern of PLC ζ expression continues beyond first mitosis by exogenously expressing PLC ζ at later stages of embryo development showing a continuation of PLC ζ undergoing nuclear sequestration repeatedly in mitotic proliferation. Thus, cytoplasm/nucleus shuttling of PLC ζ is related to the turning on and off of Ca²⁺ oscillations in

a cell-cycle dependent manner suggesting a possible role for PLC ζ in mediating the cell-cycle dependency of Ca²⁺ oscillations in the early embryo and possibly other roles (Sone *et al.*, 2005). Since no other species share the ability of mouse PLC ζ to translocate between nucleus and cytoplasm, it remains unclear whether this is a widespread phenomenon in mammals and whether it holds any physiological importance.

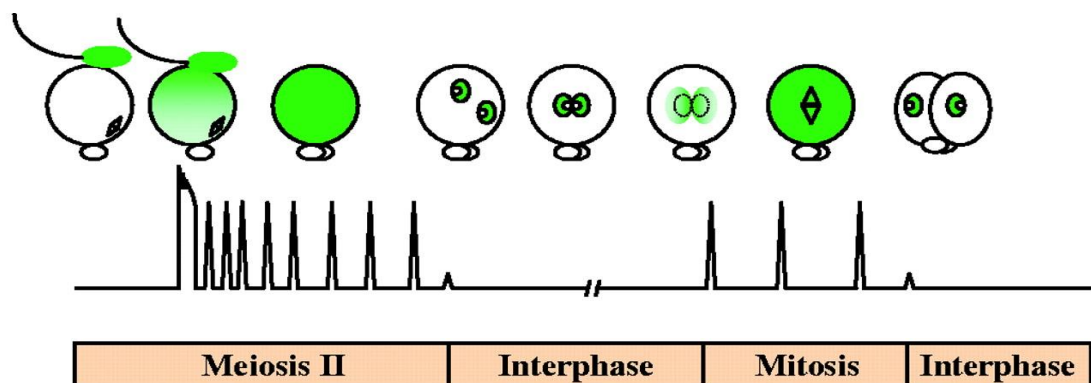


Figure 1.10 | **PLC ζ (green) translocates into male and female PN upon which Ca²⁺ oscillations cease (Interphase).** Upon PN envelope breakdown, PLC ζ is dispersed into the cytoplasm leading to the resumption of Ca²⁺ oscillations (Mitosis).

Adapted from Larman et al. (2004)

Consistent with its function as the oocyte activating factor, PLC ζ remains in an inert state within sperm, and once introduced into the oocyte by gamete membrane fusion, becomes active enzymatically and able to generate IP₃ (Swann *et al.*, 2006). It is presently unclear how such a physiologically active protein is held in an inert form inside the sperm. Furthermore, there appears to be species-specific differences in the concentration and activity of PLC ζ delivered into oocytes during fertilisation, allowing for the different specific requirements of each species, and the specific Ca²⁺ oscillation pattern leading to embryo development (Cooney *et al.*, 2010).

PLC ζ has been identified in the sperm of many species, and is localised to distinct regions in the sperm head, with suggestions of differential functional roles for each population. Three distinct populations of PLC ζ have been identified in the human sperm head – acrosomal, equatorial and post-acrosomal (Grasa *et al.*, 2008; Yoon *et al.*, 2008; Heytens *et al.*, 2009), whereas in mouse, two populations have been identified – acrosomal and post-acrosomal (Fujimoto *et al.*, 2004; Young *et al.*, 2009) [Figure 1.11].

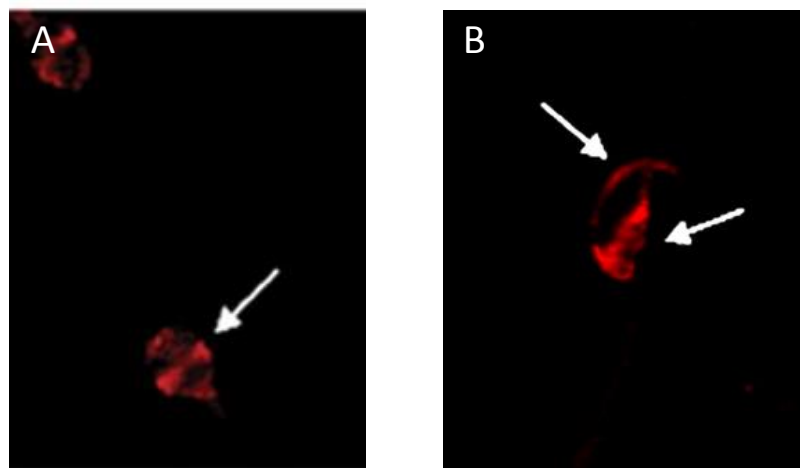


Figure 1.11 | **PLC ζ localisation patterns** in **A** | human sperm, showing an acrosomal, equatorial (arrow) and post-acrosomal PLC ζ populations; and **B** | mouse sperm showing the acrosomal and post-acrosomal populations.

Adapted from Grasa et al. (2008) and Young et al. (2009)

There are suggestions that PLC ζ may undergo dynamic changes within the sperm head. Young *et al.* (2009) showed dynamic changes in the localisation pattern of PLC ζ in mouse sperm following capacitation and the acrosome reaction. Prior to capacitation, both populations were present, with the acrosomal population being more prominent, but following capacitation, sperm exhibited post-acrosomal localisation only (Young *et al.*,

2009). Based on the latter discovery, the authors hypothesised a possible role for the acrosomal PLC ζ population in the acrosome reaction and for the post-acrosome population in oocyte activation. Grasa *et al.* (2008) echoed the findings of Young *et al.* (2009) in capacitated and non-capacitated fertile human sperm, but described three distinct populations of PLC ζ suggesting differential roles for each population (Grasa *et al.*, 2008). Furthermore, a study by Bi *et al.* (2009) identified an isoform of PLC ζ , inexplicably designated NYD-SP27, found in the acrosome of human and mouse sperm. The authors suggest that this protein is necessary for capacitation and the acrosome reaction, functioning as an intrinsic decapacitation factor (Bi *et al.*, 2009).

With significant debate over localisation and functional significance, it has been proposed that the endogenous oocyte activation factor in sperm should be localised in a region of the sperm that would allow direct and rapid release into the oocyte following gamete fusion (Lawrence *et al.*, 1997; Sutovsky *et al.*, 2003). It is therefore expected that PLC ζ , the proposed putative endogenous Ca²⁺ oscillation-inducing factor, be located in the post-acrosomal region of the mature sperm cell since the contents of this region have rapid access to the ooplasm upon sperm-oocyte fusion (Mandahar and Toshimori, 2003; Sutovsky *et al.*, 2003). Indeed, a post-acrosomal PLC ζ population has been identified in human, mice, hamster and bull sperm (Fujimoto *et al.*, 2004; Yoon and Fissore, 2007; Grasa *et al.*, 2008; Young *et al.*, 2009). Furthermore, the presence of this post-acrosomal population following *in-vivo* induced acrosome reaction provides further credence to not only PLC ζ 's role in oocyte activation, but also for this specific population, as the oocyte activation factor would be expected to be retained following this important physiological process (Grasa *et al.*,

2008; Young *et al.*, 2009). However, speculation still remains as to the specific functional roles of the differential PLC ζ populations, and further investigation is therefore required.

PLC ζ mRNA expression, and the initiation of translation have, been identified during the early and late stages of spermatogenesis in mice and pigs (Yoneda *et al.*, 2006; Young *et al.*, 2009; Heytens *et al.*, 2010). However, PLC ζ mRNA is not only expressed and translated during spermatogenesis, but as well as other functional paternal mRNA delivered into the oocyte upon fertilisation (Ostermeier *et al.*, 2004; Miller *et al.*, 2005; Gur and Breitbart, 2006; Boerke *et al.*, 2007), PLC ζ mRNA has also been identified in ejaculated sperm (Lalancette *et al.*, 2008). Indeed, injection of PLC ζ cRNA into the oocyte induces PLC ζ protein production (Saunders *et al.*, 2002) but the functional role of PLC ζ mRNA, and other paternal mRNAs, in the zygote remains to be ascertained, and despite the translation of mRNA, it is generally accepted that the highly condensed nuclei of sperm are largely or completely inactive (Publicover *et al.*, 2007). Considering there is a considerable difference in sperm mRNA content between fertile and infertile individuals (Garrido *et al.*, 2009), this suggests that similar significant differences may apply to PLC ζ , with potential repercussions on fertility, but this requires specific investigation.

1.4 Links between PLC ζ and male-factor infertility

Much remains ambiguous in the field of developmental biology on the precise events that ensue and precede the life-forming fusion of the mature sperm and oocyte. Much work has focused on ascertaining the events that underlie this process, the physiological state of the oocyte before the sperm approaches; how the sperm fertilises the

oocyte on fusion; and what happens thereafter. Many emerging strategies for contraception and for the diagnosis and treatment of infertility are based on expanded knowledge of the mechanism of the crucial union of these specialised cells.

It is now believed that infertility affects one in seven couples globally (Boivin *et al.*, 2007; Ombelet *et al.*, 2008, Kashir *et al.*, 2010), an incidence so significant that it has led to infertility becoming classified as a disease by the World Health Organisation (WHO; Zegers-Hochschild *et al.*, 2009). The WHO expects infertility and sterility to be the third most serious disease worldwide in the twenty-first century, after cancer and cardiovascular diseases (Cai *et al.*, 2011). In the UK, 1.5% of all births are the result of assisted reproductive technology (ART) (HFEA, 2010), and up to 7% of births in other developed countries (Kashir *et al.*, 2010; Nasr-Esfahani *et al.*, 2010). While *in-vitro* fertilisation (IVF) offers effective treatment for many infertile couples, this technique is not effective for several conditions including severe male infertility, which accounts for 19-57% of cases (Wilkes *et al.*, 2009). In these cases, intracytoplasmic sperm injection (ICSI), whereby a single spermatozoon is directly injected into the oocyte (Palermo *et al.*, 1992), is implemented. Despite this revolutionary approach, 1-5% of ICSI cycles still fail (Yanagida *et al.*, 2008; Nasr-Esfahani *et al.*, 2010). Considering that approximately 52% of all IVF cycles in the UK involves ICSI (HFEA, 2010), this represents a significant problem, and on average would affect over 1200 couples per year, based on current HFEA (2010) figures.

Since PLC ζ plays a fundamental role in fertilisation, it follows that deficiency or abnormality of this crucial protein may be associated with human infertility. Indeed, a deficiency in the mechanism of oocyte activation is regarded as the principal cause of fertilisation failure following ICSI, accounting for 40% of failed cases (Sousa and Tesarik,

1994; Rawe *et al.*, 2000; Mahutte and Arici, 2003; Swann *et al.*, 2004; Kashir *et al.*, 2010). ICSI addresses only one of the necessary requisites for successful fertilisation, that of sperm penetration, whereas a plethora of post-penetration events are crucial for successful fertilisation (Swain and Pool, 2008). The aetiology of the failure of ICSI in these cases is likely to be multi-factorial and may be also attributable to factors in the oocyte since the inherent quality of the oocyte is greatly important for successful fertilisation. Such factors would thus be reliant upon the fidelity of oocyte maturation, such as the inadequate sensitisation of IP₃R and the regulation of ER Ca²⁺ concentration for optimal sperm-initiated Ca²⁺ release (Mehlmann and Kline, 1994; Xu *et al.*, 1994; Jones *et al.*, 1999), or a reduction in the number of CG due to premature release, resulting in the early establishment of block to polyspermy prior to insemination (Ducibella *et al.*, 1995).

Sperm defects however, are presently considered to represent the leading cause of activation failure, and given the role of PLCζ as the oocyte activating factor, it is highly plausible that defective forms of PLCζ, or abnormal function of PLCζ, may be the underlying cause of certain types of male-factor infertility and oocyte activation failure. Indeed, much evidence now exists to highlight PLCζ's role in fertility and in male-factor infertility. Studies have shown that sperm from infertile men who consistently fail IVF and ICSI, fail to induce Ca²⁺ oscillations upon injection into mouse oocytes, or cause abnormal patterns of Ca²⁺ release when compared with those of fertile males (Yoon *et al.*, 2008; Heytens *et al.*, 2009) [Figure 1.12]. Such sperm also showed an undetectable or abnormal localisation pattern of PLCζ [Figure 1.13] and in some cases an abnormally low molecular weight of PLCζ (Yoon *et al.*, 2008; Heytens *et al.*, 2009).

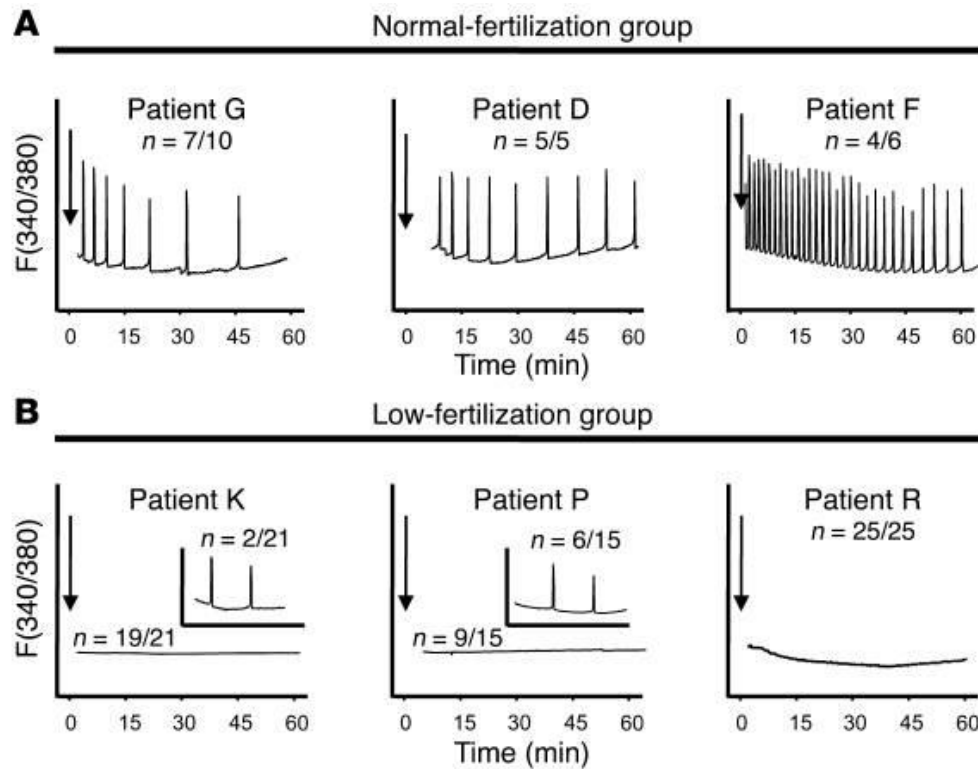


Figure 1.12 | Ca^{2+} oscillation profiles following the injection of sperm from A | normal fertile humans and B | infertile patients who had previous failure or demonstrated low fertilisation rates following ICSI.

Adapted from Yoon et al. (2008)

Other underlying factors have been identified recently with studies reporting genetic links to PLC ζ deficiency. The first such link was reported by Heytens *et al.* (2009) who identified a substitution mutation in an infertile male diagnosed with oocyte activation deficiency (OAD). This involved the substitution of a Histidine to a Proline residue within the Y domain of the catalytic site of the protein (PLC ζ_{H398P}). Micro-injection of PLC ζ_{H398P} cRNA into mouse oocytes failed to induce Ca^{2+} oscillations, whilst the injection of sperm possessing this mutation resulted in an atypical pattern of Ca^{2+} oscillations (Heytens *et al.*,

2009). Moreover, sperm from infertile patients exhibited a significantly lower level of PLC ζ compared with fertile controls (Heytens *et al.*, 2009).

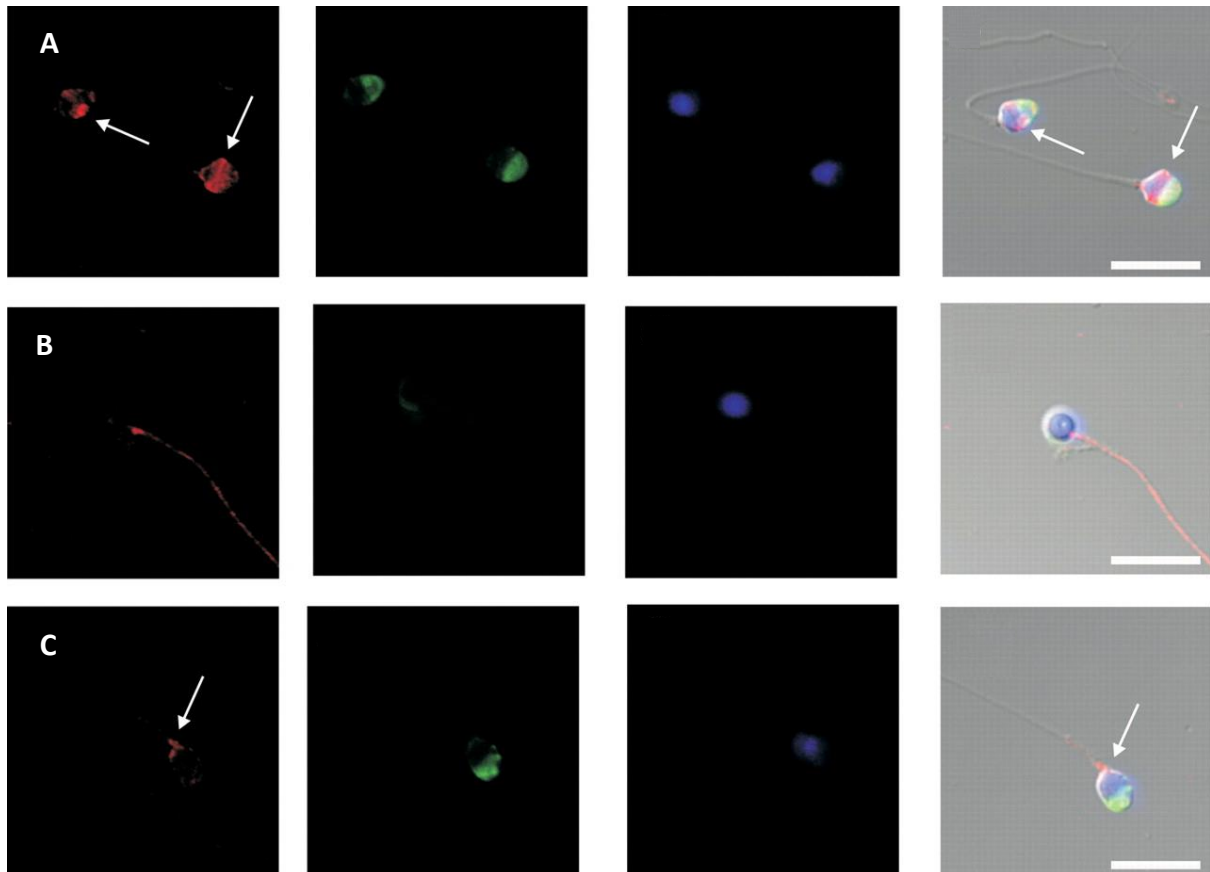


Figure 1.13 | Sperm from A | fertile controls immunostained with PLC ζ (red), compared with reduced PLC ζ immunostaining in sperm from infertile ICSI-failed patients exhibiting B | abnormal morphology (globozoospermia) and C | normal morphology. FITC-conjugated peanut agglutinin (FITC-PNA)-lectin staining (green) identifies the acrosome, while Hoescht-33342 staining (blue) identifies the nuclei. Scale bars indicate 5μm.

Adapted from Heytens et al. (2009)

Sperm of patients with globozoospermia, a rare condition affecting 0.1% of infertile men (Dam *et al.*, 2007), characterised by round-headed sperm which lack the acrosome, have been shown to be either devoid of PLC ζ or exhibit abnormal localisations (Yoon *et al.*, 2008). These findings were further demonstrated in the wobbler mouse, a neurological mouse model whose sperm are globozoospermia-like in morphology (Heytens *et al.*, 2010). Having a mouse model of globozoospermia made it possible to evaluate the possibility of abnormalities during spermatogenesis, and indeed analyses indicated that PLC ζ , although expressed in the testis in areas representing later stages of spermatogenesis, was of lower abundance than in wild-type mice (Heytens *et al.*, 2010). This correlated with significant down-regulation of PLC ζ mRNA in the testis of wobbler mice, probably due to a reduced number of maturing sperm cells in the testis as indicated by the down-regulation of protamine-1 mRNA, a marker for post-meiotic spermatids (Heytens *et al.*, 2010). This mouse model may represent a very useful tool with which to investigate the aetiology and development of artificial oocyte activation (AOA) strategies to treat globozoospermia.

However, the clinical correlates of PLC ζ and OAD remain limited and there is an urgent need to increase the number of samples screened for PLC ζ in OAD patients and fertile controls. Furthermore, whether factors such as paternal age are associated with PLC ζ functionality requires investigation to further our understanding of male fertility.

1.5 The potential use of PLC ζ as a therapeutic agent in ART

Cases of oocyte activation failure can currently be treated by AOA methods such as Ca²⁺-ionophore, strontium chloride, or even ethanol (Nakada and Mizuno, 1998; Eldar-Geva *et al.*, 2003; Heindryckx *et al.*, 2008; Yanagida *et al.*, 2008; Taylor *et al.*, 2010). In Ghent University Hospital (Belgium), ICSI-AOA using ionomycin is a commonly-used procedure, treating approximately 30 patients per year, reporting the restoration of fertilisation rates to normal in most patients with a history of previously failed or low fertilisation (Heindryckx *et al.*, 2005, 2008). Taylor *et al.* (2010) demonstrated high rates of fertilisation, and a successful pregnancy and delivery, in PLC ζ -deficient globozoospermic patients using a Ca²⁺ ionophore to artificially activate oocytes following ICSI. However, there is significant concern of how such chemicals may be detrimental to embryo viability and future health due to potential cytotoxic, mutagenic and teratogenic effects upon oocytes and embryos (Nasr-Esfahani *et al.*, 2010). Ca²⁺ oscillation patterns following ionophore treatment do not mimic those observed during normal fertilisation, where a single Ca²⁺ transient is often produced rather than a series of oscillations (Heytens *et al.*, 2009) [Figure 1.14]. Altering the fertilising Ca²⁺ signal may have long-term effects on both gene expression during activation and embryogenesis, and development to term. Consequently, the abnormal Ca²⁺ signal induced by AOAs represent a potential threat to ensuing development (Ozil, 1990; Ozil *et al.*, 2006) with potential repercussions on epigenetic processes (Ciapa and Arnoult, 2011). Moreover, in cases where AOA is used in conjugation with abnormal sperm such as in cases of globozoospermia, the threat is greatly manifested since a high percentage of sperm DNA fragmentation is already associated with this pathology (Taylor *et al.*, 2010; Avendaño and Oehninger, 2011; Ciapa and Arnoult, 2011).

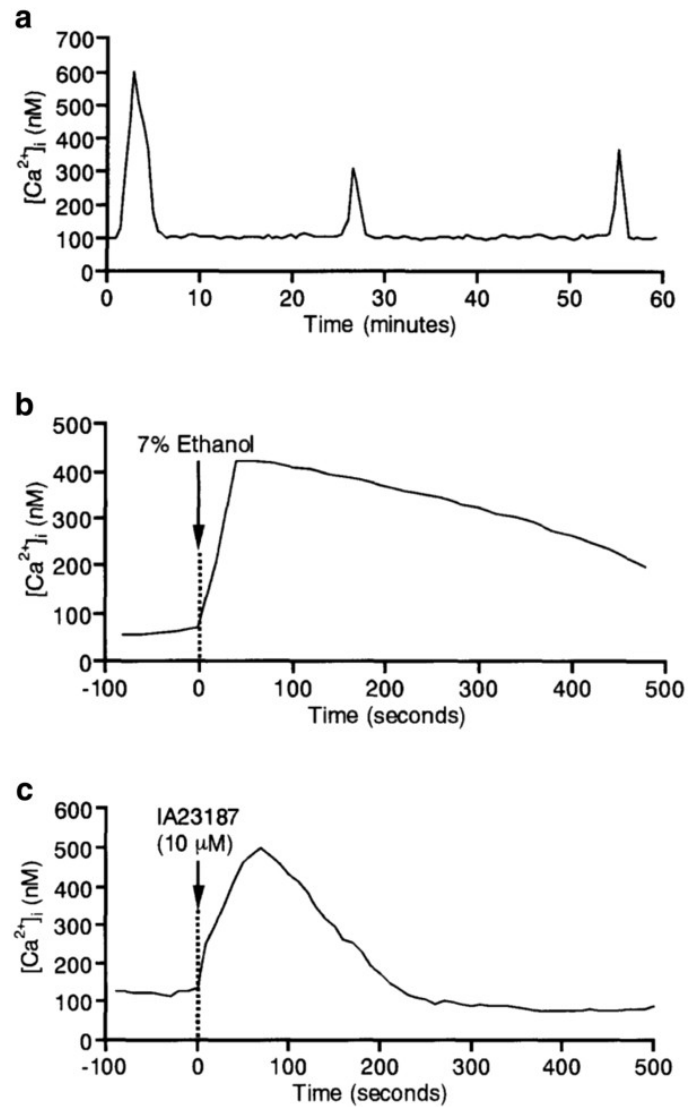


Figure 1.14 | Representative Ca^{2+} responses of bovine oocytes treated with A | sperm (normal fertilisation), B | 7% ethanol, and C | Ca^{2+} ionophore IA23187.

Adapted from Nakada and Mizuno (1998)

Given the incidence of ICSI failure and the concern over the use of AOAs, there is significant hope for PLC ζ as a more physiological therapeutic option, to substitute or augment endogenous PLC ζ activity. Indeed, Yoon *et al.* (2008) showed that the failure of sperm exhibiting abnormal PLC ζ localisation to activate an oocyte could be rescued upon co-injection with mouse PLC ζ mRNA (Yoon *et al.*, 2008) [Figure 1.15], while Rogers *et al.* (2004)

showed that it was possible to parthenogenetically generate blastocysts following the injection of PLC ζ cRNA into aged human oocytes (Rogers *et al.*, 2004), providing significant credence to the clinical use of PLC ζ as a therapeutic. However, the therapeutic use of PLC ζ cRNA is not a viable option since the uncontrollable transcription of PLC ζ may impede normal pre-implantational development through gene expression irregularities, with developmental defects observed in some embryos (Ducibella *et al.*, 2002; Ozil *et al.*, 2006; Rogers *et al.*, 2006). Moreover, injected PLC ζ RNA could potentially be reverse transcribed into cDNA which may then incorporate into the genome (Spadafora, 2004; Kashir *et al.*, 2010). It follows therefore, that an active, purified, recombinant version of PLC ζ would be the ideal alternative for therapeutic application in the clinic, as it would function in a safe and endogenous manner.

Significant attempts have been made over the past decade to develop an active recombinant form of PLC ζ protein. Thus far, it has proved exceptionally difficult to produce an active purified form of recombinant PLC ζ protein in a reproducible manner, and prior to commencing this project, only one study yielded a hint of success. Kouchi *et al.* (2004) successfully purified a mouse recombinant PLC ζ protein which exhibited high Ca²⁺ sensitivity and induced Ca²⁺ oscillations on injection into mouse oocytes. However, these oscillations were of a much higher frequency than those seen during normal fertilisation and results have proved difficult to replicate thereafter.

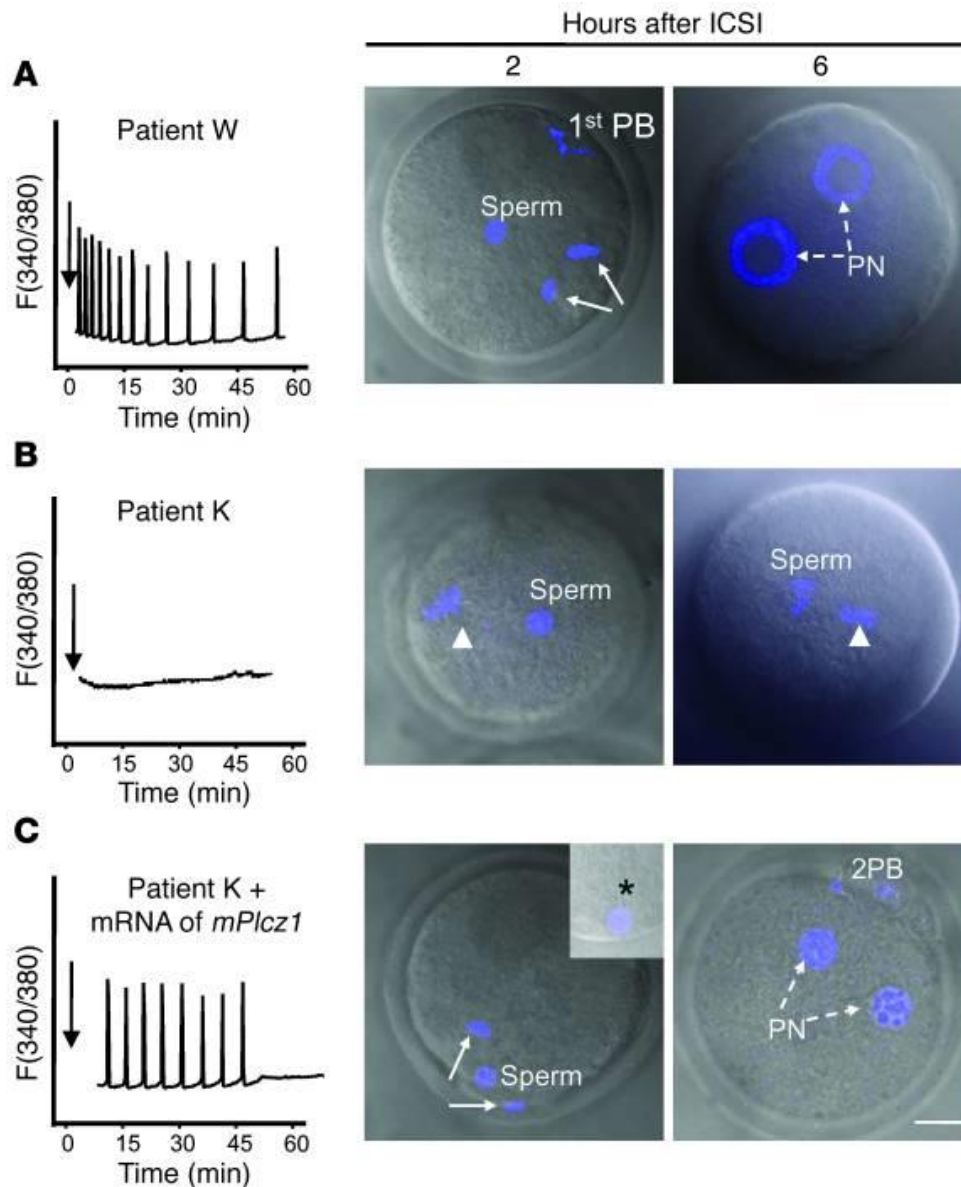


Figure 1.15 | **A** | Injection of sperm from a fertile control elicits Ca^{2+} oscillations and activates mouse oocytes. **B** | Sperm from an infertile patient with a history of ICSI failure was unable to elicit Ca^{2+} oscillations, and was unable to activate mouse oocytes following injection, **C** | Ca^{2+} oscillations were elicited following the co-injection of PLC ζ mRNA. Arrows on graphs denote time of sperm injection; solid white arrows on images denote meiosis resumption (anaphase II); broken arrows denote PN formation; arrow heads denote MII chromatin; 1st PB – first polar body; 2PB – second polar body. TO-PRO-3 staining (blue) was used to stain chromatin. Asterisk in inset points to the persistence of the human sperm tail in mouse oocytes. Scale bar indicates 10 μ m.

Adapted from Yoon et al. (2008)

Given the huge potential for clinical translation, with the significant incidence of ICSI failure and concern over the use of AOA, the synthesis of a pure and active recombinant protein remains a key goal in the field. Purification in particular has been tenuous thus far with previous studies showing inactivation of the protein following purification suggesting the absence or disruption of post-translational modifications (PTM) or protein-folding during the purification process (Cooney *et al.*, 2010). Hence, the optimisation of a successful purification protocol for recombinant human PLC ζ , whilst maintaining the functional active nature of the protein, is imperative.

1.6 The potential use of PLC ζ as a biomarker of fertility

Sperm analysis based on concentration, motility and morphology has been used for the diagnosis of male fertility for many years, with standardised criteria for normal semen parameters established by the WHO (2010) (Cooper *et al.*, 2010). However, a significant number of patients with normal parameters in terms of sperm concentration, motility and morphology still undergo difficulty in achieving successful pregnancy (Garrido *et al.*, 2004). This reflects the complexity of the events involved in sperm function, indicating that analysis based on concentration, motility and morphology is not the most effective method to assess the fertilising potential of a sperm sample. Rather, more sophisticated assessments should be developed to complement existing analytical procedures. The identification of suitable biomarkers of sperm function and viability have thus been a subject of interest in recent years, with recent studies demonstrating the potential of measuring DNA fragmentation and

protamine deficiency in sperm samples, highlighting significant differences between fertile and infertile samples (Lukanov *et al.*, 2009; Tavalaei *et al.*, 2010).

Identifying a pattern of localisation of PLC ζ that is compatible with its role as the putative endogenous oocyte activation factor provides further confirmation of its physiological relevance as a mediator of this important process. Moreover, the identification of the precise localisation pattern in fertile males that is specifically associated with oocyte activation ability would provide a benchmark against which sperm from infertile patients may be compared to, to ascertain the reasons behind their inability to fertilise, with options for therapy. Previous work using immunofluorescent techniques has already demonstrated that there appears to be a pattern of PLC ζ localisation in the sperm head consistent with fertile sperm (Grasa *et al.*, 2008; Heytens *et al.*, 2009). These sperm samples exhibited a predominantly equatorial PLC ζ localisation, whereas abnormal patterning was evident in ICSI-failed sperm (Yoon *et al.*, 2008; Heytens *et al.*, 2009), implicating a correlation between abnormal localisation patterning of PLC ζ with aberrant function and thus infertility. This suggests that analysing the expression of PLC ζ in sperm is a credible fertility index for patients attending a fertility clinic. Thus far, quantitative PLC ζ analysis performed in sperm has been undertaken using fluorescent immunocytochemistry, but this may not be an efficient system for clinics, thus the need to develop a more rapid tool. Moreover, the quantitative study of the total levels of PLC ζ protein within a sperm sample may provide an indication of the oocyte activation ability of a sample for ART.

The quantitative analysis of sperm PLC ζ using flow cytometry may represent a more rapid, useful diagnostic tool for both research and translational purposes. This technique has never been used to analyse PLC ζ expression in sperm thus far, but has been shown to

represent a potential fertility diagnostic tool by measuring sperm nuclear DNA fragmentation in patient sperm, revealing significant differences in levels between fertile and infertile sperm (Lukanov *et al.*, 2009), and also for protamine deficiency (Tavalaee *et al.*, 2009). A similar assay for oocyte activation potential by assessment of PLC ζ may also prove to be a useful clinical biomarker of fertility.

Further clinical investigation and different experimental approaches are necessary to determine appropriate sperm biomarkers that correlate with clinical reproductive success or failure and can be readily measured in a clinical scenario. Ascertaining the quantitative expression of PLC ζ , in both a temporal and spatial manner, holds much promise since vast evidence supports its role in fertility and its association with infertility and OAD. It is indeed very conceivable that such therapeutic options may be introduced into the clinic in the near future.

1.7 Aims of this study

PLC ζ holds great promise for diagnostic and therapeutic applications in the treatment of human OAD and infertility. However, there are many areas of critical understanding that have yet to be elucidated. This project aims to address some of these outstanding issues to assist the translation of PLC ζ research to the clinic. In particular, the principle aims of this thesis were to:

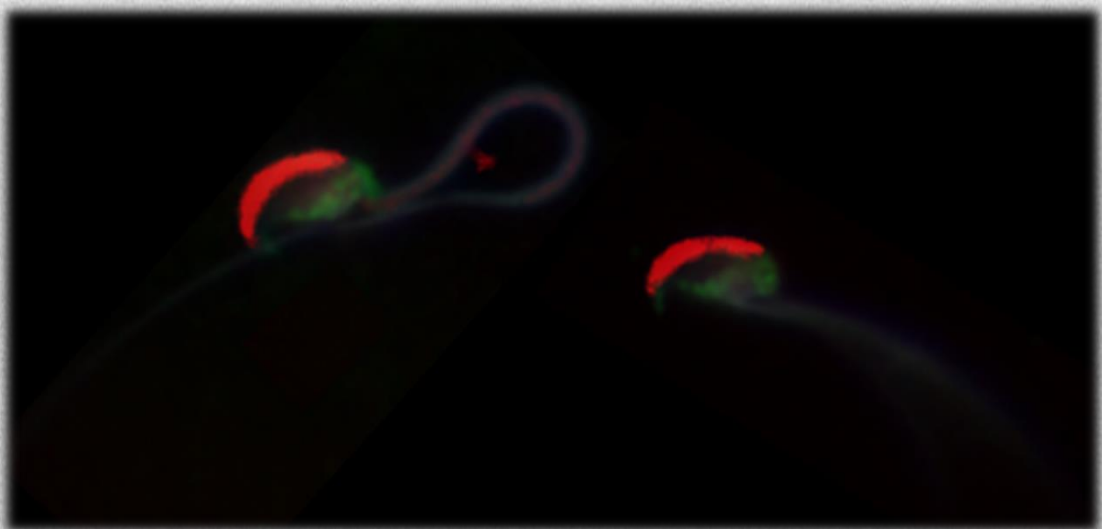
1 – Investigate the effect of spermatogenesis, epididymal progression and male age upon PLC ζ protein expression and localisation. This project investigates, for the first time in any species, the effect of age and epididymal maturation upon PLC ζ localisation and total PLC ζ levels in mouse sperm by fluorescent immunocytochemistry and immunohistochemistry. Age effects are presently unknown, and abnormalities in epididymal expression may result in clinical problems. Developmental expression may provide further clues to PLC ζ biology that may be useful clinically.

2 – Investigate the effect of male age on total PLC ζ and its localisation in human sperm. Existing studies show that increased male age causes reduced semen quality, increased frequency of morphological aberrations, and reduced genetic integrity in human sperm (Ezkenazi *et al.*, 2003; Wyrobek *et al.*, 2006; Slotter *et al.*, 2007), but whether there is a change in oocyte activation ability is currently unknown. This project will investigate, for the first time, the effect of male age upon PLC ζ localisation and total PLC ζ levels in human sperm using fluorescent immunocytochemistry techniques.

3 – Develop an understanding of the mechanism of PLCζ action and how these might be applied clinically by synthesising an active recombinant protein. This project aims to develop the therapeutic potential of PLCζ by synthesising a purified recombinant form of human PLCζ protein which retains Ca²⁺-releasing ability. This study will also endeavor to study the subcellular localisation and stability of wild-type and mutant forms of recombinant human PLCζ using fluorescently-tagged human recombinant PLCζ in a mammalian cell line (HEK293T).

4 – Assess and develop tools for research and the ART clinic. Despite clear evidence of abnormal PLCζ levels or localisation pattern in OAD and infertile patients, it is not yet known at which point in sperm development such detrimental changes arise. As yet, there has been no attempt to map PLCζ expression or localisation in human testicular tissue, largely due to ethical restrictions and supply. This study, will for the first time, attempt to use immunological techniques to investigate PLCζ expression in human testicular biopsies obtained by aspiration in a collaborating ART unit. Additional studies aim to develop a biomarker assay for oocyte activation ability using flow cytometry to assess levels of PLCζ in human sperm. Moreover, further to the significant debate over localisation patterns in the literature with criticism regarding antibody specificity, this study will endeavor to improve antibody specificity and lay the foundation for the development of a monoclonal antibody. A monoclonal antibody would provide a powerful research tool with significant diagnostic potential for the ART clinic, and the improved specificity of such an antibody may provide significant insight into the current debate over the existence of multiple PLCζ localisation patterns.

Chapter 2 | THE EFFECT OF MALE AGE AND EPIDIDYMAL PROGRESSION UPON MOUSE PLC ζ



2.1 Introduction

Fertile sperm arise from a successive series of morphological and surface modifications in the testis and epididymis. The main morphological modifications occur during spermatogenesis where round germ cells turn into highly organised cells of a specific morphology, with a greatly condensed inactive nucleus and a flagellar apparatus. Spermatogenesis occurs in the testis and exiting sperm move via the rete testis to the epididymis. The epididymis is a series of highly convoluted tubules through which sperm must pass in order to acquire the functional capacities of progressive motility and the ability to fertilise an oocyte (Gatti *et al.*, 2004). The epididymis is divided morphologically into three segments: the caput (the proximal part), the corpus, and the cauda [Figure 2.1.1]. Sperm gathers full fertilisation competence as it travels through the epididymis until it reaches the cauda, which acts as a sperm reservoir, and where sperm is stored until ejaculation. This maturation process requires the interaction of sperm with proteins synthesised and secreted by the epididymal epithelium into the lumen in a region-dependent manner (Gatti *et al.*, 2004). As sperm move along the epididymis, they undergo various physiological changes in the constantly changing environment from the testis, through to the cauda (Cornwall, 2009; Leahy and Gadella, 2011). On reaching the cauda, sperm will have acquired the properties that will enable it to fertilise and activate an oocyte.

Strong evidence now exists to indicate that the sperm-specific enzyme PLC ζ is the physiological agent responsible for IP $_3$ -mediated Ca $^{2+}$ release in activating oocytes (Cox *et al.*, 2002; Saunders *et al.*, 2002; Kashir *et al.*, 2010). Studies involving micro-injection of PLC ζ cRNA, micro-injection of recombinant PLC ζ protein, immunodepletion of PLC ζ from sperm extracts, or the inhibition of PLC ζ expression in sperm using transgenic RNA interference, all

support the concept that PLC ζ is indeed the oocyte activation factor (Cox *et al.*, 2002; Saunders *et al.*, 2002; Kouchi *et al.*, 2004; Rogers *et al.*, 2004; Knott *et al.*, 2005; Swann and Yu, 2008).

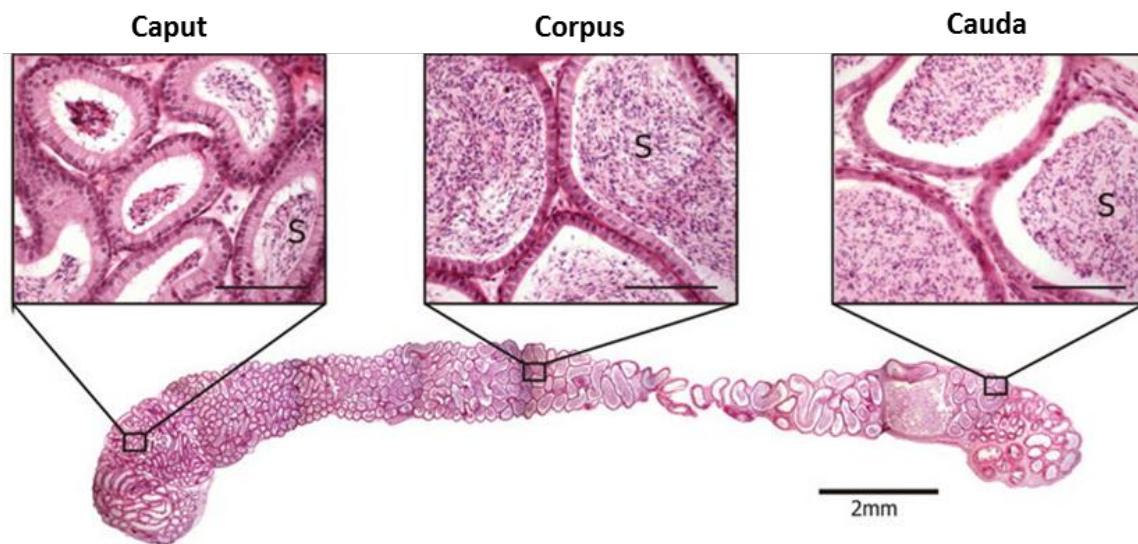


Figure 2.1.1 | **The mouse epididymis showing the different regions – caput, corpus, cauda – whereby sperm undergo epididymal maturation through its passage.** The epididymis ends at the vas deferens (not shown). S = sperm.

Figure adapted from Borg et al. (2010)

Recent studies have addressed the localisation of PLC ζ in sperm from various species and suggested possible differential functional roles for different populations. Three distinct populations of PLC ζ have been identified in the human sperm head – acrosomal, equatorial and post-acrosomal (Grasa *et al.*, 2008), whereas in the mouse, two populations have been identified – acrosomal and post-acrosomal (Fujimoto *et al.*, 2004; Young *et al.*, 2009).

It has been postulated that PLC ζ undergoes dynamic changes in localisation pattern within the sperm head. Young *et al.* (2009) identified dynamic changes in the localisation pattern of PLC ζ in mouse sperm following capacitation and the acrosome reaction. Prior to capacitation, both populations were present, with the acrosomal population being more prominent, while following capacitation, sperm showed post-acrosomal localisation only consistent with the loss of the acrosomal contents. Based on this data, the authors hypothesised a possible role for the acrosomal PLC ζ population in the acrosome reaction and the post-acrosomal population in oocyte activation. Furthermore, a study by Bi *et al.* (2009) identified an isoform of PLC ζ , named NYD-SP27 by the authors, which was localised in the acrosome of human and mouse sperm. The authors suggested that this isoform was necessary for capacitation and the acrosome reaction and functioned as an intrinsic decapacitation factor, defects of which might be linked to infertility.

Abnormal localisation patterns of PLC ζ have been identified in human sperm that are unable to activate oocytes (Yoon *et al.*, 2008; Heytens *et al.*, 2009), implicating a correlation between abnormal localisation and abnormal function/infertility. Thus, the characterisation of PLC ζ localisation and expression within the sperm head based on immunofluorescent antibody detection may provide a useful benchmark to study the pattern of localisation of PLC ζ in infertile patients compared with fertile males and thus develop a potential diagnostic test of oocyte activation ability for clinical use as a biomarker for fertility. However, much remains unresolved in our understanding of the precise mechanism of action and the functional role of PLC ζ . Given the importance the level and localisation of PLC ζ may have upon the oocyte activating potential of sperm, and its potential as a biomarker, it is of great importance that the localisation properties of PLC ζ in sperm is

determined and verified, and that quantitative analysis is undertaken on the overall level and localisation of PLC ζ in fertile sperm to act as a benchmark against which sperm from infertile patients may be compared to.

Epididymal sperm maturation may underlie the the existence of different populations of PLC ζ , whereby PLC ζ may undergo various forms of PTMs. Such modifications may be vital for enhancing the oocyte-activating potency in sperm as it matures. Approximately 40% of infertile men exhibit idiopathic infertility, a fact which may reflect sperm maturational disorders (Cornwall, 2009). Of more concern is that in these cases ICSI may be performed by sperm retrieved from the testis or epididymis, thus utilising sperm independent of maturational status, which may lead to increased risks of genetic abnormalities being transmitted to the offspring (Coward *et al.*, 2007; Fedder *et al.*, 2007; Sanchez-Albisua *et al.*, 2007). PLC ζ expression in sperm of reduced maturity may also lead to developmental abnormalities since such sperm may lack PTM which would normally occur at later stages of maturity.

Using a mouse model, this study investigates, for the first time, the effect of age and epididymal maturation upon the level and localisation of PLC ζ by immunofluorescence and immunohistochemistry techniques. Moreover, no previous work has attempted to demonstrate the developmental expression of PLC ζ during spermatogenesis within the testis. Consequently, a secondary aim was to study the localisation of PLC ζ during spermatogenesis, and whether this was influenced by male age. This study endeavored to attain these aims by employing fluorescent immunocytochemistry on epididymal swim-out sperm, and immunohistochemistry on testicular and epididymal tissue to investigate levels and localisation patterns of PLC ζ , and examine the potential effect of male age.

As well as shedding light upon the significance of epididymal maturation upon the oocyte activation ability of sperm, should a correlation exist between the level and localisation of PLC ζ and age, this study would provide the first evidence for reduced oocyte activating capability with age and develop our understanding of how PLC ζ is expressed within developing sperm.

2.2 Materials and Methods

2.2.1 Animals

The British Home Office approved the maintenance and procedures undertaken in this study. C57BL/6 male mice were housed in the Biomedical Services unit (BMS) at the John Radcliffe Hospital, Oxford. Mice aged 6 weeks (n=6), 16 weeks (n=6) and 32 weeks (n=6) were sacrificed via cervical dislocation by fully trained and licensed personnel at the BMS. Fur was removed by shaving the hair from the surgical area and the surgical site cleaned with 70% ethanol. The entire male reproductive tract was exposed via an abdominal incision and the reproductive system dissected out (testes, epididymis, seminal vesicles, and prostate). Testes and epididymi were dissected and each region (caput, corpus, cauda) separated [Figure 2.2.1].

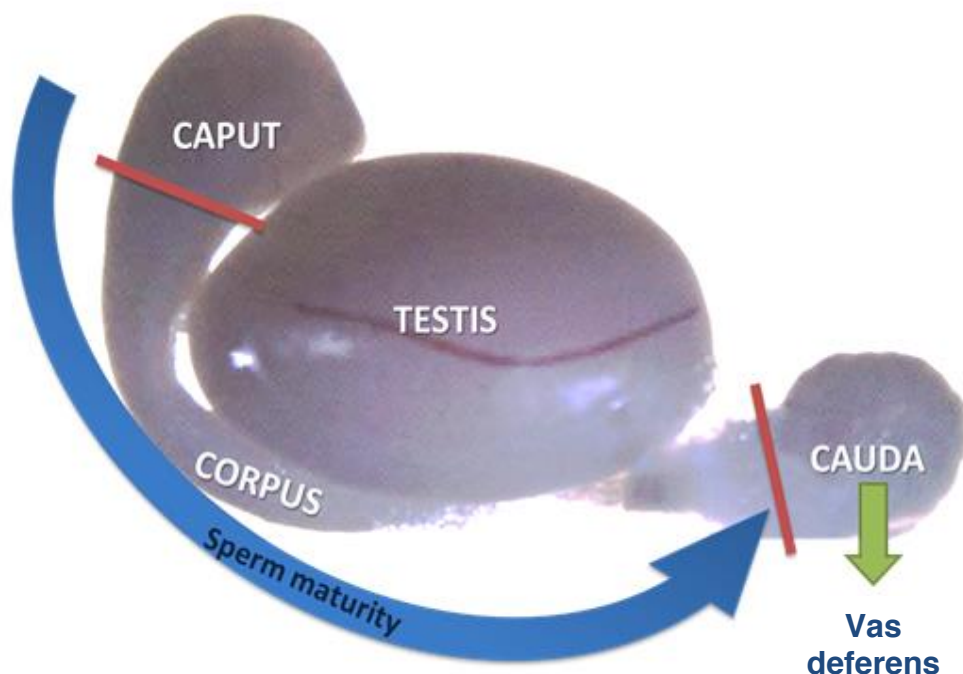


Figure 2.2.1 | Mouse epididymis showing different regions (caput, corpus and cauda).

2.2.2 Experimental design

Mouse epididymis and testis were treated in different ways according to the experiment for which they were to be used. Figures 2.2.2 and 2.2.3 provide an overview of the experiments undertaken, and what procedures were undertaken on the dissected tissue.

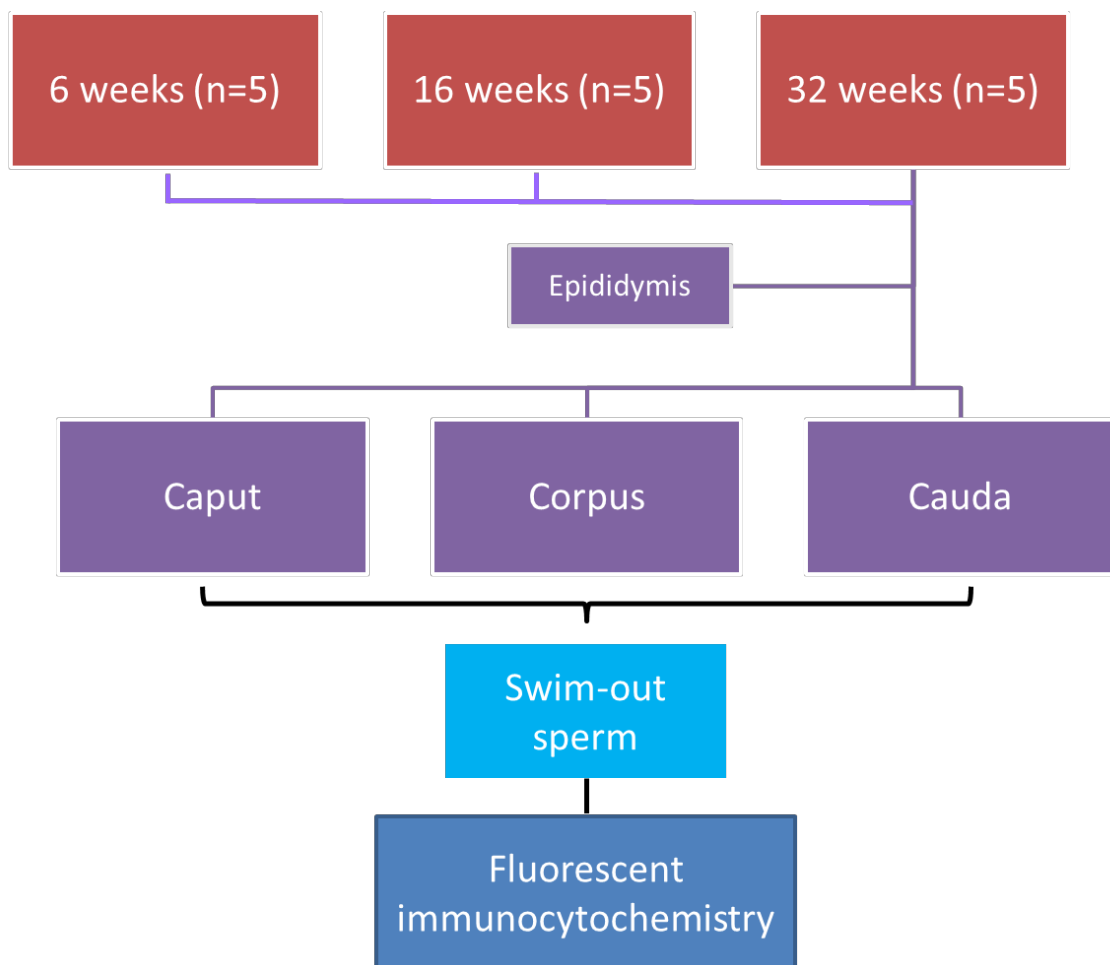


Figure 2.2.2 | For the fluorescent immunocytochemical analyses of the total levels and localisation of PLC ζ in the epididymis, swim-up sperm was derived from the three regions of the epididymis (caput, corpus and cauda) of 6 week (n=5), 16 week (n=5) and 32 (n=5) mice.

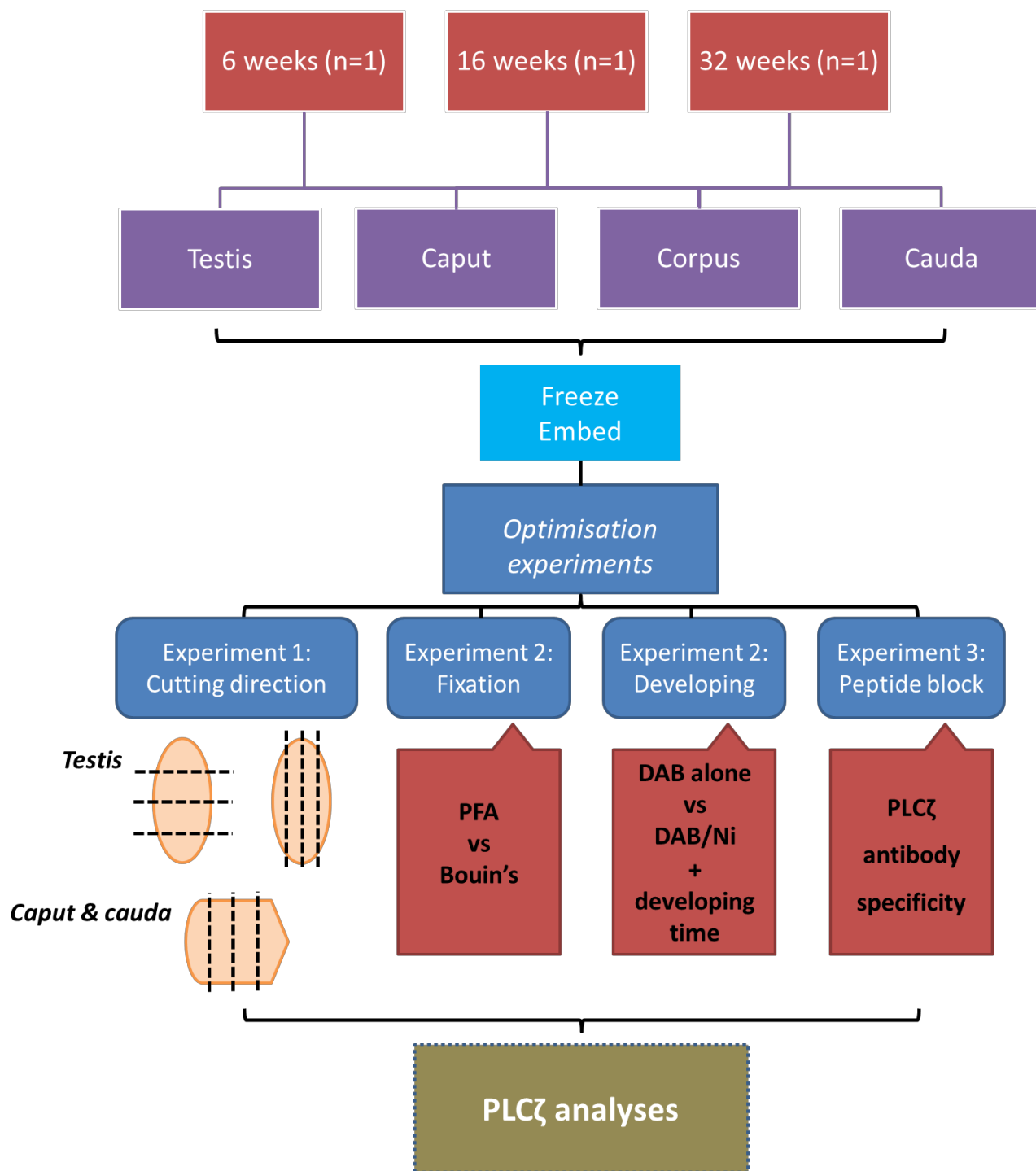


Figure 2.2.3 | For the immunohistochemical analyses of the localisation of PLC ζ during spermatogenesis and epididymal progression, the testis and epididymis (caput, corpus and cauda) of 6 week (n=1), 16 week (n=1) and 32 (n=1) mice was frozen upon dissection and embedded. Experiments for the optimisation of the immunohistochemical protocol were undertaken prior to the analyses of PLC ζ . (PFA – paraformaldehyde; DAB – diaminobenzidine; DAB/Ni – with nickel enhancement)

2.2.3 Isolation of epididymal swim-out sperm

Sperm was collected from regions of the epididymi perforated with a 25 gauge needle into equal volumes (200µl) of pre-warmed phosphate buffered saline (PBS; Sigma, UK), at 37°C for 30 minutes to allow sperm to swim out. Sperm concentration and motility was assessed using computer assisted sperm analysis (CASA) with a Hamilton Thorne sperm analyser [Appendix 1]. Swim-out sperm were pelleted by centrifugation at 800g for 3 minutes, washed by centrifugation twice in 200µl PBS, fixed in PBS/4% paraformaldehyde (PFA; Sigma, UK) for 30 minutes at room temperature (RT), followed by two further washes in 200µl PBS by centrifugation.

2.2.4 Immunofluorescence studies on swim-out sperm

Fixed sperm suspensions were added on PAP moulds drawn by a hydrophobic barrier pen (Immedge Pen, Vector laboratories, UK) on slides pre-coated with 0.01% poly-L-lysine (Sigma, UK), air dried, and permeabilised for 20 minutes with PBS/0.5% Triton-X100 (Sigma, UK) in a humidifying chamber at RT. Slides were washed twice with PBS, and non-specific binding blocked with PBS/5% bovine serum albumin (BSA; Sigma, UK) for 1 hour at RT. Samples were then incubated overnight at 4°C with 25µg/ml anti-PLCζ antibody, used in the studies by Young *et al.* (as described by Young *et al.*, 2009) in PBS/0.05% BSA. Negative controls were incubated in the absence of primary antibody. Slides were subsequently washed twice with PBS and incubated for 1 hour at RT in the dark in 5µg/ml secondary fluorescent antibody (Alexa Fluor 488 F(ab')₂ goat anti-rabbit IgG, Invitrogen, UK), washed three times with PBS, then incubated for 1 hour in 40µg/ml TRITC-PNA diluted in PBS/1%

BSA for acrosome visualisation at RT in the dark. Finally, samples were washed three further times with PBS and mounted with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vectashield, UK) for nuclear staining and 22mm glass cover slips for analysis.

Images were captured and the distribution of PLC ζ in accordance with Young *et al.* (2009) in the different samples visualised under a fluorescent Nikon Eclipse 80i microscope. A FITC filter (488nm) was used to visualise PLC ζ fluorescence (green) and a TRITC filter (560nm) for acrosomal fluorescence (red). Images were captured using a Nikon DS-Ri1 digital camera and NIS-Elements BR 3.0 software. Captured fields of view were randomly selected using dark field, and 100 random cells, where the head and tail were intact, and the head was clearly visible and unobstructed, were evaluated per sample [Figure 2.2.4]. Relative overall levels of fluorescence among samples, as well as the distribution of PLC ζ localisation patterns, were assessed using the region of interest (ROI) manager in Image J software (<http://rsbweb.nih.gov/ij/>). The ROI (sperm heads) were selected in the dark field image for each field of view, and then transferred to its corresponding fluorescent image, and the colour histogram tool used to measure the relative level of green fluorescence in each sperm head, and the localisation for each sperm head noted [Appendix 2].



Figure 2.2.4 | **An example of a captured field of view in dark field.** Sperm heads highlighted with an asterisk (*) denote sperm not used for analyses: white asterisk shows a sperm not lying in the plane of view; red asterisks show sperm heads obstructed by the tail; and the green asterisk shows a sperm head without the tail intact. The other sperm heads in this field of view would be used for analyses.

2.2.5 Immunohistochemistry studies on epididymal and testicular sections

The reproductive system of 6 week, 16 week and 32 week old male mice were dissected and each region (testis, caput, corpus, cauda) separated and snap frozen immediately in liquid nitrogen before storage at -80°C. Frozen tissues were then embedded in Bright CRYO-M-BED embedding compound (Bright Instrument Company Ltd, UK) and frozen using isopentane (VWR, UK) by immersion in the gas phase of liquid nitrogen, to prevent the formation of ice crystals. The frozen embedded sections were then stored in a -80°C freezer to await sectioning.

Frozen tissue were cut into 5µm sections using a cryostat-microtome (Leica, UK) and thaw-mounted onto glass slides pre-coated with poly-L-lysine. Slides were fixed in Bouin's solution (Sigma, UK) for 20 minutes, followed by a wash in PBS. Optimisation experiments also used PFA fixation for 30 minutes. Slides were then treated with ethanol/0.3% hydrogen peroxide for 5 minutes to eliminate endogenous peroxidase activity. Thereafter, slides were washed for 5 minutes in PBS and air-dried. Following drying, PAP moulds were drawn around each section and allowed to dry. Sections were then permeabilised for 20 minutes with PBS/0.5% Triton-X100 in a humidifying chamber followed by two washes in PBS. Non-specific binding was blocked with 10µl/ml PBS/goat serum according to instructions accompanying the Vectastain Elite ABC Kit, for 1 hour at RT. Samples were then incubated overnight at 4°C with 25µg/ml mouse anti-PLCζ antibody in PBS/goat serum, whereas negative controls were incubated in the absence of primary antibody. Sections were washed twice in PBS and incubated in 20µl/ml universal secondary antibody (biotinylated goat anti-rabbit; Vector laboratories, UK) for 1 hour, and the preformed Avidin and Biotinylated horseradish peroxidase macromolecular Complex (ABC) reagent (20µl/ml

Avidin DH solution and 20µl/ml biotinylated enzyme) was then applied for 45 minutes according to instructions accompanying the Vectastain Elite ABC Kit. Slides were thereafter washed twice in PBS and antibody binding visualised using an immunoperoxidase reaction where sections were incubated in diaminobenzidine (DAB) with nickel enhancement (Vector Elite ABC kit, Vector laboratories, UK). The sections were counterstained with haematoxylin (Sigma, UK) for 3 seconds for visualisation of nuclei and washed in distilled water. Slides were allowed to air-dry before mounting using DBX mounting media (Fisher Scientific, UK). Images were captured using a Nikon DS-Ri1 digital camera and NIS-Elements BR 3.0 software. Specificity of the anti-PLCζ rabbit polyclonal antibody was tested by pre-incubating the primary antibody with an excess of the corresponding peptides used for affinity-purification of the antibody [see Chapter 5, Table 2.2.1 and Appendix 3] as previously performed by Young *et al.* (2009).

Table 2.2.1 | Mouse PLCζ peptide sequences used for specificity testing.

Mouse PLCζ	
Peptide 1	MEMRWFLSKIQDEFRGGKI
Peptide 2	CMNKGYYRRVPLFSK

2.2.6 Statistical analysis

Correlations between the localisation and relative fluorescence intensity in each epididymal region at each age were determined using one-way ANOVA followed by Bonferroni *post hoc* testing using Prism 5.0 (Graphpad, USA) [Appendix 4]. One hundred sperm cells were analysed per region/mouse. Data are presented as the mean of 5 mice ± SEM (standard error of the mean) and a *p* value of ≤ 0.05 was considered statistically significant.

2.3 Results

2.3.1 The effect of epididymal maturation and male age upon sperm motility and concentration

CASA showed a statistically significant increase ($p<0.05$) in sperm concentration with epididymal progression [Figure 2.3.1A]. There was an increase in sperm concentration as male age increased from pubertal age (6 weeks) to sexual maturity (16 weeks), with a subsequent decline in sperm concentration as the male attained old age (32 weeks) [Figure 2.3.1B].

Coincident with increased concentration with epididymal maturity and age, there was an increase in sperm motility [Figure 2.3.2A], also determined using CASA. Progressive motility is the most important parameter of interest, representing the proportion of sperm that swim in a straight line (Simon and Lewis, 2011), considered to be the most mature and best able to fertilise. The proportion of progressive sperm increased significantly as sperm matured through epididymal progression, attaining highest levels in the cauda epididymis [Figure 2.3.2B]. There was also a significant increase in progressive motility between 6 weeks and 16 weeks of age, but a subsequent decline as male age approached 32 weeks [Figure 2.3.2B]. Immotile or static sperm constituted a high proportion of sperm in the caput epididymis across all ages [Figure 2.3.2A] signifying the immaturity of sperm at this stage of development, with an inverse correlation of motility stage as sperm mature.

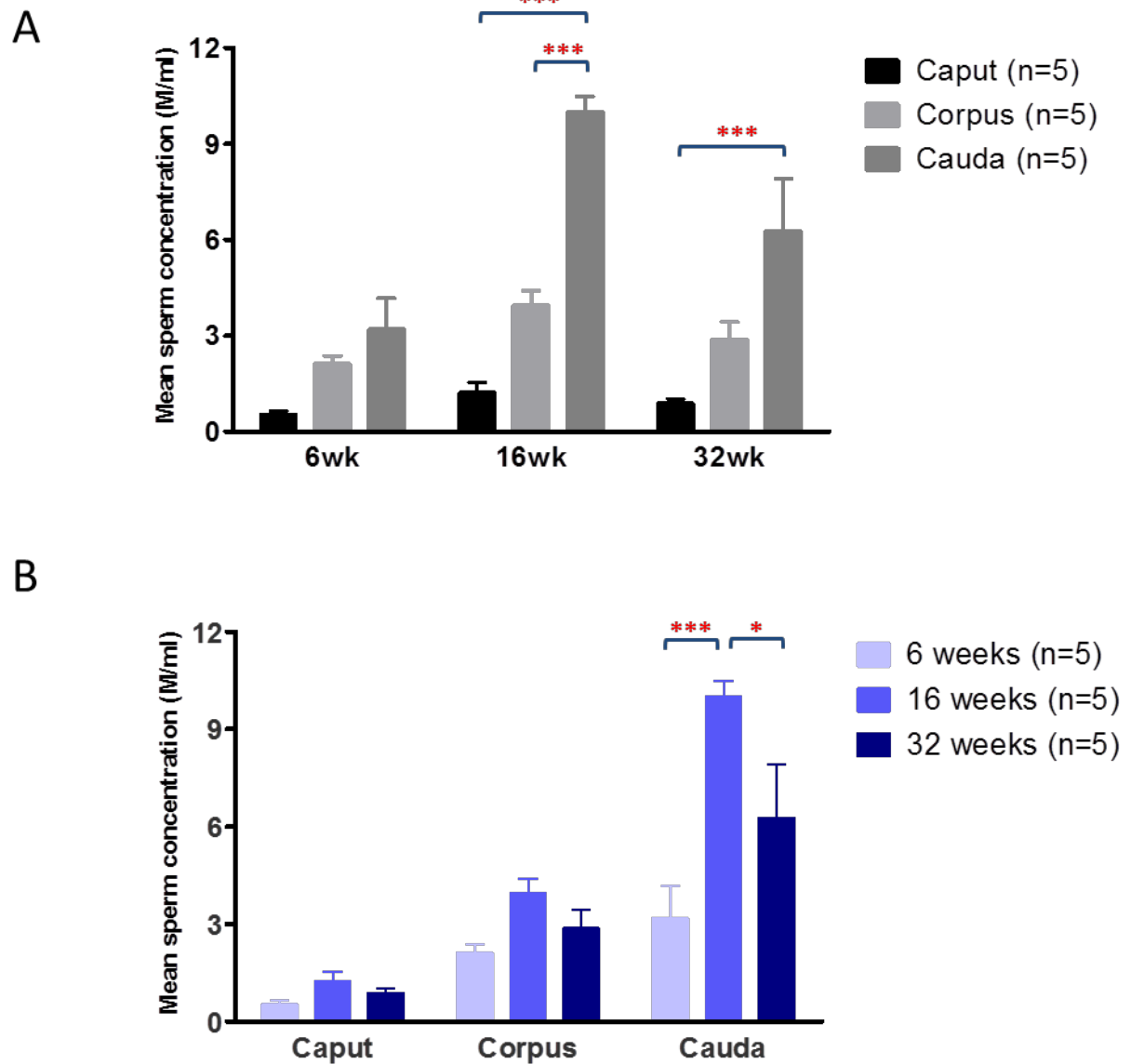


Figure 2.3.1 | Quantitative analysis of the mean sperm concentration of mouse (n=5) sperm obtained from different epididymal maturation stages at different ages. **(A)** Sperm concentration increased significantly with epididymal progression ($p < 0.05$) and **(B)** with age in the cauda epididymis there was an increase from 6 week to 16 week, followed by a significant decrease from 16 weeks to 32 weeks.

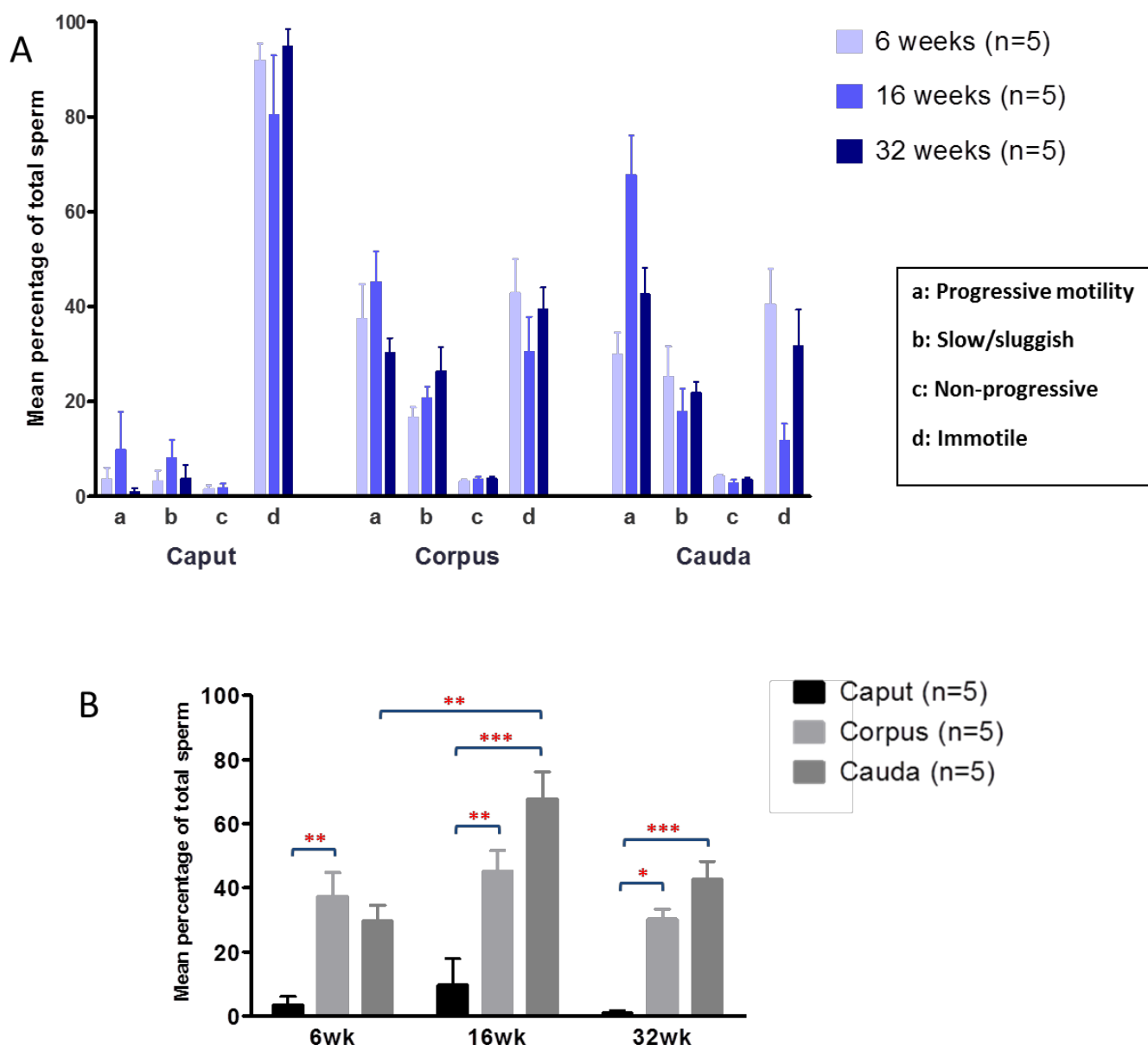


Figure 2.3.2 | Quantitative analysis of the motility of mouse (n=5) sperm obtained from different epididymal maturation stages at different ages. (A) Types of motility as assessed by the WHO criteria, and the effect of maturity and age. **(B)** The effect of maturity and age upon progressive motility, with a significant increase in progressive motility with increasing sperm maturity and increasing male age ($p < 0.05$) correlating with increased concentration observed in Figure 2.3.1A.

2.3.2 Fluorescent immunocytochemistry on swim-out mouse sperm

2.3.2.1 Acrosome integrity evaluation

Owing to an existing debate of whether the washing procedures undertaken for preparation of the samples for fluorescent immunocytochemistry had an effect on the acrosome, an assessment of acrosomal integrity was undertaken. Table 2.3.1 shows the mean percentage of intact acrosomes in each sample, and demonstrates that 76-98% of sperm retain their acrosome despite the aggressive nature of the washing procedures during preparation for immunofluorescence. Moreover, these analyses indicate that whilst the proportion of sperm with intact acrosomes remains the same in 6 week mice, in the 16 week and 32 week sperm, there was a 22% and 10% decrease respectively in the proportion of sperm with an intact acrosome as sperm moved from the caput to the cauda.

Table 2.3.1 | **Mean percentage of sperm with intact acrosomes, in 100 random sperm cells per sample in each area of epididymal sperm development, at each age analysed.** Intact acrosomes were identified by positive immunolabelling of the conjugated TRITC-PNA-lectin antibody.

Mean percentage of sperm with intact acrosomes			
	6 weeks (n=5)	16 weeks (n=5)	32 weeks (n=5)
Caput	97	98	98
Corpus	91	93	98
Cauda	94	76	88

2.3.2.2 The effect of epididymal maturation and male age upon total levels and proportions of PLC ζ

In order to ascertain the effect of epididymal maturation and male age upon total PLC ζ levels, and the proportion of sperm exhibiting PLC ζ , immunofluorescence studies were carried out on swim-out sperm from all samples. Different epididymal regions from mice of different ages were analysed by immunofluorescence and compared statistically. Findings indicated that despite fluctuations in total level of PLC ζ with epididymal progression and increasing male age, there was no statistically significant trend with either maturity or age [Figures 2.3.3 and 2.3.4]. However, there appeared to be a statistically significant increase in the total levels of PLC ζ in sexually mature 16 week mice, as sperm matured from the caput epididymis to the cauda epididymis [Figure 2.3.4B], as well as a decline in total level in the caput epididymis as mouse age increased from 6 weeks to 16 weeks [Figure 2.3.4D].

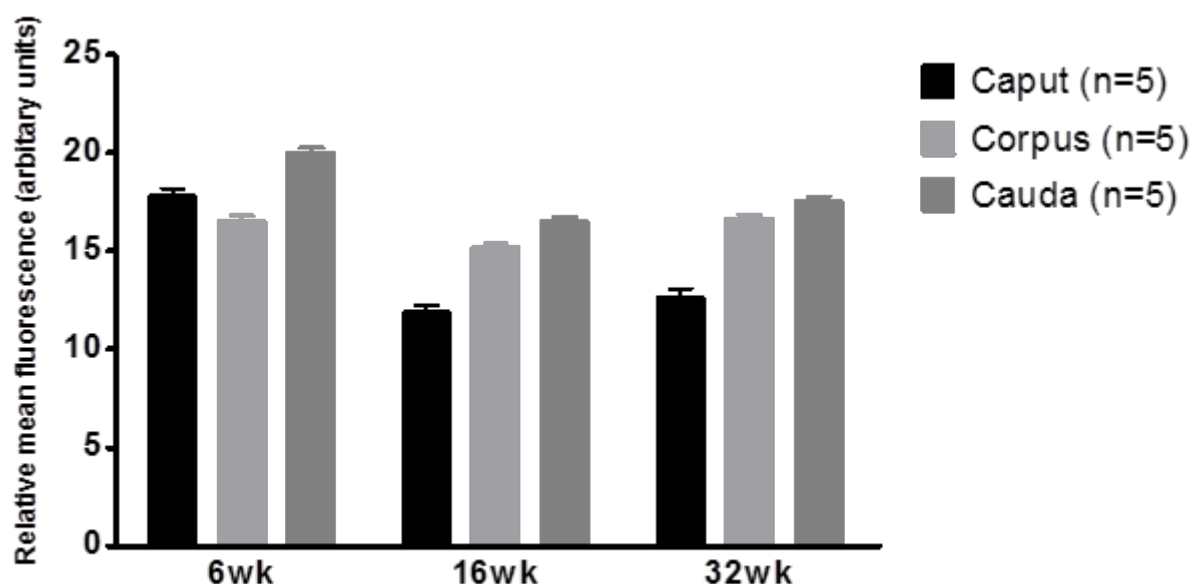


Figure 2.3.3 | Quantitative analysis of total PLC ζ in swim-out mouse sperm obtained from the different epididymal maturation stages at different ages, showing the relative mean fluorescence of 100 sperm heads per sample. Despite minor fluctuations in levels of relative fluorescence, no statistical differences in total PLC ζ levels were detected in relation to epididymal maturation or male age.

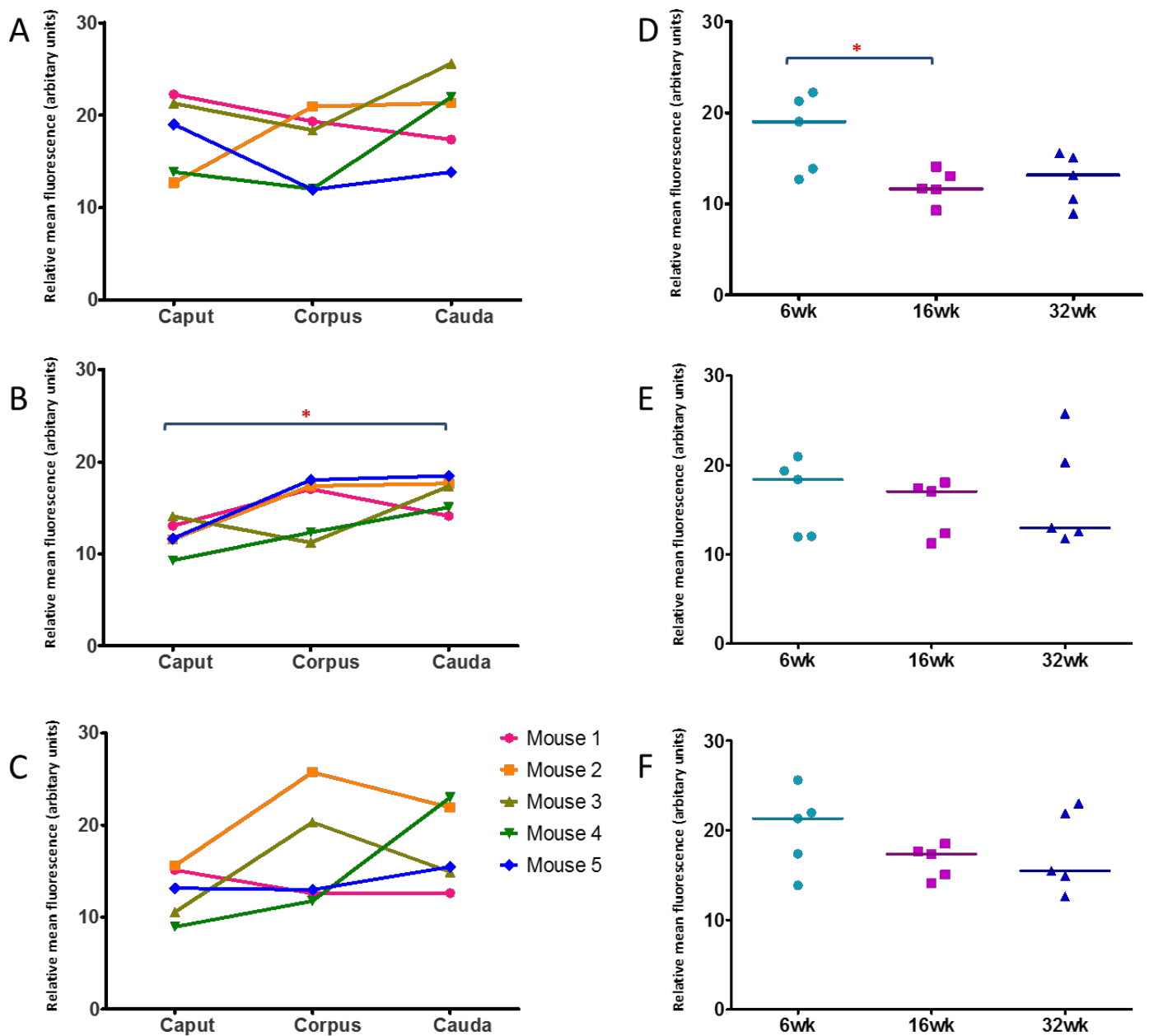


Figure 2.3.4 | Quantitative analysis of fluorescent levels in sperm from individual mice from different epididymal maturation stages at different ages. (A-C) shows the effect of maturation on the fluorescent levels in sperm at 6 weeks (A), 16 weeks (B), and 32 weeks (C); (D-F) show the effect of age on the fluorescent levels in the individual caputs (D), corpuses (E), and cauda (F).

2.3.2.2 The effect of epididymal maturation and male age upon PLC ζ localisation

Immunofluorescence studies of swim-out sperm using anti-PLC ζ antibody indicated three patterns of localisation of PLC ζ in the mouse sperm head [Figure 2.3.5], namely acrosomal alone, post-acrosomal alone, or both acrosomal and post-acrosomal populations simultaneously. PLC ζ was not detected in 0-4.8% of sperm analysed. All patterns of localisation were present in all regions of the mouse epididymi and at all ages.

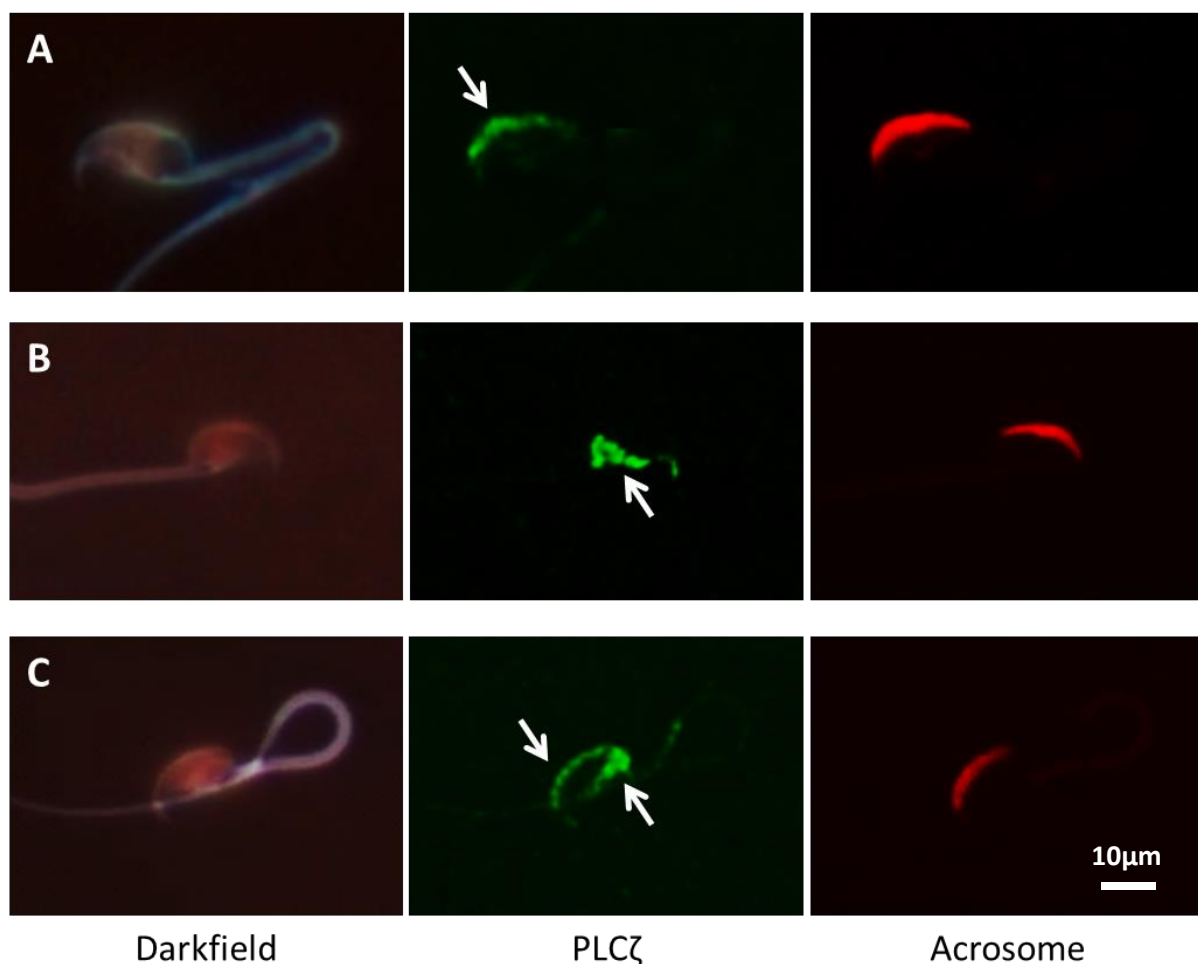


Figure 2.3.5 | **Immunofluorescent images showing the localisation patterns of PLC ζ in swim-out mouse sperm.** The anti-mouse anti-PLC ζ antibody was used to immuno-label PLC ζ in the sperm head using a FITC secondary antibody (green); and a conjugated TRITC-PNA-lectin antibody identified the acrosome in the same sperm (red). Some sperm exhibited an acrosomal PLC ζ population alone (**A**); some exhibited the post-acrosomal population alone (**B**); and others exhibited both populations simultaneously (**C**); in addition, it was noted that some sperm showed no PLC ζ expression.

Despite no significant relationship between total PLC ζ and sperm maturation and mouse age, there was a significant change ($p<0.05$) in PLC ζ localisation pattern with epididymal progression [Figure 2.3.6]. There was a significant shift ($p<0.05$) to post-acrosomal localisation as sperm matured, with caudal sperm constituting the largest proportion of post-acrosomal PLC ζ [Figure 2.3.6B and E]. This shift was from a predominant acrosomal and post-acrosomal localisation in the caput [Figure 2.3.6C and F] to a predominant post-acrosomal localisation in the cauda. The increase in the proportion of sperm that exhibited post-acrosomal PLC ζ localisation as sperm matured [Figure 2.3.6B] was correlated with a significant reduction in the number of sperm exhibiting both PLC ζ populations [Figure 2.3.6C]. Findings showed an increase in the proportion of sperm exhibiting post-acrosomal PLC ζ in the cauda epididymis as mouse aged from 6 week to 16 week, with a reduction in caudal post-acrosomal PLC ζ as mice entered old age at 32 weeks [Figure 2.3.6E]. Acrosomal PLC ζ expression did not change significantly with either age or epididymal maturity [Figures 2.3.6A and D].

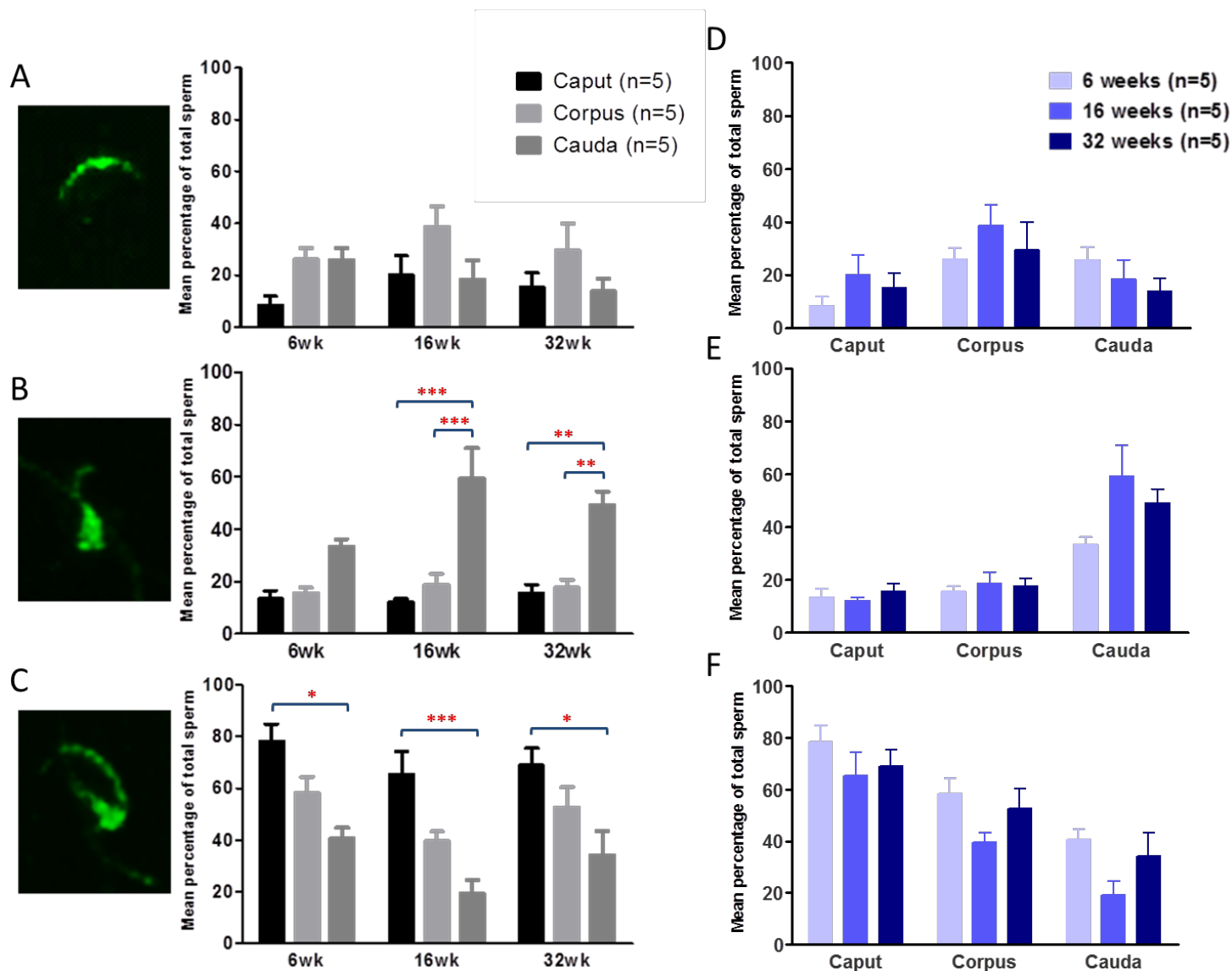


Figure 2.3.6 | Quantitative analysis of the different PLCζ immunopatterns in mouse sperm obtained from the different epididymal maturation stages at different ages. Sperm exhibited three patterns of localisation: The effect of maturity (A-C) and age (D-F) on the mean percentage of sperm exhibiting the acrosomal PLCζ population only (A and D); post-acrosomal PLCζ population only (B and E); or both acrosomal and post-acrosomal populations (C and F). In some sperm, PLCζ was not detected at all (not shown). In less mature sperm, PLCζ was localised to both acrosomal and post-acrosomal regions. With maturation, PLCζ became significantly more prominent in the post-acrosomal region ($p < 0.05$).

2.3.3 Optimisation of immunohistochemical protocol

2.3.3.1 Experiment 1: Optimisation of cutting direction

To illustrate the immunolocalisation of PLC ζ during spermatogenesis and epididymal progression, mouse testicular and epididymal tissue was sectioned and stained with anti-PLC ζ antibody. The epididymis was analysed to confirm immunofluorescence studies on epididymal swim-up sperm. Prior to such analysis, the immunohistological protocol required optimisation as shown in Figure 2.2.3.

Testicular tissue was first stained with haematoxylin to determine morphological integrity when the testis was sectioned either transversally or longitudinally. Moreover, in each direction, sections were cut from the anterior portion of the testis, the mid part and the posterior part as illustrated in Figure 2.2.3. Figure 2.3.7 illustrates representative images at various magnifications from these studies. Images indicated that both transverse and longitudinal sectioning of the testes provided good morphological information of the testicular structure with clearly evident seminiferous tubules showing sperm from all stages of sperm development. Comparison of the sections from the anterior, mid and posterior show that sections obtained from the mid-part of the testis were most suitable to visualise seminiferous tubules more precisely as the concentration of sperm in the lumen were less dense than either the proximal or distal regions, where the rete testis would lie. The identification of sperm from various stages of sperm development in a section of a seminiferous tubule is illustrated in Figure 2.3.8.

A

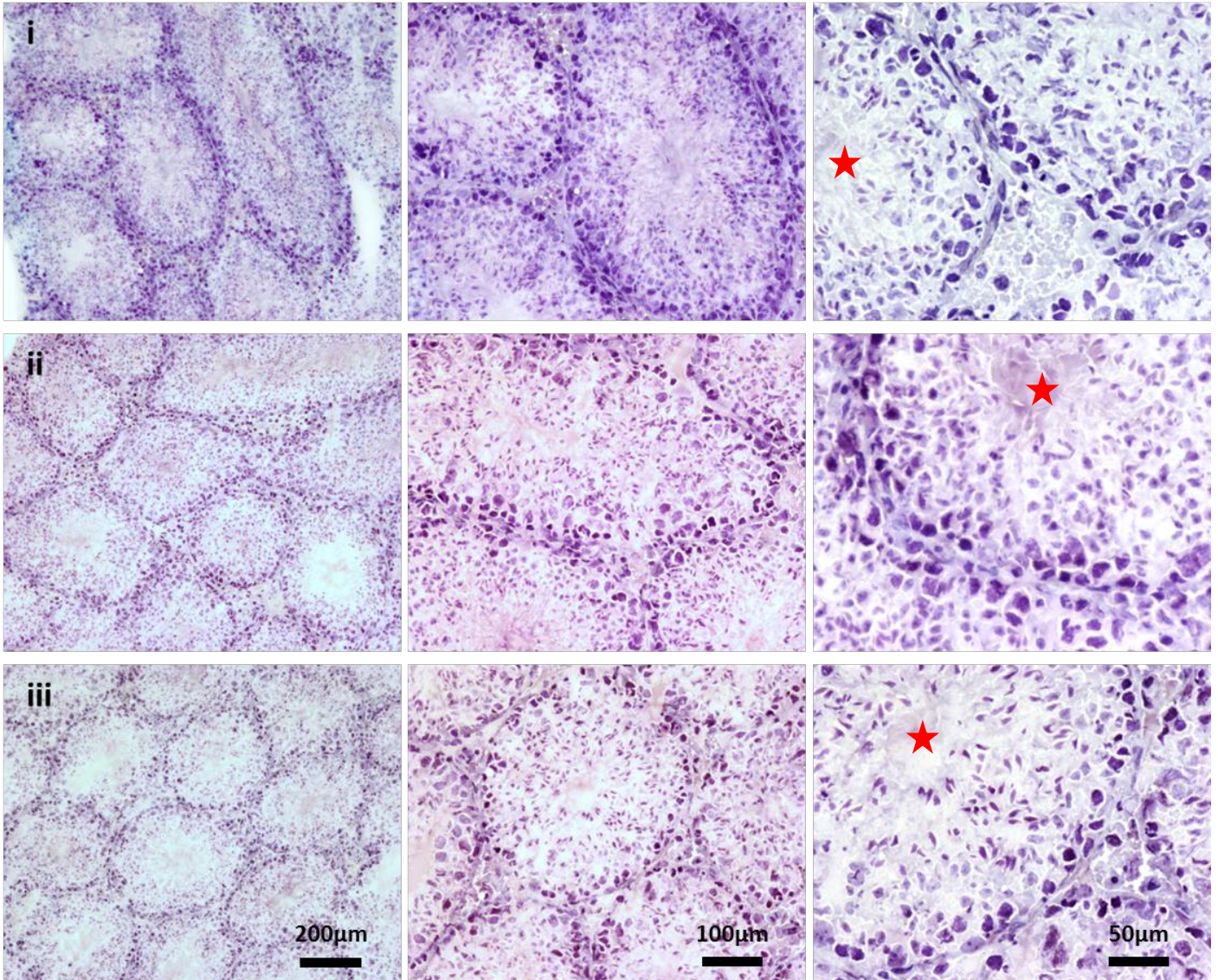


Figure 2.3.7 (A) | **Morphology of mouse testis cut transversally, compared with longitudinal sectioning (B).** 5µm sections were cut from the anterior (i), mid (ii) and posterior (iii) part of the testis, stained with haematoxylin and images captured at x10, x20 and x40 magnifications. Images show similar morphology of testis cut transversally or longitudinally, and at either anterior, mid-testis or posterior (red star denotes lumen of seminiferous tubule).

B

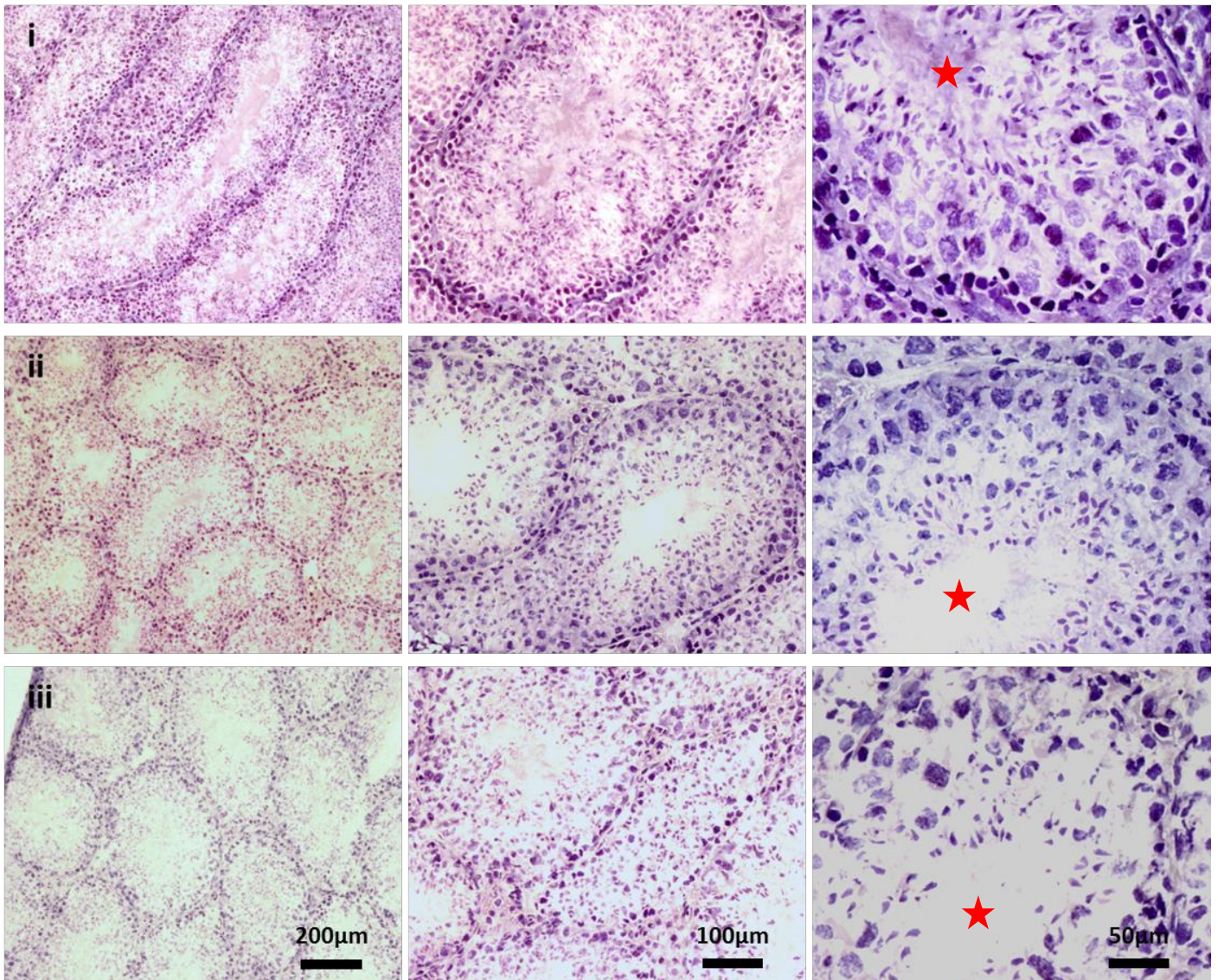


Figure 2.3.7 (B) | **Morphology of mouse testis cut longitudinally, compared with transversal sectioning (A).** 5µm sections were cut from the anterior (i), mid (ii) and posterior (iii) part of the testis, stained with haematoxylin and images captured at x10, x20 and x40 magnifications. Images show similar morphology of testis cut transversally or longitudinally, and at either anterior, mid-testis or posterior (red star denotes lumen of seminiferous tubule).

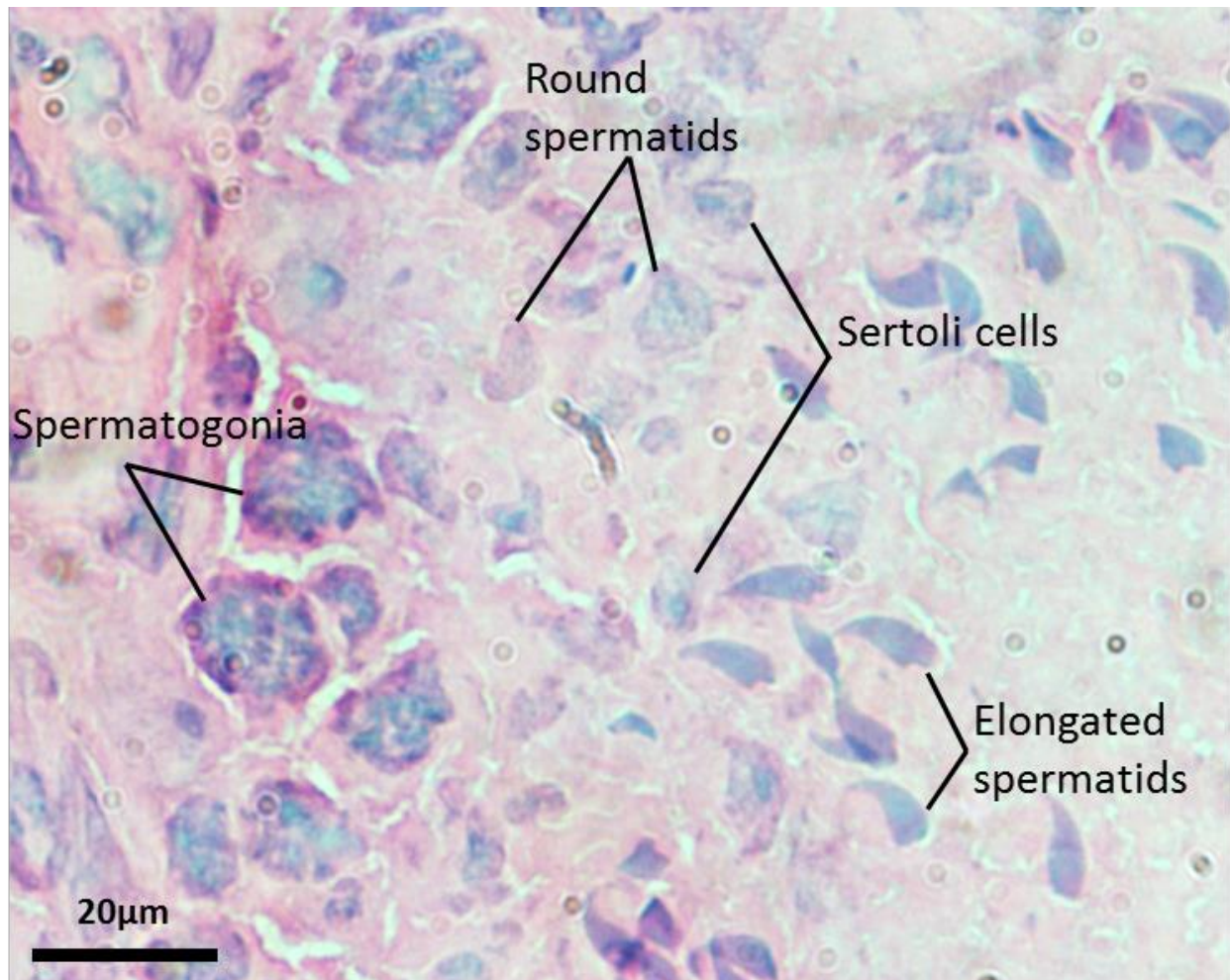


Figure 2.3.8 | Section through a seminiferous tubule of a 16 week mouse testis stained with haematoxylin (blue; nucleus) and eosin (red; cytoplasm) showing sperm at different levels of development.

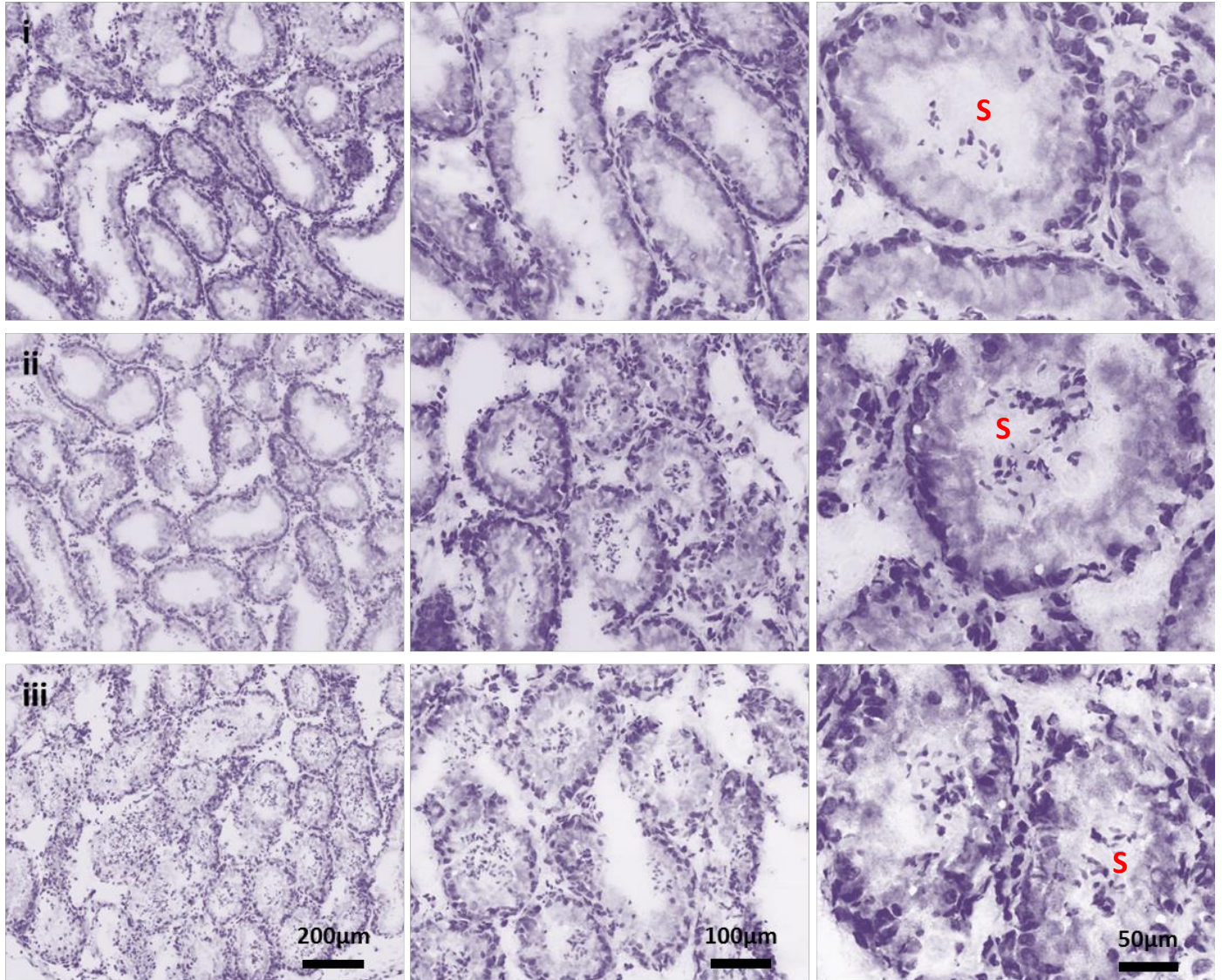
The different stages of sperm development occurring in the testis (spermatogenesis) [Figure 2.3.8] were identified using the characteristic features by which each cell type can be identified [Table 2.3.2]. Spermatogonia exhibit a characteristic large round irregular shape with chromatic granules, and are found on the basal lamina of the seminiferous tubule. Round spermatids and sertoli cells were difficult to differentiate since they were similar in shape and light in colour, but what distinguishes the sertoli cells is the dark nucleolus. Elongated spermatids were easily identified by their distinct morphology and dark nucleus.

Table 2.3.2 | **Characteristic features for identification of the different stages of sperm development in the testis.**

Sperm cell type	Features for identification
Spermatogonia	- Rounded nucleus with chromatin granules - Always in contact with basal lamina of tubule
Primary spermatocytes	- Larger than spermatogonia - Large numbers present
Secondary spermatocytes	- Smaller than primary spermatocytes - Seldom seen in histological sections (rapidly enter and complete MII)
Round spermatids	- Small; light (often eccentric) nucleus
Elongated spermatids	- Nucleus smaller and stains darker (chromatin condenses)
Sertoli cells	- Has a dark nucleolus

Directional analyses were undertaken on epididymal tissue comparing sections obtained from the anterior, mid and posterior region of the caput and cauda epididymis. It was very difficult to undertake such analyses on the corpus due to its very fragile and delicate nature. Figure 2.3.9 presents representative haematoxylin-stained sections from each region of the epididymis illustrating the morphology of each part at various magnifications. Sperm was found in only sparse concentrations in the anterior portion of the caput [Figure 2.3.9Ai], whereas the mid and posterior regions exhibited significantly more sperm [Figure 2.3.9Aii]. The cauda epididymis presented a very condensed population of sperm regardless of the portion of the tissue analysed [Figure 2.3.9C].

A



B

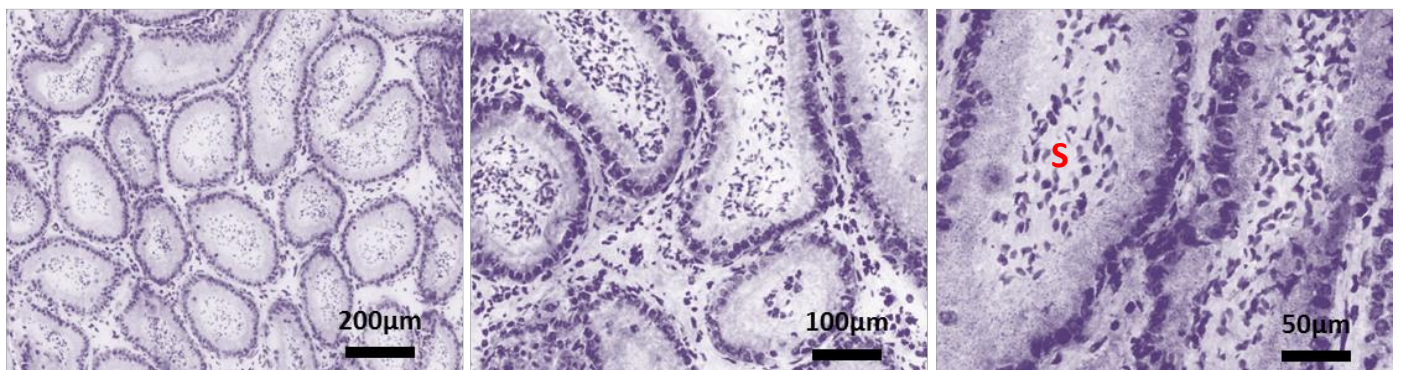


Figure 2.3.9 | For legend see page 65.

C

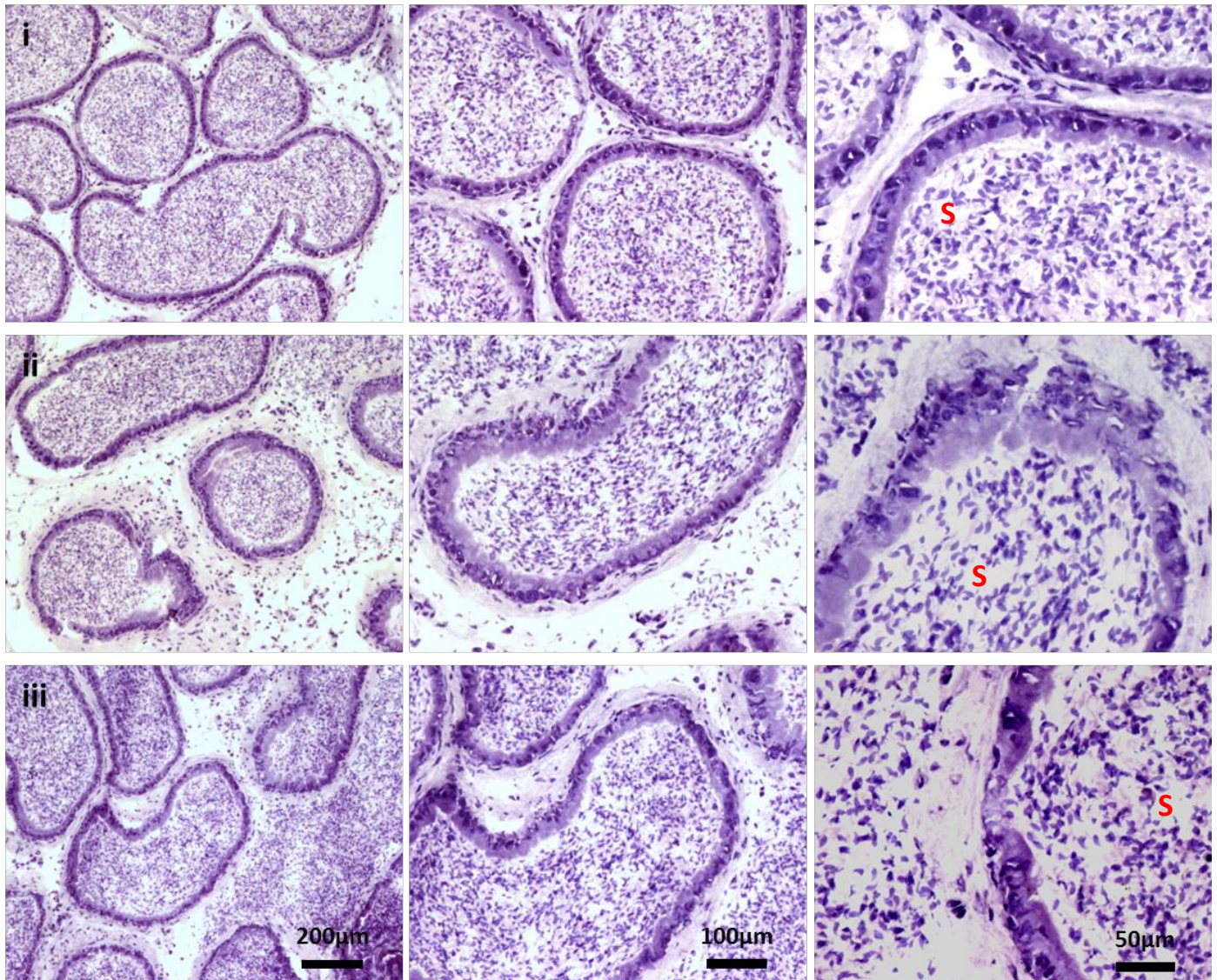


Figure 2.3.9 | **Morphological comparison of mouse epididymis cut at different levels (anterior, mid and posterior).** Embedded caput (A) and cauda (C) were sectioned at 5µm from the anterior (i), mid (ii) and posterior (iii) part of the tissue, stained with haematoxylin and images captured at x10, x20 and x40 magnifications. The corpus (B) was difficult to determine the different regions due to its delicate nature. Images show similar morphology of the caput and cauda cut at whichever level and sperm is identified clearly at all levels (S denotes sperm in the epididymal tubule). The anterior of the caput presented very little sperm so sectioning mid-posterior was postulated to present a better representation of sperm in this level of development.

2.3.3.2 Experiment 2: Optimisation of fixation and developing of sections for PLCζ analysis

Previous studies using immunohistochemistry on testicular and epididymal tissue have used a variety of different fixatives prior to immunostaining [See Appendix 5]. To ascertain which fixative was optimal for use with the anti-PLCζ antibody, caudal tissue sections fixed with Bouin's solution were compared with those fixed with PFA. Moreover, visualisation using the standard immunoperoxidase method (positive signals appearing as brown) was compared with that using additional nickel enhancement (positive signals appearing as black). Findings illustrated in representative images [Figure 2.3.10A] suggested that fixation of tissue with PFA caused considerable background noise either with or without nickel enhancement. This background was more intense in the epithelial intraluminal lining as well as sperm tails, and was even evident in negative control sections [Figure 2.3.10A].

Fixation with Bouin's solution however, appeared to exert a milder effect on tissue sections, showing very little background noise and only minimal background staining [Figure 2.3.10B]. The latter was more noticeable in negative controls where the only signal was from haematoxylin-stained nuclei. Furthermore, nickel enhancement produced better signal amplification showing clear black positive signals, compared with the lighter, more subtle, brown signal that occurred with a standard DAB chromogen without nickel enhancement [Figure 2.3.10B].

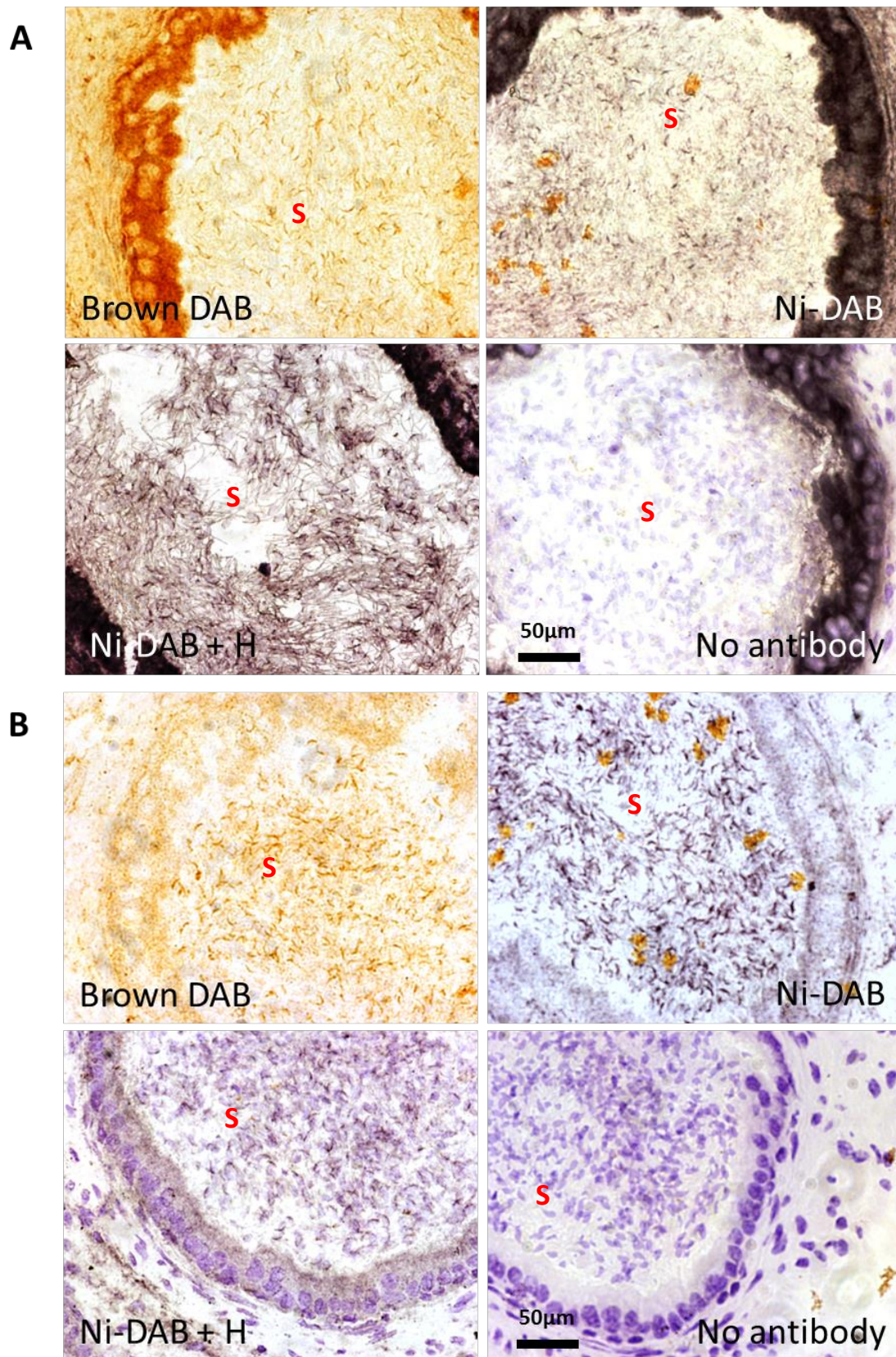


Figure 2.3.10 | Comparison of the morphology of caudal epididymis fixed with PFA (A) and Bouin's solution (B). Epididymi were stained with anti-PLC ζ antibody and developed with DAB (brown) or DAB/Ni (black). Bottom panels show counterstaining with haematoxylin (H) to visualise tissue integrity and morphology (S denotes sperm in the epididymal tubule; PLC ζ is exhibited in all sections as the brown/black positive signals).

It was necessary to determine the time of development for the DAB reaction which provided the optimal signal amplification for PLC ζ staining in the mouse reproductive tissues. Time-points, from 2 minutes to 13 minutes incubation in the DAB reaction with nickel enhancement mixture, were analysed [Figure 2.3.11] using caudal epididymal sections. Representative images captured from each time-point suggested that the signal gained in under 11 minutes of development was weak and difficult to discern, whilst the signal revealed after more than 11 minutes was very distinct, but with considerably more background noise. Therefore, a developing time of 11 minutes was chosen to be the most suitable time for development with the anti-PLC ζ antibody.

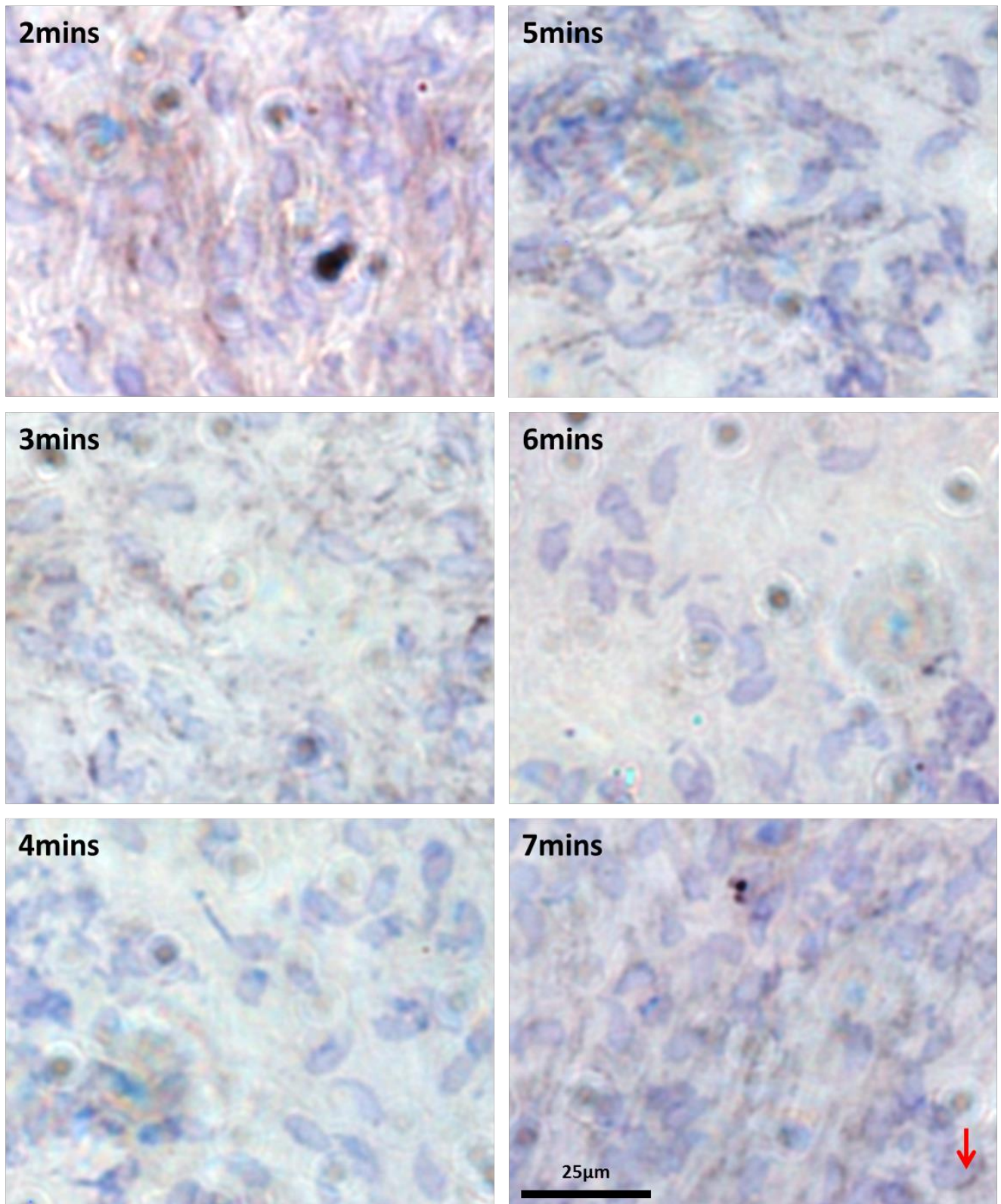


Figure 2.3.11 | Mouse caudal epididymis sections stained with anti-PLC ζ and developed with DAB/Ni. The optimal developing time was ascertained by undergoing a 2-13 minutes time series in DAB/Ni. Optimal time was determined to be 11 minutes after which the background staining increased rapidly, and before which positive staining was somewhat weaker. Sections were counter-stained with haematoxylin to stain the nuclei of the sperm heads (blue). Red arrows indicate to positive PLC ζ immunolabelling.

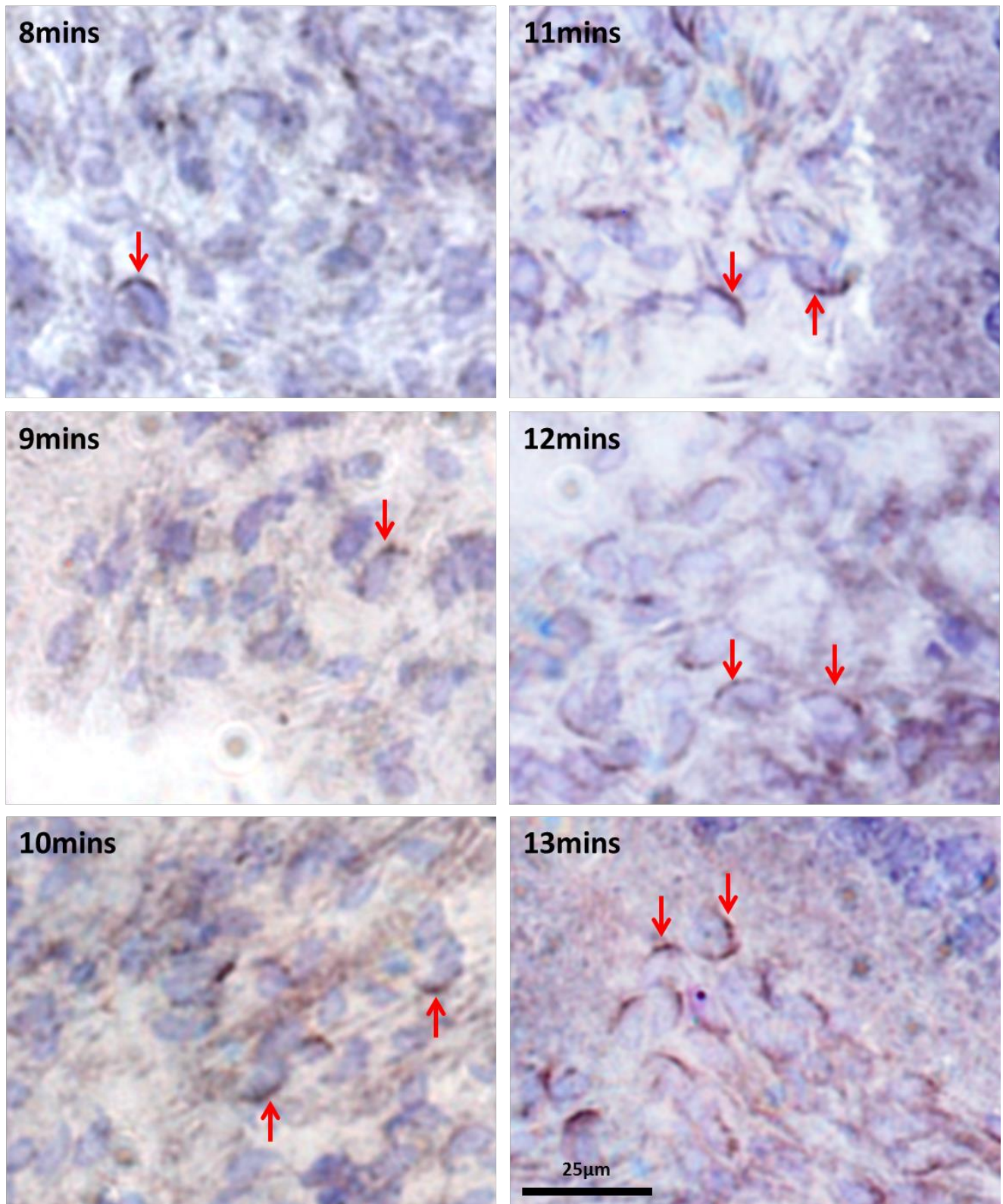


Figure 2.3.11 | Mouse caudal epididymis sections stained with anti-PLC ζ and developed with DAB/Ni. The optimal developing time was ascertained by undergoing a 2-13 minutes time series in DAB/Ni. Optimal time was determined to be 11 minutes after which the background staining increased rapidly, and before which positive staining was somewhat weaker. Sections were counter-stained with haematoxylin to stain the nuclei of the sperm heads (blue). Red arrows indicate to positive PLC ζ immunolabelling.

2.3.3.3 Experiment 3: Testing PLC ζ antibody specificity for immunohistochemistry

To determine specificity of the anti-PLC ζ antibody for immunohistochemistry, an immunising peptide blocking experiment was performed on a corpora epididymis from a 16 week mouse (middle aged mouse corpus where sperm concentration was not as dense as cauda, and not as sparse as the caput). Staining with the anti-PLC ζ antibody showed specific staining in sperm, particularly in the acrosomal region which was easily visualised. However, sections co-incubated with the peptide showed no positive staining in the sperm cells [Figure 2.3.12], correlating with immunofluorescent findings obtained by Young *et al.* (2009) [Appendix 6]. Both sections showed background staining in the epithelial intraluminal epididymal lining which was assumed to be non-specific background staining.

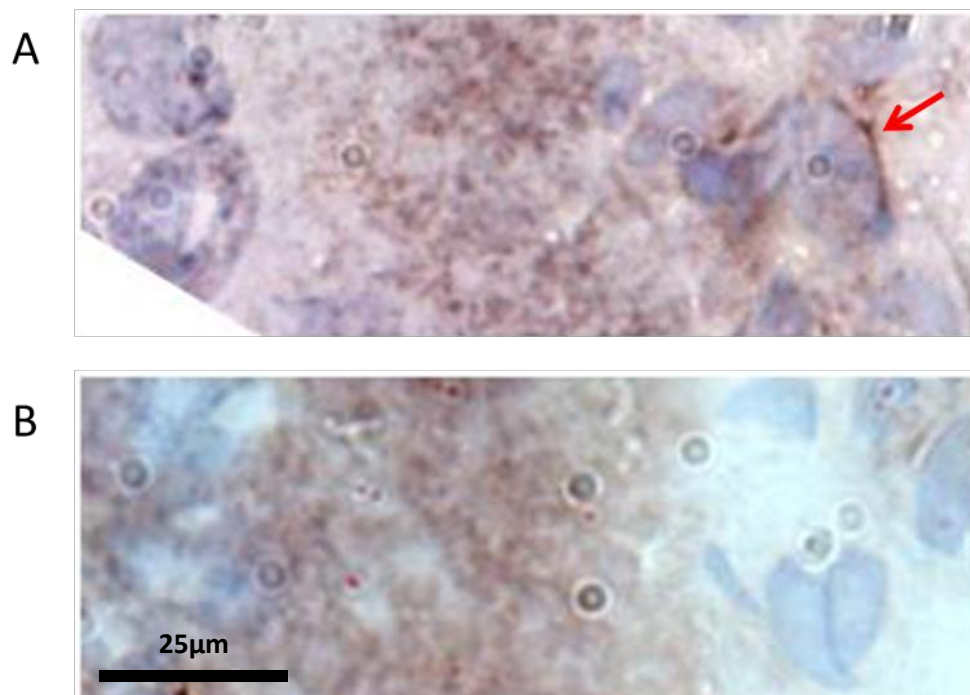


Figure 2.3.12 | Section through a 16 week corporal epididymal tubule stained with anti-PLC ζ antibody only (A) and with peptide (B). Section was counterstained with haematoxylin, staining the nuclei blue. Red arrow denotes positive PLC ζ immunolabelling.

2.3.3.4 Experiment 4: Determining PLCζ localisation patterns exhibited using the immunohistochemistry protocol

Current investigations showed a very clear and distinct staining of PLCζ in the acrosome in sperm that exhibit PLCζ in testicular and epididymal sections, however, the post-acrosomal population was very difficult to discern unlike the fluorescent immunocytochemical studies carried out on swim-out sperm. The protocol optimised herein was applied to swim-out sperm to investigate whether the immunohistochemical protocol did not allow for the detection of the post-acrosomal region. Swim-out caudal sperm were applied to poly-L-lysine coated slides as with immunocytochemistry, and the same protocol used for immunohistochemical sections employed. Findings show as with the histological sections, very strong acrosomal staining, but only faint staining in the post-acrosomal region [Figure 2.3.13].

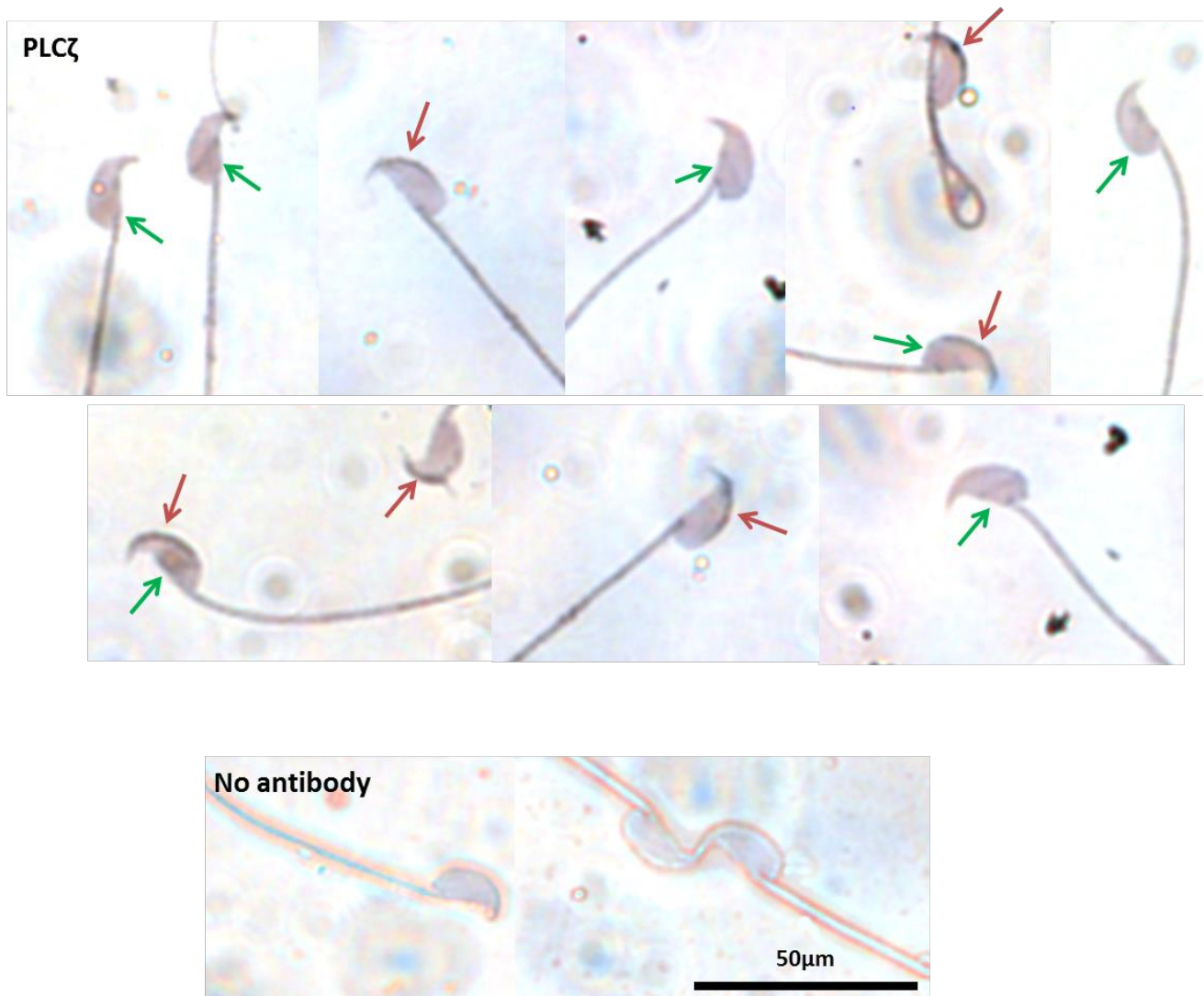


Figure 2.3.13 | **Swim-out caudal sperm from a 16 week mouse stained with anti-PLC ζ antibody and treated with DAB/Ni.** Sperm was treated in the same manner as the immunohistochemical sections described in Section 2.2.5. Sperm was counterstained with haematoxylin for nuclei visualisation. Acrosomal localisations (red arrows) were very strongly stained, compared with post-acrosomal populations (green arrows) which were far less distinct.

2.3.4 The localisation of PLC ζ in testicular tissue and the effect of age

In order to investigate the developmental expression of PLC ζ during mammalian spermatogenesis, sections from testicular tissue of mice (n=3) of the three ages (6, 16 and 32 weeks) were immunostained with anti-PLC ζ antibody. The latter was undertaken in triplicate and a fourth section for each sample was incubated in the antibody diluent alone as a negative control. Sections were visualised to determine the presence or absence of PLC ζ and elucidate the specific localisation pattern of such staining. Figure 2.3.14 shows representative images of areas that showed positive staining.

PLC ζ was exhibited at all ages, however, in the 6 week testis, PLC ζ immunostaining was very sparse. Positive PLC ζ expression was exhibited in the round spermatid and elongated spermatid stages of sperm development [Table 2.3.3]. Specific localisation was observed in both the acrosomal and/or post-acrosomal regions. Many of the sperm examined in the 16 week and 32 weeks sections showed positive immunostaining. The acrosomal PLC ζ localisation was always more evident, but closer inspection and higher magnification visualisation revealed the presence of PLC ζ in the post-acrosome of some sperm.

PLCζ

No antibody

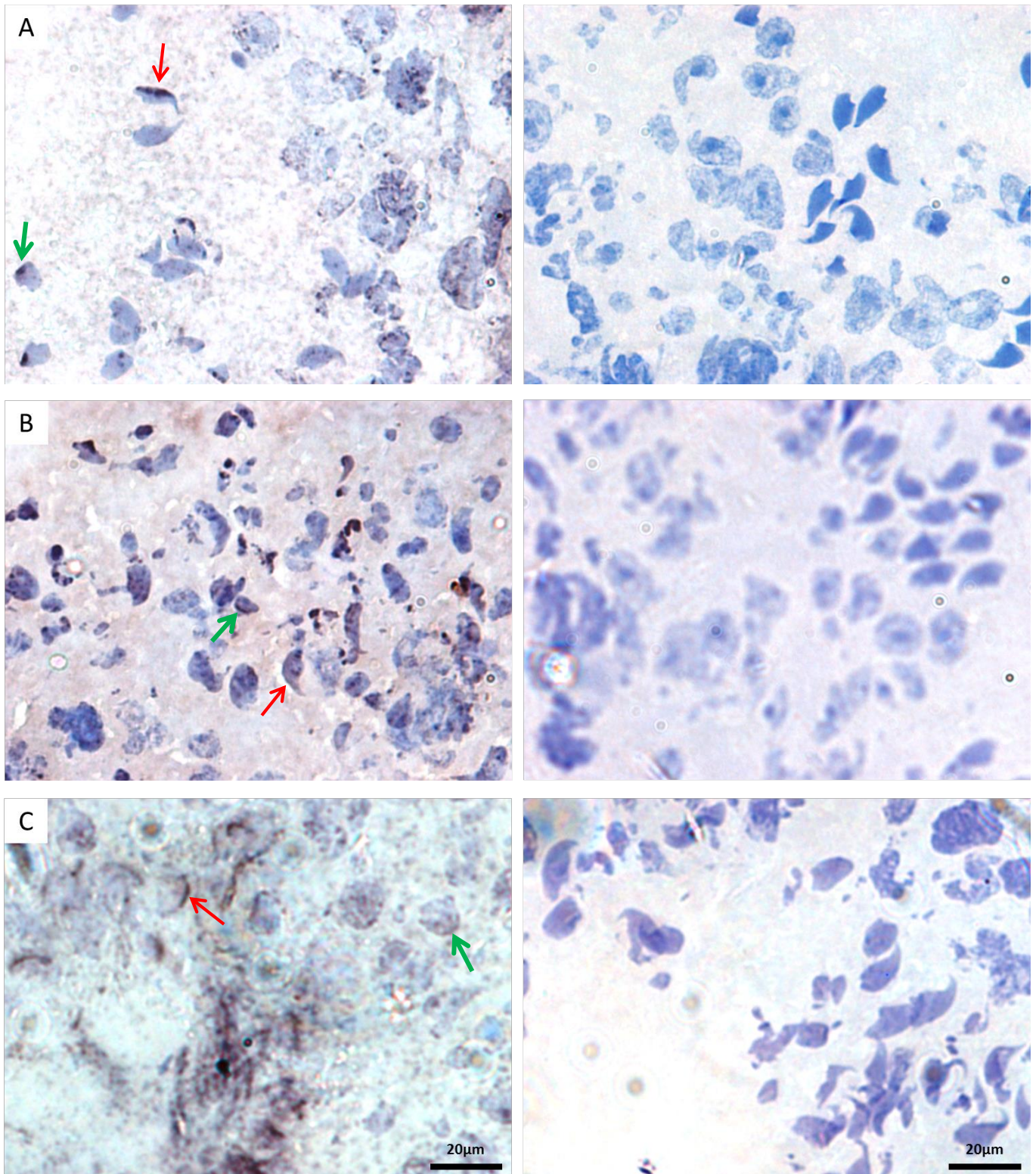


Figure 2.3.14 | Testicular mouse tissue sections showing immunolocalisation of PLCζ compared with negative controls. 5μm sections of testicular tissues from mice aged **(A)** 6 weeks, **(B)** 16 weeks, and **(C)** 32 weeks, were stained with anti-PLCζ antibody to show the localisation of PLCζ at these ages (Red arrows highlight examples of PLCζ in elongated spermatids; Green arrows highlight examples of PLCζ in round spermatids). Sections were counter-stained with haematoxylin staining the nuclei (blue).

Table 2.3.3 | **Summary of PLCζ staining in stages of mouse spermatogenesis** (neg: no staining detected, +++/- : predominantly positive staining, some cells show no staining, +++ : predominantly positive staining).

Cell type	PLCζ staining
Spermatogonia	neg
Spermatocyte	neg
Round spermatid	+++/-
Elongated spermatid	+++

2.3.5 The effect of epididymal maturation and male age upon PLCζ localisation in epididymal tissue

Qualitative immunohistochemical studies of epididymis supported immunofluorescence studies indicating the presence of PLCζ at all stages of sperm maturity and at all ages. Findings suggest that very few sperm in the 6 week mouse expressed PLCζ [Figure 2.3.15A] particularly in the caput and corpus, with more positive staining identified in the cauda. In the epididymis from the older mice (16 week and 32 weeks), there was considerably more sperm exhibiting PLCζ, particularly in the cauda which was highly populated and clearly showed a significant proportion of sperm exhibiting PLCζ. Through these qualitative studies, there appeared to be a clear relationship between the proportion of sperm exhibiting PLCζ and age, and with epididymal progression.

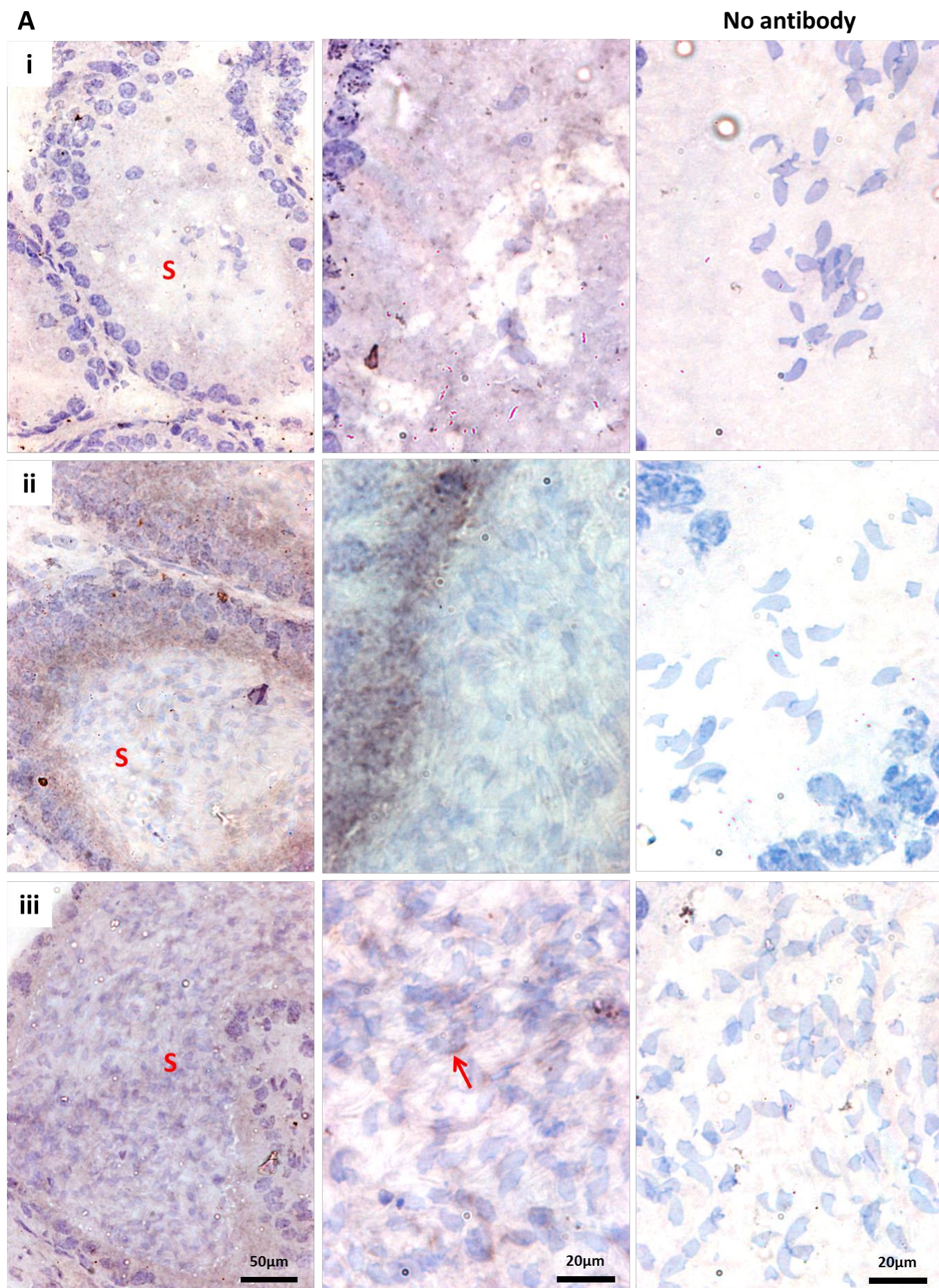


Figure 2.3.15 | For legend see page 80.

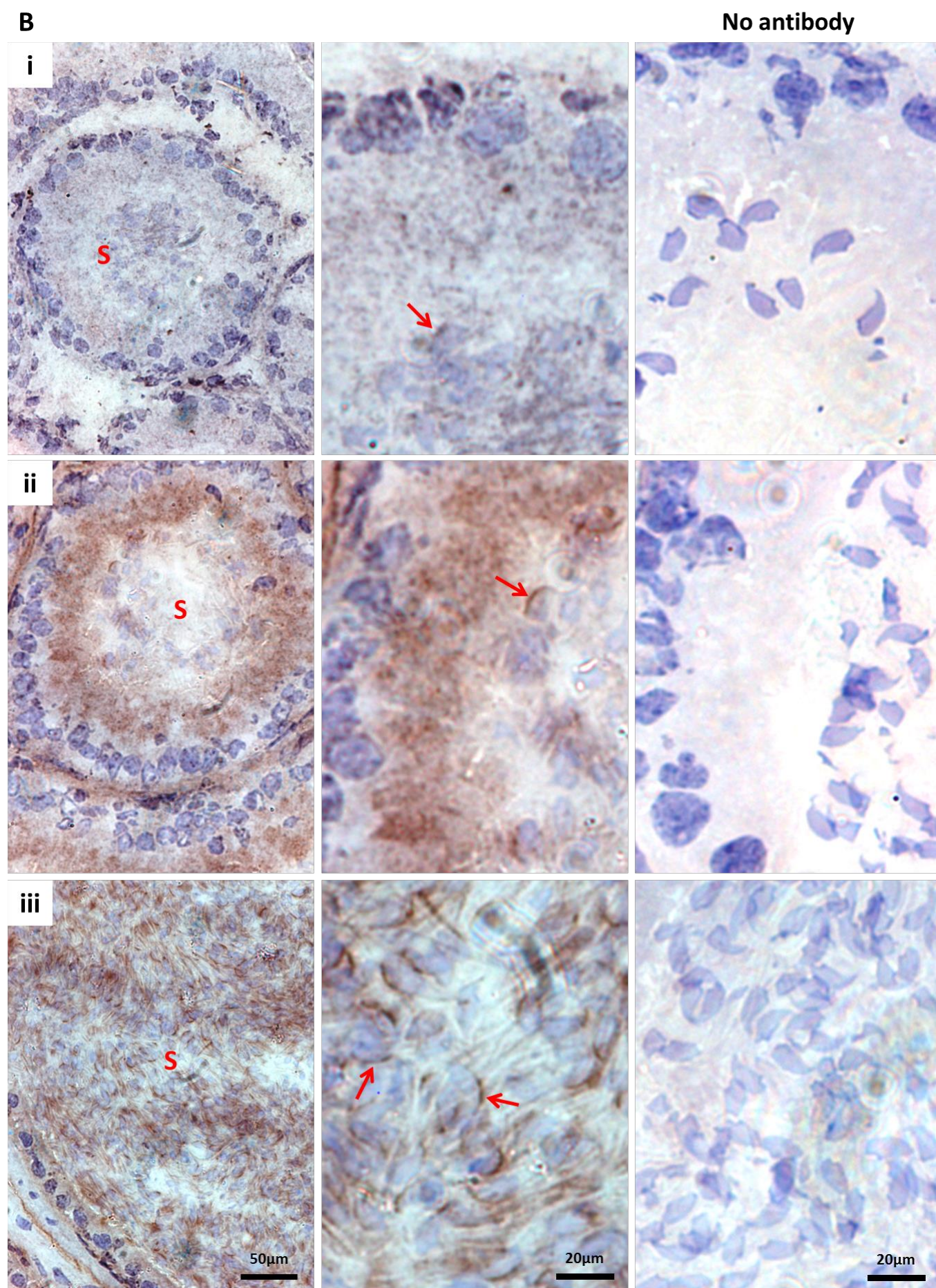


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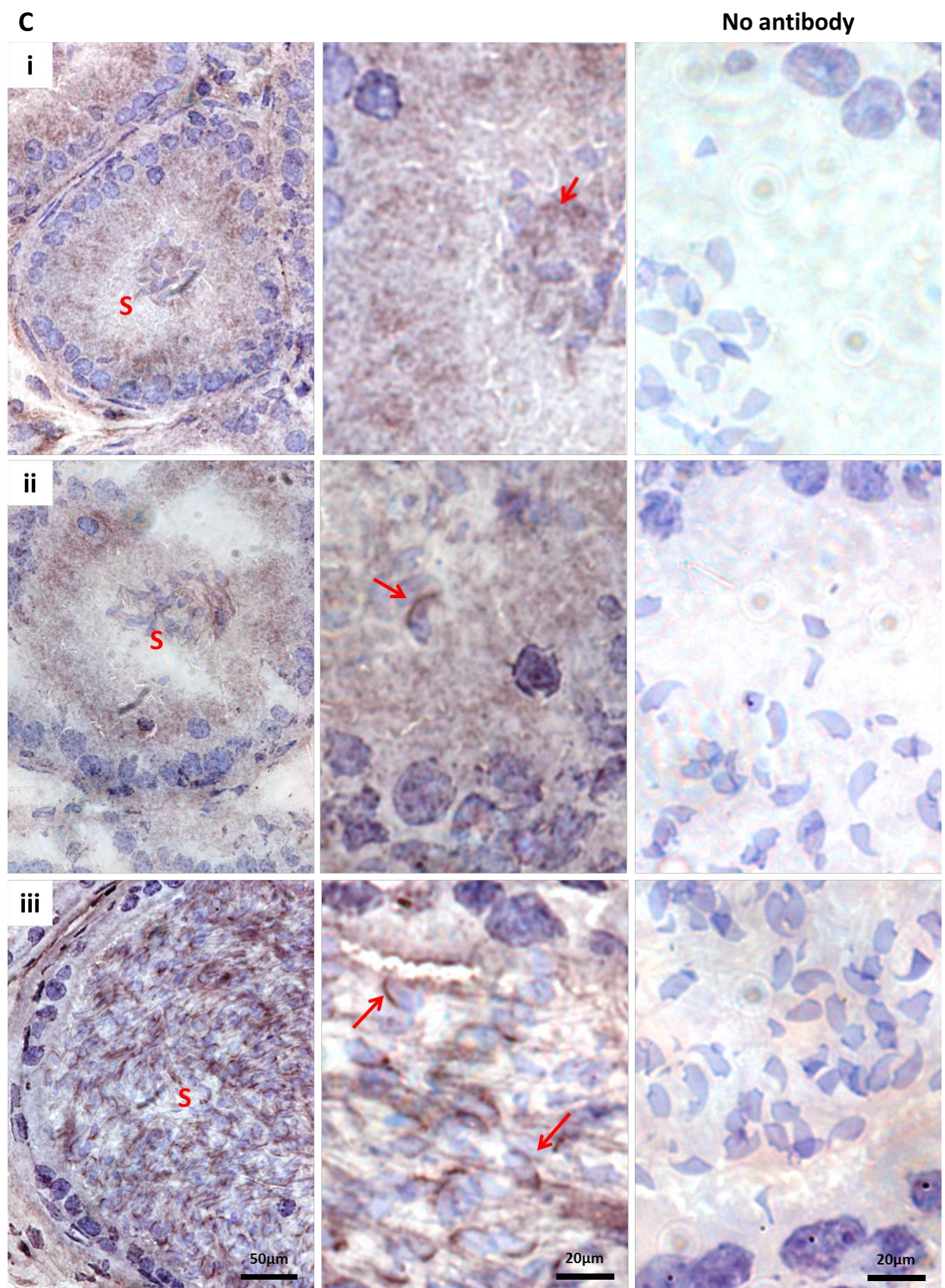


Figure 2.3.15 | For legend see page 80.

Figure 2.3.15 | Representative images showing the immunolocalisation of PLCζ in mouse epididymal tissue, counter-stained with haematoxylin. 5μm sections of epididymal tissues from mice aged **(A)** 6 weeks, **(B)** 16 weeks, and **(C)** 32 weeks, from the caput (i), corpus (ii) and cauda (iii), were stained with anti-PLCζ antibody to show the localisation of PLCζ at these ages and stages of sperm maturation. A negative control in which the section was not incubated in the antibody was produced for each tissue. Images were captured at x40 and x100 magnification (scale bars as shown). Red arrows indicate examples of PLCζ immunostaining (**S** denotes sperm in the epididymal tubule lumen).

2.4 Discussion

Previous work has established that the ability of sperm to activate oocytes depends on the expression and localisation of the sperm factor PLC ζ . It has been established that mouse sperm possess two populations of PLC ζ , namely in the acrosome and in the post-acrosome (Fujimoto *et al.*, 2004; Young *et al.*, 2009). Moreover, it has been suggested that these populations may play differential functional roles in the process of fertilisation (Young *et al.*, 2009) though evidential data to support this are currently lacking. Such findings would increase our knowledge of the mechanism of oocyte activation and further our understanding of male-factor infertility and why some sperm fail to activate an oocyte, a problem that has now been classified as a disease by the WHO (Zegers-Hochschild *et al.*, 2009).

Sperm mature in their transit from the caput to the cauda, a process which takes 10-13 days in rodents, and 2-6 days in humans (Amann and Howards, 1980; Cornwall, 2009), transforming immature immotile sperm cells into mature cells which have acquired the function of progressive motility and the ability to capacitate and fertilise an oocyte. They do this by undergoing various distinct morphological, biophysical and biochemical changes, including changes in protein composition and modifications which may be important for the acquisition of motility as sperm mature (Baker *et al.*, 2005; Aitken *et al.*, 2007; Cornwall, 2009; Leahy and Gadella, 2011).

The present study investigates for the first time the effect of the maturation process through epididymal transit, as well as male age, upon sperm concentration and motility in a mouse model. It also investigates firstly, whether a relationship exists between such factors

and the total PLC ζ levels in the sperm head, and secondly, whether the precise localisation of PLC ζ was affected. Through this, this study aimed to address previous suggestions of the possible functional roles of the different populations of PLC ζ . Moreover, this study aimed to explore for the first time the developmental expression and localisation of PLC ζ during spermatogenesis in the testis, and ascertain whether age influenced these mechanisms.

Ascertaining the stages of sperm maturation and at what stage they acquire the physiological state that would be most suited for their role in oocyte activation and fertilisation is important not only for the understanding of the mechanisms underlying sperm maturity and development, but also, to optimise ART strategies, such as ICSI using testicular- or epididymal-derived sperm. Previous studies have analysed the mRNA expression profile of mouse, hamster and pig PLC ζ during spermatogenesis and found it is first expressed in the round spermatid stage and most likely translated in elongated spermatids (Yoneda *et al.*, 2006; Young *et al.*, 2009). However, no study has investigated the protein expression during spermatogenesis and sperm maturation thus far.

2.4.1 There is a significant increase in sperm concentration and progressive motility with epididymal progression

Current findings indicate a significant increase in sperm concentration with epididymal progression. Similar increases were also seen in the proportion of progressive sperm with epididymal maturation, where sperm was mostly progressive in the cauda, and relatively immotile in the caput. These increases were particularly significant in post-pubescent mice. It is also worth noting that sperm in the cauda epididymis of 16 week mice

were at the highest concentration across all ages studied, with the highest proportion of progressively motile sperm. This age lies during the period of highest fertility in male mice, when they are most sexually active.

2.4.2 Total levels of PLC ζ do not change either with age or with epididymal progression, but a change was evident in the predominant localisation pattern

The present study identified two discrete populations of PLC ζ in mouse sperm as observed previously (Fujimoto *et al.*, 2004; Young *et al.*, 2009). Both Fujimoto *et al.* (2004) and Young *et al.* (2009) suggested differential roles for the two different populations of PLC ζ . Whereas the former suggested that the peripheral population was responsible for oocyte activation and the post-acrosomal population responsible for the modulation of pronuclear activity (Fujimoto *et al.*, 2004; Larman *et al.*, 2004; Yoda *et al.*, 2004); Young *et al.* (2009) suggested that the peripheral population, associated with the acrosomal region, may be associated with the acrosome reaction, while the post-acrosomal PLC ζ population was responsible for oocyte activation. The present study extended these previous immunolocalisation studies by investigating how these localisation patterns might change with epididymal maturation and male age, as well as total PLC ζ expression in the sperm head.

Quantitative fluorescence analysis indicated that the total level of PLC ζ in the sperm head did not change significantly, either with male age or with epididymal maturation. However, there was a significant increase in PLC ζ levels in 16 week mice as sperm developed maturity, between the caput and cauda epididymis. It is indeed plausible that the

differential populations are involved in distinct roles, but unlike previous work, the findings of this study suggest that the acrosomal PLC ζ population is unlikely to play a functional role in the process of fertilisation. It was suggested by Young *et al.* (2009) that the acrosomal PLC ζ population may play a role in the acrosome reaction as this population disappeared following induced *in-vitro* capacitation. These findings were further supported by Bi *et al.* (2009) who discovered a truncated form of PLC ζ localised in the acrosome, and the authors propose a role for this population in antagonising the PLC activity of PLC ζ .

The findings herein show that on average, only 43% of sperm reaching full maturity in sexually-mature mice exhibited the acrosomal PLC ζ population. It would be expected that if this population played a key role in fertilisation, then the majority of sperm reaching full maturity would possess acrosomal PLC ζ . Moreover, the present localisation studies failed to detect changes in the proportion of sperm exhibiting acrosomal PLC ζ with either maturity or male age. On the other hand, on average, 81% of sperm reaching full maturity in sexually mature mice exhibited the post-acrosomal PLC ζ population making it highly probable that this particular population plays the predominant functional role at fertilisation.

2.4.3 The post-acrosomal population of PLC ζ appears to be responsible for oocyte activation

It was proposed that a sperm factor responsible for oocyte activation would be localised in a region of the sperm that would allow direct and rapid release into the oocyte following gamete fusion (Lawrence *et al.*, 1997; Sutovsky *et al.*, 2003). The finding of predominantly post-acrosomal PLC ζ in caudal mouse sperm therefore represents an

appropriate distribution for the putative endogenous Ca^{2+} oscillation-inducing factor since the contents of this region have swift access to the ooplasm following sperm-oocyte fusion (Mandahar and Toshimori, 2003; Sutovsky *et al.*, 2003). Indeed, a post-acrosomal PLC ζ population has been identified not only in mouse sperm, but also in human, hamster, bull, pig and boar sperm (Fujimoto *et al.*, 2004; Yoon and Fissore, 2007; Grasa *et al.*, 2008; Young *et al.*, 2009; Kaewmala *et al.*, 2011; Nakai *et al.*, 2011). The direct involvement of the post-acrosomal PLC ζ population in oocyte activation however has yet to be confirmed. Studies involving the micro-dissection of the post-acrosome and subsequent injection into oocytes may consolidate this prediction should oocyte activation occur.

The acrosomal population may indeed play a functional role, but a role in the acrosome reaction appears to be highly doubtful. It is unlikely that the shift to a predominant post-acrosomal localisation pattern with epididymal maturation is due to the physical loss of acrosomes or unintentional experimentally-induced *in-vitro* acrosome reaction, since double-labelling with a conjugated acrosomal marker, PNA, indicated that the majority (76-88%) of acrosomes remain intact following analysis, thus further work is required to investigate what the function of the acrosomal population of PLC ζ may be.

2.4.4 PLC ζ may translocate in the sperm head with epididymal progression

This study has found, for the first time that despite there being a change in the localisation pattern of PLC ζ as sperm mature, the actual total level of PLC ζ in the sperm head remains relatively stable. This suggests that the PLC ζ protein within the sperm head does not undergo further translation with epididymal maturation, in line with the concept of

sperm being translationally inert (Baker *et al.*, 2005; Galeraud-Denis *et al.*, 2007), but does appear to suggest that the PLC ζ protein may translocate within the sperm head to position itself in the location most apt for its subsequent role at its most mature state, that of oocyte activation.

As sperm pass through the epididymis, they encounter sequential changes in the composition of luminal fluid created by the epididymis (Gatti *et al.*, 2005; Guo *et al.*, 2007). Through this microenvironment in the lumen, where interactions between epithelial cells and sperm take place via the fluid they interact with, they are altered by this immediate environment, undergoing a myriad of encounters that enable them to become competent for fertilisation success (Chan and Zhang, 2005; Leahy and Gadella, 2011). It is indeed plausible to suggest that the observed changes in the localisation of PLC ζ with sperm maturity may be instigated by post-translational modifications (PTM) occurring within the sperm induced by such extracellular encounters in the epididymis. Indeed there are many examples in the literature of sperm proteins undergoing PTM during transit through the epididymis. Baker *et al.* (2005, 2012a) have shown examples of PTM of several proteins, such as Izumo1, a sperm-egg fusion protein, and a mitochondrial enzyme, F1-ATPase, which are both phosphorylated as they undergo epididymal maturation (Baker *et al.*, 2005; 2012a). Furthermore, Guo *et al.* (2007) demonstrated the upregulation of sperm proteins as sperm traverse the epididymis to be expressed more in the cauda than the caput. Since there are many such examples, it would be conceivable to advocate that sperm undergo morphological, biochemical, and physiological modifications (Guo *et al.*, 2007; Baker *et al.*, 2012b) in their journey of acquiring the characteristics required for fertilisation, and that PTM drives this unique cell into a state of functional maturity (Baker *et al.*, 2012ab).

Moreover, a model has been suggested by Baker *et al.* (2012a) suggesting that such PTMs are likely to act as a scaffold for the association of adaptor proteins with the modified proteins in preparation for fertilisation. Further work is thus required in order to determine the precise PTMs that occur with PLC ζ during their transit through the epididymis in order to acquire fertilisation potential.

2.4.5 Variability in semen parameters and total PLC ζ was observed between mice of the same age and epididymal maturation

It is important to note that although only five mice were analysed, one hundred sperm cells of each region and at each age group were assessed, so localisation and expression studies were carried out on 500 sperm cells per region/age. However, individual mouse results indicate notable variability between mice of the same age in all measured parameters of sperm obtained from epididymal regions (i.e., concentration, motility, fluorescence intensity). Reasons for this may be due to natural variability between mice, or experimental judgements, as analysis of the localisation patterns involved the objective assessment of the localisation of fluorescence in the sperm head. Physiologically, such variations may be due to the time-span between the sacrifice of the mouse and the last time of mating, hence previous ejaculation. Previous studies showed that the frequency of semen-collection in boars influenced the quality of sperm in boar ejaculates (Pruneda *et al.*, 2005). These authors found that a high frequency of semen collection led to altered sperm concentrations, brought about by an altered resorption and secretion pattern of epididymal fluid, an increased frequency of defective sperm maturation and abnormal development of sperm motility (Pruneda *et al.*, 2005), which may in turn also affect the expression and

localisation of PLC ζ . It would be conceivable therefore to suggest that the sperm from a mouse which had recently mated would not be equivalent to sperm from a mouse which had not mated for a period of time. Future studies may aim to eliminate this variability by keeping this abstinence time constant in all subjects.

Another reason for this variability may be the precise accuracy in dissecting the different epididymal regions since epididymal regions are not discrete. Overlap may have easily occurred when the regions were separated, so swim-up sperm from the caput may be contaminated by sperm from the corpus, corpus sperm with caput and caudal sperm, and caudal sperm with sperm from the corpus. Future work may aim to cut smaller pieces of tissue, removing the inter-regional tissue to prevent potential contamination. Alternatively, the epididymis may be separated into more regions for analysis, incorporating those inter-regional regions. Indeed the mouse epididymis has been previously divided into seven epididymal zones by Takano (1980; Aitken *et al.*, 2007).

2.4.6 Developmental expression of PLC ζ begins at the round-spermatid stage of development and continues until full maturation

Determining the stage of development at which PLC ζ is first expressed is important for understanding its development and the mechanisms involved. This study aimed to investigate this, and for the first time, considered the developmental expression and localisation of PLC ζ protein during spermatogenesis in the testis and during epididymal maturation, and investigated the potential effect of age on localisation and expression. PLC ζ was exhibited from as early as the round spermatid stage of spermatogenesis, which

represent the most immature haploid male germ cells at which the chromosomal and genomic changes required for male gamete function have been completed (Ogura *et al.*, 2005). A recent study by Aarabi *et al.* (2012) also identified the earliest expression of PLC ζ during mouse spermatogenesis to be in the round spermatid stage. Furthermore, a recent study looking at PLC ζ in equine spermatogenesis identified the round spermatid stage to be the stage of earliest expression of PLC ζ (Bedford-Guaus *et al.*, 2011) and these round spermatids were found to be enzymatically active in activating oocytes when injected into mouse oocytes (Bedford-Guaus *et al.*, 2011). Moreover, human and hamster-derived round spermatids are also capable to causing Ca²⁺ oscillations, thus oocyte activation, when injected into mouse oocytes (Kimura and Yanagimachi, 1995; Yazawa *et al.*, 2001). However, unlike Aarabi *et al.* (2012), who state that little or no immunostaining was detected in elongated spermatids, this study identified substantial immunolabelling in elongated spermatids, particularly in the acrosome and in post-pubescent mice. This is clearly evident in the representative images shown herein. An age-related relationship appears to be present in the developmental expression of PLC ζ with the 6 week pubescent mice exhibiting little or no PLC ζ during spermatogenesis, but in the 16 week and 32 week testicular tissue, findings herein show this expression very clearly.

Furthermore, to consolidate findings attained from the immunofluorescence studies, immunohistochemical analysis of the epididymis was undertaken. Findings complimented the immunofluorescence results by establishing the presence of PLC ζ in all areas of the epididymis. Unlike the immunofluorescence findings, immunohistochemical findings indicated a relationship between the proportion of sperm exhibiting PLC ζ and sperm maturity and age. Sperm from the pubescent mouse did not exhibit much PLC ζ although the

immunofluorescence data did not show any significant changes in either the levels of PLC ζ and proportions of sperm exhibiting PLC ζ . It may be that since the sperm derivation technique of swim-up used for immunofluorescence analysis selects the motile sperm in the tissue, it is the motile healthy sperm that exhibit PLC ζ , so while swim-up sperm are the best sperm in the tissue, they do not represent the whole population inside the region. Immunohistochemical findings also indicate that as sperm mature, the proportion of sperm exhibiting PLC ζ increases. This was seen clearly across the ages, particularly the post-pubescent ages.

Controversially, a recent study by Aarabi *et al.* (2012) suggested that PLC ζ is an epididymal secretory protein secreted by the epididymal epithelium which then binds to the surface of the sperm passing through the epididymal lumen (Aarabi *et al.*, 2012). Our findings negate Aarabi *et al.*'s findings in their suggestion of PLC ζ being an epididymal protein. Indeed the study herein identified PLC ζ from the round spermatid stage of spermatogenesis and all the way through to the most mature stage of sperm development, that found in the cauda epididymis. It is important however to note that Aarabi *et al.* (2012) arrived at their conclusions on the epididymal expression of PLC ζ by analysing mRNA expression and not the translated protein itself. Moreover, unlike Aarabi *et al.* (2012) this study was able to replicate Young *et al.*'s (2009) findings in identifying two clear populations of PLC ζ in mouse sperm. Peptide blocking using the same peptides that were used for the affinity purification of the antibody were used to demonstrate that the staining found in the epididymal epithelium was background staining and not real.

Indeed, it is important to note that the specificity of the antibody has been questioned due to its polyclonal nature exists, and may explain the variance in localisation

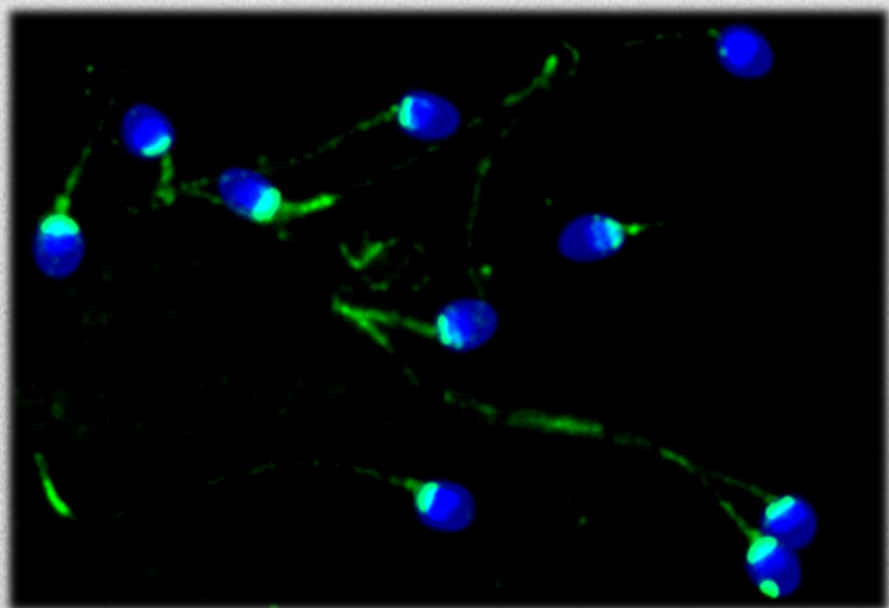
found between published studies. It is therefore imperative that a more specific monoclonal antibody against PLC ζ is synthesised in order to provide a more powerful research tool and obtain more conclusive determination on the localisation of PLC ζ in sperm.

The findings of the present study support previous studies identifying PLC ζ as the sperm-specific oocyte activation factor. Caudal sperm, which is responsible for fertilisation, in the most sexually-active mice in this study (16 weeks), exhibited significantly higher levels of PLC ζ than less mature sperm at the same age, and the majority of sperm possess a post-acrosomal population of PLC ζ , the population in the region thought to be involved in sperm-oocyte fusion. Although the increase in total PLC ζ was significant, but not consistent across ages, a higher sample size might allow for more robust analysis. Furthermore, this study showed for the first time, that there are dynamic changes of PLC ζ with epididymal maturity, showing that sperm appear to adapt a localisation pattern most apt for their functional role.

2.4.7 Summary of findings

- There was a significant increase in sperm concentration and progressive motility with epididymal maturation
- There was no effect of age or epididymal progression upon total levels of PLC ζ
- There was a significant shift to a predominant post-acrosomal localisation of PLC ζ as sperm reach the cauda epididymis
- It is unlikely that the acrosomal PLC ζ population plays a role in oocyte activation
- Developmental expression of PLC ζ begins at the round spermatid stage
- PLC ζ is expressed at all levels of sperm development from the round spermatid stage onwards
- Immunohistochemical studies suggest that the proportion of sperm within the epididymis expressing PLC ζ increase with age and with epididymal progression.

Chapter 3 | THE EFFECT OF MALE AGE UPON TOTAL LEVELS AND LOCALISATION OF PLC ζ IN HUMAN SPERM



3.1 Introduction

It is becoming more and more socially acceptable to delay parenthood (Ventura *et al.*, 1997). Fecundity, the physiological ability to bear a child, has long been recognised to decline markedly with female age, due to reduced fertilisation and implantation rates, higher rates of spontaneous abortions, and genetic and chromosomal abnormalities (Schramm *et al.*, 2002; Wilton, 2002; Mastenbroek *et al.*, 2007). Moreover, pregnancies amongst older women (>40 years) are associated with more complications, premature births, congenital malformations and interventions at birth (ESHRE, 2005). However, similar associations with male age are less well characterised (Sloter *et al.*, 2004). It is, however, becoming increasingly important to understand how male age affects these risks.

The principle aspect of female reproductive senescence is the decline in oocyte quality and loss of functional oocytes with age, which is inevitable and irreversible (ESHRE, 2005; Tesarik *et al.*, 2005; Perheentupa and Huhtaniemi, 2009). This is not the case for males however, where spermatogenesis continues well into advanced age, allowing men to reproduce during senescence (Kidd *et al.*, 2001; Eskenazi *et al.*, 2003). A broad range of abnormal reproductive and genetic outcomes have recently been implicated with advanced paternal age. These include diminished semen quality, such as semen volume, concentration and morphology, with the largest effect on sperm motility (Kidd *et al.*, 2001; Eskenazi *et al.*, 2003; Slotter *et al.*, 2006; Frattarelli *et al.*, 2008; Duran *et al.*, 2010). Such quantitative findings would suggest implications on fertility for men who choose to delay fatherhood. However, routine semen analysis provides only limited prognostic information on fertility (Barratt *et al.*, 2011). Increasing studies on the integrity of DNA have provided findings indicating increased risk of genetic integrity with increasing male age, such as

increased incidence of DNA fragmentation, reduced chromatin integrity, increased sperm aneuploidy and incidence of *de novo* germinal mutations (Sloter *et al.*, 2004; Tesarik, 2006; Wyrobek *et al.*, 2006). High levels of sperm DNA damage has been reported to affect fertility potential, with increased risk of recurrent miscarriage, decreased chances of successful implantation, developmental defects and genetic diseases among offspring (Kidd *et al.*, 2001; Rochebrochard and Thonneau, 2003; Sloter *et al.*, 2004, 2007; Benchaib *et al.*, 2007; Bungum *et al.*, 2007; Schmid *et al.*, 2007). Paternal age has also been associated with a range of birth defects including cleft lip and palate, achondroplasia, oncogenesis for conditions such as prostate and breast cancer, non-familial schizophrenia, Klinefelter syndrome, XYY syndromes and autism (Bille *et al.*, 2005; Aitken and De Iulius, 2007; De Souza *et al.*, 2010; Wiener-Megnazi *et al.*, 2012). Such age-related mechanisms may include the accumulation of environmental damage, reduced efficiency of DNA repair, increased genomic instability, genetic factors, suppressed apoptosis, hormonal influences or decreased effectiveness of micronutrients and antioxidants (Sloter *et al.*, 2004).

The changes in genetic integrity, endocrine and semen parameters do not necessarily indicate a direct association with a reduced fertility in males with advancing age. However, significant reduction in reproductive outcome, demonstrated by a decline in fertilisation, blastocyst formation, implantation rates, as well as increased time to attain successful pregnancy, and increased sub-fecundity, have all been associated with advanced male age (Kidd *et al.*, 2001; Luna *et al.*, 2009). A recent study which controlled for female age, and used an oocyte donation model, showed that whilst indeed fertilisation rate decreased with advanced male age, embryo quality, clinical pregnancy, implantation, miscarriage and live births remained unaffected (Duran *et al.*, 2010).

In the testis, age-related changes have been identified, with a decrease in number of the testosterone-producing Leydig cells (Chen *et al.*, 2009; Midzak *et al.*, 2009). Age-related changes also occur in the basal membranes and the seminiferous tubules with disarrangement of the seminiferous epithelium, including germ cell degeneration and alterations in both spermiogenesis and Sertoli cell structure (Morales *et al.*, 2004; Perheentupa and Huhtaniemi, 2009; Sartorius and Nieschlag, 2010).

The oocyte activation ability of a sperm sample represents its fertile potential. The absence, reduced levels, or abnormal localisation patterns of PLC ζ in human sperm has been linked to certain types of human male factor infertility (Yoon *et al.*, 2008; Heytens *et al.*, 2009). Thus the analysis and quantification of total PLC ζ and its localisation in the sperm head would, theoretically, represent a useful indicator of the oocyte activation ability of a given sample. Grasa *et al.* (2008) have identified three populations of PLC ζ in the human sperm head – acrosomal, equatorial and post-acrosomal, with the predominant PLC ζ localisation in fertile control sperm in the equatorial region. The authors further suggested that there may be differences in oocyte activation abilities between individuals or within an ejaculate since there appeared to be clear variability in PLC ζ localisation (Grasa *et al.*, 2008).

Whether the level and localisation of PLC ζ change with the advent of male age is currently unknown. Such a relationship may provide clues as to how the aging process may influence oocyte activation ability in males. In this chapter, the analysis and quantification of total PLC ζ and its specific localisation pattern in sperm samples from fertile controls was investigated using samples obtained from the Oxford Fertility Unit (OFU) and a collaborative IVF centre in Venezuela (Ferti Aguerrevere Fertility Unit). The samples were categorised

into discrete age groups in order to ascertain whether there was a relationship between age and PLC ζ expression and localisation.

Moreover, the applicability of using the quantitative analysis of PLC ζ in a given sperm sample as a fertility biomarker was assessed by studying a sperm sample from an infertile patient who had a history of failed ICSI cycles. It was hoped that by comparing this sample with control samples in terms of the total levels of PLC ζ , the proportion of sperm exhibiting PLC ζ relative to those that do not, as well as the localisation of PLC ζ within the sperm head might shed light upon the potential for such analyses in the fertility clinic as a novel biomarker for oocyte activation ability.

3.2 Materials and Methods

3.2.1 Samples and ethics

Sperm samples collected for screening were obtained from control (n=6) and OAD (n=1) patients visiting the Oxford Fertility Unit (OFU), aged 23-42 years. Samples were also obtained from control patients recruited from the Ferti Aguerrevere Fertility Unit (Venezuela) in two separate batches. The first batch (n=13; Table 3.2.1), aged 23-43 years, was used for a preliminary experiment, followed by a second batch (n=14; Table 3.2.1), aged 21-48 years, with an adapted protocol. Control subjects were defined as men whose sperm were able to result in successful fertilisation and oocyte activation following ART, and whose semen parameters fulfilled normal WHO criteria [Table 3.2.2]. The OAD patient recruited from the OFU had a history of failed ICSI cycles. The use of samples from the OFU was ethically approved by the National Research Ethics Service Committee, South Central Oxford C (REC Ref: 10/HO606/65). The samples from Venezuela entered the UK with ethical approval from the Oxford Tropical Research Ethics Committee (OXTREC; Ref: 31-09). All individuals agreed to participate by providing informed written consent. Patients were categorised into arbitrary age groups: 20-25, 26-30, 31-35 and 36+ years of age.

Table 3.2.1 | Concentration and motility data for first and second batches of Density-Gradient Washed control sperm from the Ferti Aguerrevere Fertility Unit in Venezuela.

BATCH 1	Age (years)	Concentration 10 ⁶ /ml	Motility (%)
Control V1-1	35	92.0	98.1
Control V1-2	35	82.6	95.4
Control V1-3	43	77.2	94.8
Control V1-4	31	130.8	99.9
Control V1-5	29	125.3	99.8
Control V1-6	23	19.3	96.5
Control V1-7	29	125.3	99.8
Control V1-8	29	125.3	99.8
Control V1-9	29	125.3	99.8

BATCH 2	Age (years)	Concentration 10 ⁶ /ml	Motility (%)
Control V2-1	22	103.4	92.0
Control V2-2	22	96.7	96.3
Control V2-3	42	n-a*	n-a*
Control V2-4	29	n-a*	n-a*
Control V2-5	24	54.7	96.1
Control V2-6	35	50.2	93.9
Control V2-7	29	16.3	95.3
Control V2-8	27	213.4	99.5
Control V2-9	25	73.1	96.9
Control V2-10	21	n-a*	n-a*
Control V2-11	32	24.5	90.6
Control V2-12	48	27.8	97.3
Control V2-13	39	48.0	95.8
Control V2-14	47	18.2	83.9

* data not available

Table 3.2.2 | **The current World Health Organisation lower reference ranges for semen characteristics (5th centiles and their 95% confidence intervals) (WHO, 2010).**

Parameter	Lower reference limit
Semen volume (ml)	1.5 (1.4-1.7)
Total sperm number (x10 ⁶ /ejaculate)	39 (33-46)
Sperm concentration (10 ⁶ /ml)	15 (12-16)
Progressive motility (%)	32 (31-34)
Total motility (%)	40 (38-42)
Vitality, live spermatozoa (%)	58 (55-63)
Normal morphology (%)	4 (3-4)

3.2.2 Sperm Density-Gradient Washing (DGW)

All samples (except 4 samples in batch 1 of samples from Venezuela) were subjected to DGW using PureSperm 40/80 (Nidacon, Sweden) as described in Figure 3.2.1 before analysis to select the highest quality sperm in the sample and in accordance with normal clinical practice prior to ART. Samples from the OFU underwent DGW in the Coward laboratory whereas samples from Venezuela underwent DGW in the Ferti Aguerrevere Fertility Unit. The first batch of control samples from Venezuela (n=13) were delivered to Oxford as frozen fresh DGW samples on dry ice. Upon receipt, samples from the OFU and the first batch from Venezuela were fixed in PBS/4% PFA for subsequent analysis. The

second batch (n=14) from Venezuela was transported to the Oxford laboratory following DGW and fixation to prevent damage caused to the samples should a delay in shipment occur as occurred with the first batch.

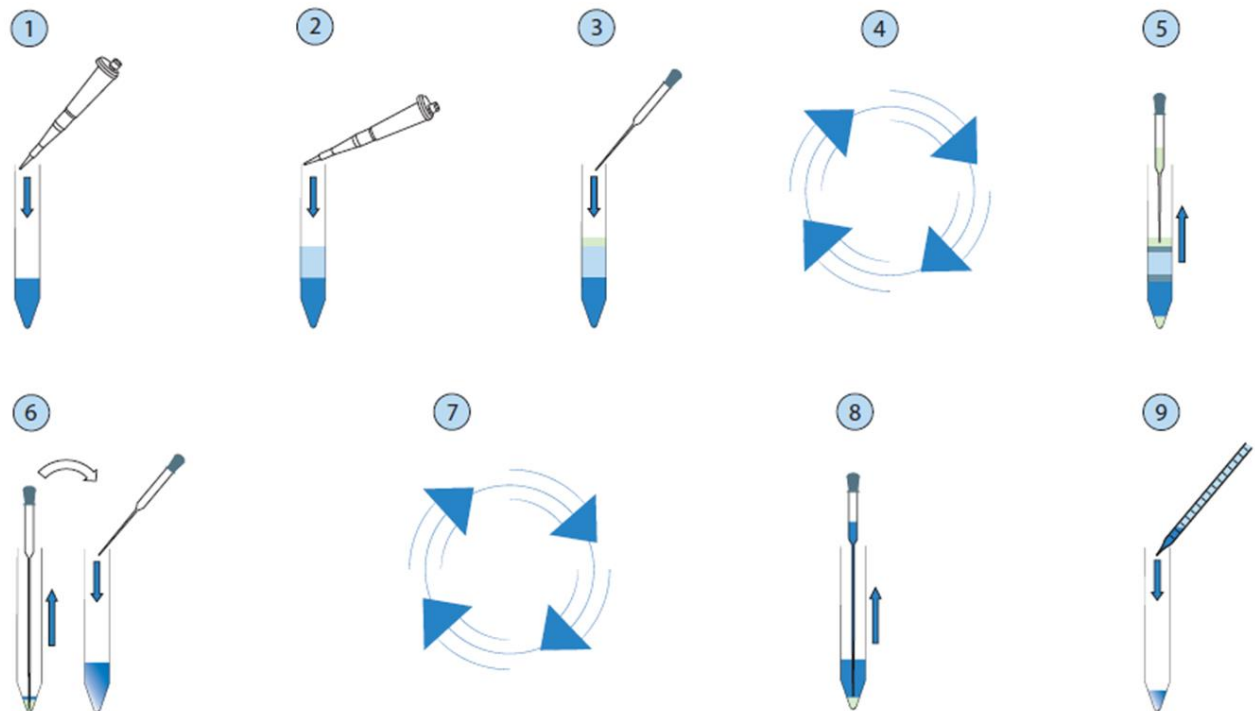


Figure 3.2.1 | Procedure of the Density-Gradient Wash (DGW): (1) 2ml PureSperm 80 was added to a centrifuge tube; (2) 2ml PureSperm 40 was layered on top of the PureSperm 80; (3) Liquefied sperm was layered on top; (4) Suspensions were centrifuged at 300g for 20 minutes; (5) Supernatant above pellet was aspirated or lowest 0.5ml was left; (6) Pellet was aspirated and transferred into a new tube and resuspended in 5ml PureSperm Wash; (7) Suspensions were centrifuged at 500g for 10 minutes; (8) PureSperm Wash supernatant was aspirated leaving the pellet or the lowest 0.25ml fluid in the tube; (9) Pellet was resuspended in PBS.

Figure adapted from Nidacon PureSperm 40/80 Instructions (www.nidacon.com)

3.2.3 Determination of PLC ζ immunofluorescence in sperm

Fixed sperm suspensions were added on to PAP moulds drawn on slides pre-coated with 0.01% poly-L-lysine, air dried, and permeabilised for 20 minutes with PBS/0.5% Triton-X100 in a humidifying chamber. Slides were then washed twice with PBS, and non-specific binding blocked with PBS/3% BSA for 1 hour at RT. Samples were then incubated overnight at 4°C with 25 μ g/ml anti-PLC ζ antibody (as recommended by Grasa *et al.*, 2009) in PBS/0.05% BSA, whereas negative controls were incubated in the absence of primary antibody. Slides were subsequently washed twice with PBS and incubated for 1 hour at RT in 5 μ g/ml secondary fluorescent antibody (Alexa Fluor 488 F(ab')₂ goat anti-rabbit IgG). Finally, samples were washed three times with PBS and mounted with Vectashield mounting medium containing DAPI and 22mm glass cover slips for analysis. Some initial samples underwent further staining with 40 μ g/ml TRITC-PNA diluted in PBS/1% BSA to confirm acrosome integrity.

Images were captured and the distribution of PLC ζ within the sperm head visualised under a fluorescent Nikon Eclipse 80i microscope. A FITC filter (488nm) was used to visualise PLC ζ fluorescence (green) and a TRITC filter (560nm) for acrosomal fluorescence (red). Images were captured using the Nikon DS-Ri1 digital camera and NIS-Elements BR 3.0 software. Captured fields of view were randomly selected using bright field, and 100 random cells, where the head and tail were intact, and the head was clearly visible and unobstructed, were evaluated per sample as described in Section 2.2.4. Relative overall levels of fluorescence among samples, as well as the distribution of PLC ζ localisation patterns, were assessed using the region of interest (ROI) manager in Image J software (<http://rsbweb.nih.gov/ij/>). The ROI (sperm heads) were selected in the bright field image

for each field of view, and then transferred to its corresponding fluorescent image, and the colour histogram tool used to measure the relative level of green fluorescence in each sperm head, and the localisation for each sperm head noted.

3.2.4 Statistical analysis

Correlations between mean levels of total PLC ζ fluorescence (total fluorescence in the sperm head including all PLC ζ populations) between individuals and age categories were determined, as well as correlations between proportions of sperm with or without PLC ζ immunofluorescence. The proportions of sperm exhibiting the discrete localisation patterns previously reported by Grasa *et al.* (2008) were also quantified and analysed between age categories. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple-comparison *post hoc* test using Prism 5.0 (Graphpad, USA) [Appendix 4]. The student's *t*-test was used to determine statistical significance between control and OAD groups. Data are presented as the mean $\pm$ SEM and a *p* value of ≤ 0.05 was considered statistically significant.

3.3 Results

3.3.1 Immunolocalisation of PLC ζ

Sperm from fertile controls exhibited PLC ζ immunolocalisation in the acrosomal, equatorial and post-acrosomal regions of the sperm head, showing various combinations of all three localisation patterns. Figure 3.3.1 shows representative images of human sperm exhibiting characteristic patterns of PLC ζ immunofluorescence described previously by Grasa *et al.* (2008).

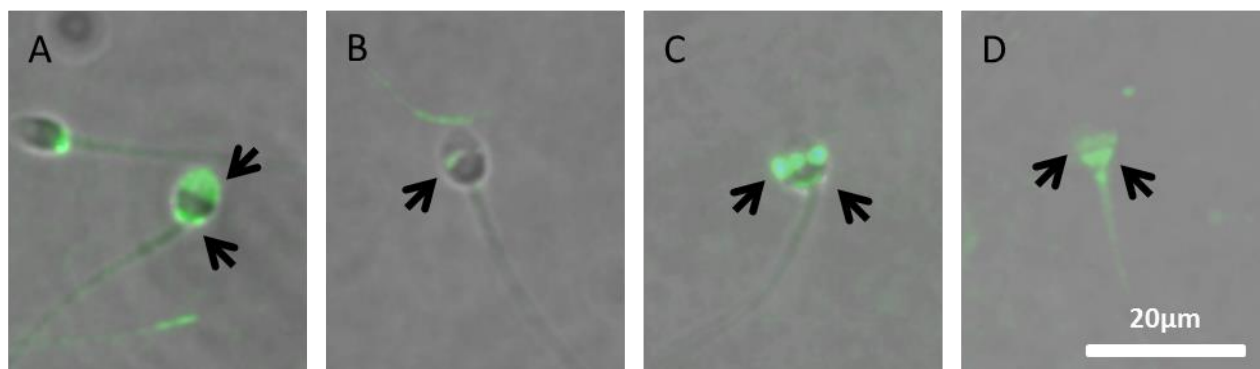


Figure 3.3.1 | **Representative images of PLC ζ immunofluorescence in fertile human sperm exhibiting characteristic localisation patterns in localisations previously identified by Grasa *et al.* (2008).** Images were captured at x40 magnification and represent overlays of PLC ζ (green) and phase images. Arrows indicate distinct PLC ζ populations: (A) acrosomal and post-acrosomal; (B) equatorial; (C and D) equatorial and post-acrosomal.

3.3.2 Quantitative analysis of PLC ζ immunofluorescence in OFU subjects

Quantitative immunofluorescence analysis of control samples obtained from the OFU showed considerable variance in the total levels of PLC ζ [Figure 3.3.2], varying from a mean of 16.5 – 119.8 arbitrary units of fluorescence, whilst the OAD patient had a mean of

37.7 arbitrary units of fluorescence. However, 67% (4/6) of control samples exhibited significantly higher levels of PLC ζ compared with the OAD patient ($p<0.05$ as indicated by the t -test) [Figure 3.3.2].

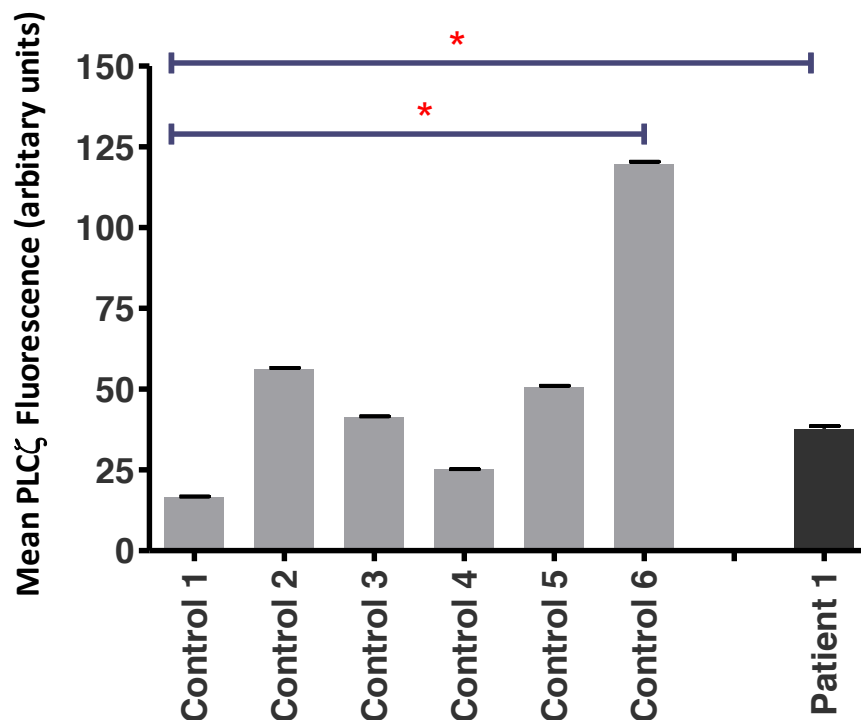


Figure 3.3.2 | **Mean relative total PLC ζ fluorescence levels in controls (n=6) and on OAD patient (n=1) following DGW.** Fluorescence intensity was measured in arbitrary units using Image J software. Asterisks (*) denote statistically significant differences between the control samples and the OAD patient ($p<0.05$), as well as among the control samples. Error bars denote SEM.

Proportional analysis of sperm exhibiting PLC ζ immunoreactivity indicated that a significantly larger proportion of control sperm exhibited PLC ζ immunoreactivity ($p<0.05$ as indicated by the t -test) compared with sperm from the OAD patient, with a mean of 95% in the control samples and 61% in the OAD patient exhibiting PLC ζ immunoreactivity [Figure 3.3.3]. Among the control samples, the variation between proportions of sperm within a sample exhibiting PLC ζ immunoreactivity was 80-100%.

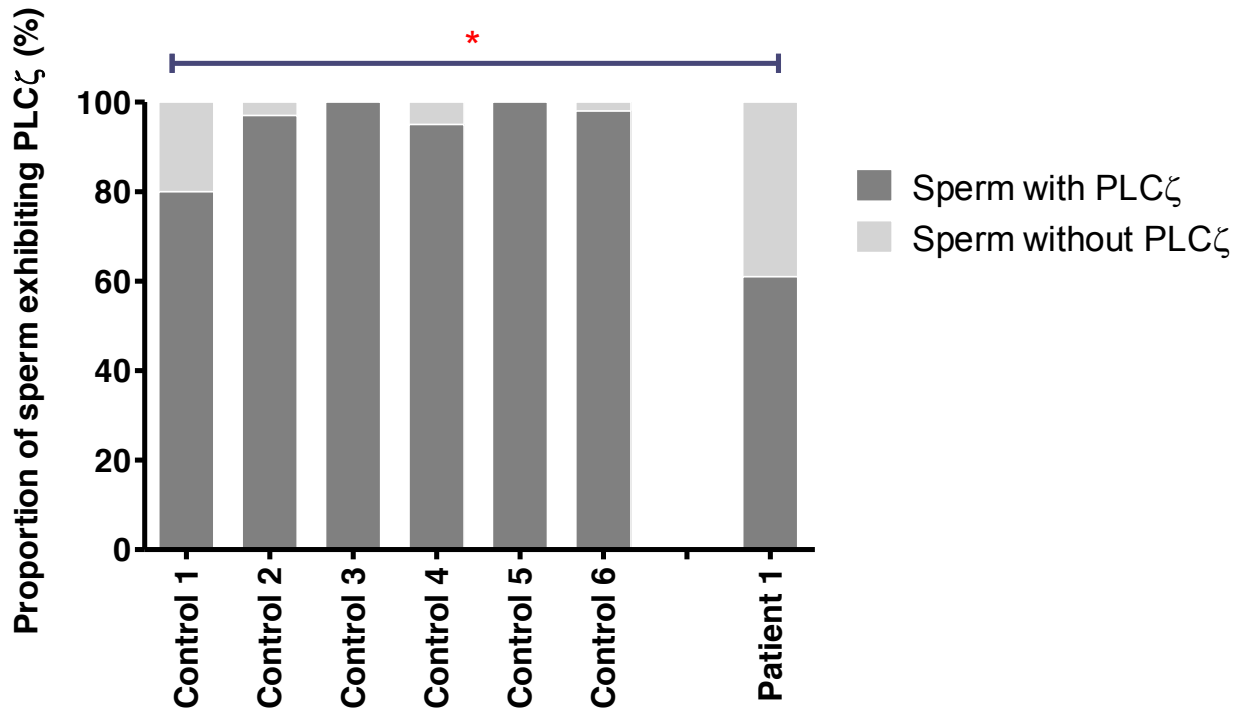


Figure 3.3.3 | Proportions of sperm (percentages) exhibiting PLC ζ immunofluorescence from control subjects and the OAD patient following DGW. Asterisk (*) denotes statistical significance ($p < 0.05$) between the controls and OAD patient, showing a significant decrease in the proportion of sperm exhibiting PLC ζ in the OAD patient compared with the fertile controls.

Analysis of the discrete populations of PLC ζ localisation patterns in sperm from individual subjects showed significant variation in the relative proportions with no predominant localisation pattern revealed amongst all samples [Figure 3.3.4]. The localisation patterns of PLC ζ observed were: acrosomal; post-acrosomal; acrosomal and post-acrosomal; equatorial; acrosomal and equatorial; post-acrosomal and equatorial; and all three localisations simultaneously. However, a pattern with post-acrosomal localisation, either alone, or in combination with other localisations is predominant in each sample.

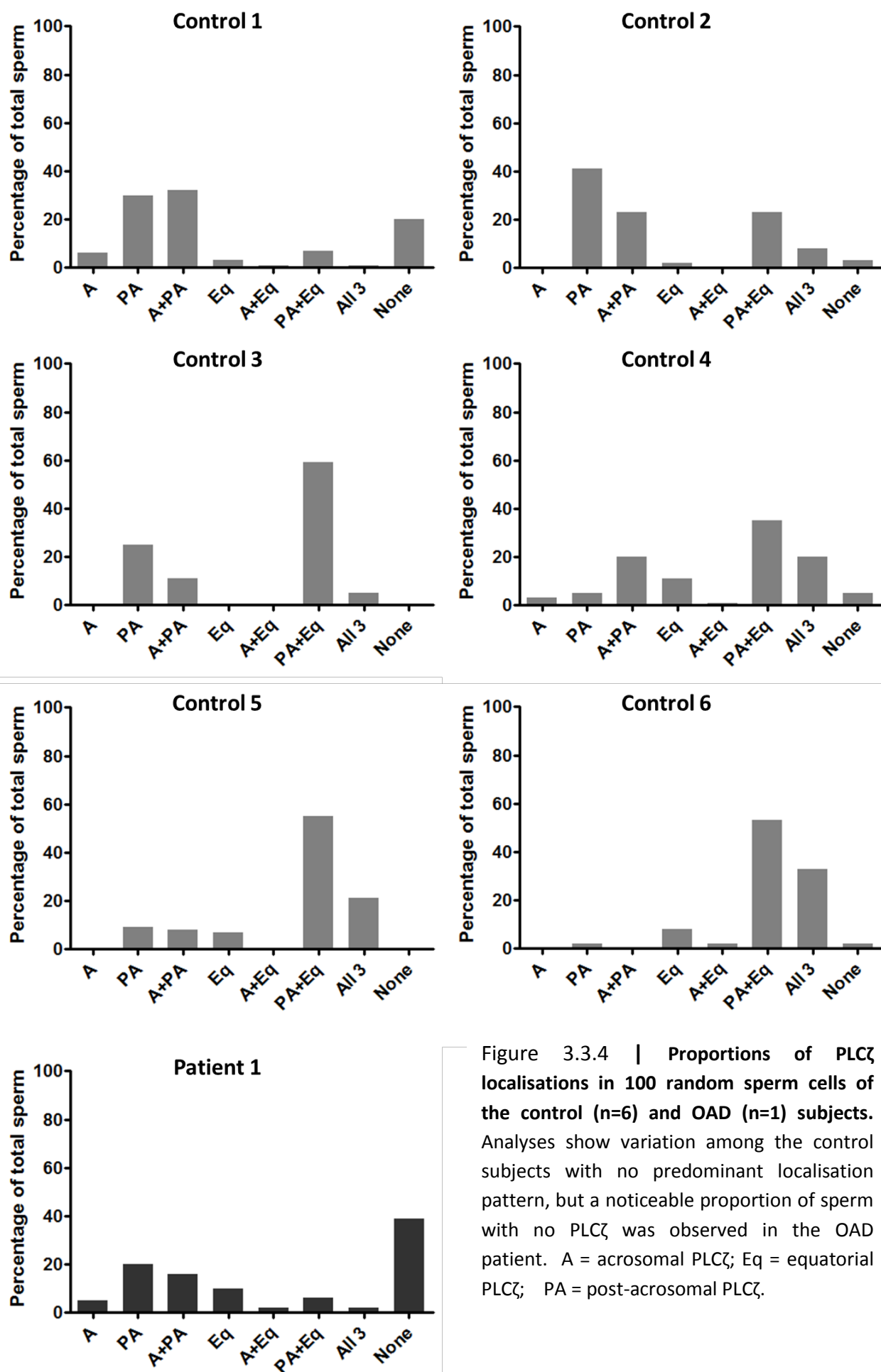


Figure 3.3.4 | Proportions of PLC ζ localisations in 100 random sperm cells of the control (n=6) and OAD (n=1) subjects. Analyses show variation among the control subjects with no predominant localisation pattern, but a noticeable proportion of sperm with no PLC ζ was observed in the OAD patient. A = acrosomal PLC ζ ; Eq = equatorial PLC ζ ; PA = post-acrosomal PLC ζ .

3.3.3 Preliminary quantitative analysis of PLC ζ immunofluorescence in control subjects from Ferti Aguerrevere Fertility Unit, Venezuela (batch 1)

Quantitative immunoanalysis was undertaken on the first batch of sperm samples (n=13) aged 23-43 years from Venezuela. Out of the 13 controls, 9 had undergone DGW in Venezuela, whereas 4 were raw un-washed semen. Immunofluorescence analysis indicated that total levels of PLC ζ in the DGW samples were significantly higher than unwashed raw samples ($p<0.05$ as indicated by the t test) [Figure 3.3.5].

Amongst all the control samples, as with the OFU control samples, a large degree of variability was observed in total PLC ζ levels, ranging from 27.4 arbitrary units in Control V1-5 to 79.9 arbitrary units in Control V1-1 in the DGW group, and from 10.6 arbitrary units in Control V1-13 to 28.9 arbitrary units in V1-10 in the non-DGW group. However, when the DGW samples only were categorised into age groups (20-25, 26-30, 31-35, 36+ years of age) and mean fluorescence was analysed per age category, there appeared to be a reduction in the total levels of PLC ζ with increasing age [Figure 3.3.6]. The sample numbers per age group however were too small for appropriate statistical analysis to be carried out. Indeed there was only one sample in the upper and lower age groups respectively, so whilst there did appear to be a potential trend, no conclusions could be made unless a larger sample size was obtained.

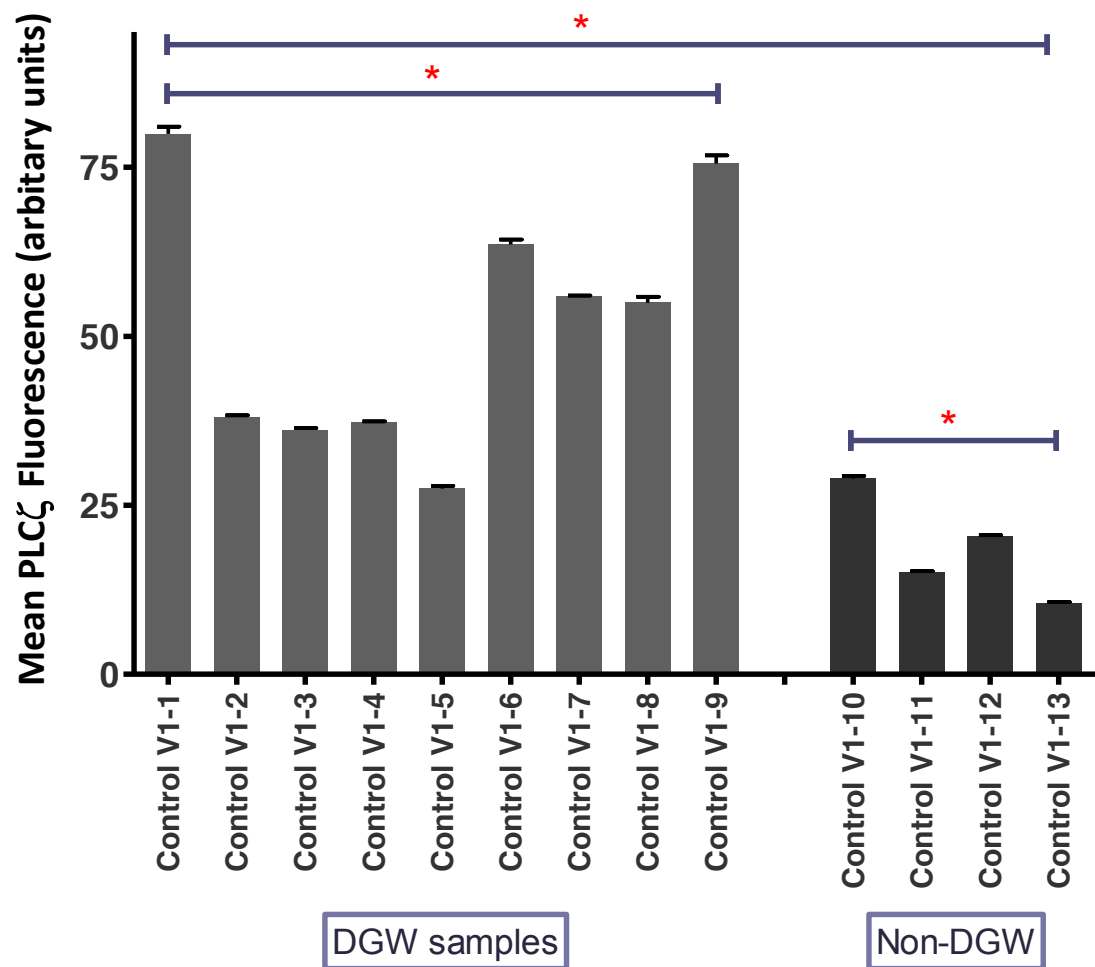


Figure 3.3.5 | Mean relative total PLC ζ fluorescence levels in DGW control (n=9) and non-DGW controls (n=4). Fluorescence intensity was statistically significant between and within both groups when analysed by a *t*-test (Asterisk * denotes $p < 0.05$). Mean total levels of PLC ζ levels in the DGW control samples were significantly higher than the mean levels of the non-DGW control samples. Error bars denote SEM.

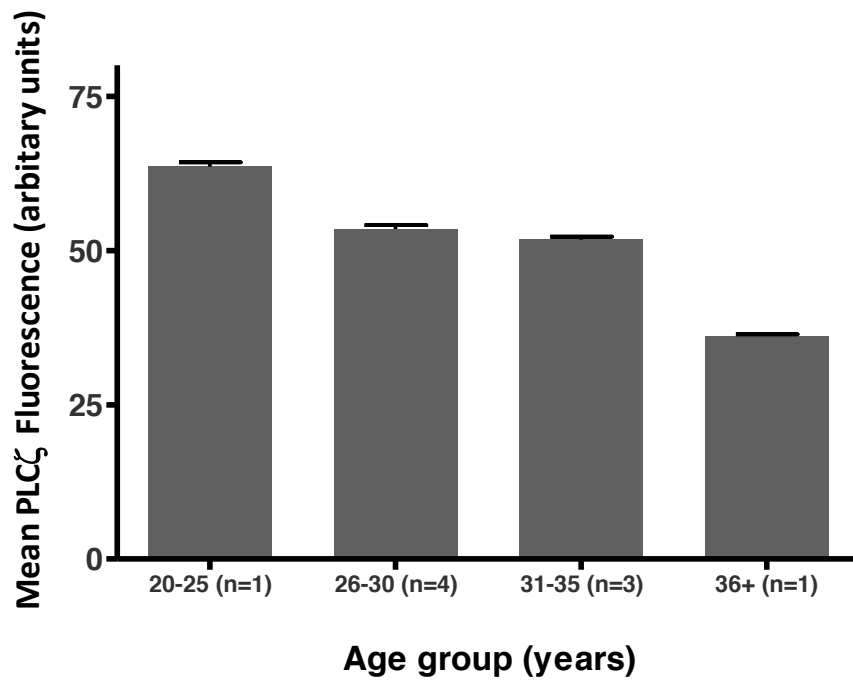


Figure 3.3.6 | **Mean relative total PLC ζ fluorescence levels in control DGW samples from Venezuela categorised into age groups.** Findings indicate a reduction in the total levels of PLC ζ with increasing age, but due to the small sample size of the individual age groups, no appropriate statistical analyses was carried out. Error bars denote SEM.

Proportional analysis of sperm exhibiting PLC ζ immunoreactivity in the different age groups suggested a potential reduction in the proportion of sperm exhibiting PLC ζ with increasing age, although the single sample in the highest age category showed the highest proportion of sperm exhibiting PLC ζ [Figure 3.3.7]. In this instance, sample size was not sufficient to derive any firm conclusions as to the effect of age on levels of total PLC ζ .

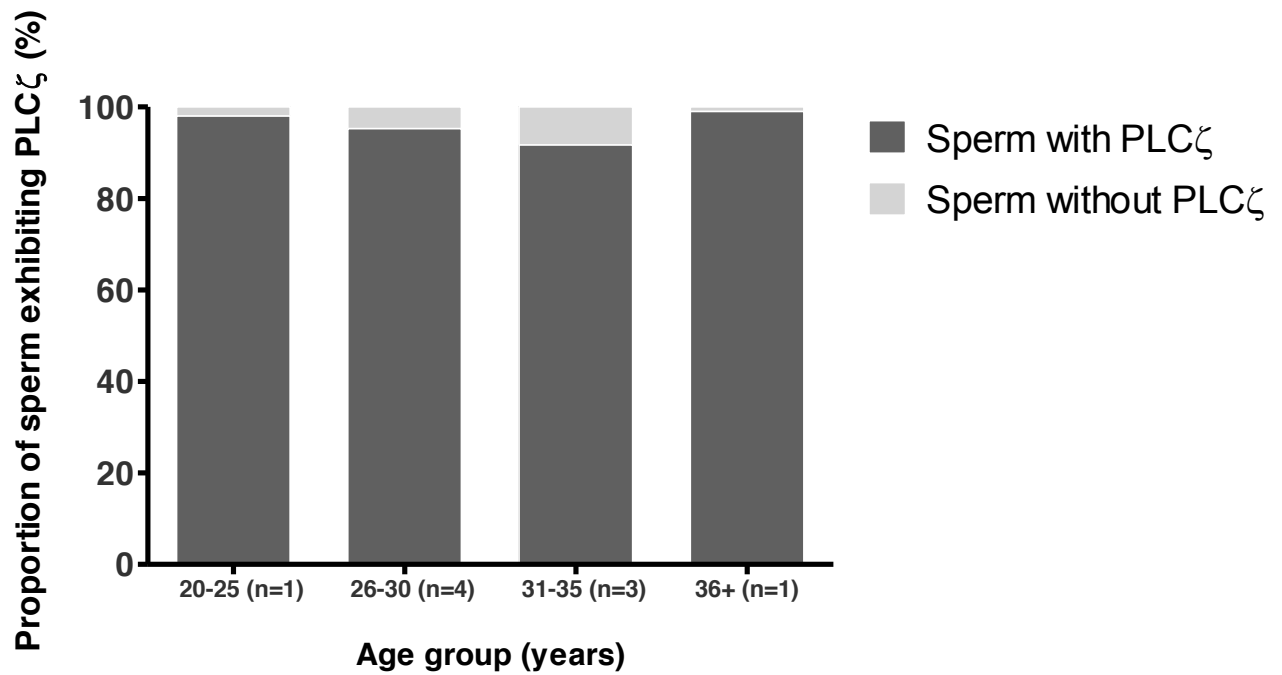


Figure 3.3.7 | Proportions of sperm (percentages) exhibiting PLC ζ immunofluorescence from in control DGW samples from Venezuela categorised into age groups. Findings suggest a downward trend in the proportion of sperm exhibiting PLC ζ immunofluorescence with increasing age, but sample sizes of each age group is too small to carry out any appropriate statistical analysis.

Analysis of the discrete populations of PLC ζ localisation patterns in the different age groups show sperm exhibiting different localisation patterns. Figure 3.3.8 shows clearly that the predominant localisation pattern in all age groups was the post-acrosomal localisation region. Localisation to the post-acrosomal region alone was the most predominant in most age groups, and localisation of PLC ζ to the post-acrosomal region, whether alone or in combination with other localisations patterns, was evident in 91.33 – 99% of all sperm analysed across all age groups [Figure 3.3.8].

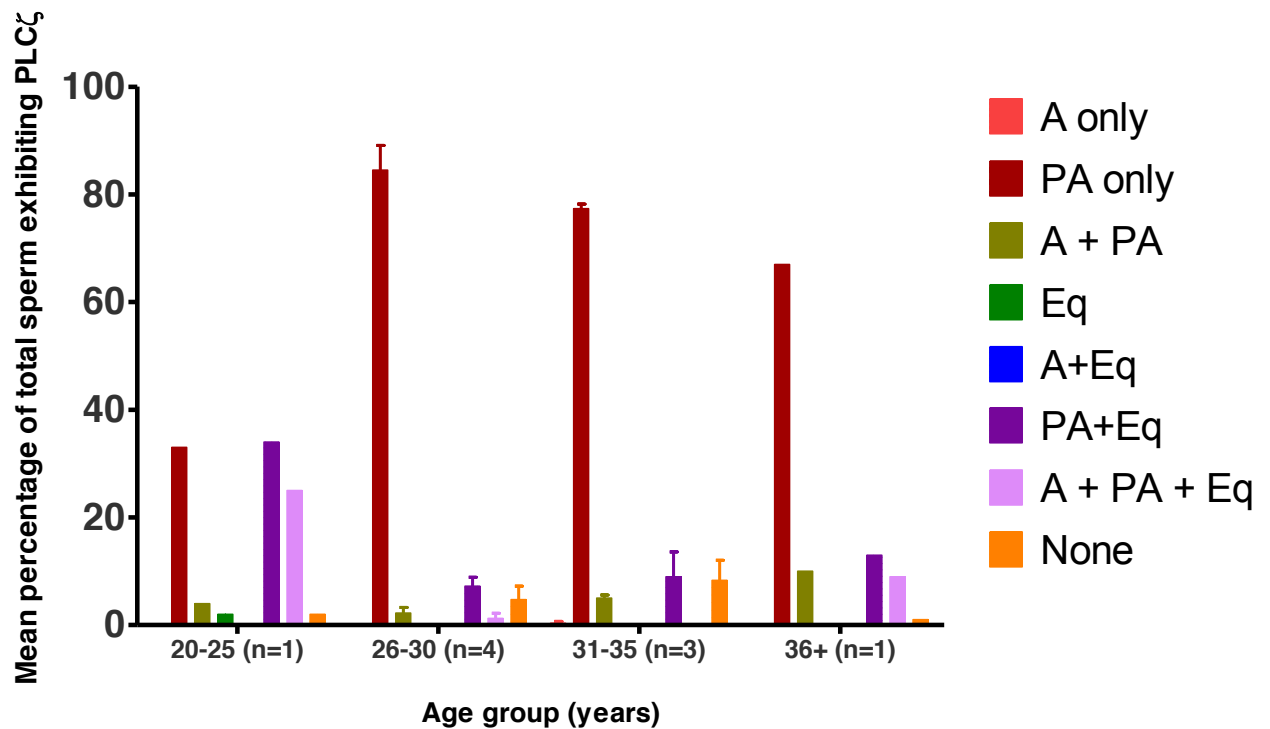


Figure 3.3.8 | **Representation of the various mean localisation patterns of PLC ζ in sperm from control DGW samples from Venezuela of different age categories.** Seven localisation patterns of PLC ζ were identified: A = acrosomal; Eq = equatorial; PA = post-acrosomal. Immunolocalisation of PLC ζ in the post-acrosomal region appeared to be the predominant localisation pattern across all age groups. Error bars denote SEM.

The samples were frozen upon collection in Venezuela and thawed upon arrival in the UK followed by fixation. Difficulties were encountered in the transport of the frozen samples by air from Venezuela to the UK which meant the samples may have thawed during transit allowing protein degradation within the sperm since the samples were unfixed. Ideally the samples should not undergo any freezing and be fixed soon after collection, therefore a further batch of samples was requested which would undergo DGW and fixation following collection. Furthermore, logistical issues prevented the use of liquid nitrogen, thus necessitating the use of dry ice for batch 1.

3.3.4 Quantitative analysis of PLC ζ immunofluorescence in control subjects from Ferti Aguerrevere Fertility Unit, Venezuela (batch 2)

New samples (n=14) were obtained from males aged 21-48 years attending the Ferti Aguerrevere Fertility Unit. Samples underwent DGW and fixation upon collection in Venezuela, and were delivered fixed to the UK ready for analysis. Quantitative immunoanalysis of control samples exhibited a large degree of variation in the total levels of PLC ζ immunofluorescence [Figure 3.3.9] ranging from 13.8 arbitrary units in Control V2-04 to 76.3 arbitrary units in Control V2-12.

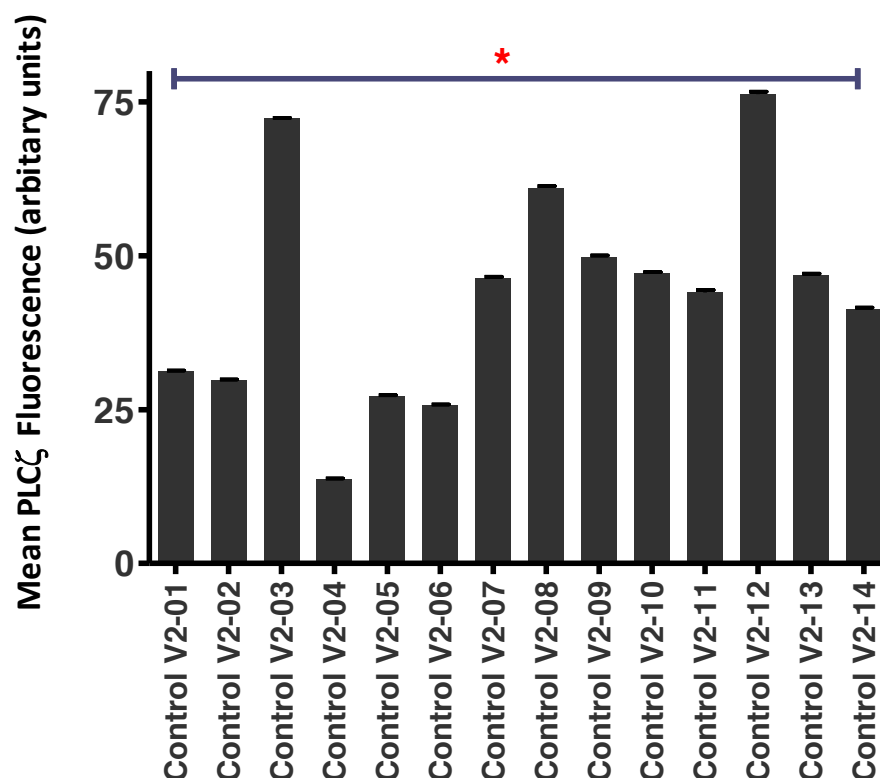


Figure 3.3.9 | Mean relative total PLC ζ fluorescence levels in control DGW samples from Venezuela (n=14; batch 2) of males aged 21-48 years, showing a large degree of variation between control samples. Error bars denote SEM.

The samples were allocated into the same age categories as Section 3.3.3 (20-25, 26-30, 31-35, 36+ years of age). Mean sperm concentration and percentage motility was calculated for each category using the data provided in Table 3.2.1 [Table 3.3.1]. Figure 3.3.10 demonstrates the effect of age on the mean concentration and motility of DGW sperm from this selection of control subjects. With the small sample size per age category, there was large variation within age groups for both parameters. However, the student *t*-test analysis indicated a statistically significant reduction in the concentration of DGW sperm between the lowest age group (20-25 years) and the highest age group (36+) with $p<0.05$ [Figure 3.3.10]. There were no significant changes in motility between the age groups in this selection of subjects.

Table 3.3.1 | **Mean concentration and motility of control DGW samples from Venezuela (batch 2) categorised into age groups.**

Age group (years)	Mean concentration 10 ⁶ /ml	Mean motility (%)
20-25 (<i>n</i> =4)	82.0	95.3
26-30 (<i>n</i> =2)	114.9	98.9
31-35 (<i>n</i> =2)	37.4	92.3
36+ (<i>n</i> =3)	31.3	92.4

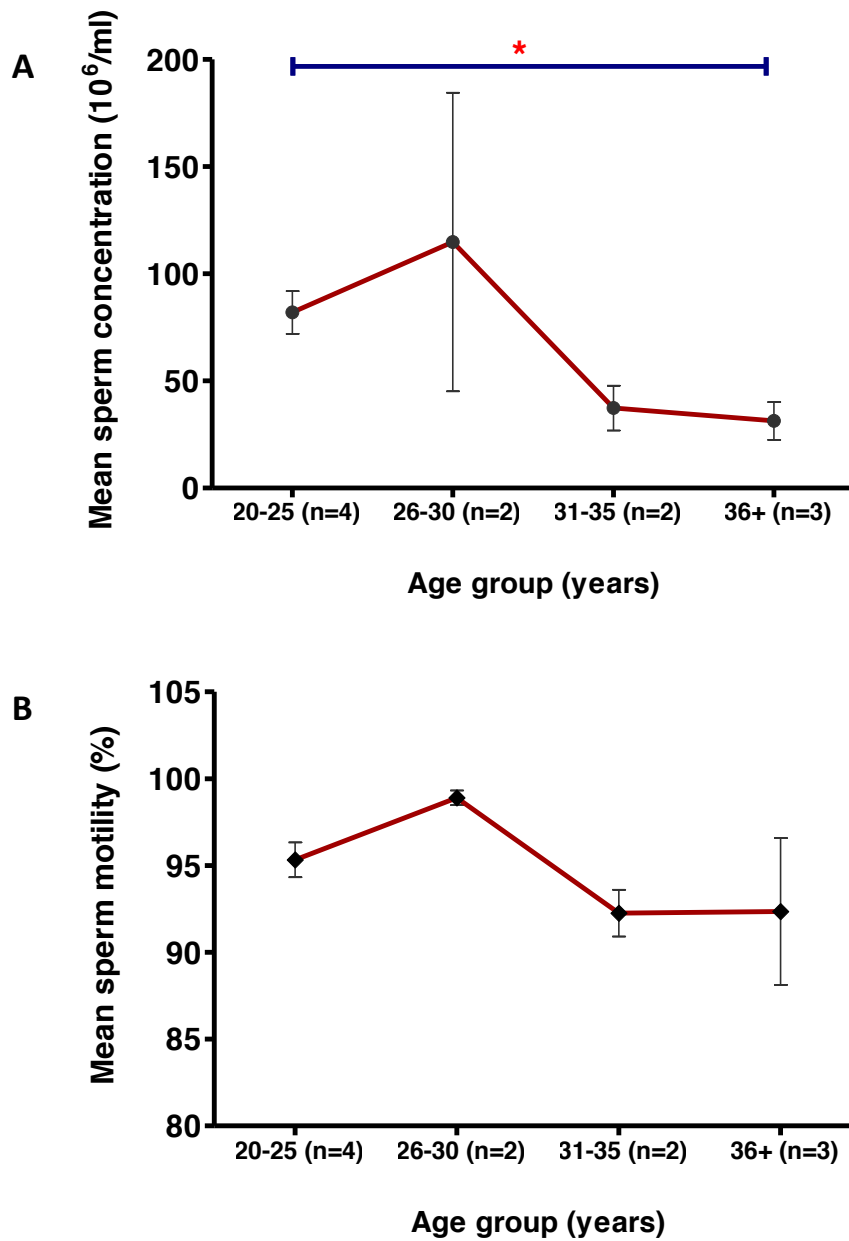


Figure 3.3.10 | Mean concentration (A) and motility (B) of control DGW samples from Venezuela (batch 2) categorised into age groups, showing a significant reduction in sperm concentration from the 20-25 year age group to the 36+ age group ($p<0.05$). There was no effect of age on motility in this selection of control subjects. Error bars denote SEM.

Further to the mean relative total PLC ζ fluorescence levels analysed in Figure 3.3.9, mean fluorescence was analysed per age category [Figure 3.3.11]. One-way ANOVA indicated no statistically significant changes in the total levels of PLC ζ immunofluorescence between age groups.

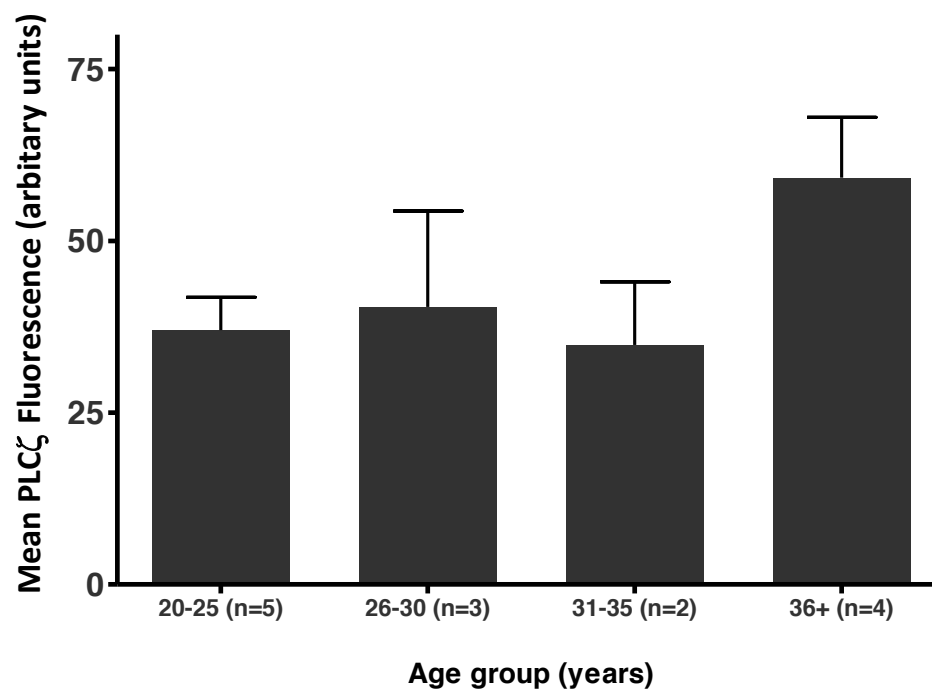


Figure 3.3.11 | **Mean relative total PLC ζ fluorescence levels in control DGW samples from Venezuela (batch 2) categorised into age groups.** Analyses indicate no statistically significant changes in the total levels of PLC ζ immunofluorescence between age groups. Error bars denote SEM.

Proportional analysis of sperm exhibiting the characteristic PLC ζ immunofluorescence patterns in control samples did not show any relationship between the proportion of sperm exhibiting PLC ζ and age; the proportion of sperm exhibiting PLC ζ was 95-99.5% across all age groups [Figure 3.3.12].

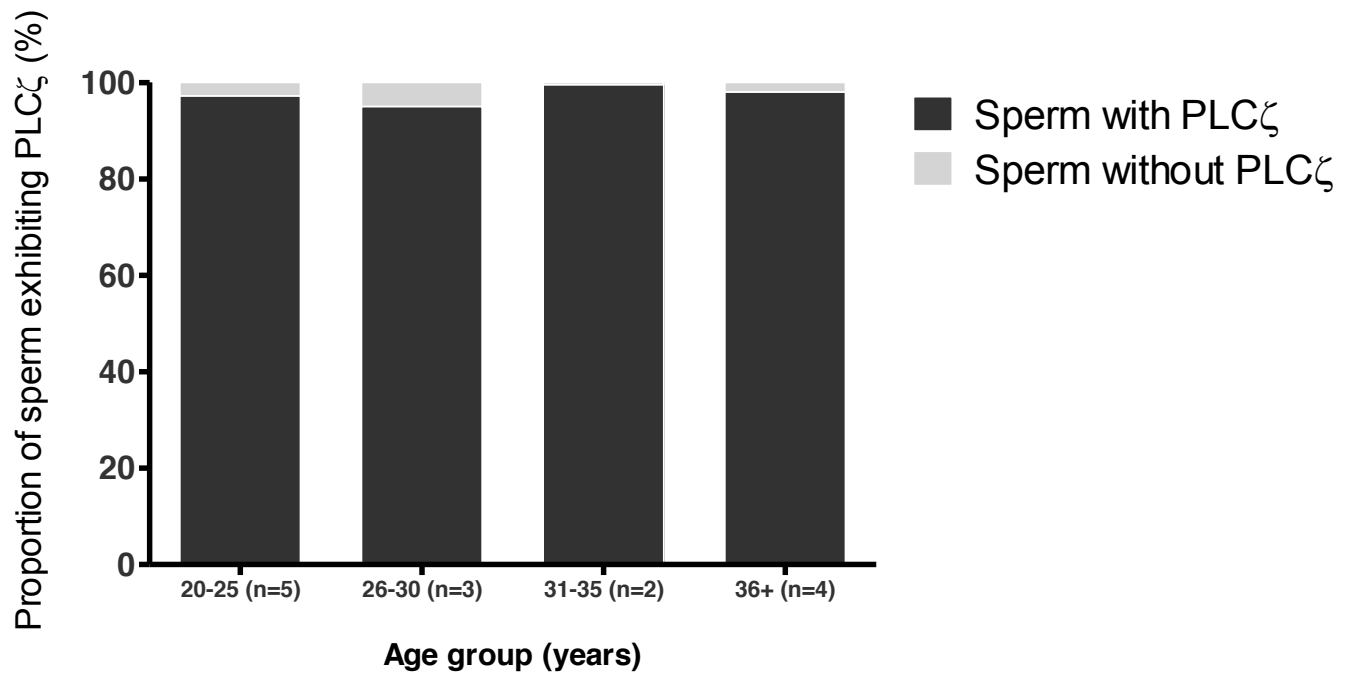


Figure 3.3.12 | Proportions of sperm (percentages) exhibiting PLC ζ immunofluorescence from in control DGW samples from Venezuela (batch 2) categorised into age groups. Findings indicated no relationship between age and the proportion of sperm exhibiting PLC ζ .

Moreover, analysis of the discrete populations of PLC ζ localisation patterns in the different age groups again, as with Sections 3.3.2 and 3.3.3, showed sperm exhibiting different localisation patterns. Figure 3.3.13 shows that the predominant localisation pattern in all age groups was the joint equatorial and post-acrosomal localisation pattern (45.4-62.5%), followed by a combination of all three localisations (16-32.3%). Further analysis showed that amongst all samples, the proportion of sperm exhibiting PLC ζ in the post-acrosomal and equatorial regions, whether alone or in combination with other populations, was 91-99% and 76.7-93.5% respectively. Statistical analysis did not, however, indicate that there was any trend in the effect of age upon the localisation pattern of PLC ζ in sperm.

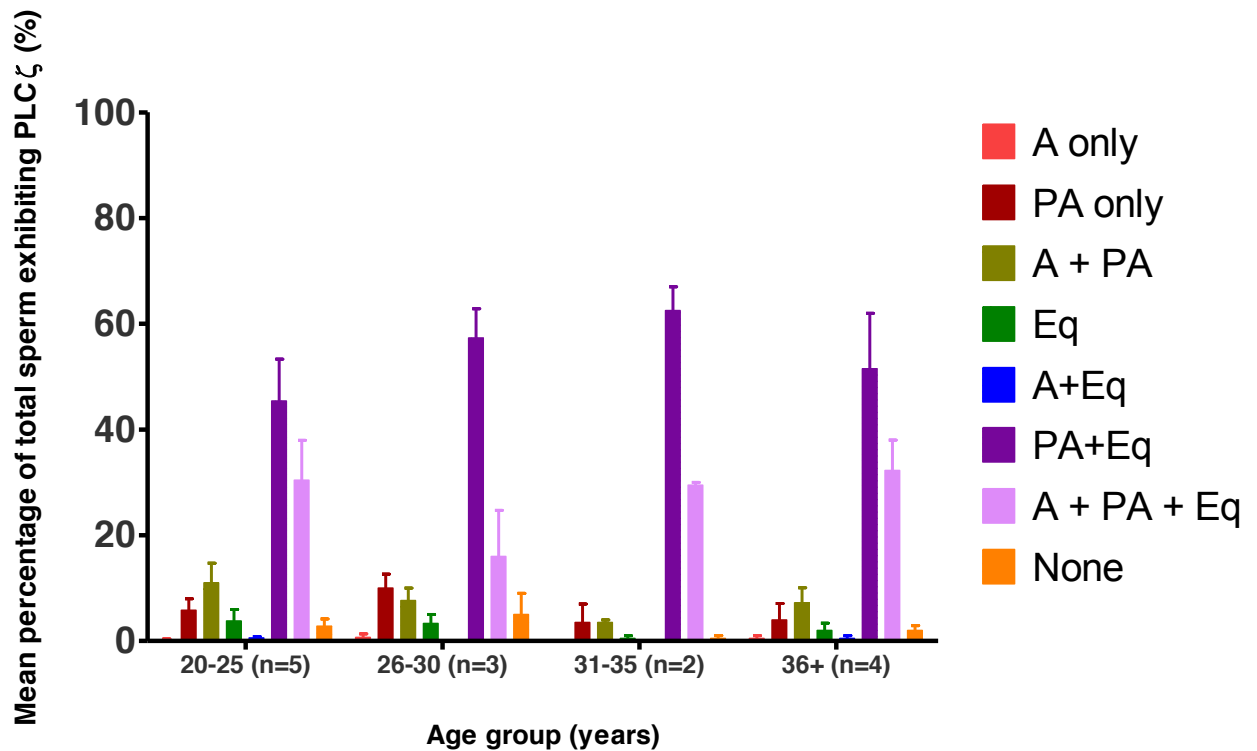


Figure 3.3.13 | Representation of the various mean localisation patterns of PLC ζ in sperm from control DGW samples from Venezuela (batch 2) of different age categories. Seven localisation patterns of PLC ζ were identified: A = acrosomal; Eq = equatorial; PA = post-acrosomal. Immunolocalisation of PLC ζ simultaneously in the equatorial and post-acrosomal regions appeared to be the predominant localisation pattern across age groups. Error bars denote SEM.

Finally, quantitative immunoanalysis of all samples in this study – samples from the OFU and both batches from the Ferti Aguerrevere Fertility Unit – were collated and categorised into age groups to assess the overall effect of age on total PLC ζ levels and its localisation. One-way ANOVA indicated that age did not have a significant effect on total levels of PLC ζ as shown in Figure 3.3.14. Collated localisation analyses indicated that the predominant localisation pattern of PLC ζ in all age categories was the post-acrosomal localisation, whether alone, or in combination with other localisation patterns [Figure

3.3.15; Table 3.3.2], with the proportion of sperm exhibiting a post-acrosomal localisation, either alone or in combination with other localisation patterns ranging from 91.6 to 94.4%, whilst sperm exhibiting an equatorial localisation, whether alone or in combination with other localisation patterns, ranged from 37.7 to 80.1% [Table 3.3.2].

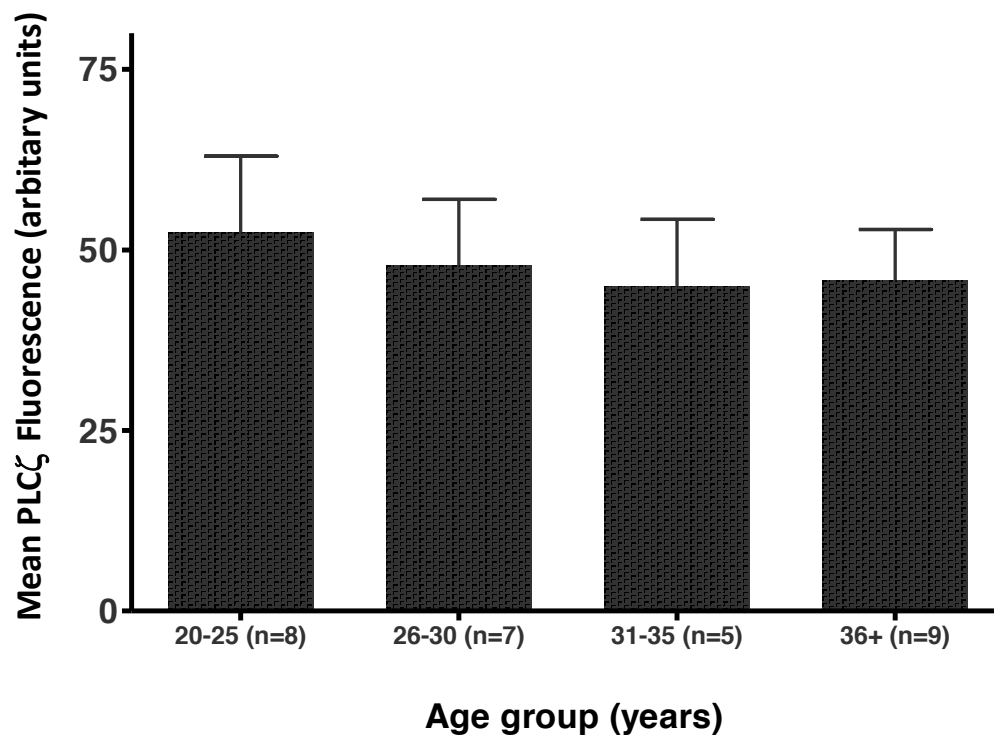


Figure 3.3.14 | **Mean relative total PLC ζ fluorescence levels in all control samples from this study (samples from the OFU and both batches from the Ferti Aguerrevere Fertility Unit) categorised into age groups.** Statistical analyses of the collated data indicated there was no relationship between total levels of PLC ζ and age. Error bars denote SEM.

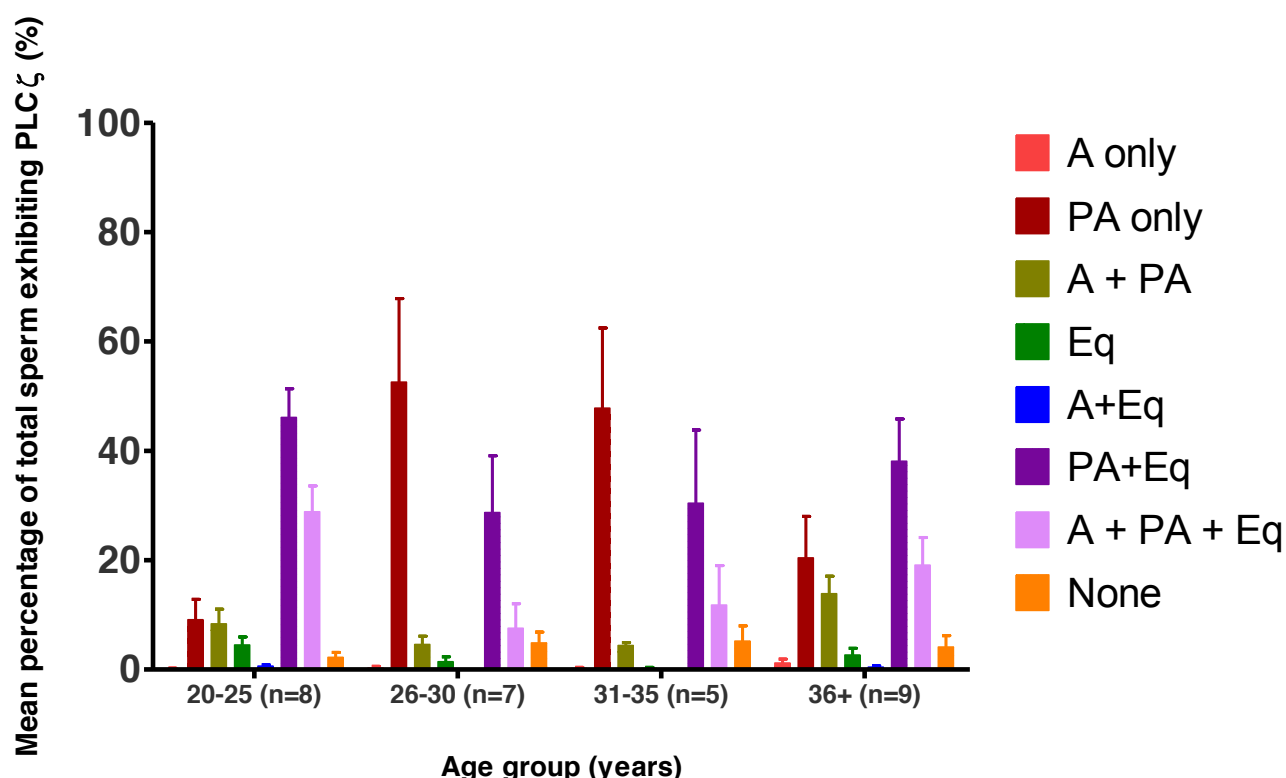


Figure 3.3.15 | Representation of the various mean localisation patterns of PLC ζ in all control samples of this study (samples from the OFU and both batches from the Ferti Aguerrevere Fertility Unit) categorised into age groups. A = acrosomal; Eq = equatorial; PA = post-acrosomal. Immunolocalisation of PLC ζ in the post-acrosomal regions, whether alone or in combination with other localisations, appeared to be the predominant pattern across age groups. Error bars denote SEM.

Table 3.3.2 | Mean percentages of sperm exhibiting the different localisation patterns of PLC ζ .

Mean percentages of sperm exhibiting PLC ζ (%)								
	A only	PA only	A + PA	Eq	A+Eq	PA+Eq	All 3	None
20-25 (n=8)	0.13	9.13	8.38	4.50	0.63	46.13	28.88	2.25
26-30 (n=7)	0.29	52.57	4.57	1.43	0	28.71	7.57	4.86
31-35 (n=5)	0.20	47.80	4.40	0.20	0	30.40	11.80	5.20
36+ (n=9)	1.22	20.44	13.89	2.67	0.44	38.11	19.11	4.11
<i>A = acrosomal; Eq = equatorial; PA = post-acrosomal</i>								

3.4 Discussion

Much evidence suggests that the increasing trend of fathering at an older age may have significant effects on the viability and genetic health of pregnancies and offspring (Kidd *et al.*, 2001; Slotter *et al.*, 2004, 2007; Wyrobek *et al.*, 2006; Schmid *et al.*, 2007; Brahem *et al.*, 2011; Matorras *et al.*, 2011; Alio *et al.*, 2012; Kong *et al.*, 2012), but the underlying mechanisms and roles of male aging reproductively are poorly understood (Ventura *et al.*, 1997). Understanding the effects of male aging on fertility and sperm quality is important and especially relevant for older men attending fertility clinics seeking ART in order to father children (Schmid *et al.*, 2013). An obvious issue for investigation is the effect of aging upon the oocyte activation ability of sperm.

No previous work thus far has ascertained whether there is a physiological decline in the oocyte activation ability of sperm with increasing age. The present study aimed to investigate whether male age is likely to influence the oocyte activation ability of a sperm sample by quantitatively analysing the sperm-specific oocyte activation factor PLC ζ in sperm from human males from different age-groups. Furthermore the study aimed to further investigate the specific localisation pattern of PLC ζ in human sperm and ascertain whether there is a predominant localisation pattern in control sperm which have normal oocyte activation ability, and compare with an OAD patient, to shed light on the possibilities of using quantitative PLC ζ analyses as a fertility biomarker.

3.4.1 Quantitative and proportional analyses of PLC ζ in fertile controls and an OAD patient

Samples were obtained from the OFU for PLC ζ screening with appropriate ethical consent. These included six controls (men with proven oocyte activation ability) and a single OAD patient (who had previous ICSI-failed cycles). Quantitative analysis of total PLC ζ in the samples showed significant variability, with 3 out of the 6 control subjects (50%) exhibiting total PLC ζ levels similar to the OAD patient. Although the relative mean level of PLC ζ in all 6 control subjects was significantly higher than the level found in the OAD patient, this would not indicate that quantifying total PLC ζ levels would serve as a reliable diagnostic biomarker for oocyte activating ability.

The findings herein correlate with a recent study recently published by our laboratory which examined a different population of 16 control subjects from the OFU. Analysis indicated that sperm from these subjects exhibited significant variability in total levels of PLC ζ (Kashir *et al.*, 2013). Moreover, the study also found OAD patients (62.5%) to have similar levels of total PLC ζ as that found in control subjects although the mean levels of all control subjects was significantly greater than the mean levels of the OAD patients (Kashir *et al.*, 2013) mirroring the findings found herein. Collectively, these findings concur with those of Grasa *et al.* (2008) regarding the variability of PLC ζ within control subjects. However, this is intriguing since previous studies have implicated the absence or reduced levels of PLC ζ in cases of OAD. The findings revealed both in this study and by Kashir *et al.* (2013), however, owing to the great variability observed, invalidate the use of the quantification of total levels of PLC ζ in a sample as a biomarker for oocyte activation ability.

More significantly, the relative proportions of sperm within a sample exhibiting PLC ζ immunofluorescence was significantly higher in the sperm of control subjects compared with the OAD patient (95% and 61% respectively). Similar findings were also derived from the study by Kashir *et al.* (2013) who found the proportion of sperm exhibiting PLC ζ immunoreactivity in control subjects was significantly greater than in OAD patients (82.6% and 27.4% respectively). This increased proportion would theoretically lead to an increased chance of successful oocyte activation, and similarly the low proportion evident in the OAD patient may indicate a reduced chance of successful oocyte activation.

Grasa *et al.* (2008) indicated that the distribution of localisation patterns of PLC ζ within fertile controls varied with the predominant localisation occurring within the equatorial region of the sperm head. The present localisation studies in OFU controls also exhibited variance in localisation with no single localisation pattern amongst the seven permutations being predominant. However, out of the three populations, the PLC ζ population that was the most predominant in all control samples, whether alone or in combination with other populations, was the post-acrosomal population. Indeed, contrary to Grasa *et al.* (2008), none of the samples from the OFU had a predominant equatorial PLC ζ localisation, unless it was in combination with a post-acrosomal population. Indeed, 67% (4/6) of the samples had a simultaneous post-acrosomal and equatorial localisation pattern as the predominant pattern in the sperm analysed within the respective sample. Out of the 61% of sperm in the OAD patient that did exhibit PLC ζ , again sperm exhibiting all the different localisation patterns were observed, and the predominant localisation pattern was the post-acrosomal population alone (20%) followed by the simultaneous post-acrosomal and acrosomal populations (16%). Kashir *et al.* (2013) also showed that the predominant

localisation within the OAD patients that exhibited PLC ζ to be the post-acrosomal population, and on this basis, the authors suggested that this population may not be sufficient for successful oocyte activation. The authors also concur with Grasa *et al.* (2008) regarding the equatorial population as the predominant localisation amongst fertile controls. The present study disagrees with both studies, instead suggesting a stronger role for the post-acrosomal population for oocyte activation.

3.4.2 DGW selects for sperm exhibiting PLC ζ

Further control sperm samples were obtained from a collaborative IVF clinic in Venezuela in two separate batches. The first batch was frozen upon collection in Venezuela and thawed and fixed upon arrival at our laboratory in the UK. Among the 13 control samples, 9 underwent DGW and the remaining 4 were frozen without DGW. Quantitative analysis of total PLC ζ levels was undertaken in both groups, and statistical analysis indicated a highly significant increase in total levels of PLC ζ in the DGW washed samples compared with the raw samples. Moreover, a greater proportion of non-DGW sperm possessed sperm that did not exhibit PLC ζ (mean of 14% among the 4 samples) compared with DGW sperm (5.2% among the 9 samples). These findings concur with a recent study, also undertaken in our laboratory, which found that DGW significantly elevated the proportion of sperm which exhibited PLC ζ (Kashir *et al.*, 2011), and since DGW selects the most motile and morphologically normal sperm (Allamaneni *et al.*, 2005), it is not surprising why these DGW selected sperm would have the highest proportion of the oocyte activation factor.

3.4.3 Preliminary quantitative analyses suggest a decline in total PLC ζ levels with increasing male age

Control DGW samples from the first batch delivered from Venezuela were categorised into age groups and mean relative immunofluorescence representing total PLC ζ levels were analysed for each age category. Interestingly, albeit a very small sample size, a downward trend was observed in the total levels of PLC ζ with increasing age, but no appropriate statistical analysis was undertaken due to the small sample size. These preliminary findings warranted further work using a larger sample size, and since logistical difficulties were encountered on transporting cryopreserved samples by air, it was requested that samples be washed and fixed upon collection. Indeed, this was important in order to avoid factors which may have influenced PLC ζ levels in the samples, particularly freezing. Indeed studies have shown that cryopreservation has detrimental effects upon PLC ζ levels (Kashir *et al.*, 2011), and other proteins (Gadea *et al.*, 2011) as well as sperm viability, motility and morphology (Ozkavukcu *et al.*, 2008). Cryopreservation has also been shown to induce DNA fragmentation and oxidative damage (Zribi *et al.*, 2010).

In this preliminary investigation using the first batch of samples from Venezuela, the proportion of sperm exhibiting PLC ζ did not change with advancing age, nor did specific localisation patterns. Similar to the OFU samples, the predominant localisation pattern in these samples was the post-acrosomal region. However, unlike the OFU samples, it was this population alone, not in combination with other populations. Findings from this batch formed a good preliminary base on which to further investigate the effect of age on total PLC ζ levels and localisation patterns.

3.4.4 Male age does not affect total PLC ζ levels in human sperm and the predominant PLC ζ population is the post-acrosomal population across all age groups

The second batch of samples from Venezuela provided a larger sample size with which the effect of age could be examined further. These samples underwent DGW and fixation upon collection in Venezuela and hence were not frozen/thawed in the UK laboratory. Samples underwent the same fluorescent immunocytochemistry and quantitation protocols. Again, total PLC ζ levels showed a wide range of variation amongst the samples in relative mean fluorescence. However, when samples were categorised into age groups, unlike the first batch, there was no apparent trend in PLC ζ immunofluorescence with increasing age. Statistical analysis using one-way ANOVA showed there was no significant difference between any of the age groups. Furthermore, proportional analysis did not show any significant effect of age upon the proportion of sperm exhibiting PLC ζ relative to those that did not. Localisation studies, despite showing variability in PLC ζ localisation patterns, showed that there were two predominant localisation patterns, the simultaneous post-acrosomal and equatorial populations, and sperm that exhibited all three populations. Indeed, 91-99% of sperm exhibited the post-acrosomal population, whether alone or in combination with other populations.

Grasa *et al.* (2008) reported that the predominant localisation pattern in control human sperm occurred in the equatorial region and Kashir *et al.* (2013) agreed with this suggestion. The present study, however, strongly suggests that the post-acrosomal population is the predominant population in control sperm. This may be in combination with other populations or alone. In fact, the equatorial population appears to be the most common localisation that is expressed simultaneously with the post-acrosomal population

with 77-94% of sperm exhibiting this population. Intriguingly, the study by Kashir *et al.* (2013), which also analysed OFU samples but of a larger sample size, as well as a sample from Belgium, found that there was no predominant localisation pattern amongst control subjects, but a predominant post-acrosomal localisation in OAD patients. The former was also evident in the present study amongst the control subjects from the OFU.

Further work is warranted as to why there appears to be predominant localisation patterns in the samples from Venezuela but not in the samples in Oxford. Multiple factors may be responsible. The team in Venezuela undertook the DGW and fixation protocol according to instructions delivered to them by our laboratory. Whether there are differences in the solutions or techniques used remains unknown. Previous work has shown that there are bio-geographical variations in semen quality (Swan *et al.*, 2003; Maya *et al.*, 2009). Whether such changes may also influence PLC ζ localisation patterns remains unknown, although total levels of PLC ζ immunofluorescence was similar in samples obtained from Oxford and Venezuela.

It may indeed be that a predominant localisation pattern is not required for successful oocyte activation, but rather its mere presence in sperm regardless of its localisation. Nevertheless, the present study does support the notion that the predominant localisation of the oocyte activation factor in fertile sperm would be on the lateral side of the sperm head, at the site of gamete fusion (post-acrosomal and equatorial), which this study has shown clearly. Indeed, this population would thus be most suited to contain the oocyte activation factor being the site of gamete fusion, thus allowing direct and rapid release into the oocyte following fusion (Sutovsky *et al.*, 2003).

3.4.5 Sperm concentration reduced significantly with age

This study concurred with previous studies in detecting a decline in sperm concentration with increased age. A significant decrease was found from the lowest age group to the highest aged group ($p < 0.05$). Many studies have identified similar reductions in both this semen parameter as well as motility (Sloter *et al.*, 2006; Maya *et al.*, 2009), but findings herein did not find any significant changes in motility in the sample size that was provided. It would be interesting to investigate this further with a larger sample size, particularly with the recent observations by Kashir *et al.* (2013) who found that sperm samples that displayed significantly decreased motility amongst the fertile controls, as well as all the OAD patients, also exhibited the least proportion of sperm exhibiting PLC ζ . This correlates with a previous study by Stalf *et al.* (2005) who demonstrated that ICSI using immotile sperm resulted in a lower rate of successful fertilisation.

3.4.6 Limitations of the present study

Whilst previous work pertaining to the effect of age on sperm quality does show that there is a deleterious effect on the genetic integrity and semen parameters on sperm with increasing male age, similar effects were not evident on the expression of PLC ζ . This study, however, did not include males of advanced male age owing to clinical limitations, and whilst previous studies have examined males over an extended range of ages, our age range was limited to 21-48 years. Examining males at a more advanced age, as well as increasing sample size, would provide more conclusive findings in future.

The polyclonal anti-PLC ζ antibody used in this study was the same antibody used by Grasa *et al.* (2008) and Kashir *et al.* (2013). Grasa *et al.* (2008) confirmed specificity by producing a series of peptide blocking experiments. However, the polyclonal nature of this antibody may underlie the observed variance in localisation patterns amongst studies. Indeed, such variance has not only been observed in fluorescent immunocytochemical studies, but in immunoblotting studies too. Grasa *et al.* (2008) and Heytens *et al.* (2009), using the same antibodies used for immunofluorescence studies, have shown immunoblots of sperm preparations from fertile controls (Grasa *et al.*, 2008) and from globozoospermic patients (Heytens *et al.*, 2009), exhibiting various-sized bands of molecular weight which were lower than that expected for PLC ζ . It is unknown whether these differences in size are real or due to the antibody (Grasa *et al.*, 2008; Heytens *et al.*, 2009; Kashir *et al.*, 2013). The synthesis of a monoclonal antibody is imperative in order that it can be determined whether immunofluorescence, or immunoblotting, or both, can be useful clinically.

More pertinent to the aims of this study, is the repetition of the observations by Kashir *et al.* (2013), in which some of the present control samples exhibited similar PLC ζ levels to OAD patients. Thus, clinical application as a diagnostic is not just a simple case of quantifying total PLC ζ levels as previously thought. In fact, it has been shown both by Kashir *et al.* (2012) and in this study that proportional analysis of sperm exhibiting PLC ζ relative to those that do not would provide a far better biomarker of fertility. However, repetition of these quantitative studies is required with a more specific monoclonal antibody to confirm findings.

3.4.7 Future directions

Further work which time did not allow for in the present study should include immunoblot analysis of sperm extracts using an anti-PLC ζ antibody to ascertain whether the different localisation patterns are reflected in different-sized bands. Moreover, activity assays using sperm from each age group and ascertaining the resultant Ca²⁺ release profile following micro-injection into oocytes would be very informative, along with assessment of the ability of sperm to successfully activate oocytes. Recently, a new collaboration has been formed by Dr Kevin Coward (Oxford University) and Professor Karl Swann (Cardiff University), and with an approved HFEA licence and appropriate ethical authorities, will set up such a screening assay further to the current PLC ζ screening service which Dr Kevin Coward has instigated for the first time in the UK in his laboratory which at present relies upon immunofluorescent and genetic mutation analysis only.

Maternal age is generally correlated with paternal age, and the contributions of each factor are challenging to separate (Whitcomb *et al.*, 2011). Since there is no effect of male age on the levels of total PLC ζ or the proportion of sperm exhibiting PLC ζ , it would thus be important to investigate maternal factors within the oocyte that interact with PLC ζ . More and more evidence is surfacing regarding the importance of oocyte factors in the fertilising potential of an oocyte. Already, evidence has shown that the oocyte undergoes cellular arrangement and modifications during maturation to optimise the internal environment for maximal Ca²⁺ responses on sperm fusion (Wakai *et al.*, 2012), and dysfunction of this arrangement, such as that occurring during aging of the oocyte, leads to an impedance of the fertilising process (Zhang *et al.*, 2011). An advanced understanding may allow the

assessment of Ca^{2+} dynamics within the oocyte for oocyte viability (Ajduk *et al.*, 2011; Swann *et al.*, 2012).

Furthermore, malformations in the non-genomic pathway of inheritance – epigenetic processes – such as parental imprinting may have serious detrimental effects on offspring, both pre- and post-natal and even into adulthood (Weiner-Megnazi *et al.*, 2012). Indeed epigenetic imprinting, which alters the levels of gene transcription, may contribute to offspring of older Fathers having diseases including neurocognitive disorders such as autism (Croen *et al.*, 2007), schizophrenia (Sipos *et al.*, 2004), bipolar disorders, or even malignancy (Weiner-Megnazi *et al.*, 2012). Indeed, associations between advanced paternal age and such diseases have been ascertained, and require further investigation.

More recently, a study analysing the elemental composition of human semen has identified significant differences in concentrations of certain elements in sperm and seminal plasma (Schmid *et al.*, 2013). The authors found that older men have significantly higher concentrations of copper, Ca^{2+} and zinc in their sperm, and higher levels of sulphur in their seminal plasma. These elements were quantitatively associated with increased risks for poorer semen quality and genomic sperm defects (Schmid *et al.*, 2013).

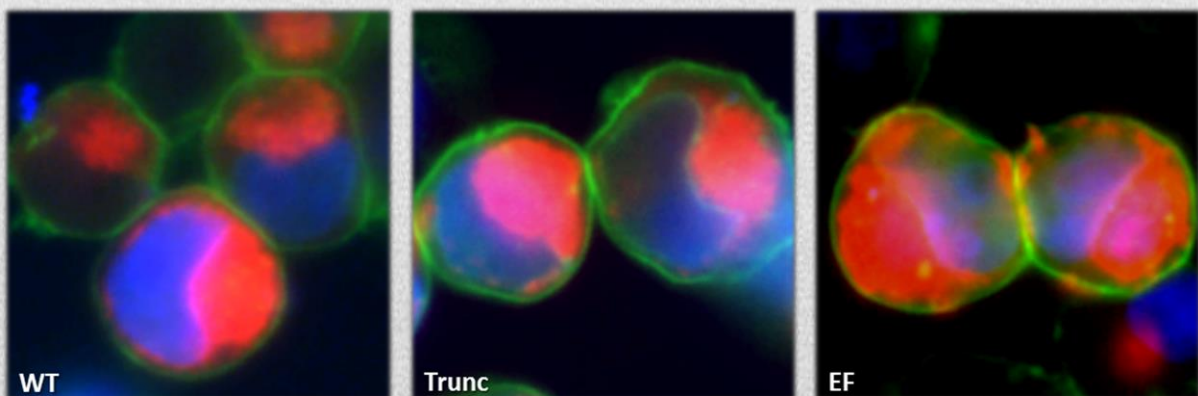
In view of the growing social phenomena of delaying family planning and contemplating parenthood at advanced maternal and paternal ages (Schmidt *et al.*, 2012), along with the emerging indications associated with this trend, further investigations are required into the relative effects of female and male factors, as well as the combined effect of both higher female and male age on infertility and reproductive outcome. This is important to provide social and public awareness of the potential impacts of delaying

parenthood in order that couples are able to make well-informed decisions, as well as offer appropriate counselling to patients of advanced age attending fertility clinics.

3.4.8 Summary of findings

-  There was great variability in the levels of total PLCζ amongst fertile controls
-  Quantitative analysis of total levels of PLCζ may not provide an accurate fertility biomarker, as levels in some fertile controls are similar to levels found in the OAD patient
-  Proportional analysis of sperm exhibiting PLCζ relative to those that do not presented significant differences between fertile controls and an OAD patient
-  DGW selects sperm with the highest levels of PLCζ as well as the highest proportion of sperm exhibiting PLCζ
-  Age does not have an effect upon the total levels of PLCζ in sperm
-  Sperm concentration decreases with increased age
-  The predominant population of PLCζ in fertile control human sperm is the post-acrosomal population, whether alone or in combination with other populations.

Chapter 4 | THERAPEUTIC APPLICATIONS AND MECHANISM OF ACTION OF PLC ζ



4.1 Introduction

Progress in the field of ART, and in particular micromanipulation, signals a new era in the management of male factor infertility. Such techniques now offer couples, who are otherwise deemed irreversibly infertile, the chance of conceiving their own genetic child. ICSI offered a revolutionary breakthrough in the treatment of male factor infertility, where, unlike IVF which necessitates an optimal sperm number and function to successfully fertilise an oocyte, ICSI involves the direct injection of a single selected spermatozoon into the oocyte (Palermo *et al.*, 1992). ICSI may be performed using fresh or cryopreserved ejaculate sperm, or using micro surgically-extracted epididymal or testicular sperm. Despite this pioneering approach, 1-5% of ICSI cycles still fail (Yanagida *et al.*, 2008; Nasr-Esfahani *et al.*, 2010) which affects over 1200 couples a year in the UK (HFEA, 2010) thus representing a significant problem. A deficiency in the mechanism of oocyte activation is thought to be the cause of around 40% of such cases (Sousa and Tesarik, 1994; Mahutte and Arici, 2003; Swann *et al.*, 2004).

With substantial evidence advocating that PLC ζ is indeed the oocyte activating factor: previous work demonstrating that the micro-injection of both mouse PLC ζ cRNA and recombinant protein into mouse and bovine oocytes results in the initiation of oscillations and oocyte activation, stimulating embryonic development to the blastocyst stage (Cox *et al.*, 2002; Saunders *et al.*, 2002; Kouchi *et al.*, 2004; Campbell and Swann, 2006; Yu *et al.*, 2008; Ross *et al.*, 2008, 2009); the injection of human PLC ζ cRNA causes Ca²⁺ oscillations and the activation of pre-implantation development in aged human oocytes (Rogers *et al.*, 2004); and the co-injection of PLC ζ cRNA with infertile sperm triggers Ca²⁺ oscillations and

rescues activation in mouse oocytes (Yoon *et al.*, 2008), all provide significant credence to the clinical use of PLC ζ as a therapeutic. However, for PLC ζ to be used as a therapeutic it would not be possible to inject PLC ζ RNA as this has been shown to impede normal pre-implantational development through gene expression irregularities, with developmental defects observed in some embryos (Ducibella *et al.*, 2002; Rogers *et al.*, 2004; Ozil *et al.*, 2006). Moreover, it has been shown that injected PLC ζ RNA could potentially convert to cDNA which may then incorporate into the genome (Kashir *et al.*, 2010).

With current AOA options such as ionophores presenting concern as a treatment for oocyte activation failure, due to their potential detriment on gene expression and embryo development caused by their 'unnatural' Ca²⁺ oscillatory profile (Nakada and Mizuno, 1998; Ozil *et al.*, 2006); and the co-micro-injection of PLC ζ cRNA during ICSI presenting difficulties since the amount of translated PLC ζ protein will be uncontrollable, and the cRNA may lead to the transcription and insertion of cDNA into the genome; it follows therefore, that the availability of a purified, active recombinant human PLC ζ protein would provide the optimal, endogenous physiological alternative as a therapeutic application in the clinic for oocyte activation failure.

Since its identification in 2002, many studies have attempted to meet the challenge of developing an active recombinant form of PLC ζ protein. Thus far, it has proved difficult to produce an active purified form of recombinant PLC ζ protein in a reproducible manner, but its potential importance warrants significant attention. Prior to the commencement of this study, only one study provided a hint of success. Kouchi *et al.* (2004) successfully purified a mouse recombinant PLC ζ protein, using a baculovirus/Sf9 expression system, which exhibited high Ca²⁺ sensitivity and induced Ca²⁺ oscillations upon injection into mouse

oocytes (Kouchi *et al.*, 2004). However, these oscillations were of a much higher frequency than those seen during normal fertilisation and results have proved difficult to replicate.

For protein therapeutics to be effective, they must be produced in biologically active forms, requiring proper folding and PTM. Cultivated mammalian cells have become the dominant system for the synthesis of recombinant proteins for therapeutic applications, due to their capacity for proper protein folding, assembly and PTM (Wurm, 2004). The first therapeutic protein produced from recombinant mammalian cells was human tissue plasminogen activator in 1986. Today 60-70% of all recombinant protein therapeutics are produced in mammalian cells (Wurm, 2004). Several cell lines have been used for recombinant protein production, most commonly Chinese hamster ovary (CHO) cells, cells derived from mouse myeloma (NS0), baby hamster kidney (BHK), human embryonic kidney (HEK293) and human retinal cells.

Previous attempts have been undertaken by the Coward laboratory to purify PLC ζ using the mammalian HEK293T cell line. This followed attempts using a bacterial expression system which succeeded in purifying a recombinant PLC ζ protein which was inactive when injected into mouse oocytes. Efforts thus diverted to employing a mammalian expression system to allow appropriate protein folding and PTM thought to be required for protein activity, and a C-terminal His-tag was used for the purification procedure. Expressed His-tagged proteins can be purified and detected easily because the string of histidine residues binds to several types of immobilised metal ions, including nickel. However, these endeavours failed again, thought to be due to the conformational folding of the protein, leaving the C-terminal His-tag unavailable for detection by the nickel-NTA beads required

for affinity purification of the His-tagged proteins [Figure 1.9, Chapter 1] (Dr Kevin Coward, personal communication).

Given PLC ζ 's role in oocyte activation, and the research indicating links between this protein and certain types of male factor infertility, research into the function and mechanism of action of PLC ζ , and how mutations and abnormalities affect its physiological function, is fundamental for developing therapeutics capable of replacing or augmenting endogenous PLC ζ activity, thus providing a more physiological therapeutic alternative to current artificial options (such as AOA). Moreover, deeper understanding of the structure-function relationship of PLC ζ may enable the development of a male contraceptive by producing inhibitors.

A genetic mutation causing PLC ζ action deficiency was discovered by Heytens *et al.* (2009). This involved a substitution mutation of a Histidine to a Proline within the Y domain of the catalytic site of the protein (PLC ζ_{H398P}) in an infertile male diagnosed with OAD [Figure 4.1.1A]. Micro-injection of PLC ζ_{H398P} cRNA into mouse oocytes failed to induce Ca²⁺ oscillations whilst the injection of sperm possessing this mutation resulted in an atypical pattern of Ca²⁺ release (Heytens *et al.*, 2009). The equivalent mutation in mice was produced in this conserved region of the protein, producing PLC ζ_{H435P} (Nomikos *et al.*, 2011), which confirmed that the mutation caused major structural changes to the protein, resulting in the functional inactivation of PLC ζ . They also negated the suggestion by Heytens *et al.* (2009) of the H398P mutation exerting a dominant-negative inhibition behaviour on PLC ζ , leaving unexplained the rationalisation for the failure of the heterozygous infertile patient sperm to cause Ca²⁺ oscillations following ICSI (Nomikos *et al.*, 2011).

More recently, a novel point mutation was discovered in the same patient with the H398P mutation, involving a further substitution of a Histidine for a Leucine residue in the catalytic X domain of the PLC ζ protein (PLC ζ_{H233L}) (Kashir *et al.*, 2012) [Figure 4.1.1B]. Micro-injection of PLC ζ_{H233L} cRNA resulted in an abnormal Ca²⁺ release profile and like the H398P mutation, a failure to activate oocytes (Kashir *et al.*, 2012). Interestingly, and for the first time, the study showed that both the PLC ζ_{H398P} and PLC ζ_{H233L} mutations, which are heterozygous in nature, originated from different parental origins – the PLC ζ_{H398P} paternally, and the PLC ζ_{H233L} maternally – representing the first description of an autosomal point mutation resulting in male fertility through maternal origin (Kashir *et al.*, 2012).

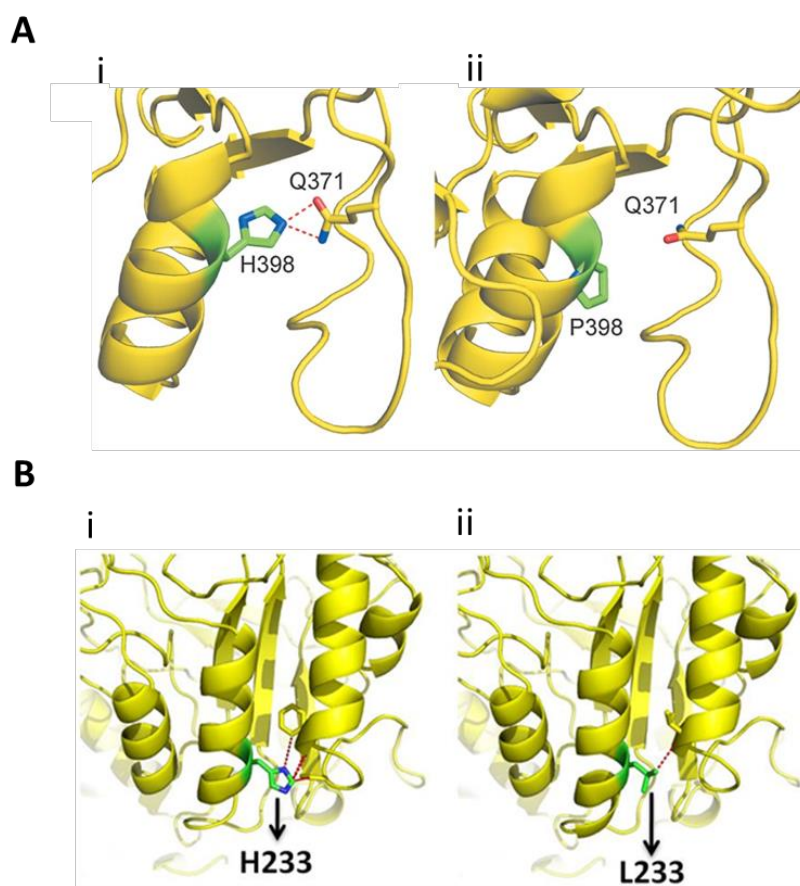


Figure 4.1.1 | Schematic representations of the effects of the Histidine > Proline (PLC ζ_{H398P}) (A) and Histidine > Leucine (PLC ζ_{H233L}) (B) point mutations identified by Heytens *et al.* (2009) and Kashir *et al.* (2012) in an infertile male patient diagnosed with OAD. For both (A) and (B), schematics represent local protein folding before mutation (i) and after mutation (ii).

Using the mammalian cell line HEK293T, this study aimed to produce a purified active recombinant wild-type (WT) PLC ζ protein. Further to previous attempts by the

Coward laboratory, the present study used an N-terminal His-tag as opposed to a C-terminal tag used previously. As well as producing a WT recombinant protein with therapeutic intentions, this study also attempted to investigate the mechanism of action of PLC ζ by characterising the relative effect of mutation upon protein structure, function and interactive properties, by producing recombinant proteins exhibiting the two known mutations (PLC ζ_{H233L} and PLC ζ_{H398P}). Furthermore, artificially-induced mutations were also deployed in the current study. A recent study by Kashir *et al.* (2011) produced an artificially-induced PLC ζ protein exhibiting a frameshift mutation (PLC $\zeta_{Frameshift}$), with a partial Y and complete C2 domain deletion of its structure. The authors found that this mutation caused total loss of activity when injected into HEK293T cells in a similar fashion to PLC ζ_{H398P} . When this mutant isoform was fluorescently tagged and expressed in HEK293T cells, it expressed a lower level of fluorescence than the WT, suggesting that it may be less stable (Kashir *et al.*, 2011). Producing a recombinant protein of this isoform may enable further understanding of the effect of the mutation, and thus the structural importance of PLC ζ . Similarly, another artificially-induced mutation lacking the EF hands was created in the present study (PLC $\zeta_{Truncated}$) in recombinant protein form. By injecting these recombinant proteins into mouse oocytes and assessing activity, it was hoped that we would gain a better understanding of the structure-function relationship of PLC ζ .

Furthermore, in the aforementioned study by Kashir *et al.* (2011), the authors used a fluorescently-tagged PLC ζ in HEK293T cells to indicate that PLC ζ does not localise to the plasma membrane, but instead localises to discrete regions within the cell cytoplasm adjacent to the nuclear envelope (Kashir *et al.*, 2011). This is important as it is not yet known how PLC ζ binds to its PIP₂ substrate given its lack of a PH domain. All other PLCs bind

to the plasma membrane via the PH domain. The present study investigated the localisation of PLC ζ expression by analysing a variety of fluorescently-tagged human PLC ζ protein isoforms in HEK293T cells as a model system. This employed WT PLC ζ (PLC ζ_{WT}), the truncated PLC ζ isoform (PLC $\zeta_{Truncated}$), and a protein formed by the EF hands alone (PLC ζ_{EF}).

The mammalian cell line HEK293T was used for all aspects of this chapter, which will be separated into two aims:

AIM 1 – Preparation of recombinant human PLC ζ protein

- Purified recombinant WT PLC ζ protein for therapeutic options
- Purified recombinant mutant PLC ζ protein (PLC ζ_{H233L} , PLC ζ_{H398P} , PLC $\zeta_{H233L+H398P}$, PLC $\zeta_{Frameshift}$ and PLC $\zeta_{Truncated}$) in order to investigate the structure-function relationship of PLC ζ .

AIM 2 – Investigating the localisation and level of expression of PLC ζ to follow on from Kashir *et al.* (2011) who suggested that PLC ζ was localised within discrete regions within the HEK293T cells. This was undertaken using:

- Fluorescently-tagged WT PLC ζ
- Fluorescently-tagged mutant PLC ζ (PLC $\zeta_{Truncated}$, PLC ζ_{EF}).

4.2 Materials and Methods

AIM 1 | Preparation of recombinant human PLC ζ protein

4.2.1 Cloning and sequencing of human PLC ζ _{WT}, PLC ζ _{H233L}, PLC ζ _{H398P}, PLC ζ _{H233L+H398P}, PLC ζ _{Frameshift} and PLC ζ _{Truncated} constructs

cDNA encoding full-length human PLC ζ _{WT}, PLC ζ with mutations PLC ζ _{H233L}, PLC ζ _{H398P}, PLC ζ _{H233L+H398P}, PLC ζ _{Frameshift} and PLC ζ _{Truncated} [Figure 4.2.1] were amplified by PCR following the program shown in Table 4.2.1 using templates previously designed in the Coward laboratory. Resultant PCR fragments were separated by electrophoresis on a 1% agarose gel in TAE stained with ethidium bromide against a 1kb DNA ladder (NEB, UK) to confirm that the desired fragments had been successfully amplified. PCR products were then purified using the QIAquick PCR purification kit (Qiagen, UK) in accordance with the manufacturer's instructions [See Appendix 7]. The resultant linearised inserts, and the vector in which they were to be cloned, the pcDNA3.1/HisA vector (Invitrogen, UK) [Figure 4.2.2], a vector used for high-level expression in mammalian cells which contains an N-terminal 6xHis-tag sequence, were cut with their respective restriction endonuclease enzymes depending on their cloning site: PLC ζ _{WT}, PLC ζ _{H233L}, PLC ζ _{H398P}, PLC ζ _{H233L+H398P} were cloned using *Eco*R1 and *Not*1 sites, PLC ζ _{Frameshift} using *Eco*R1 and *Bam*H1 sites and PLC ζ _{Truncated} using *Bam*H1 and *Not*1 sites. The insert and vector were then ligated using the Rapid DNA ligation kit (Roche Applied Science, Germany) [Figure 4.2.3].

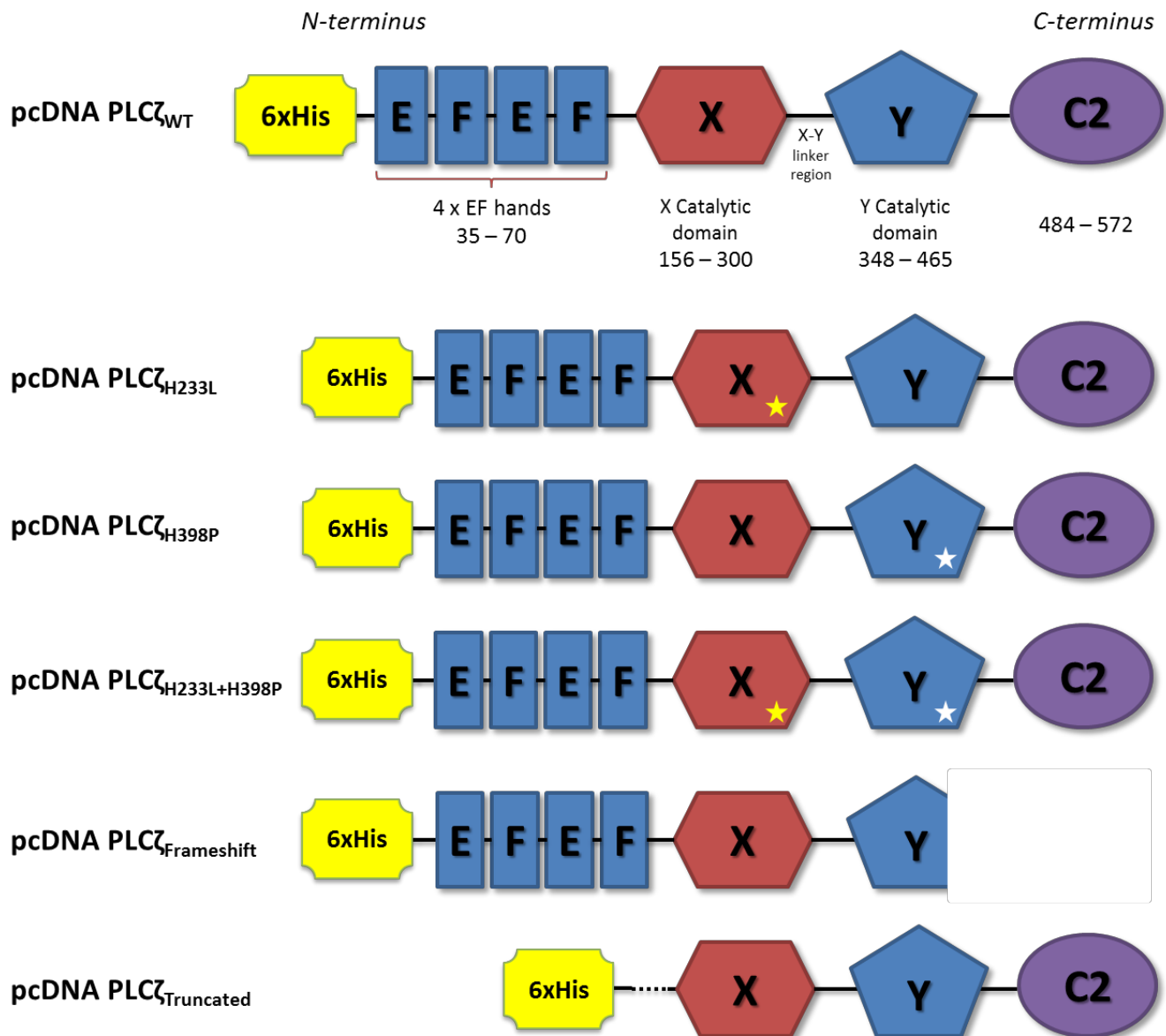


Figure 4.2.1 | **Schematic of cloned N-terminal His-pcDNA PLC ζ WT and mutated constructs.** (EF = EF hands of PLC structure; X and Y = catalytic domains; C2 = C2 terminal of PLC structure). Yellow star denotes the Histidine > Leucine point mutation (PLC ζ_{H233L}) on the X domain; White star denotes the Histidine > Proline (PLC ζ_{H398P}) point mutation on the Y domain identified in the OAD patient by Heytens *et al.* (2009) and Kashir *et al.* (2012). PLC $\zeta_{Frameshift}$ and PLC $\zeta_{Truncated}$ are artificially-induced mutations with the absence of part of the Y domain and all of C2, and EF domains respectively.

[Target sequence sizes: 1824 base pairs for PLC ζ_{WT} , PLC ζ_{H233L} , PLC ζ_{H398P} and PLC $\zeta_{H233L+H298P}$; 1278 base pairs for PLC $\zeta_{Frameshift}$; and 1614 base pairs for PLC $\zeta_{Truncated}$]

Table 4.2.1 | PCR program used to amplify human PLC ζ _{WT}, PLC ζ _{H233L}, PLC ζ _{H398P}, PLC ζ _{H233L+H398P}, PLC ζ _{Frameshift} and PLC ζ _{Truncated} constructs.

Step	Temperature	Duration
1. Initial denaturation	94°C	4 minutes
2. Denaturation	94°C	10 seconds
3. Primer annealing	58°C	1.1 minutes
4. Primer extension	72°C	2 minutes
<i>Steps 2-4 were repeated 9 times</i>		
5. Denaturation	94°C	15 seconds
6. Primer annealing	58°C	30 seconds
7. Primer extension	72°C	2 minutes
<i>Steps 5-7 were repeated 19 times</i>		
8. Final elongation	72°C	8 minutes
9. Store	8°C	

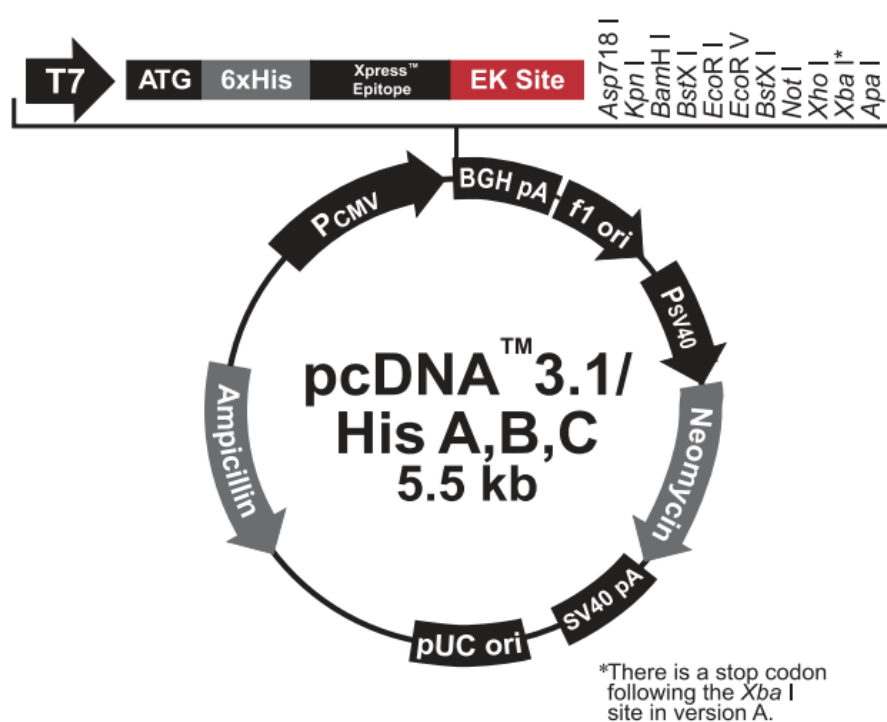


Figure 4.2.2 | Map of pcDNA3.1/His vector showing the different cloning sites. A 5.5 kb vector derived from pcDNATM3.1, with an N-terminal polyhistidine tag, designed for high-level expression and purification of recombinant proteins in mammalian hosts.

The ligated N-terminal pcDNA3.1/HisA PLC ζ constructs were then transformed into TOP10 competent cells (Invitrogen, UK) by heat shock at 42°C for 30 seconds. After culturing transformed colonies, a MiniPrep (Qiagen, UK) was performed to purify the plasmid DNA of each construct. To confirm successful cloning of the insert into the vector, a double digest using the same restriction enzymes used for cloning for each respective construct was carried out, and the product separated by a 2% agarose gel stained with ethidium bromide, after which constructs were verified by DNA sequencing (Source Bioscience UK, Oxford) using overlapping primers to cover the full length sequence of each construct [Table 4.2.2; Figure 4.2.4]. This was followed by alignment of the sequenced data against the open reading frame (ORF) of each template.

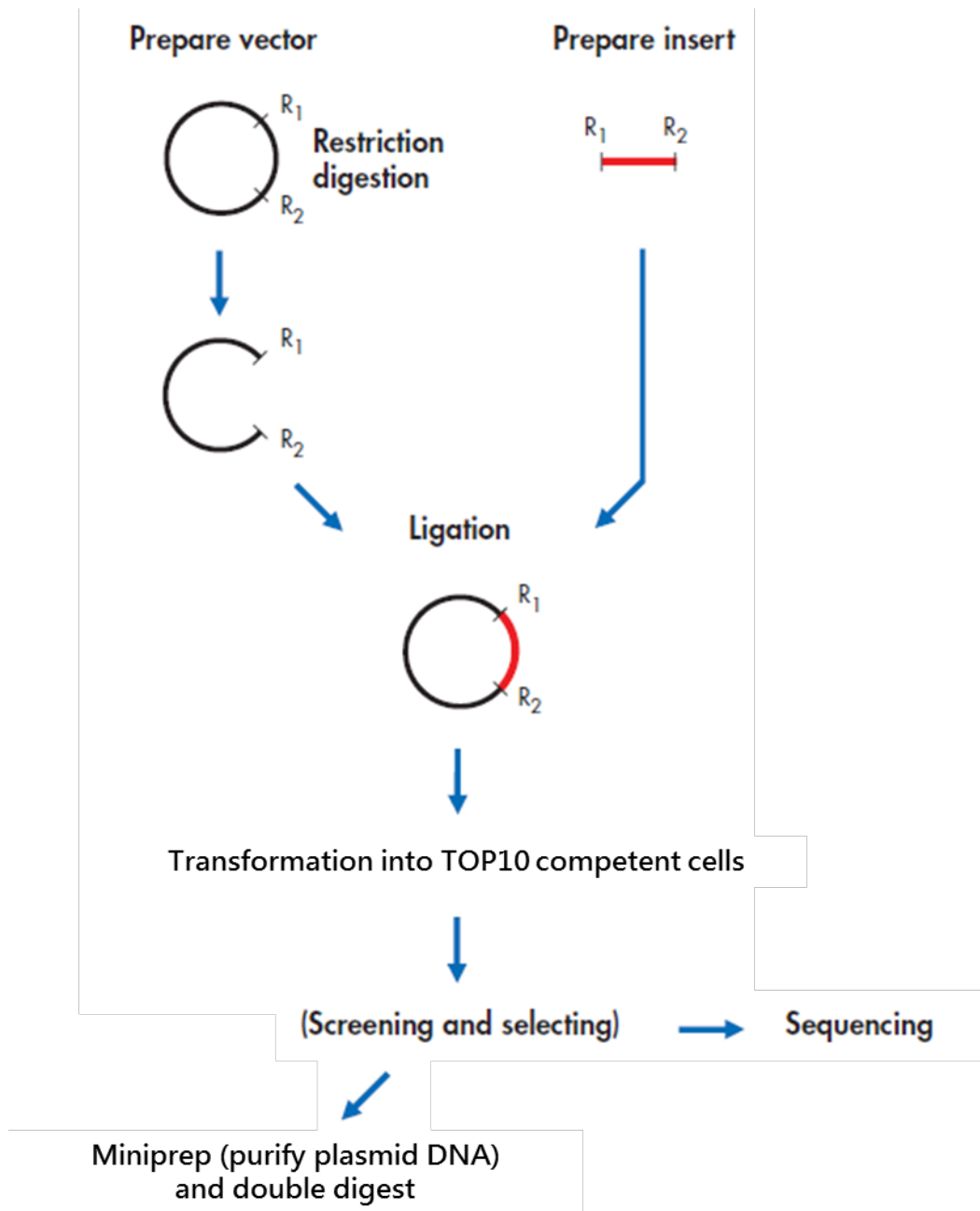


Figure 4.2.3 | Summary of the cloning procedure of the PLC ζ constructs. pcDNA3.1/HisA vector and insert were cut with their respective restriction endonuclease enzymes depending on their cloning site, then insert and vector were ligated and the plasmid DNA transformed into TOP10 competent cells. Colonies were selected and cultured, and plasmid DNA of each construct purified. A double digest was performed to determine successful cloning. Constructs were sequenced for verification.

Table 4.2.2 | Sequencing primers for PLCζ open reading frame.

Primer	Position (bases)	Sequence
T7	<i>Stock primer at Source Bioscience UK, Oxford</i>	
Zeta SEQ1	10 – 800	AGATGGTTTTTGTCAAAGATTCTAG
Zeta SEQ2	650-1400	ACACACTCACAAGCAAACCTTCTG
Zeta SEQ3	1300 – 1824	AATTTCCAGACCCCTGGTCT

>1c128039
Length=1824

Score = 1339 bits (725), Expect = 0.0
Identities = 725/725 (100%), Gaps = 0/725 (0%)
Strand=Plus/Plus

T7F primer

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Query 26  ATGGAATGAGATGGTTTTTGTCAAAGATTCTAGGATGACTTCAGAGTGGAAAAATTAAC  85
Sbjct 1   ATGGAATGAGATGGTTTTTGTCAAAGATTCTAGGATGACTTCAGAGTGGAAAAATTAAC  60
Query 86  CTAGAAAAAAGCTCAGAGGTTACTTGAAAAATAGATATTGGTGCAATTATATCATGTG  145
Sbjct 61  CTAGAAAAAAGCTCAGAGGTTACTTGAAAAATAGATATTGGTGCAATTATATCATGTG  120
Query 146  AAACAGATTTTAAAGCAATGACAGGCTGAAACAAGGAAGATCACCATAGAAGAAATTT  205
Sbjct 121  AAACAGATTTTAAAGCAATGACAGGCTGAAACAAGGAAGATCACCATAGAAGAAATTT  180
Query 206  AGAGCAATTTATCGAATTATCAGGCACAGAGAGAAATTAATGAGATTTTCAACACATAT  265
Sbjct 181  AGAGCAATTTATCGAATTATCAGGCACAGAGAGAAATTAATGAGATTTTCAACACATAT  240
Query 266  TCTGAAAAACCGAAAAATCTTTTAGCAAGTAATCTGGCTCAATTTCTGACACAAGACAA  325
Sbjct 241  TCTGAAAAACCGAAAAATCTTTTAGCAAGTAATCTGGCTCAATTTCTGACACAAGACAA  300
Query 326  TATGACAGCTGAGATGAGTAAAGCTATTGCTTTTGGATCAATCAGAAATACGAGCCTATC  385
Sbjct 301  TATGACAGCTGAGATGAGTAAAGCTATTGCTTTTGGATCAATCAGAAATACGAGCCTATC  360
Query 386  GAAGAAGTTAGGAAGACACCAAAATGTCATTAGAAGTTTTTACAAGATACATGGATTCA  445
Sbjct 361  GAAGAAGTTAGGAAGACACCAAAATGTCATTAGAAGTTTTTACAAGATACATGGATTCA  420
Query 446  CGTGAATGCTACTGTTTAAAAATGAATGTAGAAAAGTTTATCAAGATATGACTCATCCA  505
Sbjct 421  CGTGAATGCTACTGTTTAAAAATGAATGTAGAAAAGTTTATCAAGATATGACTCATCCA  480
Query 506  TTAATGATATTTTATTTTCACTTCACATACACATATTTGGTATCTGATCAATTTATG  565
Sbjct 481  TTAATGATATTTTATTTTCACTTCACATACACATATTTGGTATCTGATCAATTTATG  540
Query 566  GGACCAAGTGACCTTTGGGGATATGTAAGTGCCCTTGTGAAAGGATGCGGTGTTTGGAG  625
Sbjct 541  GGACCAAGTGACCTTTGGGGATATGTAAGTGCCCTTGTGAAAGGATGCGGTGTTTGGAG  600
Query 626  ATTGACTGCTGGGATGGAGCAAAAATGAACCTGTTGTATATCATGGCTACACACTCACA  685
Sbjct 601  ATTGACTGCTGGGATGGAGCAAAAATGAACCTGTTGTATATCATGGCTACACACTCACA  660
Query 686  AGCAAACTTCGTTTAAACTGTTATCCAAGCTATACACAAGTATGCAATCATGACATCT  745
Sbjct 661  AGCAAACTTCGTTTAAACTGTTATCCAAGCTATACACAAGTATGCAATCATGACATCT  720
Query 746  GACTA 750
Sbjct 721  GACTA 725

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Sequence 1

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CCGAGTGGTGCCTCCTTTAGAAAACTGCTGCCACTGCCCAACAAGTAATGG  660
CCGAGTGGTGCCTCCTTTAGAAAACTGCTGCCACTGCCCAACAAGTAATGG  781
Query 661  CAGACAATTTGAGGCTACTTTTGGAGAGTCTTGTCTTCTGATATGCTTGATGATTTTC  720
Sbjct 782  CAGACAATTTGAGGCTACTTTTGGAGAGTCTTGTCTTCTGATATGCTTGATGATTTTC  841
Query 721  CTGATACTCTACCATCACCAGAGGCACTAA 750
Sbjct 842  CTGATACTCTACCATCACCAGAGGCACTAA 871

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Sequence 2

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Query 121  tattagtttaaaatagaaaaataggaaacccctaaaggaacccatgaagaaaaGGTTCGT  180
Sbjct 881  TATTAGTTAAAAATAGAAAAATAGGAACCTTAAGGAAACCCATGAAGAAAAAGTTCGT  940
Query 181  ATAAGCGTGGAGACAATCAGACAAGGAAACAGGGGTAAAAAGTTACCTGGAGTAATGC  240
Sbjct 941  ATAAGCGTGGAGACAATCAGACAAGGAAACAGGGGTAAAAAGTTACCTGGAGTAATGC  1000
Query 241  TTTTCAAGAAAAAGAGACCAAGGAAAGCTTCAAAATTTGCTCTGGCCTTATCTGATCTTGCA  300
Sbjct 1001  TTTTCAAGAAAAAGAGACCAAGGAAAGCTTCAAAATTTGCTCTGGCCTTATCTGATCTTGCA  1060
Query 301  TTTATACGAAAGCTGAGAAATTCAAAAGCTTCAACATTCAAGATTATATCAGCAATTTA  360
Sbjct 1061  TTTATACGAAAGCTGAGAAATTCAAAAGCTTCAACATTCAAGATTATATCAGCAATTTA  1120
Query 361  ATGAAAAATAATTTCTATTGGGGAGACACAAGCCGAAAACTTTCAAAATTCGAGTCCATG  420
Sbjct 1121  ATGAAAAATAATTTCTATTGGGGAGACACAAGCCGAAAACTTTCAAAATTCGAGTCCATG  1180
Query 421  AGTTTATTTTTCACACAGGAGTTTCAATACCAAGATATATCCCAAGCAACAAGAGCAG  480
Sbjct 1181  AGTTTATTTTTCACACAGGAGTTTCAATACCAAGATATATCCCAAGCAACAAGAGCAG  1240
Query 481  ACTCTTCTAATTTTAAATCCCAAGAAATTTTGGAAATATAGGTTGCAATGGTGGCTTTAA  540
Sbjct 1241  ACTCTTCTAATTTTAAATCCCAAGAAATTTTGGAAATATAGGTTGCAATGGTGGCTTTAA  1300
Query 541  ATTTCCAGACCCCTGGTCTGCCCATGGATCTGCAAAATGGGAAATTTTGGATATGGTG  600
Sbjct 1301  ATTTCCAGACCCCTGGTCTGCCCATGGATCTGCAAAATGGGAAATTTTGGATATGGTG  1360
Query 601  GTTCTGGATATATTTTGAACCAACATTTCTTAAGAGAGAGTAAATCATCTTTAAACCAA  660
Sbjct 1361  GTTCTGGATATATTTTGAACCAACATTTCTTAAGAGAGAGTAAATCATCTTTAAACCAA  1420
Query 661  GTAACATAAAGAGGGTATGCCAATACATACATAAAGGCTCATCAGTGGTATCCAGT  720
Sbjct 1421  GTAACATAAAGAGGGTATGCCAATACATACATAAAGGCTCATCAGTGGTATCCAGT  1480
Query 721  TGCCCTTACTCATTATCATCTTAAACAAG 750
Sbjct 1481  TGCCCTTACTCATTATCATCTTAAACAAG 1510

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Sequence 3

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Query 181  TTTGGTGTCCAAATGATCAATGAAGCAGCAGACTCGTGAATTAAAAAAATGCTTTT  240
Sbjct 1537  TTTGGTGTCCAAATGATCAATGAAGCAGCAGACTCGTGAATTAAAAAAATGCTTTT  1596
Query 241  AGTCCAAGATGGAATGAACATTCACATTTATTATTCATGTCGCCAAGTTGGCAATTGATA  300
Sbjct 1597  AGTCCAAGATGGAATGAACATTCACATTTATTATTCATGTCGCCAAGTTGGCAATTGATA  1656
Query 301  CGTTTGTGTTGAAGGTCAAGGTTTAAAGCAGGAAATGAATTTCTTGGGCAATATACT  360
Sbjct 1657  CGTTTGTGTTGAAGGTCAAGGTTTAAAGCAGGAAATGAATTTCTTGGGCAATATACT  1716
Query 361  TTGCCACTCTATGATGAACAAAGGTTATCGTCGATATCTCTGTTTCCAGAAATGGGT  420
Sbjct 1717  TTGCCACTCTATGATGAACAAAGGTTATCGTCGATATCTCTGTTTCCAGAAATGGGT  1776
Query 421  GAGAGCCTTGAGCCTGCTTCACTGTTTGTATGTTTGGTACGTCAGA 468
Sbjct 1777  GAGAGCCTTGAGCCTGCTTCACTGTTTGTATGTTTGGTACGTCAGA 1824

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Figure 4.2.4 | Figure demonstrating how the sequencing primers overlapped to cover the whole length of the PLCζ open reading frame.

4.2.2 Transfection and purification of pcDNA-PLC ζ WT, mutant and pcDNA/HisA empty vector

A mammalian system was used in order to imitate *in-vivo* activity allowing PTM. HEK293T cells were grown in Dulbecco's modified eagle medium (DMEM) with 1% (v/v) penicillin (5000 U) -streptomycin (5mg/ml) solution (Sigma, UK), 2mM L-Glutamine, Sodium Pyruvate and 10% fetal bovine serum (FBS), to a confluency of ~70% in 175cm² flasks. Cells were incubated at 37°C with 5% CO₂. Transfection was performed in 5 x 175cm² culture flasks using 30µg pcDNA-PLC ζ _{WT} per flask and 60µl jetPEI DNA transfection reagent (Polyplus transfection, France) as per manufacturer's instructions.

Transfected cells were grown for 56 hours (media was replaced 24 hours after transfection with DMEM without FBS so cells could not continue to divide), washed with cold PBS, lysed using a 0.25mm syringe in lysis buffer (50mM NaH₂PO₄, 300mM NaCl, 10mM imidazole, 0.05% Tween 20, pH 8) containing cOmplete, Mini, EDTA-free Protease Inhibitor (Roche Diagnostics Ltd, UK). After centrifugation at 7200g for 20 minutes at 4°C, the supernatant containing the cell contents was filtered through 0.45µm syringe filters (VWR, UK) and incubated in an equilibrated column of Ni-NTA agarose beads (Qiagen, UK) which had been washed with lysis buffer. After allowing binding time for the His-tagged protein (2 hours), the beads were washed by allowing wash buffer (50mM NaH₂PO₄, 300mM NaCl, 20mM imidazole, 0.05% Tween 20, pH 8) to run over the beads in the column and the protein eluted with elution buffer (50mM NaH₂PO₄, 300mM NaCl, 250mM imidazole, 0.05% Tween 20, pH 8) [Figure 4.2.5].

A NuPAGE Novex 10% Bis-Tris Gel sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (Invitrogen, UK) was run against a Novex Sharp Pre-stained

Protein Standard (Invitrogen, UK) and stained with SimplyBlue SafeStain (Invitrogen, UK) (coomassie blue) to confirm purification. A pcDNA3.1/HisA empty vector (EV) was also produced as a control for experiments with the pcDNA3.1/HisA PLC ζ constructs.

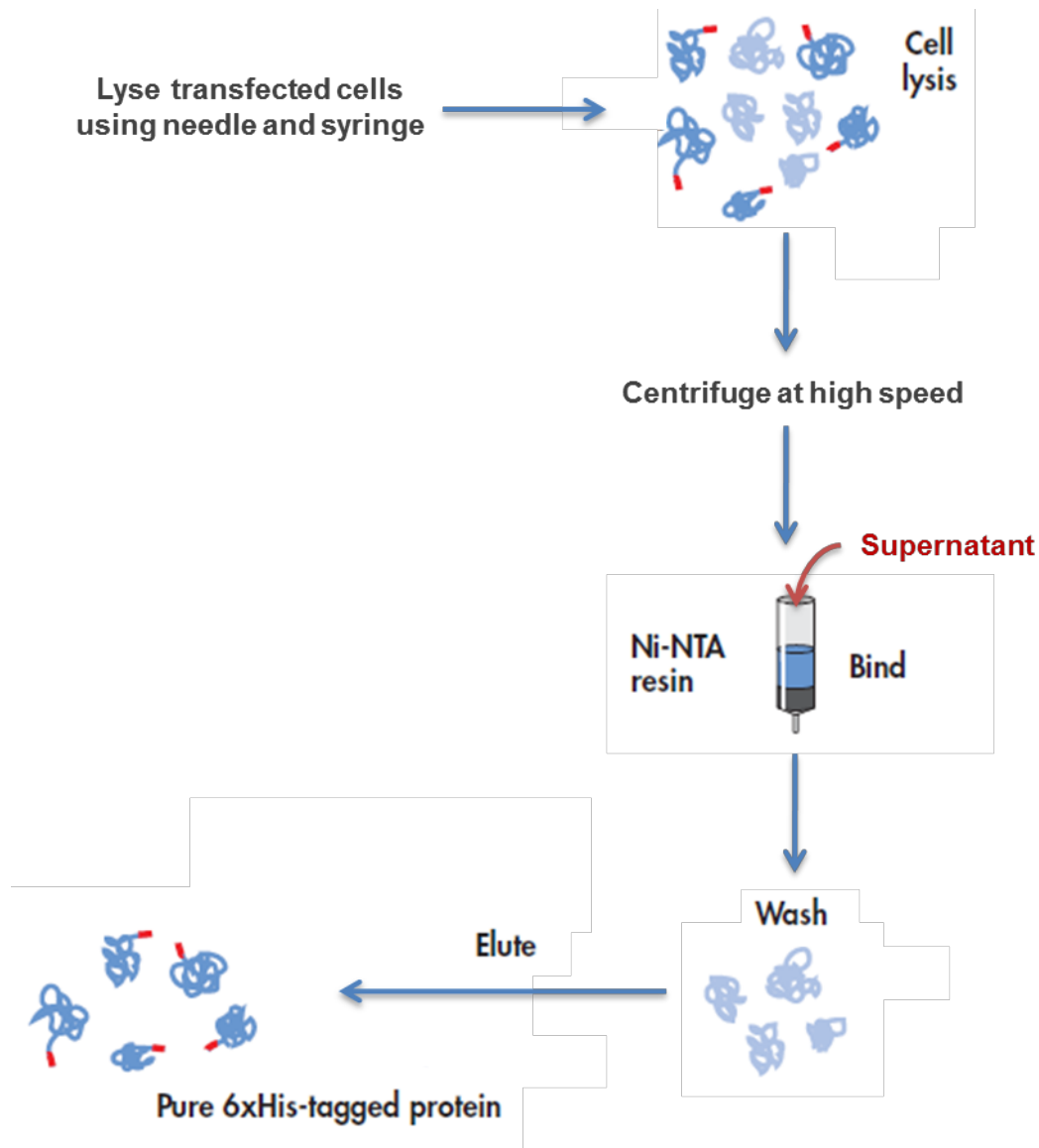


Figure 4.2.5 | Summary of the purification procedure of His-tagged pcDNA-PLC ζ proteins. Lysed transfected cells were centrifuged at high speed and the supernatant filtered. The His-tagged proteins were allowed to bind in the column containing Nickel-NTA agarose resin. The column was subsequently washed and the pure His-tagged PLC ζ proteins eluted. A separate column was used for each of the constructs (pcDNA-PLC ζ_{WT} , pcDNA-PLC ζ_{H233L} , pcDNA-PLC ζ_{H398P} , pcDNA-PLC $\zeta_{H233L+H398P}$, pcDNA-PLC $\zeta_{Frameshift}$, pcDNA-PLC $\zeta_{Truncated}$ and pcDNA/HisA empty vector).

4.2.3 Immunoblotting

Immunoblotting was used to confirm successful production and purification of recombinant human PLC ζ protein. Eluted purified protein samples were separated by SDS-PAGE on a 10% gel after denaturing at 101°C for 5 minutes with (4x) NuPAGE loading buffer (Invitrogen, UK) and (10x) NuPAGE reducing agent (Invitrogen, UK). Separated proteins were then transferred for 1 hour onto nitrocellulose membranes (GE Healthcare Life Sciences, UK) using a semi-wet transfer method. Membranes were blocked in PBS containing 0.05% Tween (Sigma, UK) and 5% dried milk powder on ice on a rocking platform for 1 hour then incubated on a rocking platform overnight at 4°C in 25 μ g/ml of the affinity-purified anti-human-PLC ζ antibody, in accordance with Heytens *et al.* (2009), made in PBS/0.05% Tween/1% dried milk powder. Membranes were then washed four times for 15 minutes in PBS/0.05% Tween and incubated for 1 hour in goat anti-rabbit secondary antibody conjugated to horse-radish peroxidase (HRP) (Thermo-Scientific, UK) applied at a dilution 1:1500 in the same diluent as for the primary antibody. The membrane was subsequently re-washed in PBS/0.05% Tween four times for 15 minutes before the antibody was visualised on the membrane using the enhanced chemiluminescence detection kit (GE Healthcare Life Sciences, UK) as per manufacturer's instructions. An HRP-conjugated His-tag antibody kit (Merck-Chemicals, UK) was also used to confirm positive immunoreaction.

4.2.4 Activity assay of pcDNA PLC ζ constructs

The purified protein which was eluted in a buffer containing NaH₂PO₄, NaCl, imidazole and Tween, was exchanged into a Ca²⁺-free medium composed of 120 mM KCl and 20 mM HEPES (pH = 7.4) using centrifugal concentrators.

AIM 2 | Investigating the localisation and level of expression of PLC ζ in HEK293T cells

4.2.5 Transfection of HEK293T mammalian cells with mCherry-PLC ζ constructs

Fluorescent mCherry tags were previously N-terminally fused by the Coward laboratory via a linker sequence to PLC ζ _{WT}, PLC ζ _{Trunc} and PLC ζ _{EF} [Figure 4.2.6]. DNA sequences encoding the three PLC ζ isoforms and mCherry alone were amplified by PCR and the High-Fidelity Master Kit (Roche Biosciences, UK). Molecular constructs were previously verified by DNA sequencing (Geneservice, Oxford).

In the present study, HEK293T cells were grown on 13mm round coverslips in DMEM with 1% (v/v) penicillin (5000 U) -streptomycin (5mg/ml) solution, 2mM L-Glutamine, Sodium Pyruvate and 10% FBS, to a confluency of ~40% in 4-well plates. Cells were incubated at 37°C with 5% CO₂. Transfection was performed using 1 μ g mCherry-PLC ζ construct per 1.92cm² well and 2 μ l jetPEI DNA transfection reagent as per manufacturer's instructions. Cells were transfected with the same construct in two sets of duplicates, for two time-points and analysed with two antibodies [Figure 4.2.7 shows what was prepared for every time-point for a single antibody (four of these were prepared for each repeat)]. Each set of duplicates were grown for 48 and 56 hours respectively as described by Kashir *et al.* (2011). Media was replaced 24 hours after transfection with DMEM without FBS so that cells could not continue to divide. At 48 and 56 hours, cells were washed three times with pre-warmed PBS and fixed for 10 minutes in 4% PFA, followed by three washes in PBS. The experiment was repeated in triplicate for analysis.

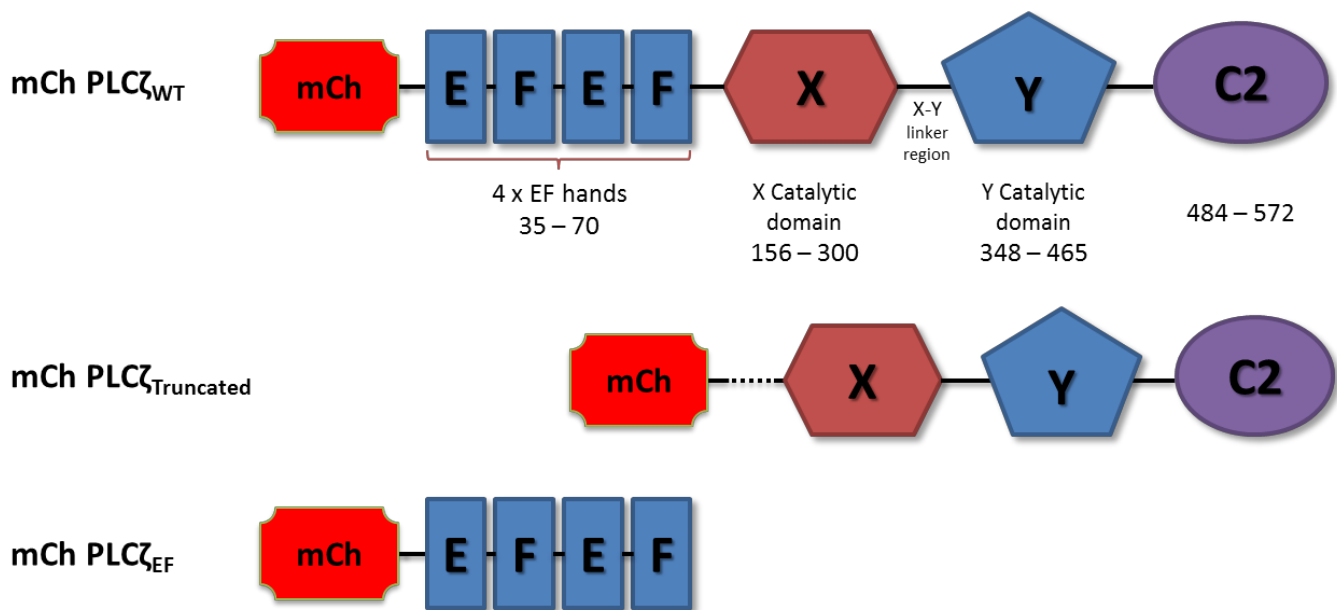


Figure 4.2.6 | **N-terminal mCherry PLCζ wild-type and mutated constructs.** (EF = EF hands of PLC structure; X and Y = catalytic domains; C2 = C2 terminal of PLC structure). PLCζ_{Truncated} and PLCζ_{EF} were artificially-induced mutations with the absence of EF domains, and the EF hands alone respectively.

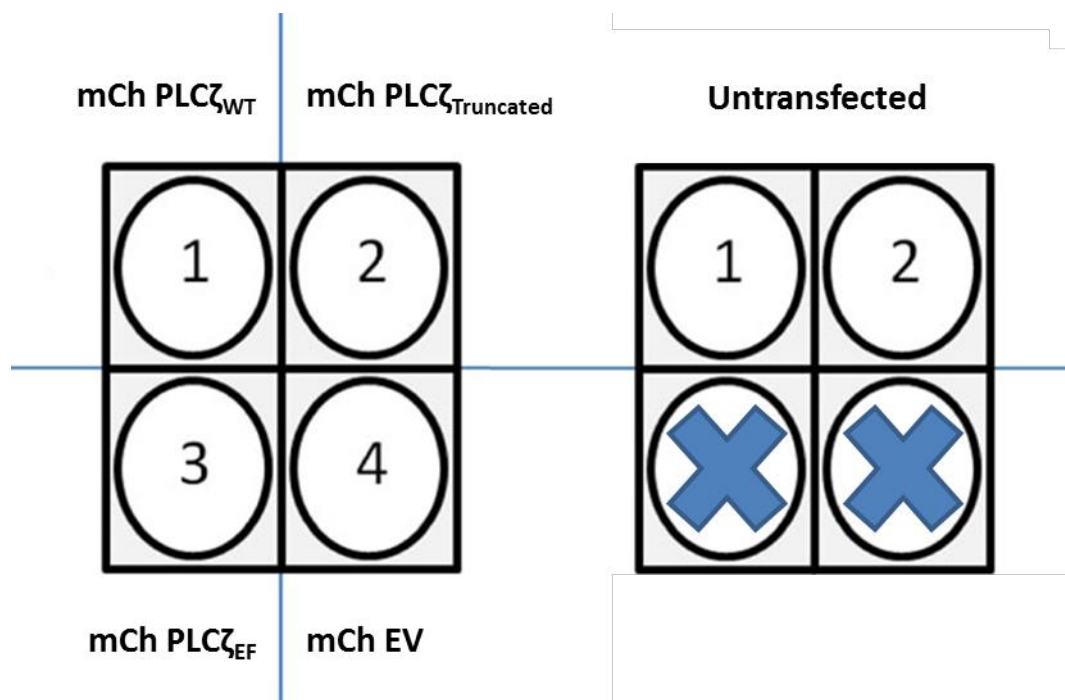


Figure 4.2.7 | **Representation of the transfection for a single time-point for a single antibody.** Four sets of this pair of 4-well plates were prepared and transfected as shown on the left-hand plate (mCherry (mCh) tagged PLCζ_{WT}, PLCζ_{Truncated}, PLCζ_{EF} and mCherry EV). Both wells in the right-hand plate were not transfected for control experiments (X denotes unused wells).

4.2.6 Immunofluorescent analysis of total mCherry PLC ζ expression and localisation in HEK293T cells

In order to stain for the plasma membrane, fixed cells were incubated for 10 minutes at RT with 10 μ g/ml Alexa Fluor 488/555 conjugate of wheat germ agglutinin (WGA, Invitrogen, UK). For the quantification studies, Alexa Fluor 488 WGA (green) was used, and Alexa Fluor 555 WGA used for the localisation studies.

For quantification studies, coverslips stained with Alexa Fluor 488 WGA were mounted with Vectashield mounting medium containing DAPI for nuclear staining. Images were captured and the distribution of PLC ζ visualised under a fluorescent Nikon Eclipse 80i microscope. A TRITC 560nm filter was used to visualise mCherry fluorescence (red) and a FITC 488nm filter for the WGA conjugate (green). Images were captured using a Nikon DS-Ri1 digital camera and NIS-Elements BR 3.0 software. One hundred random cells were evaluated per repeat (300 total), where relative levels of fluorescence among each repeat, and per isoform, were assessed using the region of interest (ROI) manager in Image J software. The colour histogram tool was used to measure the relative level of mCherry fluorescence in the cells. Only cells visibly exhibiting red mCherry fluorescence were analysed to compare the expression of protein between the different constructs, as cells exhibiting mCherry indicated cells successfully synthesising recombinant protein.

Kashir *et al.* (2011) hypothesised that PLC ζ was localising to the ER. This study aimed to confirm this. For localisation studies, selected cells were stained with 5 μ g/ml rabbit polyclonal ER membrane marker (Abcam ab22595, UK) and 10 μ g/ml rabbit polyclonal nucleolar marker (Abcam ab5821, UK). Cells were incubated in the primary antibody (overnight at 4°C for ER staining, and 1 hour at 24°C for nucleolar staining) following

permeabilisation and blocking procedures (for ER staining block and permeabilise in one step by incubation of coverslips in 1% BSA/10% goat serum/0.3M glycine in 0.1% PBS-Triton for 1 hour at RT followed by three washes in PBS; for nucleolar staining, permeabilised in 0.25% PBS-Triton for 10 minutes at RT, then block in 3% BSA/PBS for 30 minutes at 24°C, followed by three washes in PBS). After primary antibody incubation followed by three washes, coverslips were mounted with Vectashield mounting medium containing DAPI.

The experiment was repeated three times, and representative images were captured using an Olympus FV1000 Fluoview confocal microscope using a 60x/1.35 oil immersion objective, using a 405nm filter for DAPI, a FITC 488nm filter for ER/nucleoli and a 559nm filter for mCherry to examine intracellular localisation. Data were acquired and analysed using the Zeiss LSM image browser and Fiji software (<http://fiji.sc/wiki/index.php/Fiji>) respectively.

4.2.7 Statistical analysis

Statistical significance between each PLC ζ isoform was determined using one-way ANOVA followed by Bonferroni *post hoc* testing using Prism 5.0 (Graphpad, USA). One hundred cells which exhibited red mCherry fluorescence were analysed per repeat (300 in total). Data are presented as mean $\pm$ SEM and a *p* value of ≤ 0.05 was considered statistically significant.

4.3 Results

AIM 1 | Preparation of recombinant human PLC ζ protein

4.3.1 Cloning and sequencing of human PLC ζ_{WT} , PLC ζ_{H233L} , PLC ζ_{H398P} , PLC $\zeta_{H233L+H398P}$, PLC $\zeta_{frameshift}$ and PLC $\zeta_{truncated}$ constructs

Site-directed mutagenesis was used to produce WT, as well as mutant forms, of PLC ζ – mutants described previously (PLC ζ_{H233L} and PLC ζ_{H398P}) and laboratory-designed mutants (PLC $\zeta_{frameshift}$ and PLC $\zeta_{truncated}$). These were produced to allow characterisation of the relative effect of mutation upon protein structure, and to further understand the structure-function relationship of PLC ζ .

cDNA encoding the wild-type PLC ζ and PLC ζ mutant constructs (PLC ζ_{H233L} , PLC ζ_{H398P} , PLC $\zeta_{H233L+H398P}$, PLC $\zeta_{frameshift}$ and PLC $\zeta_{truncated}$) were amplified by PCR as described in Section 4.2.1, and separated by 1% agarose gel electrophoresis. Figure 4.3.1A indicates that the desired fragments were indeed amplified, as the product sizes correlate with expected sequence size [Figure 4.3.1C]. Following cloning of inserts into the pcDNA3.1/HisA vector as described in Section 4.2.1, a double restriction digest was carried out to confirm successful cloning. Figure 4.3.1B shows that all inserts were cloned successfully as the two bands relating to the vector and insert, linearised following digestion, correlate to the size of the pcDNA3.1/HisA vector at 5.5kbp and the inserts at the sizes given in Figure 4.3.1C.

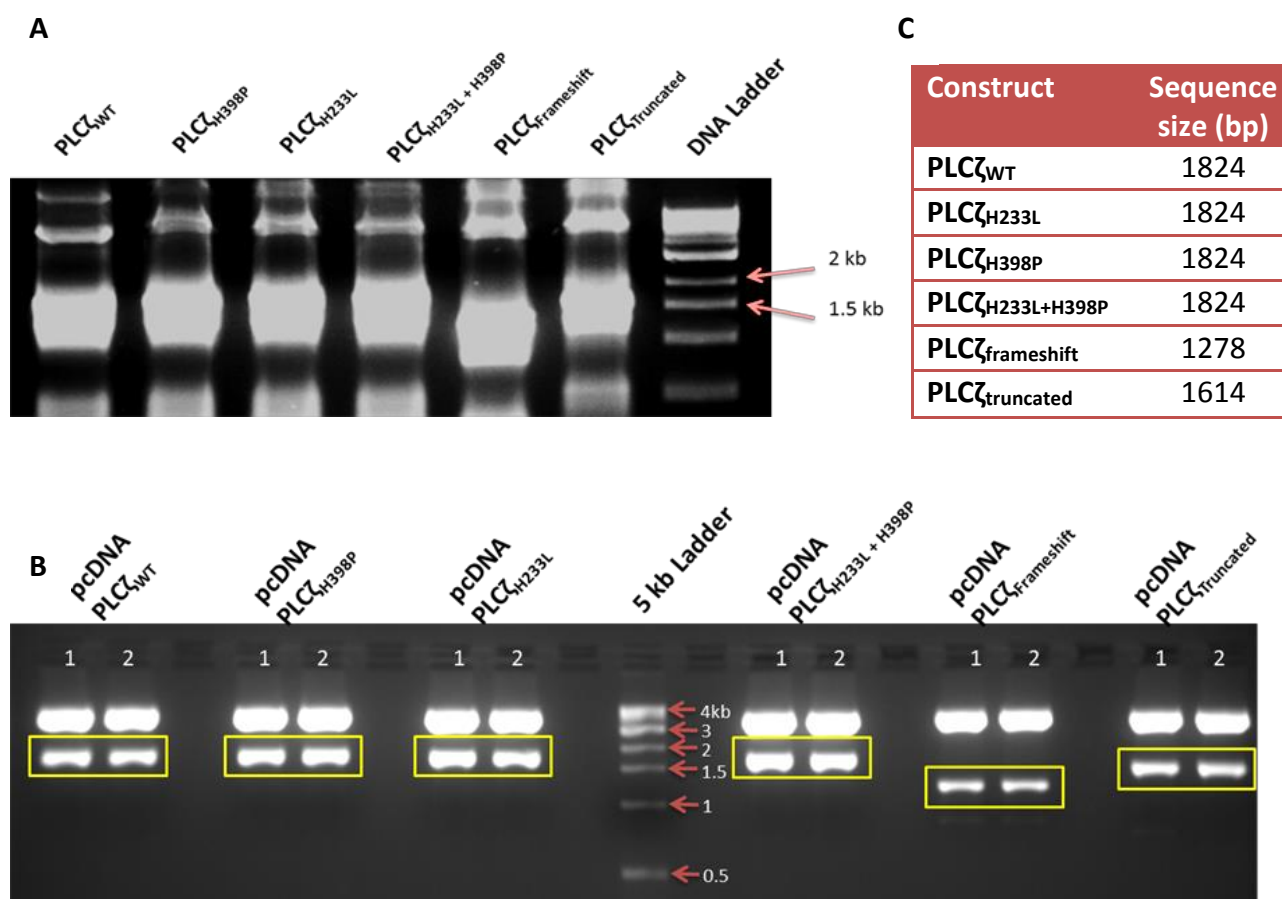


Figure 4.3.1 | PCR amplification products of all constructs on a 1% agarose gel (A), and double restriction digests of two colonies of pcDNA3.1/HisA PLC ζ constructs (B), depicting two bands for each construct: the upper band correlating to the pcDNA3.1/HisA vector at ~5.5kbp, and the lower band (highlighted) correlating to the insert, each at the correct size as shown in (C).

The successfully cloned constructs were sequenced using overlapping primers to cover the whole length of the fragment insertion as described in Section 4.2.1 and shown in Figure 4.2.4, and then aligned against the ORF of each template using bioinformatics software. Figure 4.3.2 shows an annotated example of the alignment of the sequenced PLC ζ _{WT} template using the T7 primer.



Table 4.3.1 | Alignment summary of all constructs against their respective ORF.

Construct	% Similarity to ORF
pcDNA PLC ζ_{WT}	100
pcDNA PLC ζ_{H233L}	100
pcDNA PLC ζ_{H398P}	100
pcDNA PLC $\zeta_{H233L+H398P}$	100
pcDNA PLC $\zeta_{Frameshift}$	100
pcDNA PLC $\zeta_{Truncated}$	100

4.3.2 Purification and immunoblotting

In order to overcome previous problems at purifying a recombinant PLC ζ protein using a C-terminal His tag as discussed in Section 4.1, which proved unsuccessful due to the conformational structure of the protein, this project employed an N-terminal His-tag. Recombinant PLC ζ WT and mutant protein was synthesised by transfection of HEK293T cells with pcDNA-PLC ζ . As a control, HEK293T cells were transfected with pcDNA3.1/HisA EV. Following purification as described in Section 4.2.2, protein products were separated by SDS-PAGE and stained with coomassie blue in order to confirm protein elution [Figure 4.3.3 A-G]. As Figure 4.3.3A shows, in the WT elute, a band can be seen at the expected size $\sim$ 80kDa, with a more intense band at $\sim$ 60kDa. Similar sized bands were also seen in the mutant elutes H233L, H398P, H233L + H398P [Figure 4.3.3 B-D], whilst the Frameshift and Truncated produced slightly lower sized bands as expected, the more intense band exhibiting around $\sim$ 55kDa [Figure 4.3.3 E-F]. Unexpectedly, similar sized bands were seen with the EV purification products, at $\sim$ 80kDa and $\sim$ 55kDa [Figure 4.3.3G].

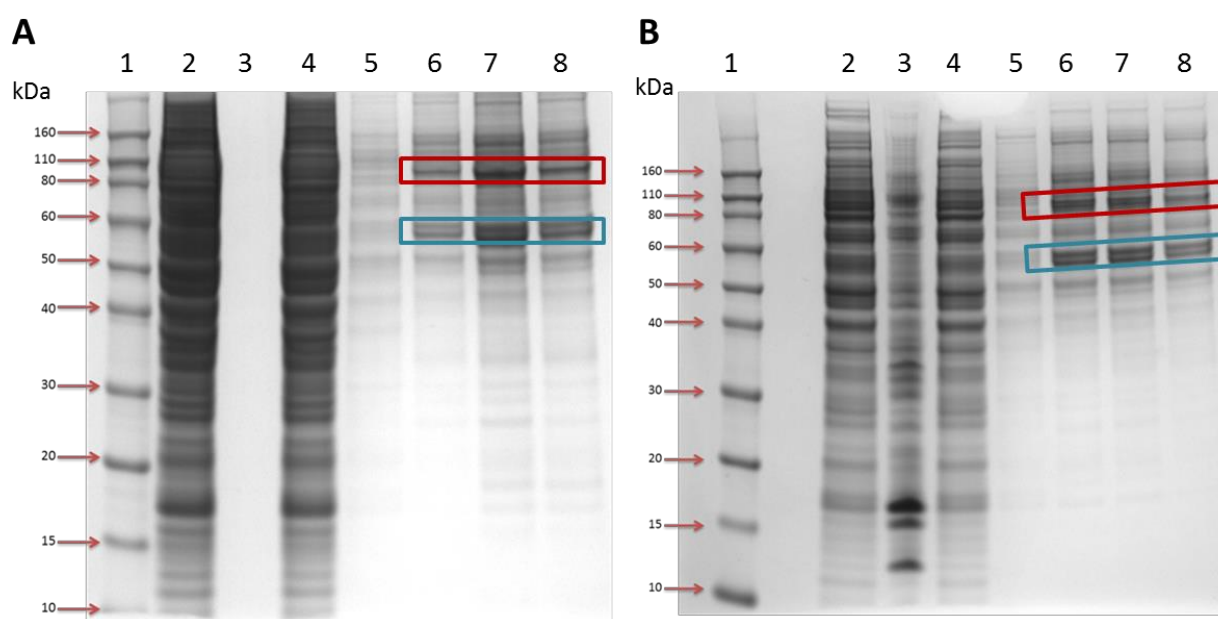


Figure 4.3.3 | Figure legend overleaf

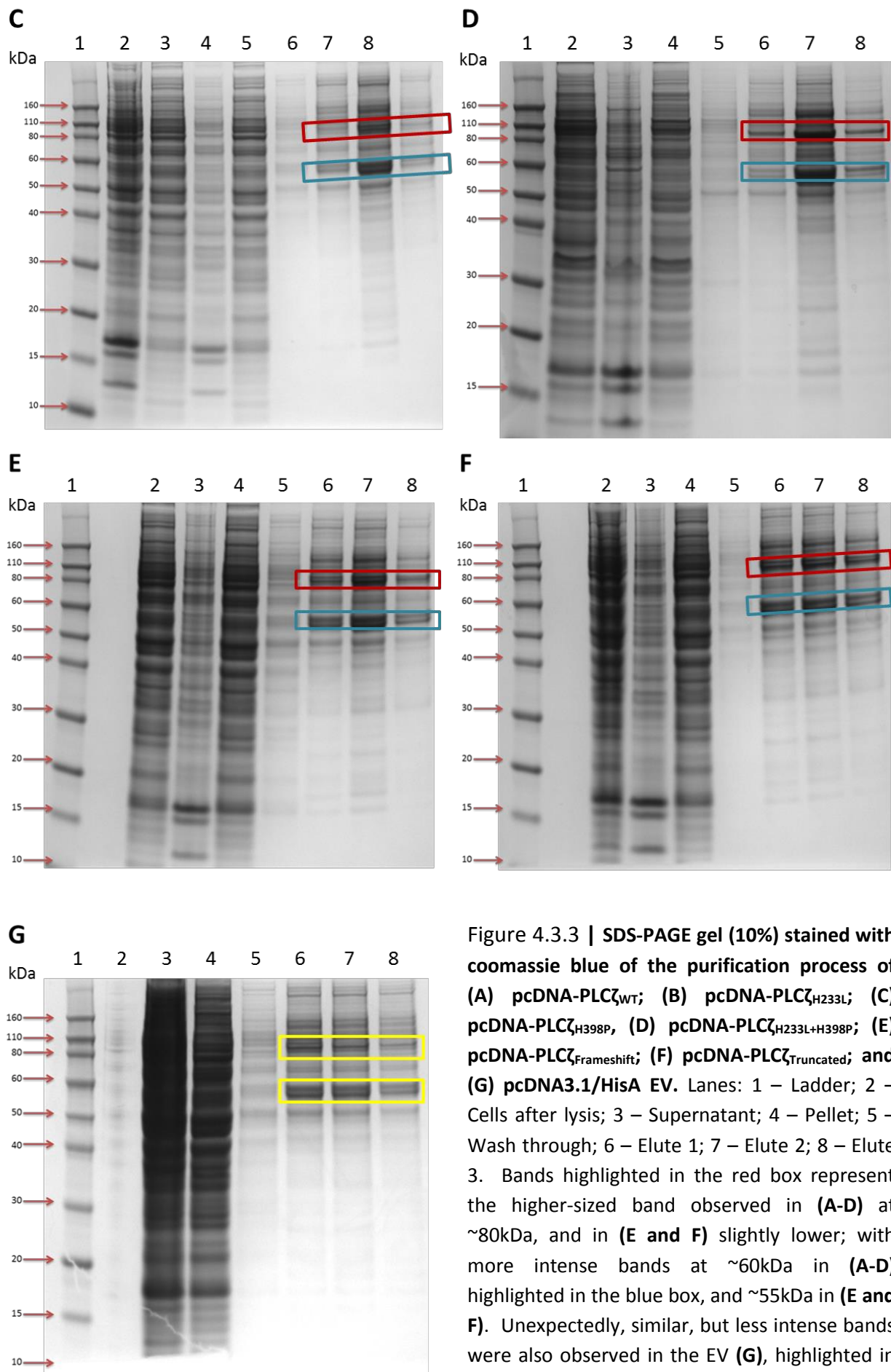


Figure 4.3.3 | SDS-PAGE gel (10%) stained with coomassie blue of the purification process of (A) pcDNA-PLC ζ _{WT}; (B) pcDNA-PLC ζ _{H233L}; (C) pcDNA-PLC ζ _{H398P}, (D) pcDNA-PLC ζ _{H233L+H398P}; (E) pcDNA-PLC ζ _{Frameshift}; (F) pcDNA-PLC ζ _{Truncated}; and (G) pcDNA3.1/HisA EV. Lanes: 1 – Ladder; 2 – Cells after lysis; 3 – Supernatant; 4 – Pellet; 5 – Wash through; 6 – Elute 1; 7 – Elute 2; 8 – Elute 3. Bands highlighted in the red box represent the higher-sized band observed in (A-D) at ~80kDa, and in (E and F) slightly lower; with more intense bands at ~60kDa in (A-D) highlighted in the blue box, and ~55kDa in (E and F). Unexpectedly, similar, but less intense bands were also observed in the EV (G), highlighted in the yellow box.

The purified protein product, elute 2 of each protein, which exhibited the strongest bands, was then subjected to immunoblotting using the anti-His antibody to confirm successful purification of the His-tagged protein. Thereafter, immunoblotting with the anti-PLC ζ antibody was undertaken to determine whether these His-tagged proteins were PLC ζ . As shown in Figure 4.3.4, the bands immunoreactive to anti-PLC ζ correlate to those immunoreactive to anti-His, indicating successful synthesis of recombinant PLC ζ protein. However, unexpectedly, the EV was also immunoreactive to the anti-PLC ζ antibody exhibiting immunoreactivity similar to the PLC ζ constructs, as well as additional bands not present in the PLC ζ protein constructs, implying that the immunoreactive bands seen in the protein blots may not be specific.

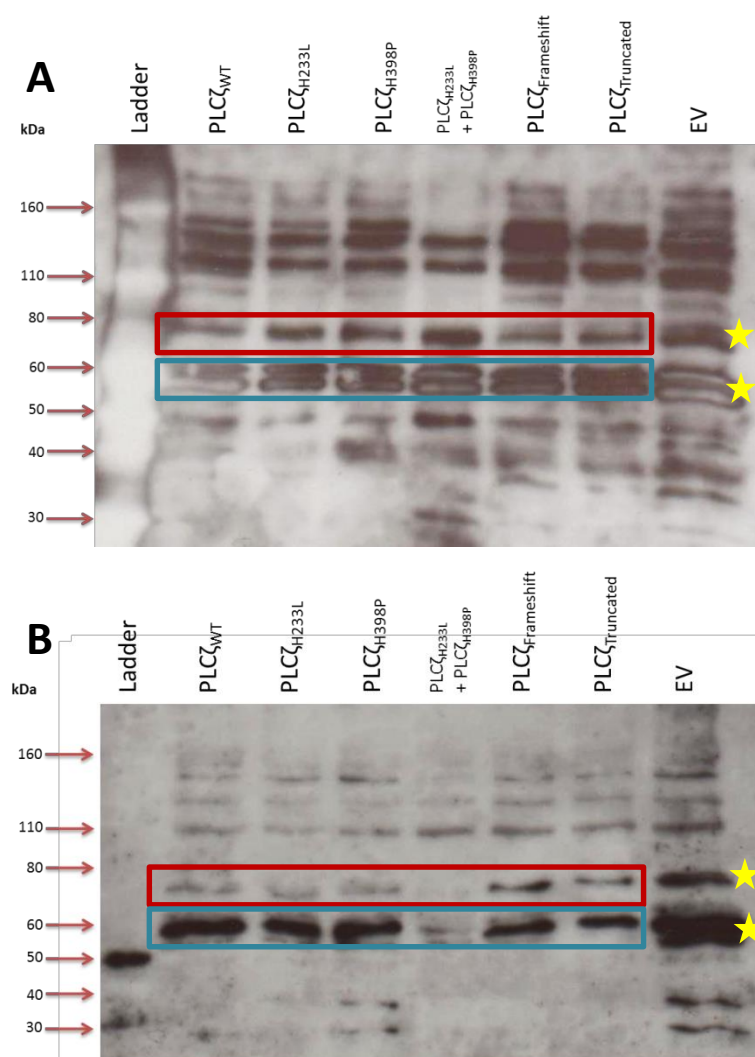


Figure 4.3.4 | Immunoblot with anti-His (A) and anti-PLC ζ (B) antibodies of the purified protein product of all the constructs. Bands around 75kDa (red box) and 60kDa (blue box) were identified in the protein product of all the constructs. Unexpectedly, similar-sized bands were also seen in the EV (star). Other non-specific bands were present in the EV elutes, not corresponding to the bands seen in the other protein products.

Owing to the therapeutic potential of purifying a recombinant PLC ζ _{WT} protein, the purification product was scrutinised more remotely. Analyzing the eluted products, both previously reported bands were observed – at ~70kDa as well as the lower band at ~57kDa [Figure 4.3.5], with the bands immunoreactive to anti-PLC ζ correlating to those immunoreactive to anti-His.

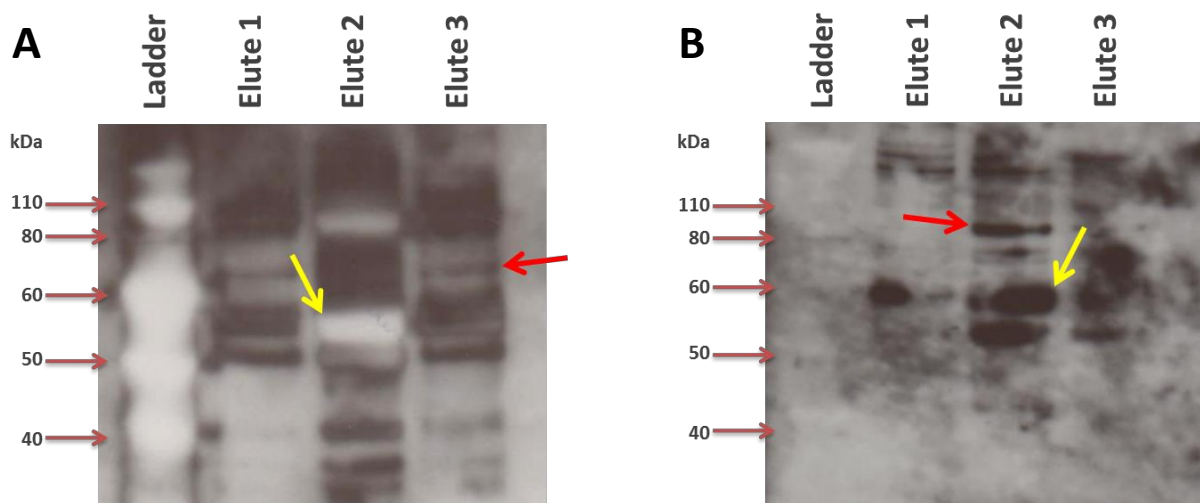


Figure 4.3.5 | Immunoblots of purified PLC ζ _{WT} protein with (A) anti-His antibody and (B) anti-PLC ζ antibody. Blots show correlating immunoreactive bands indicative of WT PLC ζ at the expected size of ~70kDa (red arrow) as well as the lower band at ~57kDa (yellow arrow), suggesting the successful purification of the His-tagged PLC ζ _{WT}.

In order to confirm this was indeed PLC ζ , the WT eluate was run alongside the EV, with equal loading of the eluates. The immunoblot was performed on a 4-12% SDS gel to separate the bands further when stained with anti-PLC ζ antibody. Figure 4.3.6 shows that when bands were separated further, a difference was found between the WT and EV proteins.

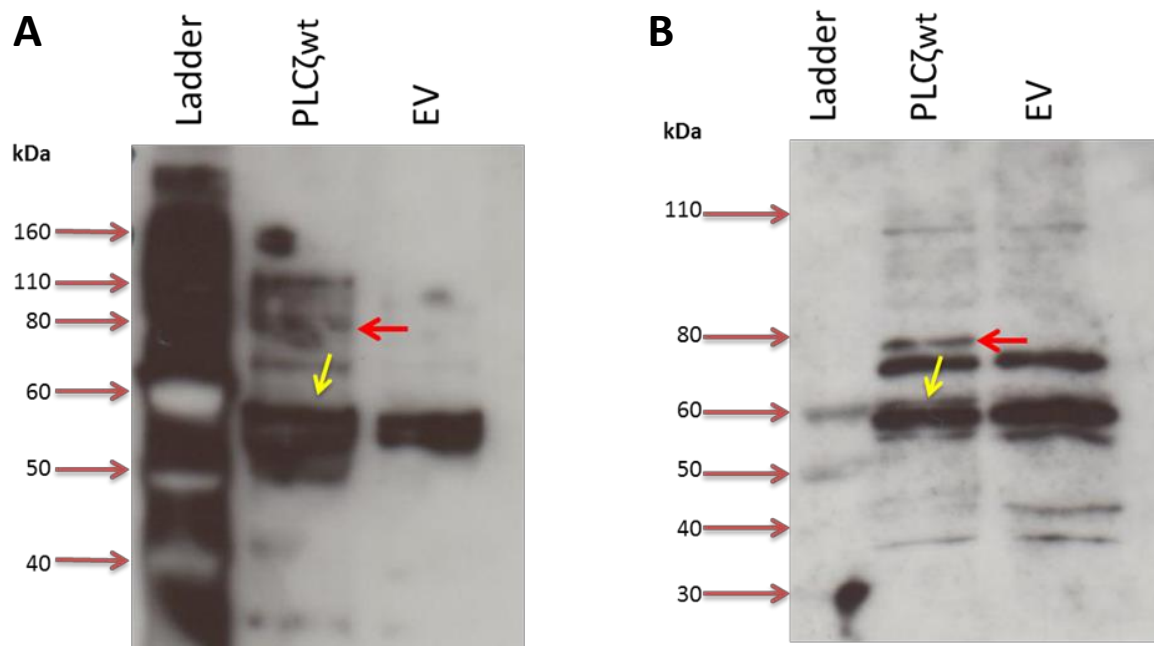


Figure 4.3.6 | Immunoblots of purified PLC ζ protein with the empty pcDNA3.1/HisA vector (EV) as a control with (A) anti-His antibody on a 10% gel and (B) anti-PLC ζ antibody on a 4-12% gel. Blots show strong correlating immunoreactive bands indicative of PLC ζ at the expected size ~70kDa (red arrow) as well as the lower band at ~57kDa (yellow arrow) which were not present in EV, suggesting successful purification of PLC ζ_{WT} protein.

Results indicated the successful synthesis and purification of wild-type recombinant PLC ζ protein by exhibiting bands at molecular sizes expected of WT human PLC ζ which were not present in the EV [Figure 4.3.6]. Before proceeding to test activity of the protein by micro-injection into mouse oocytes, it was necessary to identify these bands at the amino acid level by liquid chromatography mass spectrometry (LC/MS). In order to do this, samples were separated by SDS-PAGE, and stained with coomassie blue. The bands that were most intense at the appropriate size, were sent for identification using LC/MS (Professor Benedikt Kessler, Central Proteomics Facility, Nuffield Department of Medicine, University of Oxford).

LC/MS results [Figure 4.3.7] were not positive for PLC ζ but were positive for other proteins. The upper band was positive for a putative ATP-dependent RNA helicase implicated in pre-mRNA splicing, DHX15, while the lower band was positive for heterogeneous nuclear ribonucleoprotein L (HNRNPL), a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) complexes which provide the substrate for the processing events that pre-mRNAs undergo before becoming functional, translatable mRNAs in the cytoplasm. However, there were multiple bands at approximately the size indicative of PLC ζ [Figure 4.3.3A]. Different bands were selected on two subsequent occasions but results did not provide positive identification of PLC ζ .

A

		Score	Mass	Matches	Sequences	emPAI	
1.1	<u>Q5RAZ4</u>	1120	91643	100 (55)	33 (21)	1.73	RedName: Full=Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15; EC=3.6.4.13
1.2	<u>O17438</u>	272	51788	19 (11)	4 (3)	0.36	RedName: Full=Putative pre-mRNA-splicing factor ATP-dependent RNA helicase PRP1; EC=3.6.4.13
2	<u>P23246</u>	1060	76216	104 (47)	30 (19)	1.71	RedName: Full=Splicing factor, proline- and glutamine-rich; AltName: Full=100 kDa DNA-pairing pr
3	<u>Q86T12</u>	104	98999	4 (2)	2 (1)	0.03	RedName: Full=Dipeptidyl peptidase 9; Short=DP9; EC=3.4.14.5; AltName: Full=Dipeptidyl peptid
4	<u>Q5NVJ8</u>	85	83217	8 (3)	4 (2)	0.08	RedName: Full=ATP-dependent RNA helicase DDX1; EC=3.6.4.13; AltName: Full=DEAD box protei
5	<u>Q04724</u>	70	84061	4 (3)	4 (3)	0.12	RedName: Full=Transducin-like enhancer protein 1; AltName: Full=E(Sp1) homolog; AltName: Full
6	<u>P00761</u>	69	25078	2 (2)	1 (1)	0.28	RedName: Full=Trypsin; EC=3.4.21.4; Flags: Precursor;
7	<u>Q8BY02</u>	59	78343	5 (1)	4 (1)	0.04	RedName: Full=NF-kappa-B-repressing factor; Short=NFKB-repressing factor; AltName: Full=Trans
8	<u>Q99K67</u>	59	103650	2 (1)	2 (1)	0.03	RedName: Full=Alpha-aminoadipic semialdehyde synthase, mitochondrial; AltName: Full=LKR/SDH
9	<u>C1H563</u>	48	32828	2 (1)	1 (1)	0.10	RedName: Full=Ubiquinone biosynthesis protein COQ4, mitochondrial; AltName: Full=Coenzyme Q
10	<u>P11387</u>	39	91125	1 (1)	1 (1)	0.04	RedName: Full=DNA topoisomerase 1; EC=5.99.1.2; AltName: Full=DNA topoisomerase I;
11	<u>A1UEC6</u>	33	30142	1 (1)	1 (1)	0.11	RedName: Full=Uncharacterized oxidoreductase Mkms_1985; EC=1.-.-.-;

B

		Score	Mass	Matches	Sequences	emPAI	
1	<u>P14866</u>	919	64720	67 (40)	20 (15)	2.21	RedName: Full=Heterogeneous nuclear ribonucleoprotein L; Short=hnRNP L;
2	<u>P49368</u>	338	61066	26 (11)	15 (7)	0.43	RedName: Full=T-complex protein 1 subunit gamma; Short=TCP-1-gamma; AltName: Full=CCT-gai
3	<u>Q4R6V2</u>	204	60089	30 (9)	12 (7)	0.44	RedName: Full=T-complex protein 1 subunit epsilon; Short=TCP-1-epsilon; AltName: Full=CCT-eps
4.1	<u>P35520</u>	197	61175	25 (12)	11 (6)	0.43	RedName: Full=Cystathionine beta-synthase; EC=4.2.1.22; AltName: Full=Beta-thionase; AltNam
4.2	<u>P32232</u>	101	62157	15 (7)	5 (3)	0.22	RedName: Full=Cystathionine beta-synthase; EC=4.2.1.22; AltName: Full=Beta-thionase; AltNam
5	<u>Q99832</u>	174	59842	15 (8)	10 (6)	0.37	RedName: Full=T-complex protein 1 subunit eta; Short=TCP-1-eta; AltName: Full=CCT-eta; AltNar
6	<u>P17987</u>	168	60819	23 (8)	13 (6)	0.36	RedName: Full=T-complex protein 1 subunit alpha; Short=TCP-1-alpha; AltName: Full=CCT-alpha;
7	<u>Q5RA22</u>	95	65384	4 (2)	2 (1)	0.05	RedName: Full=Cytosolic purine 5'-nucleotidase; EC=3.1.3.5; AltName: Full=Cytosolic 5'-nucleotid
8	<u>P00761</u>	80	25078	2 (2)	1 (1)	0.28	RedName: Full=Trypsin; EC=3.4.21.4; Flags: Precursor;
9	<u>Q5FVM4</u>	59	55005	5 (2)	2 (2)	0.12	RedName: Full=Non-POU domain-containing octamer-binding protein; Short=NonO protein;
10	<u>ASPJN8</u>	45	50694	6 (2)	3 (2)	0.13	RedName: Full=Splicing factor 3A subunit 2;
11	<u>Q43776</u>	33	63758	2 (1)	2 (1)	0.05	RedName: Full=Asparaginyl-tRNA synthetase, cytoplasmic; EC=6.1.1.22; AltName: Full=Asparagin
12	<u>Q55E54</u>	17	0	2 (1)	1 (1)	0.03	RedName: Full=Coronin-B; AltName: Full=Coronin-7; Short=DdCRN7; AltName: Full=Doublecoronin

Figure 4.3.7 | LC/MS results on a band at ~70kDa (A) and a band at ~55kDa (B). (A) Identification of the higher band showing it to be a putative pre-mRNA splicing factor DHX15. (B) Identification of the lower band showing it to be a heterogeneous nuclear ribonucleoprotein L.

In order to perform the future activity assays by injecting eluted protein into mouse oocytes to ascertain activity of the WT and mutant protein products, the eluted protein needed to be stored in a Ca^{2+} -free media as the injected protein needs to be in a Ca^{2+} -free media to avoid parthogenetic activation of the oocytes in which the protein will be injected. Following exchange of buffer, a protein assay was undertaken to determine the amount of protein for the activity assay. Results showed that there was no protein present, and must have been lost or degraded during the procedure. Time restraints did not allow for the replication of protein synthesis and buffer exchange.

AIM 2 | Investigating the localisation and level of expression of PLC ζ in HEK293T cells

4.3.3 Quantification of PLC ζ immunofluorescence and localisation in transfected HEK293T cells

To determine an indication of the potential localisation of the expression of recombinant PLC ζ in mammalian oocytes, the mammalian cell line HEK293T was used as a model system and transfected with mCherry tagged PLC ζ_{WT} and mutant isoforms PLC $\zeta_{Truncated}$, PLC ζ_{EF} as well as an mCherry EV. Staining with a plasma membrane marker WGA indicated that the fluorescently-tagged PLC ζ isoforms were localising to discrete regions in the cell cytoplasm as shown previously by Kashir *et al.* (2011). Further experiments with transfected cells stained with antibodies against the ER and nucleoli were carried out to determine specific localisations. Cells were fixed at two time-points to determine protein stability properties.

Confocal microscopy was used to determine specific localisations. Figures 4.3.8 and 4.3.9 present representative images of cells transfected by each of the isoforms at the different time-points, showing immunolocalisation of the mCherry constructs in relation to the anti-ER and anti-nucleoli antibodies respectively. Figures 4.3.8 shows the localisation of PLC ζ_{WT} , PLC $\zeta_{Truncated}$ and PLC ζ_{EF} to the ER, with clear co-localisation of the fluorescently-tagged PLC ζ and the ER antibody. PLC $\zeta_{Truncated}$, which lacks the EF hand, showed localisation within the cell nucleus as well as the ER. Localisation of this latter isoform appeared to be located in the nucleoli [Figure 4.3.9B], but findings obtained from co-staining with an anti-

nucleoli antibody remained inconclusive, and requires repetition with a better antibody since fluorescent images faded much too quickly with the anti-nucleoli used here.

HEK293T cells transfected with the mCherry EV showed homogenous expression throughout the cell. Co-staining with the anti-ER and anti-nucleoli antibodies showed that this homogenous expression was less pronounced in the ER and more intense in the nucleoli. Untransfected cells were used as a negative control, and stained with the plasma membrane marker WGA and the anti-ER and anti-nucleoli antibodies, showing in Figures 4.3.8C and 4.3.9C the ER and nucleoli in HEK293T cells.

As well as ascertaining the specific subcellular localisation of the PLC ζ isoforms in the HEK293T cell line, fluorescence levels were analysed to indicate the level of protein expression between constructs. Quantitative analysis of 300 cells, from three repeats, demonstrated that fluorescence levels in the mCherry-PLC ζ_{WT} and mCherry-PLC $\zeta_{Truncated}$ transfected cells were similar [Figure 4.3.10]. Fluorescence in the mCherry-PLC ζ_{EF} transfected cells was significantly greater than the latter two isoforms, and the mCherry EV significantly greater than all the isoforms, such that $(PLC\zeta_{WT} = PLC\zeta_{Truncated}) < (PLC\zeta_{EF}) < (mCherry-EV)$. The latter was seen at both time-points except PLC ζ_{WT} and PLC $\zeta_{Truncated}$ were not similar at 56 hours where PLC $\zeta_{Truncated}$ showed a statistically significant reduction in fluorescence to PLC ζ_{WT} . There was a statistically significant increase in fluorescence levels between 48 and 56 hours in all isoforms ($p < 0.05$).

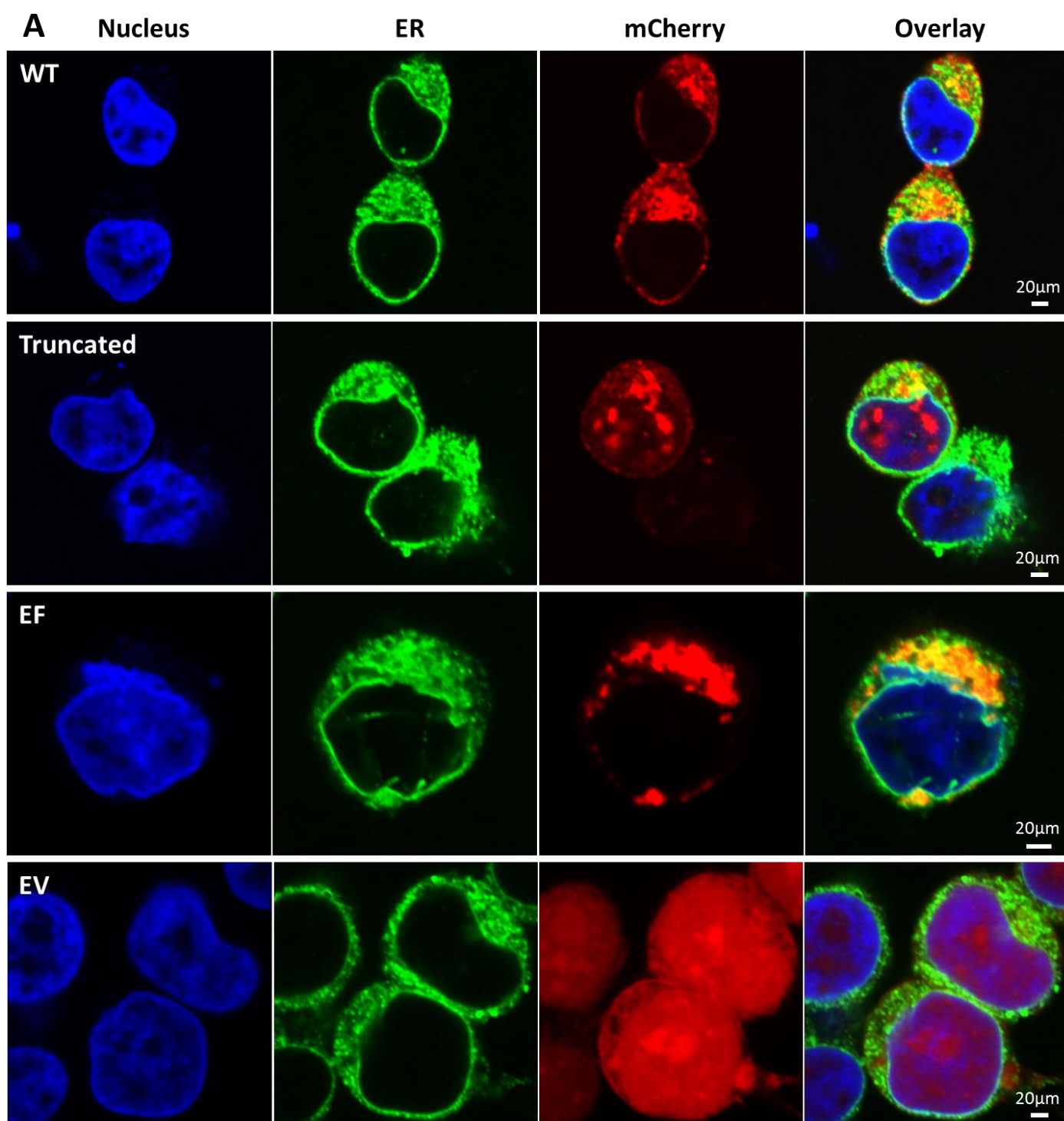
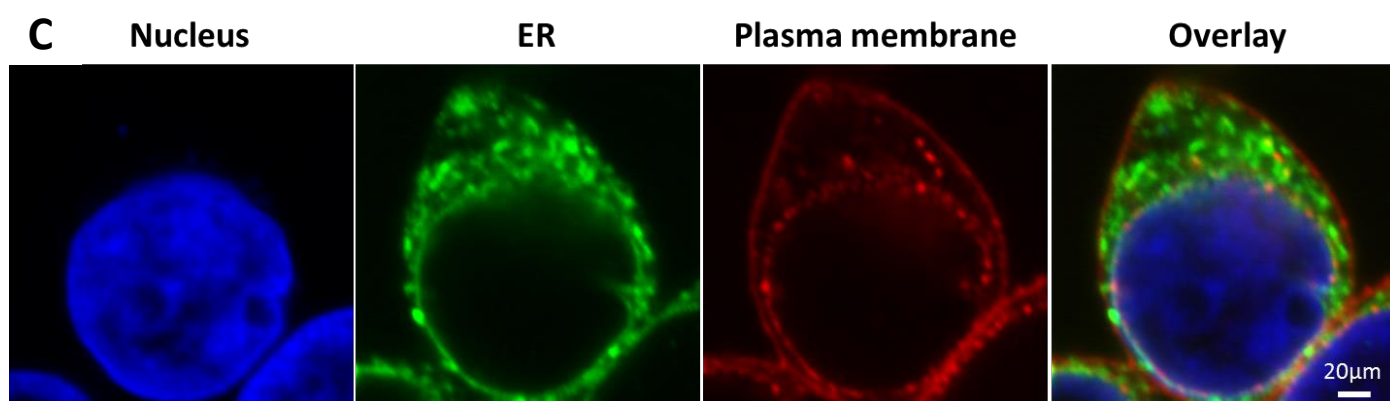
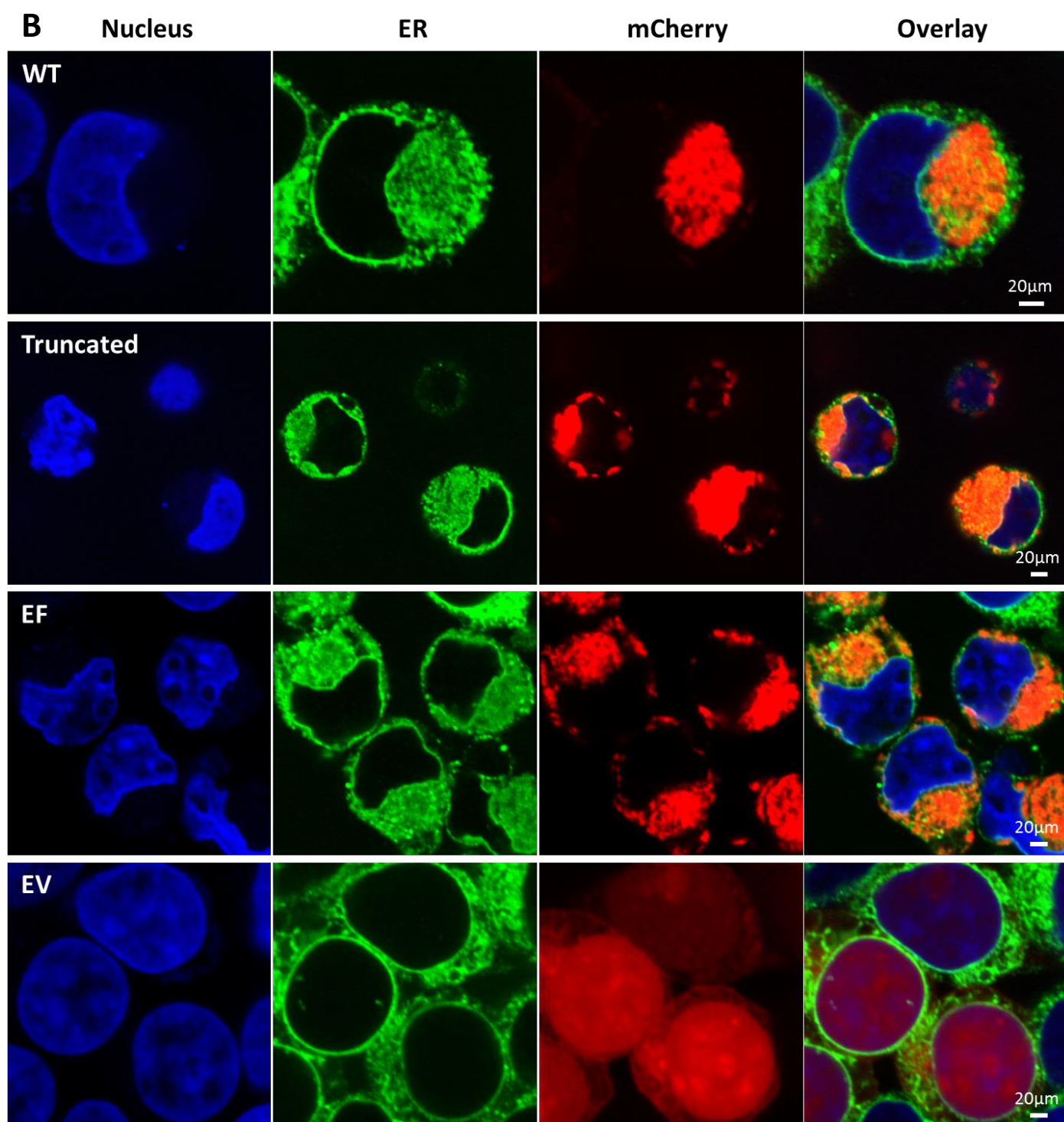


Figure 4.3.8 | Representative confocal images of HEK293T cells transfected with mCherry constructs (PLCζ_{WT}, PLCζ_{Truncated}, PLCζ_{EF}) and mCherry EV, stained with an ER membrane marker and fixed at (A) 48 hours; and (B) 56 hours, and un-transfected cells as a control stained with the plasma membrane marker WGA (C). PLCζ isoforms were expressed in the ER showing co-localisation with the ER marker. The truncated isoform showed further expression in regions within the nucleus. The mCherry EV showed homogenous expression throughout the cell.



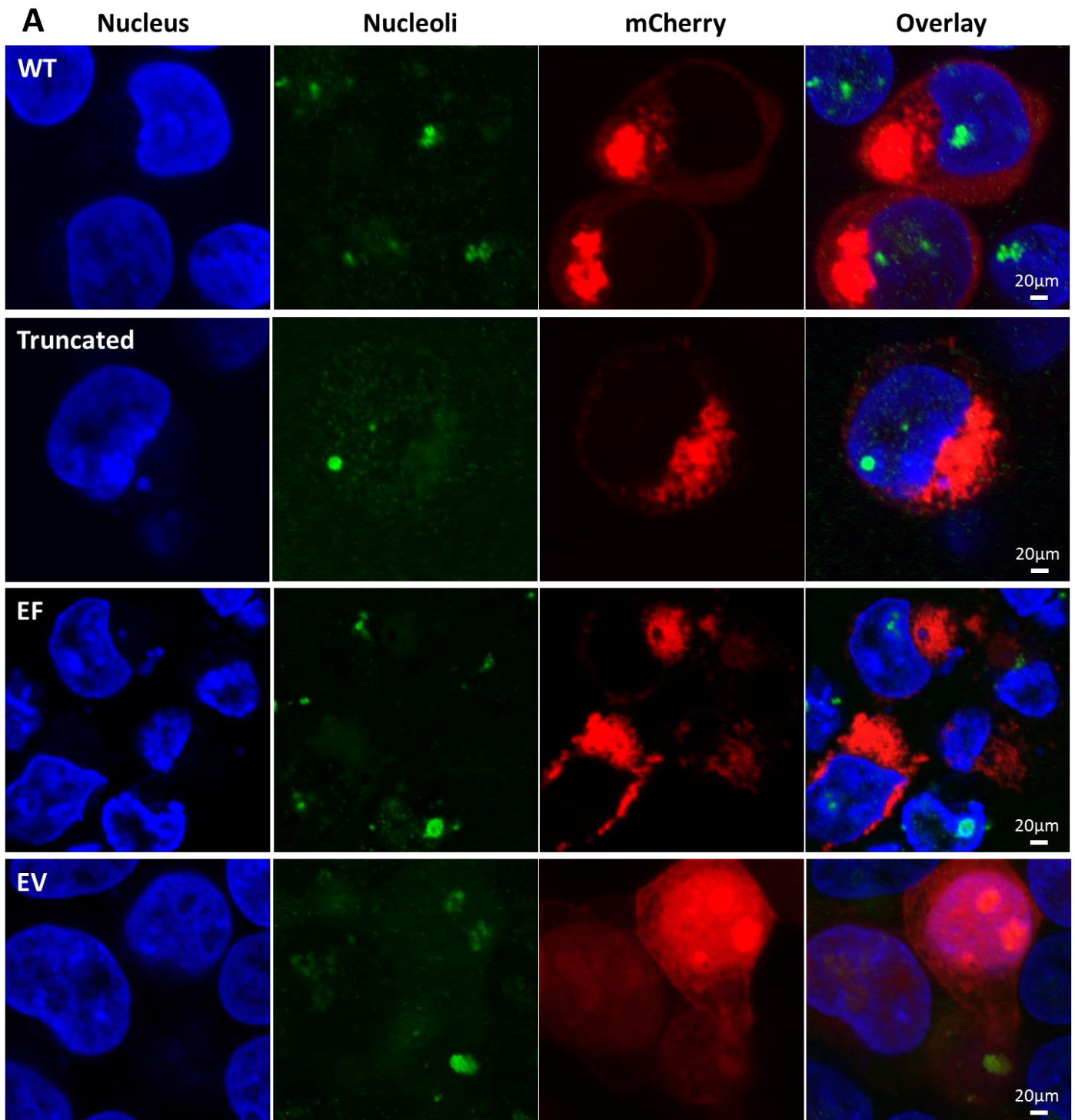
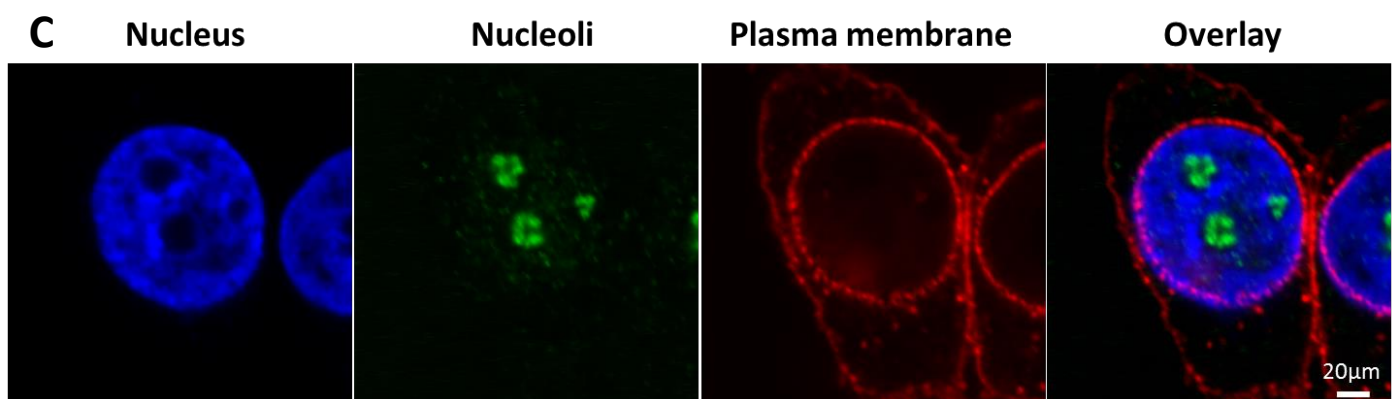
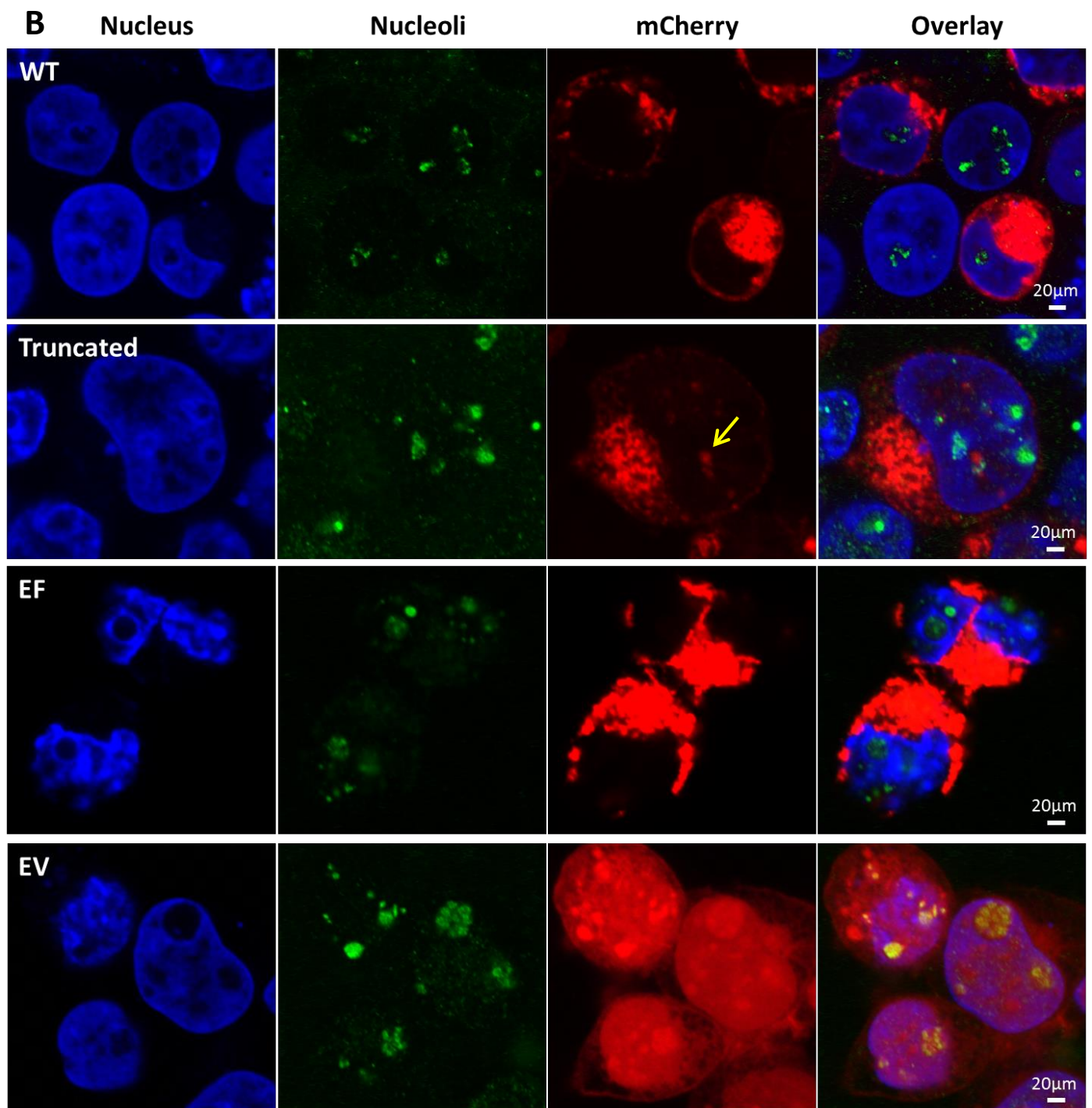


Figure 4.3.9 | Representative confocal images of HEK293T cells transfected with mCherry constructs (PLC ζ _{WT}, PLC ζ _{Truncated}, PLC ζ _{EF}) and mCherry EV, stained with a nucleoli marker and fixed at (A) 48 hours; and (B) 56 hours, and un-transfected cells as a control stained with the plasma membrane marker WGA (C). PLC ζ isoforms were not expressed in the nucleoli showing no co-localisation with the nucleoli marker. The truncated isoform however showed indications that it may be localised in the nucleoli (yellow arrow in (B)). The mCherry EV showed homogenous expression throughout the cell.



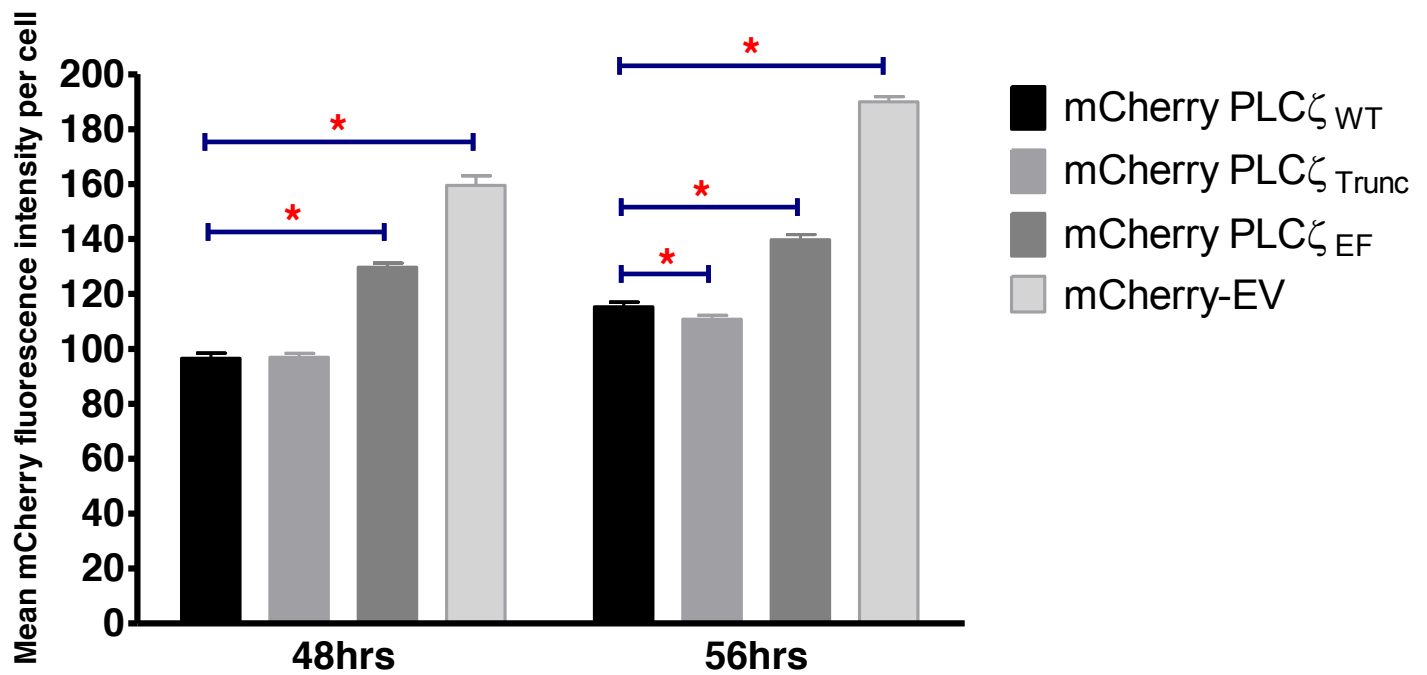


Figure 4.3.10 | Mean mCherry fluorescence intensity per cell for HEK293T cells transfected with mCherry-PLC ζ_{WT} , -PLC ζ_{Trunc} , -PLC ζ_{EF} and mCherry EV at 48 and 56 hour time points. Data shown as mean $\pm$ SEM. Asterisk (*) denotes statistical significance $p < 0.05$ using the *t*-test.

4.4 Discussion

Given the incidence of ICSI failure and the concern over the use of AOAs which use non-physiological stimuli, and the established role of PLC ζ in oocyte activation and link to male infertility, there is significant hope for PLC ζ as a physiological therapeutic option, to substitute or augment endogenous PLC ζ activity.

Provision of a synthetic recombinant form of PLC ζ protein holds great promise therapeutically for infertility, but it needs to be in a purified active form that can be stored safely. Purifying a recombinant WT PLC ζ protein has proved problematic in the hands of various researchers, although Kouchi *et al.* (2004) successfully purified a mouse recombinant protein. However, this protein exhibited Ca²⁺ oscillations of higher amplitude, and has thereafter proved difficult to replicate. This project endeavoured to produce a recombinant PLC ζ protein following on from previous efforts undertaken in the Coward laboratory using the mammalian HEK293T cell line. Previous attempts to purify had failed using a C-terminal His-tag due to suspected conformational folding of the protein making the C-terminal inaccessible during the purification process.

4.4.1 PLC ζ constructs were successfully cloned and findings suggestive of successful purification of recombinant PLC ζ protein

In this study, an N-terminal His-tag was exploited rather than the C-terminal tag that was used previously. PLC ζ constructs were successfully cloned into the pcDNA3.1/HisA vector and sequenced. Immunoblotting of the purified protein product, and staining with the anti-PLC ζ antibody as well as the anti-His antibody, did suggest that purification had

been successful. Western blotting using the affinity-purified polyclonal anti-PLC ζ , however, has proved to be very unclear presenting numerous bands besides the bands at the expected size of PLC ζ . Moreover, numerous bands were observed in the EV when stained with anti-PLC ζ antibody. Previous studies which have also used this same antibody have presented blots with several unspecific bands (Grasa *et al.*, 2008; Yoon *et al.*, 2008; Heytens *et al.*, 2009; Young *et al.*, 2009) when analysing sperm extracts of both human and mouse sperm. Indeed a pattern was recurring in previous studies with immunoblotting exhibiting two bands – one at the expected size of human PLC ζ at 70kDa and a second band strongly recognised at 52-57kDa, the latter possibly being either a proteolytic breakdown product of PLC ζ , a truncated endogenous form of PLC ζ possibly relating to one of the populations of PLC ζ , or indeed an unrelated protein altogether (Fujimoto *et al.*, 2004; Kurokawa *et al.*, 2005, 2007; Bi *et al.*, 2009). The multiple bands identified in anti-PLC ζ western blots may be attributable to the polyclonal nature of this antibody. Certainly, this antibody has proved specific in immunocytochemistry studies, but again the variance in the findings from localisation studies over past years may be attributable to the specificity of the antibody. Moreover, two recent studies using two different polyclonal anti-PLC ζ antibodies recognised a single band on an immunoblot of human sperm and of recombinant protein (Yoon *et al.*, 2012; Nomikos *et al.*, 2013). Yet again, the variance in findings renders the need for producing a monoclonal antibody not only increasingly important, but imperative.

In the present study, on comparing the WT recombinant PLC ζ protein product against the EV, findings were suggestive of successful purification, and the most intense bands obtained on a coomassie were sent for LC/MS identification. Unfortunately, these proved negative for PLC ζ on two separate attempts. Since many bands appeared at the

expected sizes for PLC ζ , future studies should aim to analyse all the bands present to ascertain which, if any, truly represent PLC ζ .

For the recombinant WT PLC ζ to be used as a therapeutic in its purified form, and to ascertain the effect of mutations and determine the mechanism of action of the mutant protein products, an activity assay must be undertaken. In order for the purified recombinant product to be injected into mouse oocytes, it would need to be in Ca²⁺-free intracellular media to avoid activation by the accidental injection of Ca²⁺. Following buffer transfer, total protein concentration of the recombinant proteins using spectrophotometry presented negative values indicative of the absence of protein. This was repeated a second time by producing and purifying more protein of all the constructs, but again no protein was present following transfer of buffer although immunoblotting of the purified eluate indicated there was protein prior to buffer transferal. Future work should aim to improve the yield as indeed there may not have been much protein present before transferring buffers. While there were bands at the expected molecular weights, many unspecific bands were also present. Moreover, immunoblotting is known to be a powerful method for immunodetection of proteins, especially those in low abundance (Kurien *et al.*, 2011). Other recombinant protein production methods may also be considered, such as Fc fusion proteins producing Fc-tagged proteins which are easily engineered. Fc fusions are providing significant advances in protein therapeutics currently by providing several benefits, such as facilitating expression and secretion of the recombinant protein into the media enabling easier purification, as well as better stability (Flanagan *et al.*, 2007; Carter, 2011).

While current work was underway, several studies emerged which have successfully produced an active recombinant PLC ζ protein. Phillips *et al.* (2011) found that mouse PLC ζ

remains inactive in the CHO cell line, and the authors demonstrated that a cell lysate of mouse PLC ζ -transfected CHO cells exhibited high recombinant protein expression, and on micro-injection into mouse oocytes, elicit Ca²⁺ oscillations indicative of oocyte activation (Phillips *et al.*, 2011).

Of greater translational interest, Kashir *et al.* (2011) produced the first recombinant human PLC ζ protein from HEK293T cells transfected with pTracer-PLC ζ . Upon injection into mouse oocytes, Ca²⁺ oscillations characteristic to those seen in normal fertilisation were elicited, indicative of the presence of active PLC ζ (Kashir *et al.*, 2011). However, this recombinant protein was in the form of a cell lysate, and thus will require purification before it can be considered as a clinical therapeutic. Nonetheless, the successful production of an active recombinant PLC ζ protein was a huge advancement in the quest of accomplishing a clinical translational application. Purification has been tenuous with previous studies showing inactivation of the protein following purification; suggestive of inactivation during the purification process, or that the laboratory processes involved did not permit key PTMs or protein-folding mechanisms (Cooney *et al.*, 2010). Therefore, the optimisation of a successful purification protocol, whilst maintaining the functional active nature of the protein, was an imperative hurdle to overcome.

More recently, two further studies succeeded in synthesising an active purified recombinant human PLC ζ protein. Yoon *et al.* (2012) produced a purified human PLC ζ recombinant protein, which upon injection into mouse and human oocytes initiated Ca²⁺ oscillations and oocyte activation, although embryonic development was not reached in the mouse model (Yoon *et al.*, 2012). Furthermore, these authors demonstrated how ICSI-failed human oocytes were rescued upon the micro-injection of this recombinant protein (Yoon *et*

al., 2012). Nomikos *et al.* (2013) also successfully produced a purified active recombinant human PLC ζ protein which, when micro-injected into human and mouse oocytes, instigated characteristic Ca²⁺ oscillations indicative of oocyte activation, and successful embryo development up to blastocyst stage (Nomikos *et al.*, 2013). Furthermore, the authors demonstrated how mouse eggs expressing mutant human PLC ζ that were unable to activate normally, were rescued by micro-injection with the WT human PLC ζ protein, initiating Ca²⁺ oscillations and embryogenesis up to blastocyst stage. These findings herald a great advancement in the quest to obtain this potentially revolutionary tool in the field of male-factor infertility management and therapeutics.

Contrary to our previous understanding that a mammalian cell line would be the best model for recombinant protein production since it allows for PTM and protein folding, both studies by Yoon *et al.* (2012) and Nomikos *et al.* (2013) employed a bacterial system to successfully produce the recombinant active PLC ζ proteins. Bacterial expression systems are often not able to produce a recombinant human protein that is identical to the naturally occurring WT since they do not possess the mechanisms required to perform PTMs present in higher organisms, such as glycosylation, and insufficiencies in proteolytic protein maturation and disulphide bond formation (Kamionka, 2011; Huang *et al.*, 2012). However, recent studies have shed light on how the lack of PTMs in bacterial cells may, to some extent, be overcome by protein engineering, and owing to the low cost and simplicity of cultivating bacteria, it offers a considerable advantage over other expression systems (Kamionka, 2011). Protein engineering is also employed to improve protein stability or modulate its biological function, such as those achieved by the genetic fusions of two proteins (Kamionka, 2011). Such genetic fusions were employed by Nomikos *et al.* (2013),

and indeed this technique facilitates protein purification and enhances target protein solubility (Kamionka, 2011). Many therapeutic proteins have been engineered by the use of genetic fusions, and using a bacterial expression system (Huang *et al.*, 2012), so this may indeed be the way forward for clinical translation.

4.4.2 PLC ζ localises within the ER of HEK293T cells

Since studies have established PLC ζ 's role in oocyte activation, it is plausible to deliberate how this knowledge can be concomitant with the generation of a novel male non-hormonal contraceptive technique for fertile males by blocking the activity of PLC ζ . An enhanced understanding is required of the precise mechanism of action of PLC ζ as well as elucidating the activating component in the oocyte that induces PLC ζ to cause Ca²⁺ release and ensuing oocyte activation. Moreover, further understanding of how mutations cause failure of activation is required in order that their action can be mimicked to bring about the inactivation of PLC ζ . Using WT PLC ζ and laboratory-formed mutations, this study endeavoured to investigate the structure-function relationship of PLC ζ and its subcellular localisation in mammalian cells as a model for the oocyte.

Until recently, it was unclear whether the PIP₂ that sperm PLC ζ binds to was in the oolemma or in an internal organelle of the oocyte, and precisely where PLC ζ is localised whilst stimulating Ca²⁺ release. Based on recent findings by Kashir *et al.* (2011) who found that mCherry-tagged PLC ζ localises to discrete regions within the HEK293T cell cytoplasm near the nuclear envelope, this study took this further to identify the identity of this discrete localisation. Microscopic analysis of the expression of fluorescently-tagged mCherry WT

human PLC ζ in HEK293T cells stained with a plasma membrane marker, WGA, indicate that indeed PLC ζ_{WT} localise to discrete regions in the cell cytoplasm adjacent to the nuclear envelope and not to the plasma membrane in accordance with the finding of Kashir *et al.* (2011). This discrete localisation was ascertained on comparison with cells transfected with the mCherry EV alone which exhibit a more homogenous expression throughout the cell. Further analyses using confocal imaging of these mCherry-PLC ζ transfected cells stained with an anti-ER antibody, determined that these discrete regions were indeed the ER.

To gain insight into the structure-function relationship of PLC ζ , HEK293T cells were transfected with an mCherry tagged truncated construct of PLC ζ which lacked the EF hands (mCherry-PLC $\zeta_{Truncated}$), and with another mCherry tagged construct which possessed the EF hands alone (mCherry-PLC ζ_{EF}). Levels of PLC ζ in cells expressing PLC $\zeta_{Truncated}$ were similar to levels of PLC ζ_{WT} when expressed in HEK293T cells. Levels of PLC ζ_{EF} however were significantly higher since it is a much smaller fragment and thus easily synthesised. Whilst the mCherry-PLC ζ_{EF} localised to the ER similar to the mCherry-PLC ζ_{WT} , mCherry-PLC $\zeta_{Truncated}$ was also localised in the ER, but was also exhibited in the nucleus of some cells. These latter localisations of the truncated PLC ζ transcript appeared to reside in the nucleoli, thus further analyses were undertaken using an anti-nucleolar marker. Whilst indeed the truncated transcript are localised in the nucleus of some cells as well as the ER, findings herein were inconclusive with regards to nucleoli localisation and further work is required with a better antibody.

EF hand domains are the Ca²⁺ binding sites for many proteins (Grabarek, 2011; Ito *et al.*, 2011), and in PLCs are thought to possess this capacity (Suh *et al.*, 2008) including PLC ζ (Ito *et al.*, 2011). Findings in this study suggest that the EF hand is required for ER

translocation; however some translocation ability is retained as seen by the expression of PLC ζ _{Truncated} in the ER as well as the nucleus in some cells. Similarly, Nomikos *et al.* (2005) showed that a truncated PLC ζ construct which had either the EF hands or the C2 domain, or both, maintained some PIP₂-hydrolysis activity, but upon micro-injection into mouse oocytes did not initiate Ca²⁺ oscillations (Nomikos *et al.*, 2005). The authors suggested that Ca²⁺-binding to the EF hands is important for the interaction of the XY domain with PIP₂ and thus PLC ζ activity. Furthermore, Kouchi *et al.* (2005) showed low levels of Ca²⁺-sensitivity when the EF hands were missing (Kouchi *et al.*, 2005). Likewise, medaka and pufferfish species express a version of PLC ζ that lacks the first 60 amino acids, comprising the first EF hand domain and part of the second (Coward *et al.*, 2005; Ito *et al.*, 2008). Injection of medaka PLC ζ cRNA into mouse oocytes initiates Ca²⁺ release in oocytes while the pufferfish cRNA does not.

Collectively, these findings confirm that the EF domain is required for the functional activity of PLC ζ . Incidentally, EF hands also appear to play a role in nuclear translocation in the mouse, where Kuroda *et al.* (2006) described how a mutation in residues residing in the EF hand domain, preclude nuclear localisation of mouse PLC ζ just as a mutation in the NLS region would (Kuroda *et al.*, 2006; Ito *et al.*, 2011). Moreover, interestingly, Bi *et al.* (2009) discovered an endogenous truncated human PLC ζ isoform which lacked the EF hands, which they identified as NYD-SP27 of 58kDa molecular weight (Bi *et al.* 2009). The authors proposed a role for this isoform in antagonising the PLC activity of PLC ζ , thus again adding credence to the role of the EF domain in the functional activity of PLC ζ , as well as nuclear localisation in the mouse (Ito *et al.*, 2011).

However, the mechanism of nuclear translocation for the truncated transcript observed in this study remains unknown. Indeed whilst mouse PLC ζ is known to have a NLS sequence present in the X-Y linker region, which is responsible for the pro-nuclear localisation of PLC ζ (Marangos *et al.*, 2003; Larman *et al.*, 2004; Yoda *et al.*, 2004; Kuroda *et al.*, 2006; Ito *et al.*, 2008), it has not yet been verified whether human PLC ζ possesses an equivalent sequence. However, unlike mouse PLC ζ , which when the EF hands are deleted, ceases nuclear translocation in mouse oocytes (Kuroda *et al.*, 2006), in human PLC ζ , deletion of the EF hands causes nuclear translocation in HEK293T cells, as observed in this study. Other PLCs have been shown to have nuclear translocation activity, such as PLC β 1 in human oocytes prior to germinal vesicle breakdown (Miyara *et al.*, 2007). Indeed it may be plausible to hypothesise that human PLC ζ does have a NLS, and that removal of the EF hands exposes this sequence by disrupting the functional three-dimensional structure of the protein. It is important that the three-dimensional structure of PLC ζ is ascertained, and now that a recombinant purified active PLC ζ protein has been produced (Yoon *et al.*, 2012; Nomikos *et al.*, 2013), this goal may be achievable.

Translocation of PLC ζ to the ER is suggestive that PLC ζ undergoes folding, assembly, and PTM in the ER which is the case with many proteins targeted to the ER (Naidoo, 2011). Chaperones, folding enzymes or accessory factors may be involved in targeting PLC ζ to the ER. Contrary to the general consensus that PIP $_2$ is located on the cell membrane only in most cells, this may not be the case in oocytes which contain many intracellular organelles, such as the ER (Helms *et al.*, 1991; Godi *et al.*, 1999; Rice *et al.*, 2000; Halet *et al.*, 2002). A recent study suggested that whilst PLC δ acts by hydrolysing PIP $_2$ in the oolemma, PLC ζ hydrolyses an intracellular membranous source of PIP $_2$ on distinct vesicular structures within

the oocyte cortex (Yu *et al.*, 2011). Findings herein suggest that the intracellular membranous source which PLC ζ targets are on the ER.

In accordance with these findings, PLC ζ remains in an inactive state and fails to induce Ca²⁺ oscillations in the CHO cell line, which exhibit an absence of PIP₂ in its organelles (Phillips *et al.*, 2011). As well as providing evidence for PLC ζ 's Ca²⁺ releasing target, this situation mimics the circumstances with the sperm and the oocyte whereby PLC ζ is inactive in sperm, but on fusion of the gametes induces Ca²⁺ oscillations. It is currently unclear how such a physiologically active protein is maintained in an inert form within sperm. This suggests that specific factors within the ooplasm may be required for PLC ζ -mediated Ca²⁺ release (Yoshida *et al.*, 2007; Kashir *et al.*, 2011; Phillips *et al.*, 2011).

4.4.3 Further work

Indeed whilst recent studies have made tremendous advances with attaining the therapeutic potential that recombinant PLC ζ possesses, post-implantation development following the use of recombinant human PLC ζ protein has not yet been evaluated. Thus, the production of an active recombinant human PLC ζ protein in a purified form suitable for clinical use remains an important issue that still warrants attention. Whilst Yoon *et al.* (2012) successfully purified an active recombinant PLC ζ , the Ca²⁺ release profiles of 50% of the activated oocytes appeared dubious. Moreover, whilst the recombinant protein produced by Nomikos *et al.* (2013) produced a more realistic advancement towards clinical application, a cause for hesitation remains at the size of the tag the authors used for purification. Recombinant human PLC ζ was expressed as a NusA-hexahistidine fusion protein, with the size of the NusA tag almost the same size as the human PLC ζ protein itself,

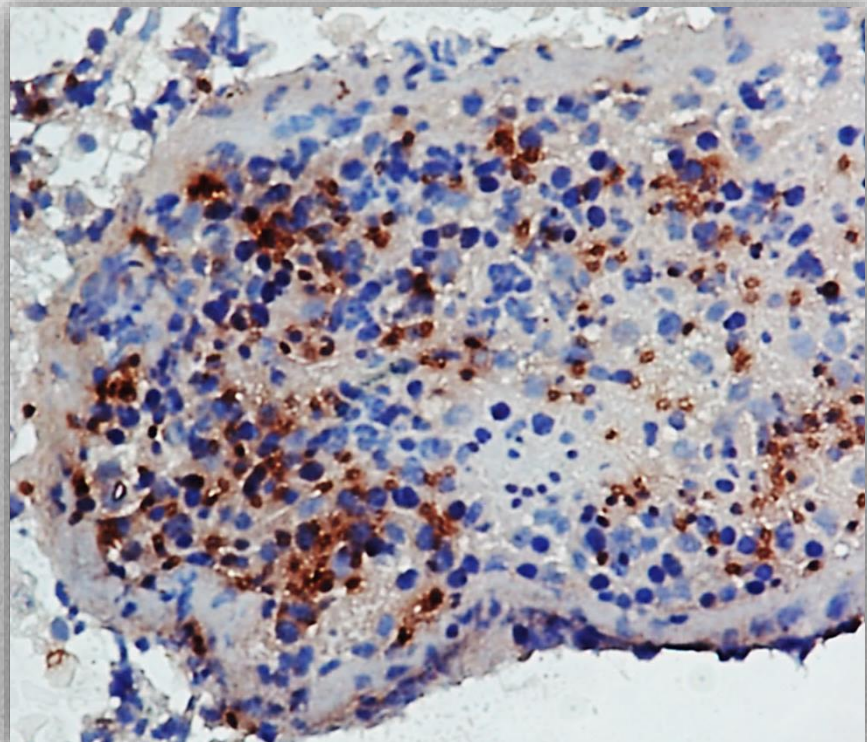
at 60kDa which limits the application of this protein somewhat, unless it can be removed by a site-specific protease. The first successful production of an active recombinant human PLC ζ protein has the only disadvantage of being in a form of a lysate (Kashir *et al.*, 2011). Should successful purification of this lysate be attained whilst maintaining activity, it would present a very realistic option. Indeed, an understanding of the modifications which the purification process disrupts may help in attaining successful purification. Moreover, the recombinant protein purified in this study must be studied further, and the continuous work to attain successful protein identification, activity and Ca²⁺ release analysis is warranted.

It is important to note that the HEK293T mammalian cell line was used as a model for human oocytes, however, findings herein, and by Kashir *et al.* (2011), cannot be conclusively related to human oocyte activity and localisations, and studies looking at localisations within human oocytes, or indeed a mouse model, would also be required in the future.

4.4.4 Summary of findings

-  PLC ζ constructs (PLC ζ _{WT}, PLC ζ _{H233L}, PLC ζ _{H398P}, PLC ζ _{H233L+H398P}, PLC ζ _{Frameshift} and PLC ζ _{Truncated}) were successfully cloned and sequenced
-  Successful purification of recombinant WT human PLC ζ protein has yet to be confirmed
-  PLC ζ localises to the ER in HEK293T cells
-  The EF hand is responsible for ER localisation in HEK293T cells.

Chapter 5 | DEVELOPING TOOLS FOR PLC ζ RESEARCH AND THE CLINIC



5.1 Introduction

The relationship between PLCζ and OAD is now firmly established and the potential for therapeutic application through the use of a recombinant purified PLCζ was discussed in depth in Chapter 4. Similarly, the scope for diagnostic applications of PLCζ have been considered widely and since the studies by Grasa *et al.* (2008) and Heytens *et al.* (2009) determined the localisation pattern and expression levels of PLCζ in fertile human sperm and in ICSI-failed sperm respectively, this aspect of potential clinical application of PLCζ has been studied more carefully with an aim to develop an oocyte activation biomarker assay to provide a definitive test to identify exactly which patients using a PLCζ therapeutic can be useful.

In current fertility clinics worldwide, the analysis of semen parameters is considered to be a diagnostic cornerstone when evaluating male fertility, and routinely include sperm concentration and the percentage of motile and morphologically normal sperm (Steger, 2003; De Jonge, 2012) using semen parameters set out by the WHO (2010). Evaluation using the light microscope has been the foundation of semen analysis in human andrology (Buckman *et al.*, 2009). However, as has been established through extensive research, many instances of idiopathic male infertility may be caused by abnormalities in testicular gene expression (Coward *et al.*, 2007; McLachlan and O'Bryan, 2010), or sperm abnormalities may occur at the molecular level (Matzuk and Lamb, 2008), sometimes in the absence of a morphological manifestation detectable by light microscopy. Therefore, it is becoming more debatable of what diagnostic and predictive value traditional semen parameters holds (Barratt, 2010; Barratt *et al.*, 2011).

Addressing this problem, a number of structural and molecular anomalies of defective human sperm are now detectable by an array of biomarkers (Agarwal *et al.*, 2008; Garrido *et al.*, 2008). These include fluorescent markers of acrosomal status, fluorochromes detecting for altered DNA integrity or sperm chromatin, and antibodies capable of detecting proteins that are either up-regulated or down-regulated in defective sperm (Evenson *et al.*, 2007; Buckman *et al.*, 2009). The revolutionary technique of flow cytometry, in contrast to light microscopy, allows for a rapid, automated and objective analysis of the relative abundance of such biomarkers in thousands of cells per sample (Gillan *et al.*, 2005).

Given the potential for PLCζ quantification as a biomarker for the oocyte activation ability of a given sample, the quantitative analysis of sperm PLCζ using flow cytometry may represent a more rapid, useful diagnostic tool for both research and translational purposes than the traditionally currently used immunofluorescent microscopy techniques. Flow cytometry has never been used to analyse PLCζ expression in sperm thus far, but has been shown as a potential fertility diagnostic by measuring sperm nuclear DNA fragmentation in patient sperm revealing significant differences in levels between fertile and infertile sperm (Lukanov *et al.*, 2009), and similarly for protamine deficiency (Tavalaee *et al.*, 2010). A similar assay for oocyte activation potential by assessment of PLCζ may also prove to be a useful clinical biomarker of fertility.

Current focus on the challenge of producing therapeutic applications of PLCζ and understanding its mechanism of action are at the forefront of research following the establishment of the connection between PLCζ deficiency with OAD. However, it is currently unknown at what stage of sperm development deficiencies arise, although

mutations in PLC ζ have been discovered in one patient (Heytens *et al.*, 2009; Kashir *et al.*, 2012). Owing to ethical and availability limitations, there is no data thus far from human testicular studies. A recent study by Heytens *et al.* (2010) using the wobbler mouse, as a model of globozoospermia, investigated the localisation of PLC ζ in WT and wobbler mice, and whilst findings suggested lower expression of PLC ζ in the wobbler mice compared with the WT, they also demonstrated the expression of PLC ζ in the inner areas of the seminiferous tubules indicating the expression of PLC ζ during the late stages of spermatogenesis. Heytens *et al.* (2010) thus demonstrated not only the developmental expression of PLC ζ in WT mice, but also demonstrated how there is a difference in PLC ζ expression between fertile and globozoospermic mice. Such findings would indicate similar findings would be observed in humans if testes (or samples thereof) were available for analyses. Such animal models are required due to their immense use and benefit for our understanding (Barratt *et al.*, 2010). In patients with azoospermia, testicular biopsies are acquired for the prognostic analysis of ICSI using sperm derived from the testis (testicular sperm extraction; TESE), namely the presence or absence of mature elongated spermatids (Zitzmann *et al.*, 2006). ICSI using surgically-recovered sperm allows such patients the chance to father children (Tournaye *et al.*, 1997).

One concern of all previous immunocytochemical and immunoblotting work relating to PLC ζ is that it has been carried out with the use of polyclonal anti-peptide antibodies generated against human and mouse PLC ζ sequences. Despite polyclonal antibodies being useful tools, they remain potentially compromised due to the fact that they can sometimes recognise multiple epitopes. Indeed the specificity of the polyclonal anti-PLC ζ antibody used by Grasa *et al.* (2008), Heytens *et al.* (2009), and indeed this study, was characterised by

Grasa *et al.* (2008), through a series of peptide-blocking experiments showing specific binding in the acrosomal, equatorial and post-acrosomal regions. However, the immunolocalisations need to be confirmed using a monoclonal antibody which is a much more specific tool, designed to recognise a single epitope. Furthermore, as discussed in Chapter 3, current immunoblotting using the polyclonal PLC ζ antibody has presented several bands – besides the one at the correct molecular weight of PLC ζ , others at lower molecular weights. It is unclear what these lower molecular weight proteins represent, although possibilities include proteolytic breakdown products of PLC ζ , a truncated endogenous form of PLC ζ relating to one of the populations of PLC ζ , or indeed unrelated proteins altogether. Interestingly, a study by Heytens *et al.* (2009) demonstrated different-sized bands between control and globozoospermic and non-globozoospermic patients, which warrant further investigations using a monoclonal anti-PLC ζ antibody.

The present study carried out a blinded study on human testicular biopsies obtained from a collaborative clinic in Belgium, ascertaining the presence/absence and localisation of PLC ζ using immunohistochemical analysis. This will represent the first attempt to map PLC ζ expression or localisation in human testicular tissue. Furthermore, this study investigated the potential for developing a biomarker for oocyte activation ability by quantitating PLC ζ in human sperm using flow cytometry. Finally, due to the debate over the localisation of PLC ζ , this study aimed to re-purify the polyclonal antibody originally used by Grasa *et al.* (2008), Young *et al.* (2009) and Heytens *et al.* (2009), and endeavor to lay the foundations for a novel monoclonal human anti-PLC ζ antibody. A monoclonal antibody would provide a powerful tool not only for research, but would also offer great potential for the ART clinic as a diagnostic.

5.2 Materials and Methods

AIM 1 | Immunohistochemical analysis of human testicular biopsies

5.2.1 Preparation of human testicular biopsies for cryosectioning

Testicular biopsy samples were obtained from patients diagnosed with obstructed and non-obstructed azoospermia at the Department of Reproductive Medicine of Ghent University, a collaborative laboratory in Belgium with informed written consent. The samples entered the UK from Belgium with ethical approval from the Oxford Tropical Research Ethics Committee (OXTREC; Ref: 31-09). Samples were obtained using the testicular sperm extraction (TESE) procedure using the methodology described by Everaert *et al.* (2006) [please refer to Appendix 8 for the method used for obtaining biopsies]. Samples were delivered to the Coward laboratory in cryo-straws on dry ice. Samples were embedded by emptying the TESE sample into a microcentrifuge tube and centrifuged at 7000rpm for 10 minutes. Tissue samples were embedded in Bright CRYO-M-BED embedding compound (Bright Instrument Company Ltd, UK) and frozen using isopentane immersed in the gas phase of liquid nitrogen to prevent formation of crystals. The frozen tissue blocks were individually labelled and stored in a -80°C freezer to await sectioning.

5.2.2 Cryosectioning and immunohistochemical analysis of embedded human testicular biopsies

Frozen tissue were cut into 8-12µm sections using a cryostat-microtome and transferred onto 0.01% poly-L-lysine coated slides. Previous studies have employed an anti-Protamine antibody as a spermatogenic marker for the identification of spermatid and spermatozoa in testicular biopsies using immunohistochemistry (Song *et al.*, 2000; Xu *et al.*, 2011). For each biopsy, slides were prepared as shown in Figure 5.2.1 to be stained with a sperm marker, anti-Protamine 2 (PM2) antibody to demonstrate the presence of sperm in the respective biopsy, as well as anti-PLCζ antibody. Slides were fixed in Bouin's solution for 20 minutes, followed by a wash in PBS. Slides were then treated with ethanol/0.3% hydrogen peroxide for 5 minutes to block endogenous peroxidase activity. Thereafter, slides were washed for 5 minutes in PBS and air-dried. On drying, PAP moulds using an Immedge Pen were drawn around each section and allowed to dry. Sections were then permeabilised for 20 minutes with PBS/0.5% Triton-X100 in a humidifying chamber followed by two washes in PBS. Non-specific binding was then blocked with 10µl/ml PBS/goat serum for sections to be stained with anti-PLCζ antibody; and 10µl/ml PBS/horse serum for sections to be stained with anti-PM2 antibody; according to instructions accompanying the Vectastain Elite ABC Kit, for 1 hour at RT.



Figure 5.2.1 | For each individual patient biopsy, two slides were prepared, with two sections on each slide. One section on each slide was stained with the antibodies anti-PLCζ or anti-Protamine 2 (PM2) antibody (ab). The second section on each slide represents a negative control for each antibody.

Samples were incubated overnight at 4°C in primary antibodies: 25µg/ml anti-PLCζ antibody diluted in blocking buffer; and 20µg/ml anti-PM2 monoclonal antibody (Briar Patch Biosciences LLC, USA) diluted in PBS, whereas negative controls were incubated in the absence of primary antibody. Sections were washed twice in PBS and incubated for 1-2 hours at RT in universal biotinylated secondary antibodies (Vector laboratories, UK): 20µl/ml anti-rabbit secondary antibody for sections stained for PLCζ; and 20µl/ml anti-mouse secondary antibody for sections stained for PM2. The preformed Avidin and Biotinylated horseradish peroxidase macromolecular Complex (ABC) reagent (20µl/ml Avidin DH solution and 20µl/ml biotinylated enzyme) was then applied for 45 minutes according to instructions accompanying the Vectastain Elite ABC Kit. Slides were thereafter washed twice in PBS and antibody binding visualised using an immunoperoxidase reaction where sections were incubated in diaminobenzidine (DAB) with nickel enhancement. Sections were counterstained with haematoxylin for 3 seconds and washed in distilled water. Slides were then allowed to air-dry before mounting using DBX mounting media (Fisher-Scientific, UK).

Images were viewed using a Nikon Eclipse 80i microscope, and representative images captured at 20x, 40x and 100x magnification using a Nikon DS-Ri1 digital camera and NIS-Elements BR 3.0 software.

AIM 2 | Quantification of PLCζ in human sperm samples using flow cytometry

5.2.3 Sample collection and preparation

A single sperm sample was collected at the Oxford Fertility Unit (OFU) from a donor aged 23 years and used for a preliminary experiment. Two control samples from patients recruited from the Ferti Aguerrevere Fertility Unit in Venezuela were also analysed, both aged 22 years. Samples were washed, prepared and fixed as described in Section 3.2.1–3.2.2. Samples were counted using a haematocytometer and a minimum of 1×10^6 sperm cells were allocated for each separate experiment.

5.2.4 Experimental design

Flow cytometric analysis essentially follows the same principle as that of fluorescent immunocytochemistry except unlike immunocytochemistry, flow cytometry is quantitative and can analyse thousands of sperm per second. The aim of this study was to determine whether flow cytometry could be used to analyse the expression of PLCζ in control and patient samples. For purposes of clarity, Figure 5.2.2 presents a brief experimental outline of the procedure used. It is very typical in flow cytometry protocols to incubate the primary antibody for 30-40 minutes, never overnight, thus the first optimisation experiment was to optimise PLCζ antibody incubation time using an OFU donor sperm sample.

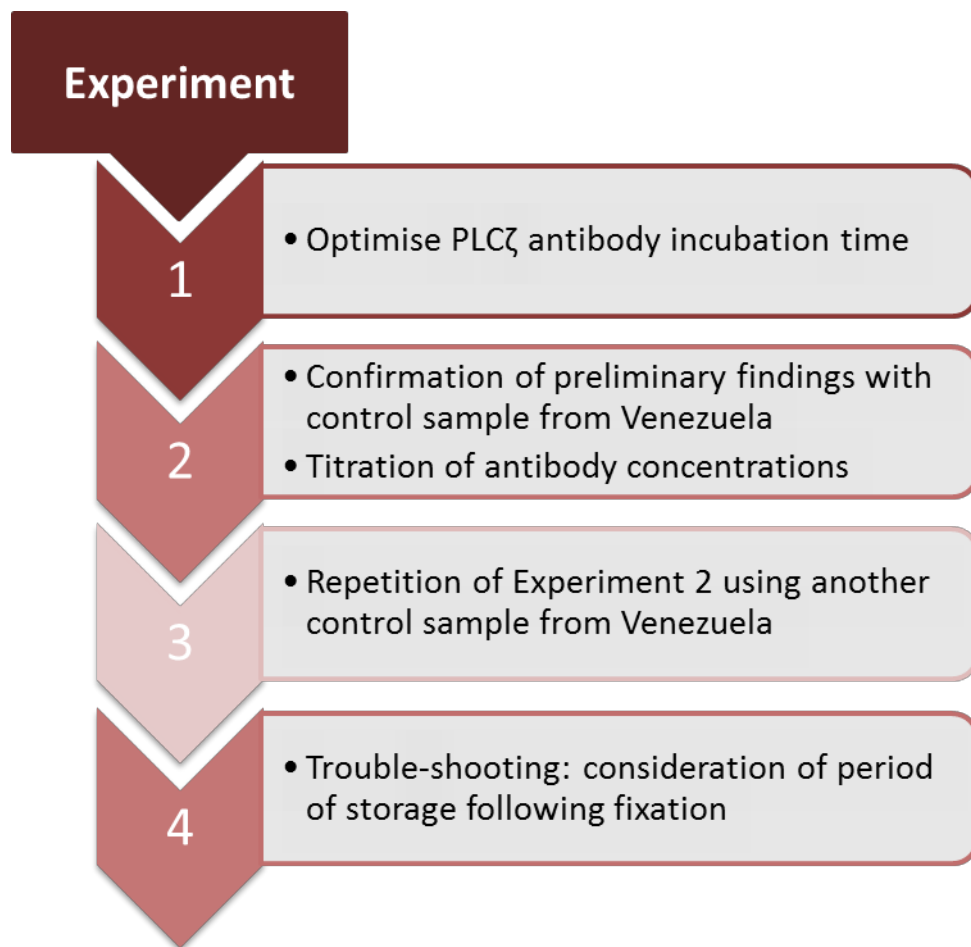


Figure 5.2.2 | An outline of the experimental undertakings to optimise a protocol for the quantitative analysis of PLCζ using flow cytometric analysis.

5.2.5 Flow cytometry analysis

After washing by centrifugation in PBS, pelleted sperm cells were permeabilised in 200μl PBS/0.5% Triton-X100 for 20 minutes. Following two washes in PBS by centrifugation at 800g for 5 minutes (all washes were performed at this speed/time) pelleted sperm cells were incubated for 30 minutes in 200μl PBS/3% BSA to block non-specific binding. Thereafter, half of the sample was washed twice by centrifugation and incubated with a titration of anti-PLCζ antibody [5-25μg/ml] and the other half washed and incubated with

non-immune sera IgG used for antibody purification in Section 5.2.6 (at the same concentrations as the anti-PLC ζ antibody). Both samples were incubated for 30 minutes at RT before they were washed by centrifugation twice with PBS. The resulting pelleted sample was resuspended and incubated for 30 minutes using a titration of secondary fluorescent antibody [1-5 μ g/ml] (Alexa Fluor 488 F(ab')₂ goat anti-rabbit IgG), and washed twice in PBS.

Samples were analysed by flow cytometry using a BD LSRII Flow Cytometer (BD Biosciences, Oxford UK) equipped with a 488nm laser. The flow cytometer was set to analyse 10,000 events (sperm) per sample (fluorescence conjugated PLC ζ ; fluorescence conjugated IgG negative control; and unlabelled sperm cells). A gate was drawn around the distinct sperm population. The sperm labelled with PLC ζ was directly compared with the negative control (gate set at 1-2 %). All data were analysed using FACS DIVA 5.03 software (BD Biosciences, Oxford, UK). These experiments were performed with the help of Dr Rebecca Dragovic (Flow Cytometer Operator, Nuffield Department of Obstetrics and Gynaecology).

AIM 3 | Developing molecular tools for the study of PLCζ

5.2.6 Re-purification of anti-PLCζ antibody

Two immunogenic peptide sequences, identified in the human PLCζ amino-acid sequence by Grasa *et al.* (2008) and in the mouse PLCζ amino-acid sequence by Young *et al.* (2009) [see Table 5.2.1], and their corresponding crude final bleed sera, were obtained from Dr John Parrington (Department of Pharmacology, University of Oxford). For each species, sera was affinity-purified to both peptides using a SulfoLink Immobilisation Kit for Peptides (Thermo Scientific UK) in accordance with the manufacturer's instructions, and eluted antibody collected after allowing for overnight binding. The concentration of the final affinity purified antibodies was determined by optical density at 280nm (OD₂₈₀) using the spectrophotometer. A 10% SDS-PAGE gel was ran against a pre-stained protein standard and stained with coomassie blue to confirm purification. Antibodies were aliquoted and stored at -20°C in 50% glycerol.

Table 5.2.1 | Human and mouse PLCζ peptide sequences.

	Human PLCζ	Mouse PLCζ
Peptide 1	RESKSYFNPSNIKE	MEMRWFLSKIQDEFRGGKI
Peptide 2	ETHERKGSDKRGDN	CMNKGYYRRVPLFSK

5.2.7 Preparation of a monoclonal antibody against human PLC ζ

Production of a novel powerful developmental tool in the form of a monoclonal antibody against PLC ζ was attempted. cDNA encoding for full-length human PLC ζ was ligated into the pGEX (6P-2) glutathione S-transferase (GST) fusion protein expression vector (GE Healthcare Life Sciences, UK) [Figure 5.2.3] previously by Dr John Parrington's group, and the construct verified by DNA sequencing. The resultant construct was subsequently transformed into BL21-Star (DE3) pLysS One Shot competent cells (Invitrogen, UK). Bacteria were cultured in Luria broth supplemented with 17mg/ml chloramphenicol and 100mg/ml ampicillin overnight at 37°C with vigorous shaking, then diluted and allowed to grow until the OD₆₀₀ was between 0.6-0.7, after which protein expression was induced for 4 hours at 37°C using 0.5M isopropyl- β -D-thiogalactopyranoside (IPTG; Sigma, UK).

Induced cultures were centrifuged at 6000rpm for 10 minutes; the supernatant removed, and pelleted cells resuspended in PBS. Phenylmethylsulfonyl fluoride (PMSF; Sigma, UK) protease inhibitor was added to the cells and lysed on ice by sonication. PBS/1% Triton-X100 was added and the sonicated lysate was spun at 19,000rpm for 20 minutes at 4°C. The supernatant was subsequently filtered through 0.45 μ m syringe filters (VWR, UK) and added to an equilibrated column with glutathione sepharose beads (GE Healthcare Life Sciences, UK) for GST-tagged PLC ζ fusion, which were then washed with PBS, PBS/1% Triton-X100, TNE wash buffer (50mM Tris pH8.0, 150mM NaCl, 10mM EDTA) then with TNED (TNE with dithiothreitol (DTT)) before incubating overnight at RT in TNED with 3c PreScission protease (GE Healthcare Life Sciences, UK) to cleave the GST tag from PLC ζ . Protein was eluted in TNED and a 10% SDS-PAGE gel was run against a pre-stained protein standard and stained with coomassie blue to confirm purification.

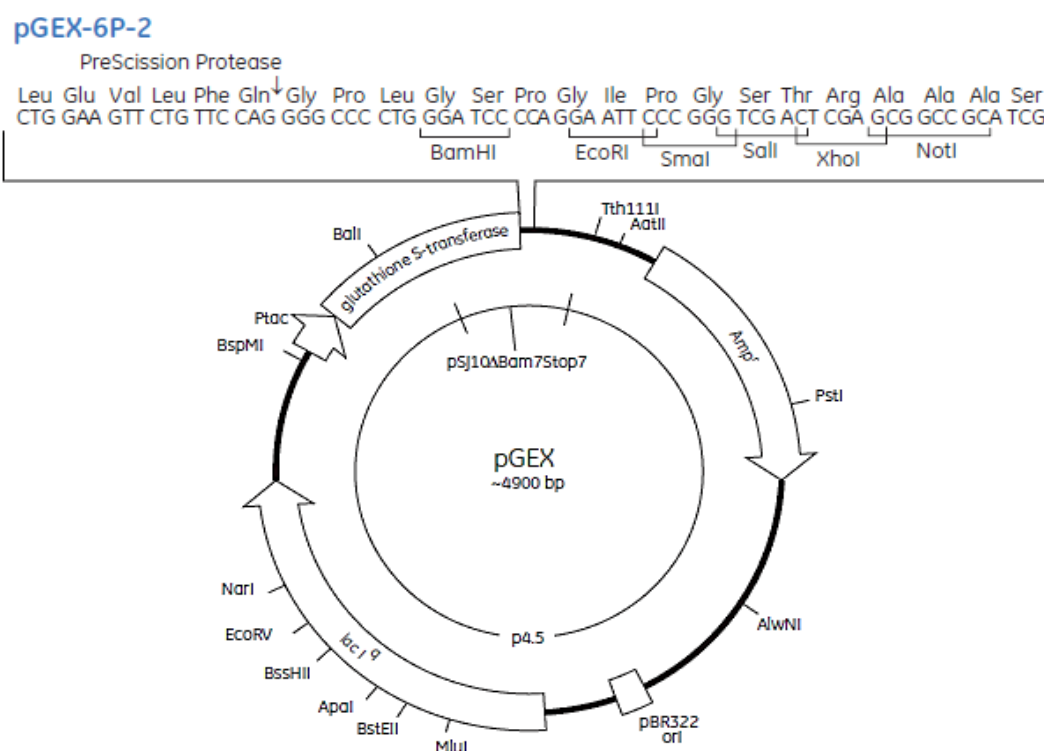


Figure 5.2.3 | Map of pGEX glutathione S-transferase (GST) fusion expression vector showing the different cloning sites. The pGEX-6P Expression Vector permits convenient site-specific cleavage and simultaneous purification on Glutathione Sepharose beads.

It was planned that the resultant purified GST-free PLC ζ protein would be sent to Covalab UK (1mg), who would perform four injections of the protein in solution (one immunisation injection followed by three boosters), to stimulate the production of antibodies within the mice to the injected antigen. The antibody-forming cells would then be isolated from the mouse's spleen. Monoclonal antibodies are produced by fusing single antibody-forming cells to tumour cells grown in culture, producing a hybridoma. On cultivation, hybridoma produce large quantities of identical antibody molecules which will identify the specific protein, PLC ζ , which induced its formation.

5.3 Results

AIM 1 | Immunohistochemical analysis of human testicular biopsies

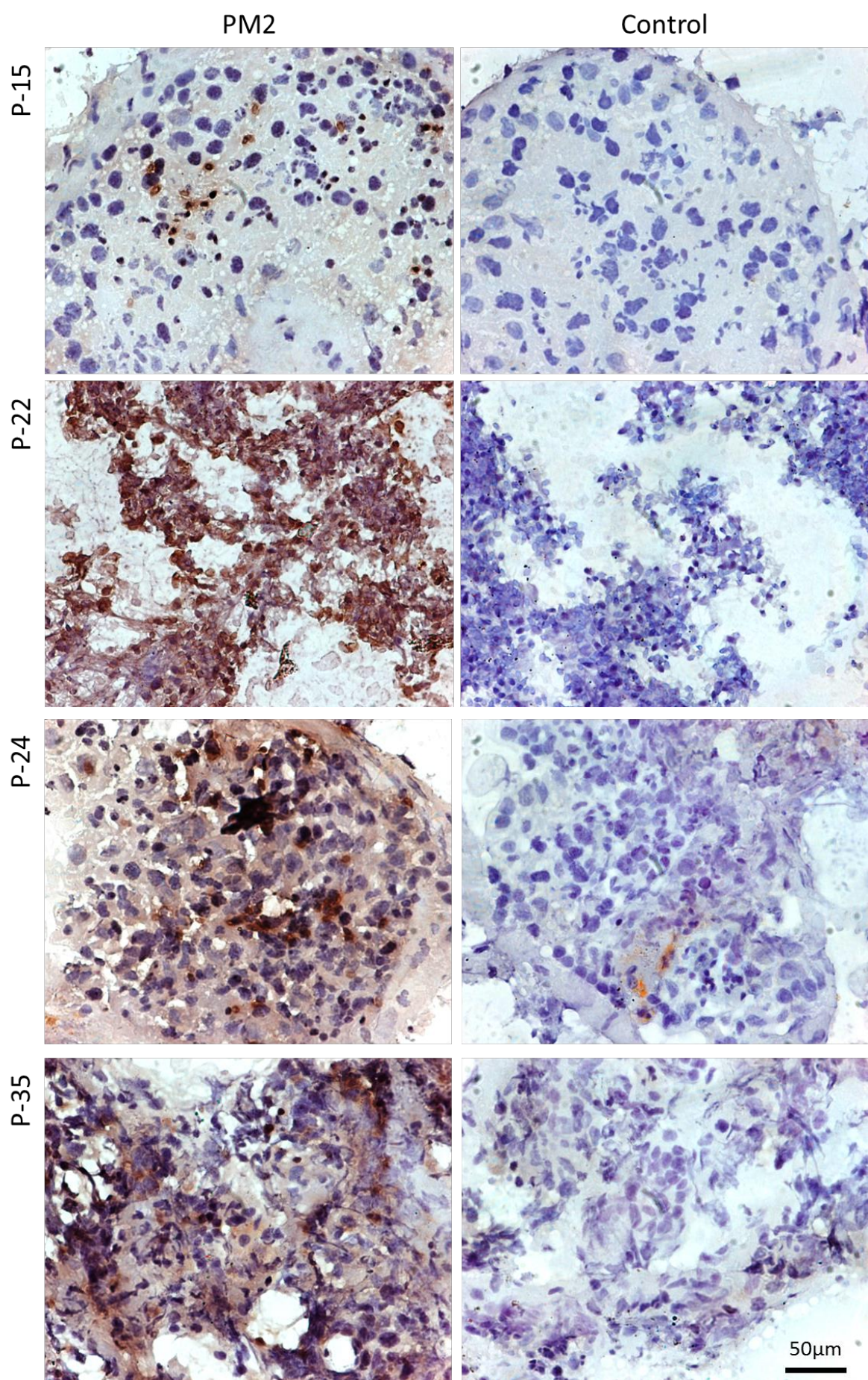
Human testicular biopsies (n=29) were analysed for PLC ζ localisation. Patients were coded with “P” (patient) followed by a number, e.g. P-15, P-24.

5.3.1 Optimisation of immunohistochemical technique

This study used an anti-PM2 antibody to confirm whether sperm were present in the testicular biopsies before determining the presence and localisation of PLC ζ . Staining of a variety of different testicular biopsies revealed that PM2 was present in many of the biopsies, and the biopsies analysed in this study were only the ones that exhibited positive PM2 staining (n=29). Sections were counter-stained with haematoxylin to assess tissue integrity and morphology. Due to the nature of collection, manipulation and preparation of the biopsies, some biopsies had a very disorganised structure, whilst in other samples whole seminiferous tubules were identifiable. Figure 5.3.1 demonstrates representative images of biopsies showing positive PM2-stained cells. Biopsies P-22 and P-35 represent examples of disorganised biopsies with no testicular seminiferous structure identifiable, whilst biopsies P-15 and P-24 represent biopsies where seminiferous tubules were clearly identifiable [Figure 5.3.1].

Figure 5.3.1 | **Sections of human testicular biopsies stained with PM2 (dark brown / black) and nuclei counterstained with haematoxylin (blue); with control sections stained with haematoxylin alone.** Biopsies of patients P-15 and P-24 represent whole biopsies in which seminiferous tubules are distinguishable whilst biopsies of patients P-22 and P-35 represent disorganised structures in which no tubules are discernible. Images were taken at x40 magnification.

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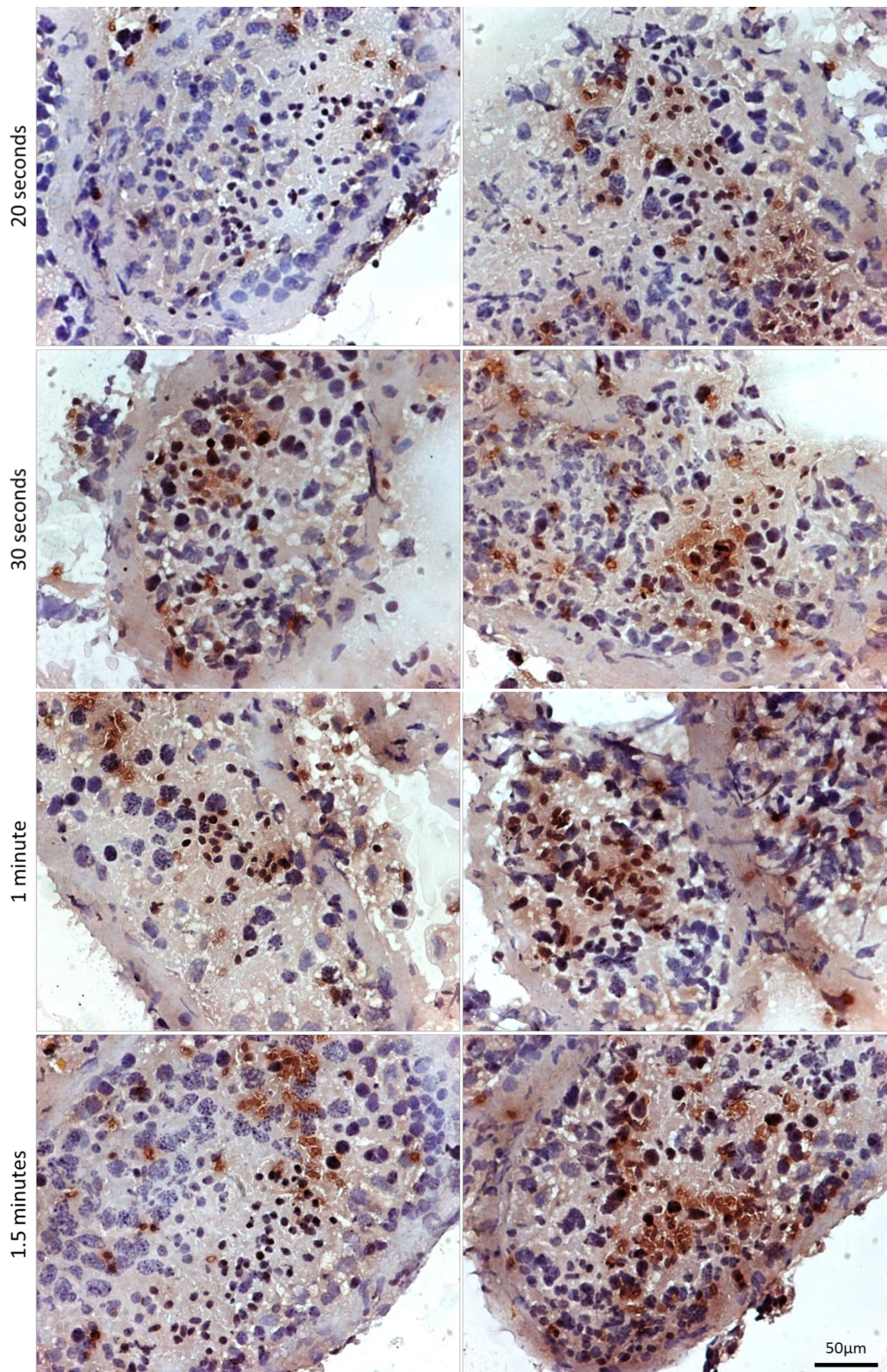


Further to ascertaining that the PM2 antibody could successfully identify sperm within human testicular biopsies, the time for DAB development which provided the best signal for PLC ζ detection was also ascertained. Various time-points, from 20 seconds to 1.5 minutes incubation in the DAB reaction with nickel enhancement were analysed [Figure 5.3.2]. Representative images captured from each time-point suggested that the signal demonstrated at 1.5 minutes was optimal, with PM2-positive sperm exhibiting a darker stain (black) as opposed to a more brownish stain exhibited in less than 1.5 minutes.

In order to determine that staining with the anti-PLC ζ antibody was specific, an immunising peptide blocking experiment was performed on sections of a testicular biopsy in which whole structures were recognisable (P-15). Whilst staining with the anti-PLC ζ antibody showed clearly specific staining in the equatorial and post-acrosomal regions, the section which was co-incubated with the peptide showed no positive staining, indicating that PLC ζ staining in un-blocked sections was genuine [Figure 5.3.3].

Figure 5.3.2 | Sections of human testicular biopsies stained with PM2 (dark brown / black) and nuclei counterstained with haematoxylin (blue) and developed with DAB/Ni. The optimal developing was ascertained by undergoing a time-series 20 seconds – 1.5 minutes in the DAB/Ni. Optimal time was determined to be 1.5 minutes. Images were taken at x40 magnification.

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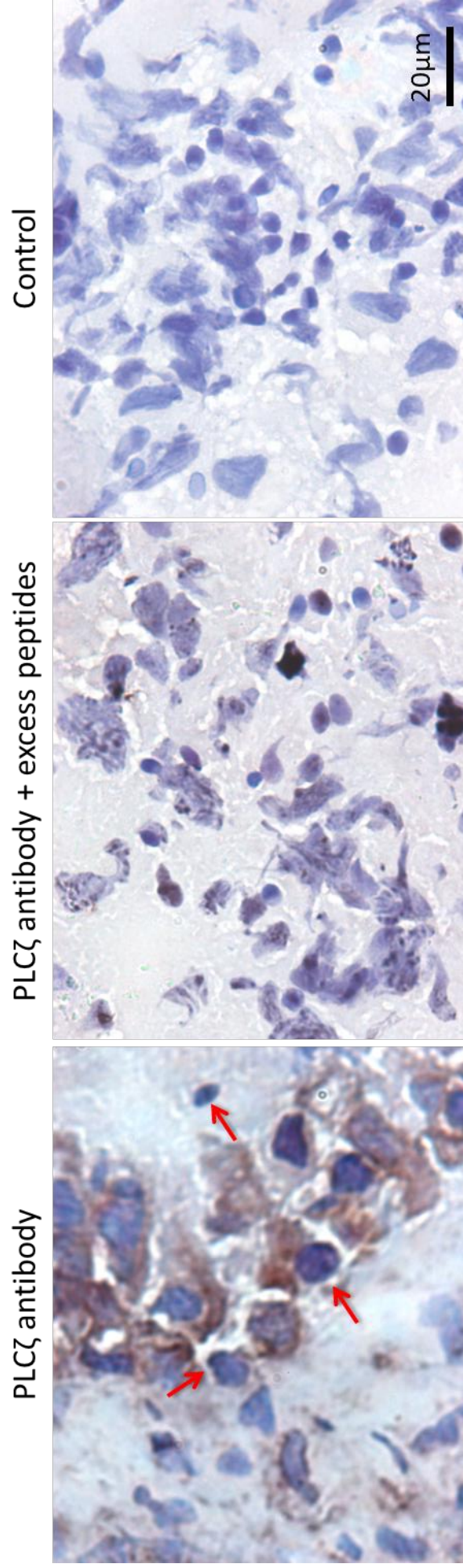


Figure 5.3.3 | Testing specificity of the anti-PLC ζ antibody using immunising peptide blocking experiment. Sections from a human testicular biopsy (P-15) stained with 25 μ g/ml anti-PLC ζ antibody only and with excess peptide showing specific staining in sperm cells in the section stained with PLC ζ antibody alone in the post-acrosomal and equatorial regions (red arrow), and no immunoreaction in the section with the neutralised antibodies. A negative control section without the addition of primary antibody was also performed. This demonstrated that staining with the anti-PLC ζ antibody is specific. Images were captured at x100 magnification.

5.3.2 Analysis of PLC ζ localisation and expression in human testicular biopsies

Following specificity testing of the PLC ζ antibody, testicular biopsies from 29 patients in which sperm tested positive with PM2 staining were analysed for the presence and localisation of PLC ζ . All sections were counter-stained with haematoxylin. Due to the nature of the biopsy, it was very difficult to cut very thin sections from the frozen embedded tissues, and sections were thus cut at 8-12 μ m thickness. Out of the 29 biopsies analysed, seminiferous tubules were recognisable in 19 biopsies, and biopsy structure was disorganised in 10. On analysis of the sections at high magnification for PLC ζ , 24 exhibited PLC ζ (82.7%), but in 9 biopsies some sperm exhibited PLC ζ whilst some did not; 5 biopsies exhibited a total absence of PLC ζ [Table 5.3.1]. In the biopsies exhibiting PLC ζ (24): 22 demonstrated equatorial localisation (91.7%), whether alone or in combination with other populations; 14 demonstrated a post-acrosomal localisation (58.3%), whether alone or in combination with other populations; and 5 demonstrated an acrosomal PLC ζ localisation (20.8%), whether alone or in combination with other localisations. Figure 5.3.4 demonstrates representative images from sections of biopsies exhibiting PLC ζ .

Table 5.3.1 | **Analysis of 29 human testicular biopsies** (A – acrosomal PLCζ; Eq – equatorial PLCζ; PA – post-acrosomal PLCζ). Staining of PLCζ was graded according to the following classification (neg – no staining detected; ++/-- – some apparent staining, some cells show no staining; +++ – predominant staining)

Patient no.	Biopsy appearance	PLCζ?	PLCζ localisation
5	Disorganised	++/--	Eq
6	Whole	neg	n/a
7	Disorganised	+++	A, Eq, PA
8	Whole	neg	n/a
9	Whole	neg	n/a
10	Disorganised	+++	Eq, PA
11	Whole	+++	Eq
12	Whole	++/--	Eq, PA
13	Whole	+++	A, Eq, PA
14	Disorganised	++/--	Eq
15	Whole	+++	Eq, PA
17	Whole	+++	Eq, PA
19	Whole	+++	Eq, PA
20	Whole	+++	A, Eq
22	Disorganised	+++	A, Eq, PA
24	Whole	++/--	Eq, PA
25	Whole	+++	PA
27	Whole	+++	Eq, PA
28	Whole	neg	n/a
30	Whole	++/--	Eq, PA
31	Disorganised	++/--	Eq
33	Disorganised	++/--	Eq, PA
34	Whole	neg	n/a
35	Whole	+++	Eq
38	Whole	+++	Eq
39	Disorganised	++/--	Eq
40	Whole	+++	Eq
42	Disorganised	+++	A, Eq
43	Disorganised	++/--	PA

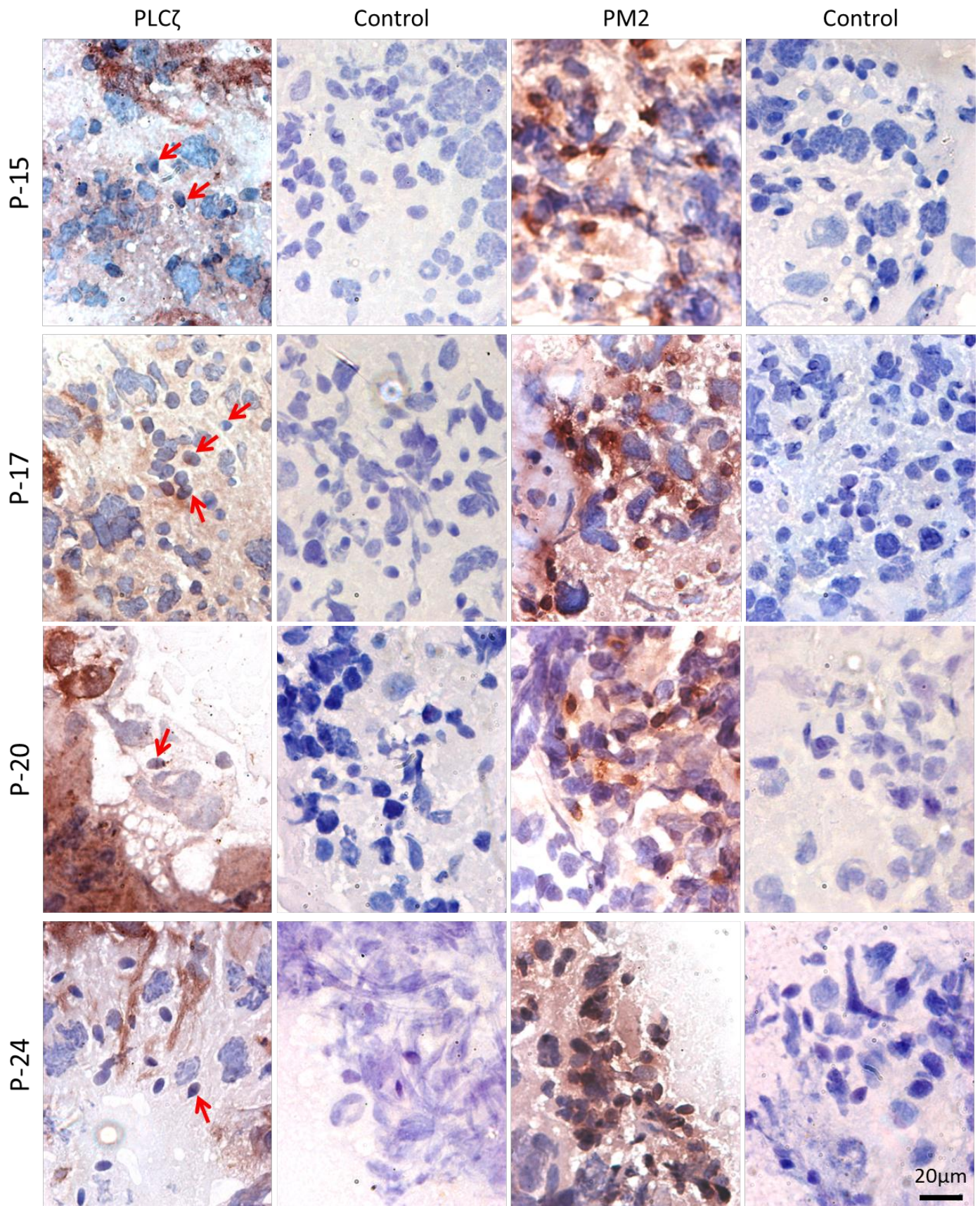


Figure 5.3.4 | **Representative images of sections of human testicular biopsies stained with PM2 and PLCζ (black / dark brown).** Sections were counterstained with haematoxylin (blue) and developed with DAB/Ni. Images were taken at 100x magnification, and negative controls were included for each antibody where the primary antibody was omitted. Arrows indicate positive immunostaining for PLCζ.

5.3.3 Developmental expression of PLCζ in human testes

The developmental expression of PLCζ was analysed from biopsies in which the seminiferous tubule structure was reasonably intact. Figure 5.3.5 shows a representation of a section from such a biopsy (P-17) indicating positive immunostaining of PLCζ. PLCζ was identified from the round spermatid stages of development [Table 5.3.2 and Figure 5.3.5], but not in earlier stages. Due to the previous manipulation of the biopsies for TESE, the structures, and thus the developmental stage of the sperm, were more than occasionally difficult to determine.

Table 5.3.2 | Summary of PLCζ staining in stages of spermatogenesis visible in human testicular biopsies (neg – no staining detected, +++/-- – predominantly positive staining, some cells show no staining, +++ – predominantly positive staining).

Cell type	PLCζ staining
Spermatogonia	neg
Spermatocyte	neg
Round spermatid	+++/--
Elongated spermatid	+++

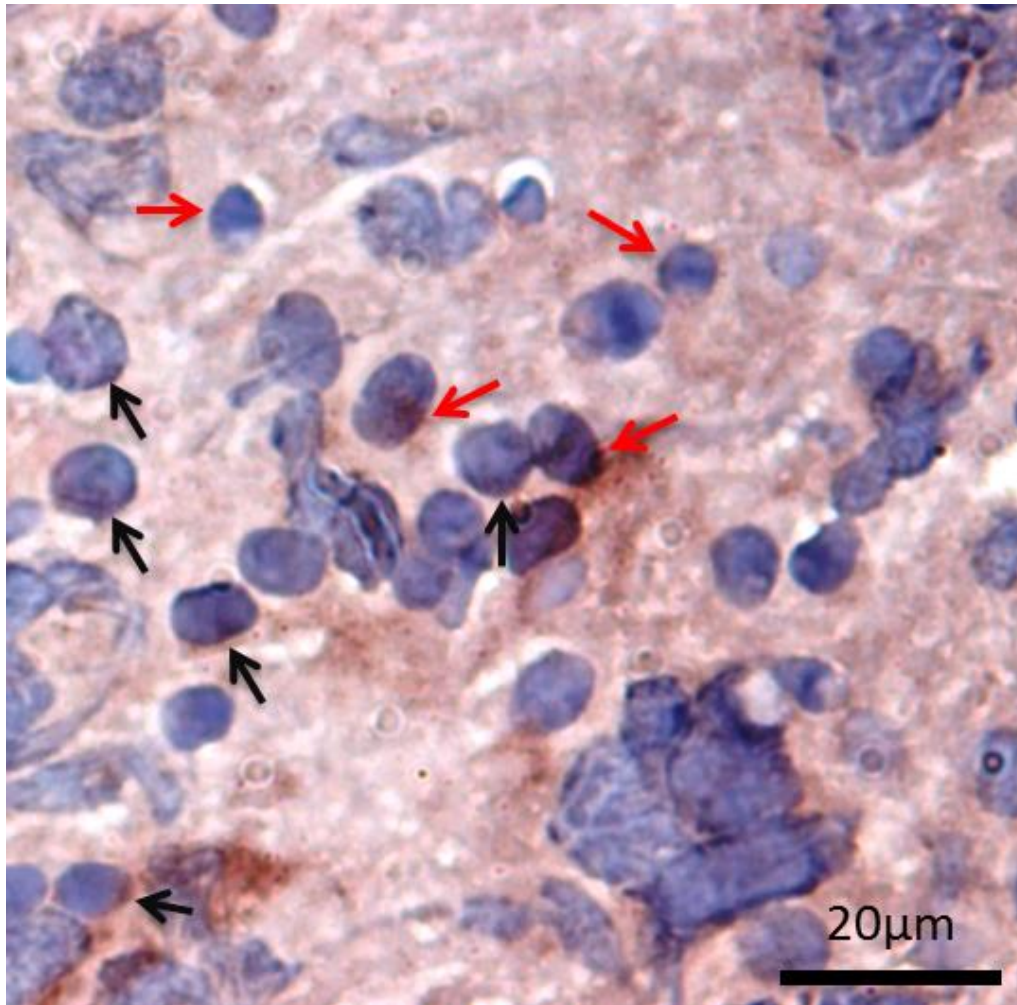


Figure 5.3.5 | **Representative image of a section of a human testicular biopsy (P-17) showing PLC ζ immunostaining** (black arrow – positive PLC ζ staining in round spermatids; red arrow – positive PLC ζ staining in elongated spermatids in the equatorial and post-acrosomal regions). Image was taken at x100 magnification.

AIM 2 | Quantification of PLCζ in human sperm samples using flow cytometry

5.3.4 Optimisation of flow cytometry technique (please refer to Figure 5.2.2)

Experiment 1:

The first optimisation experiment was designed to compare a 30 minute incubation in anti-PLCζ versus overnight. For this experiment, a donor sample obtained from the OFU was used. Both anti-PLCζ, and the anti-rabbit Alexa 488 FITC secondary (2° Ab) were used at the same concentrations used for fluorescent immunocytochemistry (at 25µg/ml and 5µg/ml respectively). Serum IgG that was used for purification of the anti-PLCζ antibody was used as a control at the same concentration as the anti-PLCζ antibody. An unlabeled control was run alongside all samples containing secondary antibody alone. The experiment was applied to a fresh control sample obtained from the OFU. Samples were prepared as follows (each box represents a tube), with one million sperm cells per tube:

Overnight PLCζ (25µg/ml) + 2° Ab (5µg/ml)	Overnight serum IgG (25µg/ml) + 2° Ab (5µg/ml)	30mins PLCζ (25µg/ml) + 2° Ab (5µg/ml)	30mins serum IgG (25µg/ml) + 2° Ab (5µg/ml)	Unlabelled sperm with 2° Ab (5µg/ml) only
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Results shown in Figure 5.3.6 indicated that the 30 minute incubation sample produced a tight control peak with serum IgG [Figure 5.3.6B] and 90.6% of the sperm showed positive staining. In comparison, in the samples that were left overnight in anti-PLCζ antibody, only 45.6% of the cells showed positive staining plus the background staining in the IgG control was higher [Figure 5.3.6C]. This may be due to over-saturation of the

cells with the antibody. This experiment showed clearly that incubation for 30 minutes in anti-PLC ζ antibody was superior when compared to overnight incubation.

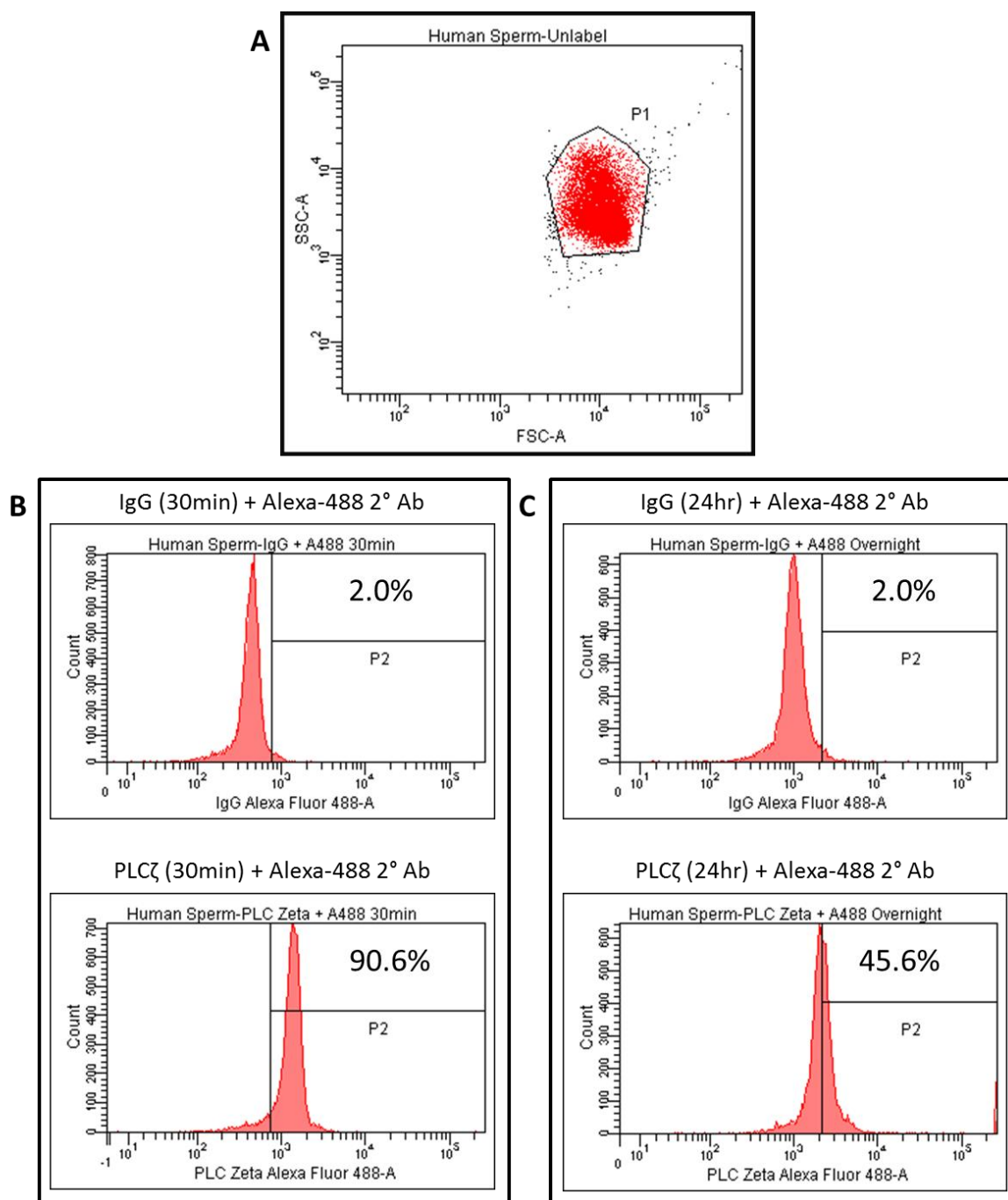


Figure 5.3.6 | Flow cytometric analysis of PLC ζ in sperm. (A) FSC. vs. SSC profile of sperm. Histograms showing the percentage of positive labelling with 25 μ g/ml rabbit serum IgG (upper panel) and 25 μ g/ml anti-PLC ζ (lower panel) comparing 30 minute (B) and overnight (C) incubation times. Cells were co-stained with 5 μ g/ml Alexa 488 FITC secondary antibody (2° Ab) for PLC ζ identification.

Experiment 2:

Next, a titration of both the primary and secondary antibodies was performed to achieve the best discrimination of the positive population from the negative control. A further control was also included to confirm whether positive staining was truly intracellular (by comparing permeabilised and non-permeabilised samples). The experiment was applied to a sample that was received from Venezuela (Section 5.2.3). Samples were prepared as follows, with one million sperm cells per tube:

Permeabilised			
5µg/ml PLCζ + 1µg/ml 2° Ab	5µg/ml serum IgG + 1µg/ml 2° Ab	5µg/ml PLCζ + 5µg/ml 2° Ab	5µg/ml serum IgG + 5µg/ml 2° Ab
10µg/ml PLCζ + 1µg/ml 2° Ab	10µg/ml serum IgG + 1µg/ml 2° Ab	10µg/ml PLCζ + 5µg/ml 2° Ab	10µg/ml serum IgG + 5µg/ml 2° Ab
25µg/ml PLCζ + 1µg/ml 2° Ab	25µg/ml serum IgG + 1µg/ml 2° Ab	25µg/ml PLCζ + 5µg/ml 2° Ab	25µg/ml serum IgG + 5µg/ml 2° Ab
Unlabelled sperm with 2° Ab (5µg/ml) only			

Non-permeabilised		
25µg/ml PLCζ + 5µg/ml 2° Ab	25µg/ml serum IgG + 5µg/ml 2° Ab	Unlabelled sperm with 2° Ab (5µg/ml) only

Unlike the previous experiment, no positive PLCζ staining was evident in any of the samples analysed [Figure 5.3.7].

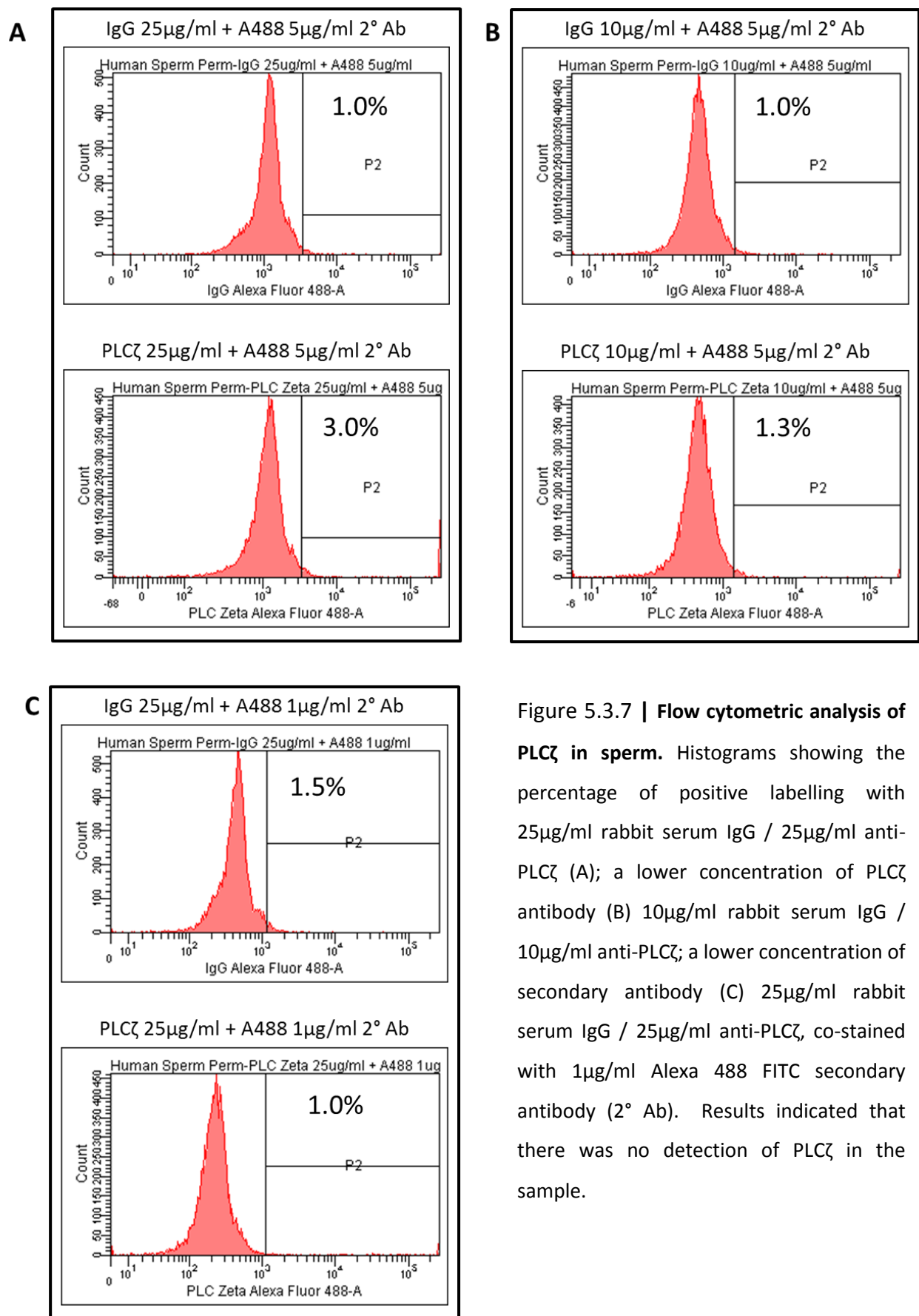


Figure 5.3.7 | Flow cytometric analysis of PLCζ in sperm. Histograms showing the percentage of positive labelling with 25µg/ml rabbit serum IgG / 25µg/ml anti-PLCζ (A); a lower concentration of PLCζ antibody (B) 10µg/ml rabbit serum IgG / 10µg/ml anti-PLCζ; a lower concentration of secondary antibody (C) 25µg/ml rabbit serum IgG / 25µg/ml anti-PLCζ, co-stained with 1µg/ml Alexa 488 FITC secondary antibody (2° Ab). Results indicated that there was no detection of PLCζ in the sample.

Experiment 3:

Fluorescent immunocytochemical analysis was undertaken on the sample used in Experiment 2 (25µg/ml anti-PLCζ) to check if background staining was abnormally high thereby masking positive signals from PLCζ-stained cells. Indeed, although specific staining was detected, there was high background. A second control sample from Venezuela was also analysed immunocytochemically which also exhibited high background fluorescence.

Experiment 1, which indicated positive PLCζ immunolabelling, was applied on sperm that was fixed immediately after collection and prepared for flow cytometry, yet the samples from Venezuela were fixed upon collection, but were stored for 1-3 months before analysis, it was considered that prolonged storage of fixed sperm may have affected the antigen therefore requiring prolonged exposure to the antibody. Overnight incubation in anti-PLCζ antibody was compared with 30 minute incubation, and the following samples were prepared with the second Venezuelan sample:

Overnight PLCζ (25µg/ml) + FITC (5µg/ml)	Overnight serum IgG (25µg/ml) + FITC (5µg/ml)	30mins PLCζ (25µg/ml) + FITC (5µg/ml)	30mins serum IgG (25µg/ml) + FITC (5µg/ml)	Unlabelled sperm with FITC (5µg/ml) only
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Results showed no change between overnight and 30 minute incubations, with no positive PLCζ staining [Figure 5.3.8].

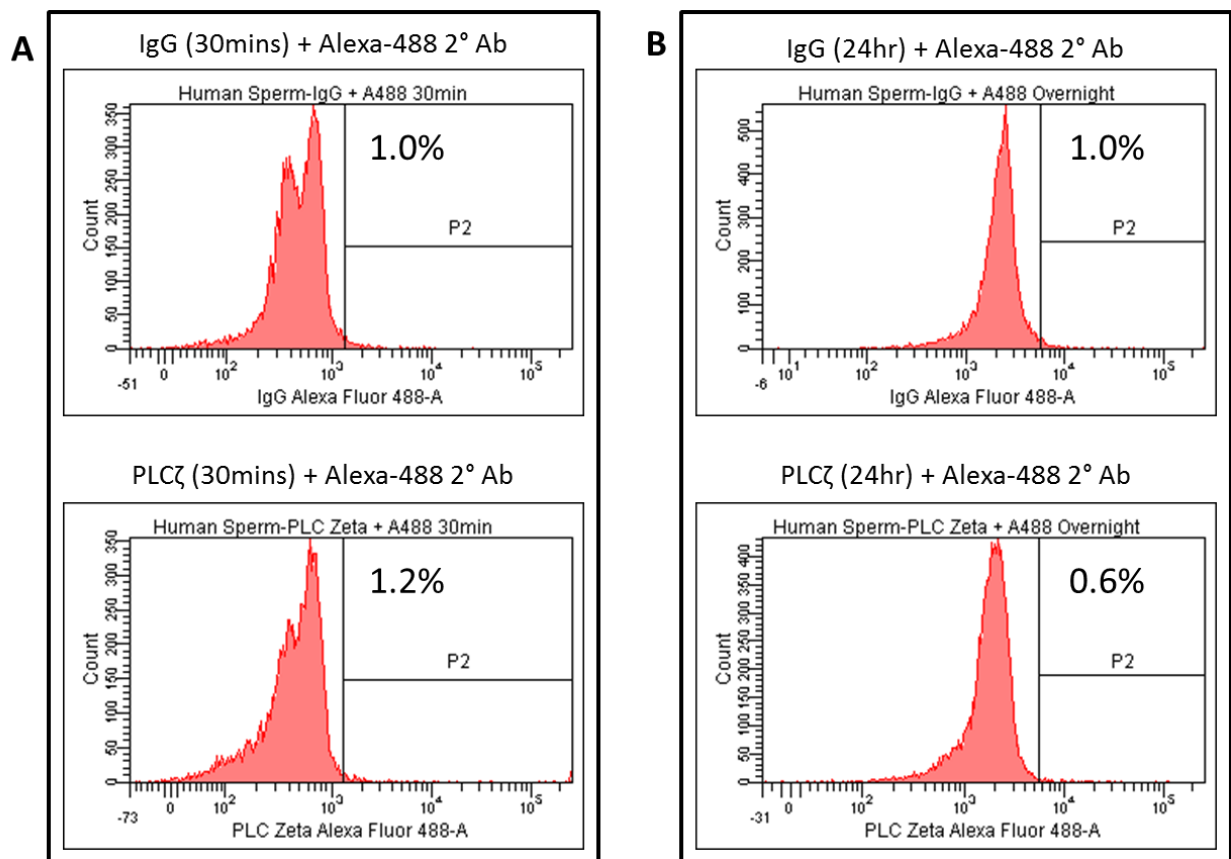


Figure 5.3.8 | Flow cytometric analysis of human sperm from a second Venezuelan sample comparing 30 minute (A) and overnight (B) incubation of sperm labelled with 25µg/ml rabbit serum IgG / 25µg/ml anti-PLCζ, co-stained with 5µg/ml Alexa 488 FITC 2° Ab for PLCζ identification. Results indicated that there was no detection of PLCζ in the sample.

Experiment 4:

To ascertain whether the Venezuelan samples were not providing any positive signals by flow cytometry as a result of prolonged storage since fixation, an immunocytochemistry experiment was carried out comparing samples received from the OFU and stored at 4°C for various periods of time, with a sample from Venezuela stored for 1-3 months. The following samples were prepared:

OFU-1 > 6 months since fixation	OFU-2 ~3.5 months since fixation	OFU-3 2.5 weeks since fixation	V-1 1-3 months since fixation
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Representative images were analysed from each sample [not shown]. Time from fixation did not appear to have any effect on the level of background fluorescence, as samples obtained from the OFU which were stored for over 6 months exhibited similar levels of background fluorescence as that seen in samples fixed for 2.5 weeks only. The sample from Venezuela however, exhibited a relatively higher level of background fluorescence in comparison to the samples from the OFU. Due to time restrictions, it was not possible to continue analysis.

AIM 3 | Developing molecular tools for the study of PLC ζ

5.3.5 Re-purification of anti-PLC ζ antibody

In order to validate previous work in human and mouse sperm reporting multiple populations of PLC ζ localised to distinct regions within the sperm head, two polyclonal antibodies for human and mouse PLC ζ that were previously used by Grasa *et al.* (2008), Young *et al.* (2009) and Heytens *et al.* (2009), were re-purified. Antibodies were purified against two PLC ζ immunogenic peptides for each respective antibody. Following affinity purification, eluted products were separated by SDS-PAGE and analysed by coomassie blue staining [Figure 5.3.9]. Expected bands corresponding to the heavy and lights chains of the IgG complex in each newly purified antibody were identified.

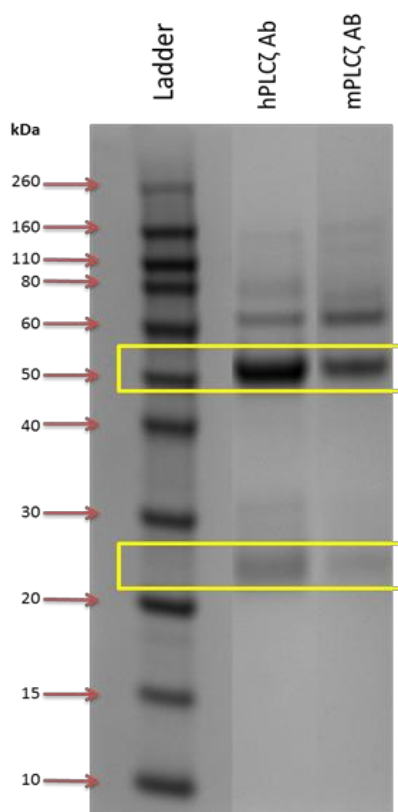


Figure 5.3.9 | SDS-PAGE gel stained with coomassie blue showing affinity-purified anti-human (hPLC ζ) and anti-mouse (mPLC ζ) antibodies, against a 260kDa protein standard ladder. The gel shows a prominent band at ~50kDa, corresponding to the heavy chain of the IgG complex, and a lighter band at ~25kDa corresponding to the light chain of the IgG.

In order to validate the re-purified antibodies, human and mouse sperm were immunostained with the re-purified antibodies to analyse the expression of PLC ζ in comparison to those reported by Grasa *et al.* (2008) and Young *et al.* (2009) respectively, using the same experimental conditions used by the previous authors. Indeed the populations reported by Grasa *et al.* (2008) in the equatorial, acrosome and post-acrosome of human sperm; and the acrosomal and post-acrosomal populations reported in mouse sperm by Young *et al.* (2009) were exhibited [Figure 5.3.10]. These re-purified antibodies was used throughout this thesis, and confirmation of the specificity with that of previous published work allows for comparison of findings obtained herein with that of previously published studies.

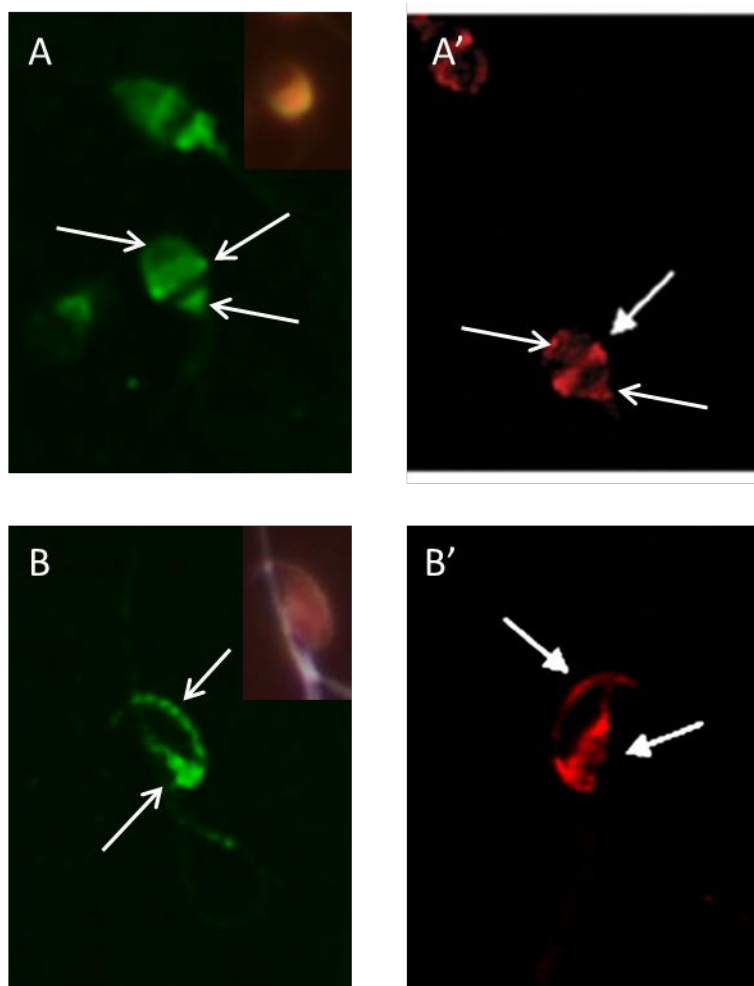


Figure 5.3.10 | Immunofluorescent localisation of PLC ζ validating the re-purification of anti-PLC ζ antibody in (A) human sperm and (B) mouse caudal sperm (green) (insert shows darkfield image). The equatorial PLC ζ localisation (arrow) as well as the acrosomal and post-acrosomal localisations in (A) correlating with (A') showing PLC ζ localisation in fertile human sperm by Grasa *et al.* (2008) (red); and the acrosomal and post-acrosomal localisations in (B) correlating with (B') in mouse sperm by Young *et al.* (2009) (red).

5.3.6 Preparation of a monoclonal antibody against PLC ζ

Since bacteria are easy to culture, grow fast and produce high yields of recombinant protein, the bacterial expression system was chosen to clone human PLC ζ ligated into the GST-tagged pGEX protein expression vector to purify protein for monoclonal antibody production. Bacteria containing pGEX-PLC ζ were cultured and pelleted bacteria sonicated to lyse cells before purification and cleavage of the GST tag. Purification products were then separated by SDS-PAGE and analysed by coomassie blue staining [Figure 5.3.11]. A band was not detected at the expected size of human PLC ζ of ~70kDa in the eluted products as shown in Figure 5.3.11. Several attempts were made to obtain a purified product at the molecular weight corresponding to PLC ζ in order that positive identification may be processed by LC/MS, but with each purification attempt, either there was no band detected, or a band was detected at the wrong molecular weight. A total of 27 litres of bacterial culture was purified, and even on concentration of the eluted product, a band at the incorrect molecular weight was identified only. BSA protein assays consistently showed that there was no protein detected, indicating a general failure to synthesise recombinant human PLC ζ protein.

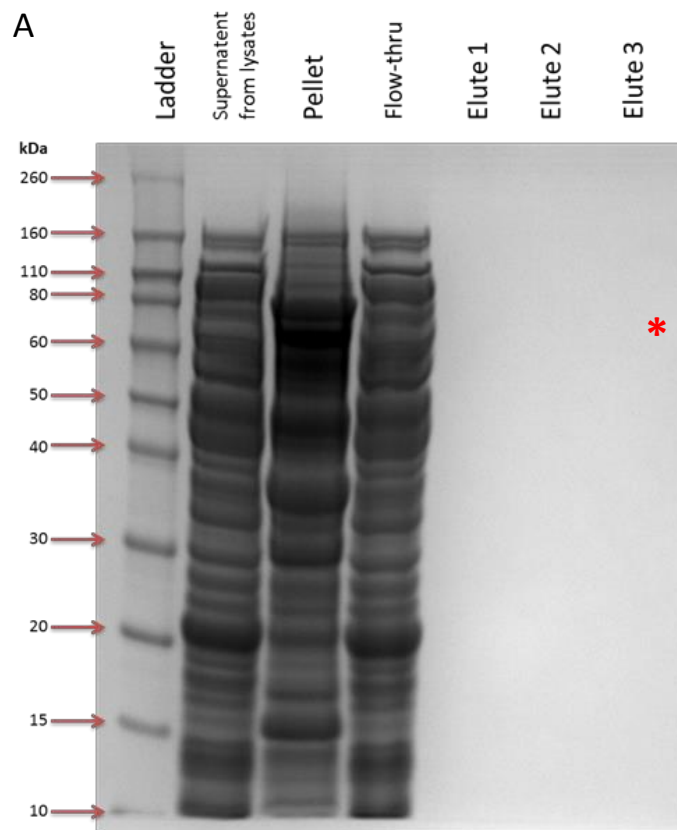
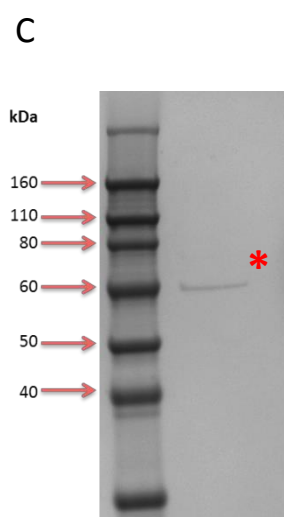
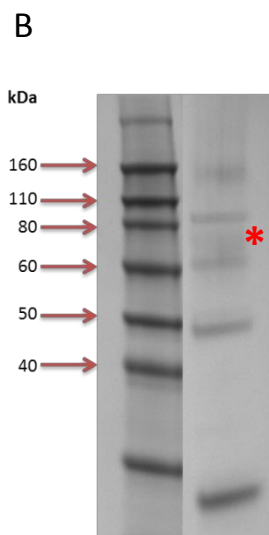


Figure 5.3.11 | **SDS-PAGE gel stained with coomassie blue showing the purification process of the pGEX PLC ζ protein (A).** There was no band present at the expected size of 70kDa (asterisk) in the eluted purified protein. Many attempts of purification were made resulting in no bands, or bands at the wrong molecular weight with the neat purified product **(B)** or after concentrating **(C)**.



5.4 Discussion

Current views in reproductive medicine and andrology have established the limited clinical value associated with the diagnostic and predictive value of semen parameter analysis (Barratt *et al.*, 2011). This limitation underpins the urgent requirement for novel methods of assessing male reproductive potential. A mouse oocyte activation test (MOAT) was first used to examine the activation capacity of sperm from an infertile patient with globozoospermia by injection of his sperm into a mouse oocyte (Rybouchkin *et al.*, 1996). This technique was later employed on a range of other patients with a history of ICSI-failure or with sperm of abnormal morphology (Tesarik *et al.*, 2002; Heindryckx *et al.*, 2005, 2008; Yoon *et al.*, 2008; Heytens *et al.*, 2009). With the establishment of the role of PLC ζ as the oocyte activation factor in sperm, and the demonstration that its deficiency or mis-localisation underlies certain forms of male infertility, PLC ζ analysis represents a powerful agent for assessing oocyte activation ability. The fact that the potency of human PLC ζ is greater than mouse PLC ζ by a hundred-fold (Cox *et al.*, 2002; Swann *et al.*, 2006), and that oocytes of different species are ‘fine-tuned’ to different levels of PLC ζ from that species (Cooney *et al.*, 2010), would explain why MOAT results cannot be extrapolated confidently into human applications as it is not sensitive enough to reveal all activation deficiencies in all sperm samples, but may only be useful in detecting extreme cases of OAD where there is complete deficiency of PLC ζ (Kashir *et al.*, 2010; Ramadan *et al.*, 2012).

It is conceivable therefore that the quantitative analysis of PLC ζ within a sperm sample represents a more precise and informative diagnostic tool for both research and clinical purposes, by providing an indicator, or biomarker, of the activation capacity of a

sample. Indeed the quantitative proportional and localisation analysis demonstrated both by Kashir *et al.* (2013) and in this study in Chapter 3 indicate that fluorescent immunocytochemical analysis possesses some diagnostic potential. However, the entire technique including sample preparation, staining and analysis is time consuming taking at least two days from sample collection to analyses, and thus is not very efficient as a daily technique for clinical use.

The quantitative analysis of sperm PLC ζ using flow cytometry may represent a rapid, useful diagnostic tool for both research and translational purposes. This technique has never been used to analyse PLC ζ expression in sperm from any species thus far, although previous studies have used this technique as a biomarker for DNA fragmentation and protamine levels, identifying differences between fertile and infertile sperm (Lukanov *et al.*, 2009; Tavalaei *et al.*, 2009). This study aimed to devise such a tool for the quantitative assessment of PLC ζ in human sperm samples. Furthermore, with the accessibility of human testicular biopsies obtained from a range of patients, including those diagnosed with obstructive and non-obstructive azoospermia, this project aimed to utilise this opportunity for assessing the possible diagnostic potential of testicular biopsies for ICSI success, as well as investigation of the developmental expression of PLC ζ in the human testis. Moreover, this project aimed to lay the foundation for a new powerful tool for research and diagnostic purposes in the form of a novel monoclonal anti-PLC ζ antibody, as well as re-purifying the polyclonal antibodies previously used by Grasa *et al.*, (2008), Heytens *et al.* (2009) and Young *et al.* (2009) to assess specificity as well as provide a supply of antibodies for the research herein.

5.4.1 Quantitative analysis of PLC ζ using flow cytometry has potential for clinical application as a biomarker for oocyte activation ability

For a fertility biomarker to be deemed applicable and efficient in the clinic, important things to consider are ease, safety, accuracy, cost-effective and speed of analysis (Barratt *et al.*, 2011). Devising such an efficient method using flow cytometry was an objective of this study. A quantitative method for analysing PLC ζ in sperm samples already exists and is currently deployed in the Coward laboratory as discussed in Chapter 3 using fluorescent immunocytochemistry. Yet, the latter technique requires at least 48 hours with laborious quantitation, so an assay that requires less time from collection to analyses would provide a more attractive option.

Flow cytometry offers a much more rapid tool for both research and translational applications. Indeed a protocol was optimised herein in which quantitative analysis can be obtained within hours of obtaining a sperm sample. In fact, much better results were obtained in the one-day protocol in which the sample was incubated for 30 minutes in anti-PLC ζ antibody compared with the overnight incubation employed in fluorescence immunocytochemistry. Indeed previous studies comparing fertile controls with infertile samples for the assessment of DNA fragmentation and protamine levels used protocols which produced their findings on the same day. The aforementioned experiment utilised a control sample obtained on-site, from the OFU, but further optimisation experiments employed samples obtained from Venezuela (Chapter 3). Unfortunately, results obtained with the OFU sample could not be duplicated with the Venezuelan samples, despite numerous attempts to optimise the protocol. Since the Venezuelan samples were not prepared in our laboratory, it is not possible to confidently state that the conditions

executed to prepare the samples were identical to those employed in our laboratory from the OFU. Time restrictions did not allow for the analysis of further samples but the initial experiment provides much hope for the clinical potential of this technique.

Traditional microscopy allows for the analysis of subcellular localisation of certain proteins within a sperm sample, but relative intensity can only be estimated on a small number of cells (100-200 per sample). It has been established from research undertaken in Chapter 3 and from recent studies (Kashir *et al.*, 2013) that the specific localisation of PLC ζ may not be vital for ascertaining the oocyte activation potential of a sample but it may be the proportion of sperm cells that exhibit PLC ζ regardless of localisation. In this way, flow cytometry is an ideal tool. Indeed, flow cytometry is now a recognised methodology for the assessment of animal sperm integrity and functionality and is used routinely for the assessment of animal semen destined for breeding programmes (Hossain *et al.*, 2011). Flow cytometry offers many advantages over microscopy techniques including speed, accuracy, objectivity, reproducibility and statistical analysis power (Tavalaee *et al.*, 2009).

Moreover, current techniques have emerged which combine fluorescence microscopy and flow cytometry combined with biomarkers for sperm quality/fertility. A high-speed quantitative imaging flow cytometer - ImageStream (Amnis Corp., Seattle, WA; George *et al.*, 2004) – which can analyse biomarkers in each passing sperm by capturing images in bright field, side scatter and fluorescence, and has quantitative image analysis tools that measure parameters related to probe intensity, size, shape, texture and localisation (Buckman *et al.*, 2009). Its application in human andrology offers alternative sperm tests and automated assays with better predictive value for ART outcome (Weber *et al.*, 2005). Using ImageStream for quantitative analysis of PLC ζ in sperm samples, and

comparing fertile controls with OAD samples is a very exciting prospect, and on optimisation of flow cytometric analysis, should be considered.

On a different note, many functional mRNAs have been shown to be delivered into the oocyte after fertilisation (Miller *et al.*, 1994; Boerke *et al.*, 2007; Lalancette *et al.*, 2008). PLC ζ mRNA has also been identified in ejaculated sperm, though its function is unknown. This presents a conundrum in current understandings since sperm is known to be transcriptionally inactive, thus whether this mRNA is functional or left-over remains unknown. However, previous work has shown the activation of oocytes of micro-injection of PLC ζ mRNA. Several previous studies have demonstrated a differential expression of some key mRNAs in infertile males compared with fertile males (Garrido *et al.*, 2004, 2009; Meseguer *et al.*, 2006), thus suggesting the potential for a fertility index through relative expression analysis. In a recent study, Aghajpour *et al.* (2011) investigated whether the level of PLC ζ mRNA in sperm may be an index of the potential of a patient sample to induce oocyte activation, and indeed the authors identified a significant reduction in levels of PLC ζ mRNA in individuals with low or failed fertilisation with ICSI, compared with fertile controls (Aghajpour *et al.*, 2011). The authors suggested that the low levels of PLC ζ mRNA may be reflective of low levels of PLC ζ protein in those patients, resulting in low or total fertilisation failure (Aghajpour *et al.*, 2011). Thus the relative expression of PLC ζ mRNA may also be used as a credible biomarker of the fertilising potential of a sperm sample. Indeed through the use of microarray analysis of mRNA expression, several authors have hypothesised that this technology may represent the future of male infertility diagnosis (Krawetz *et al.*, 1999; Krawetz, 2005; Ostermeier *et al.*, 2005; Miller *et al.*, 2005; Miller and Ostermeier, 2006; Barratt *et al.*, 2011).

Furthermore, it is currently suggested that fertility clinics adopt a genetic screening evaluation with the incidence of severe idiopathic spermatogenic failure and obstructive azoospermia presented. Indeed, it has been reported that chromosomal anomalies are found in about 7% men with idiopathic spermatogenic failure (McLachlan and O'Bryan, 2010), and routine karyotyping is recommended particularly in men that present with low sperm concentrations ($<10 \times 10^6/\text{ml}$) since aneuploidies are reported in many such cases which may lead to such chromosomal aberrations passed on to offspring should ICSI be carried out. Moreover, Y chromosome microdeletions are the most commonly identified genetic cause of infertility thus screening for this anomaly also requires attention, with highest frequency found in males presenting with azoospermia and oligozoospermia (McLachlan and O'Bryan, 2010; Stahl *et al.*, 2010).

5.4.2 Immunohistological analysis of human testicular biopsies for PLC ζ may provide indication of ICSI-success potential

Azoospermia occurs in 1% of men and in 10-15% of the infertile male population (Abdel Raheem *et al.*, 2013). Non-obstructive azoospermia (NOA) is caused by testicular failure and represents 60% of all cases of azoospermia, whilst obstructive azoospermia (OA) is caused by a blockage in the genital tract or ejaculatory dysfunction (Abdel Raheem *et al.*, 2013). The management of patients with NOA relies on TESE followed by ICSI.

Testicular biopsies can provide valuable information further to clinical evaluation and semen analysis by allowing the categorisation of men with azoospermia for purposes of prognosis and treatment (Cerilli *et al.*, 2010). However, an important issue remains

unresolved due to the lack of clinical cases and an absence of supporting literature due to the inability to interpret the variety of pathologies found in men with NOA (Cerelli *et al.*, 2010). Accurate pathological diagnosis is critical in order to determine treatment for such patients. In patients with NOA, prognostic markers for TESE are required, normally in the form of the presence or absence of mature elongated spermatids, which is successful in only 50% of these patients (Abdel Raheem *et al.*, 2013; Dohle *et al.*, 2012), whilst in patients with OA sperm retrieval rate is 100% (Dohle *et al.*, 2012), and with the advent of ICSI, these patients now have the option of fathering their own biological children. However, there is currently no assessment that can accurately predict the outcome of TESE (Samli and Dogan, 2004; Tsujimura *et al.*, 2004). Failed TESE or failed ICSI-TESE may have considerable emotional and financial implications on the couple attending clinic, so an indication of the potential outcome would serve great benefit if attained.

The pregnancy rate per treatment cycle of ICSI using testicular or epididymal-derived sperm is almost as high as with ICSI employing ejaculated sperm (Würfel, 2004), and it has been established that although ICSI results in average fertilisation rates of 70%, complete or virtually complete fertilisation failure still occurs in 1-5% of ICSI cycles (Yanagida *et al.*, 2008; Nasr-Esfahani *et al.*, 2010), and that in 40% of these cases, deficiency in oocyte activation is behind this failure (Sousa and Tesarik, 1994; Mahutte and Arici, 2003; Swann *et al.*, 2004). With PLC ζ identified as the oocyte activation factor in sperm, and its relationship with male factor infertility established, it is conceivable that a deficiency of PLC ζ is linked to failed ICSI following TESE.

Several studies have identified markers which are either up-regulated or down-regulated between testicular biopsies obtained from fertile controls and infertile patients,

such as protamine-1 (Steger *et al.*, 2003) and cyclin A (Abdou *et al.*, 2011). However, no study thus far has addressed the localisation of PLC ζ in human testicular biopsies for research or clinical purposes as a biomarker for the oocyte activation ability of sperm derived by TESE. Indeed, no study thus far has undertaken any PLC ζ immuno-analysis of human testicular or epididymal tissue to determine the developmental expression of PLC ζ .

The present study aimed to determine the presence or absence of PLC ζ in human testicular biopsies which had been previously manipulated for sperm retrieval in a fertility clinic in Belgium. Biopsies were obtained from patients diagnosed with azoospermia (NOA and OA), however, investigation was undertaken without prior knowledge of the diagnosis or clinical outcome of the biopsy being analysed. Only biopsies in which sperm was present, confirmed by positive PM2 staining, were analysed. Out of the biopsies possessing sperm, 82.7% (24/29) exhibited PLC ζ , and therefore it would be expected that sperm retrieved from these biopsies may have a greater chance of oocyte activation. Furthermore, the majority of samples expressing PLC ζ (91.7%) exhibited an equatorial localisation of PLC ζ , the population previously reported to be important for oocyte activation (Grasa *et al.*, 2008). The current study demonstrated that indeed it is possible to determine the presence and localisation of PLC ζ in a human testicular biopsy, which may provide an indication of the outcome of ICSI performed after TESE. It would be interesting to learn of the diagnosis as well as the fertility outcome of the biopsies analysed, and whether they achieved successful fertilisation, but due to time restrictions, it was not possible to obtain and decipher the biopsy codes from the collaborative Belgian clinic in sufficient time.

This study, however, has investigated for the first time the developmental expression of PLC ζ in human testicular tissue, and showed the expression of PLC ζ from the

round spermatid stage of spermatogenesis. Previous studies have identified the expression of PLC ζ mRNA and its translation during the early and late stages of mouse, equine and boar spermatogenesis (Yoneda *et al.*, 2006; Young *et al.*, 2009; Heytens *et al.*, 2010; Bedford-Guaus *et al.*, 2011; Kaewmala *et al.*, 2012). We now have early evidence that the same is true of the human.

Although TESE can indeed provide valuable information for the clinician for patients who present with azoospermia, the TESE procedure is nevertheless an invasive procedure with a small coverage of the entire testis (Schlegel and Su, 1997). In some cases, focal spermatogenesis is present in the testis of patients with NOA which is almost undetectable by current routine histological examinations following TESE (Aslani *et al.*, 2011). Molecular markers in semen have been identified which could predict spermatogenic status in such testis, presenting the probability of finding sperm in NOA testis. Signals of germ cell-specific transcripts obtained from a semen sample would thus offer a less invasive indication of sperm status in the testis of these patients as well as being a faster procedure. The presence of testis stage-specific genes DAZ and PRM2 in the semen of NOA patients can predict the presence of mature spermatids and/or sperm in testicular tissue (Song *et al.*, 2000; Steger, 2003; Haraguchi *et al.*, 2009; Aslani *et al.*, 2011), and the absence of these markers confirmed histopathological results corresponding to sertoli cell only syndrome (Aslani *et al.*, 2011). Thus, analysing these transcripts in semen would provide a non-invasive molecular diagnostic alternative to better counsel patients before undergoing TESE. Analysis of other sperm-specific, or more pertinently, activation-involving genes, such as PLC ζ may be something to be investigated further. Indeed as discussed earlier, PLC ζ mRNA has been identified in ejaculated sperm and has potential as a biomarker for fertility

(Aghajanpour *et al.*, 2011) so may indeed offer same value with NOA patients, although the true role of such RNA remains unknown, i.e. whether functional or not.

5.4.3 Production of a monoclonal PLCζ antibody is imperative

Whilst polyclonal antibodies are a collection of immunoglobulin molecules that react against a specific antigen, each identifying a different epitope, monoclonal antibodies are monospecific antibodies formed by identical immune cells that are clones of a unique parent cell. Because antibodies recognise a relatively small component of an antigen, they can therefore easily cross-react with similar epitopes of other unrelated antigens, although with less affinity (Lipman *et al.*, 2005). Monoclonal antibodies provide far better specificity since they recognise a specific epitope in the presence of other epitopes, with significantly lower cross-reactivity. This specificity renders monoclonal antibodies as powerful tools for research and for clinical applications.

Currently, all previous work undertaken on PLCζ has utilised polyclonal antibodies against PLCζ. Whilst polyclonal antibodies are highly useful for research and provide clues to possible affinities, as well as being relatively quick and inexpensive to generate, they will always remain somewhat compromised due to their nature of reacting to different epitopes. Monoclonal antibodies provide precise and consistent affinities because of their homogenous nature, and are thus much more powerful tools (Lipman *et al.*, 2005).

Findings in the field of PLCζ research have caused some debate and confusion with regards to localisation of PLCζ in sperm, and whether a different homogenous isotype of PLCζ exists at a different molecular weight, whether the different localisation patterns, if

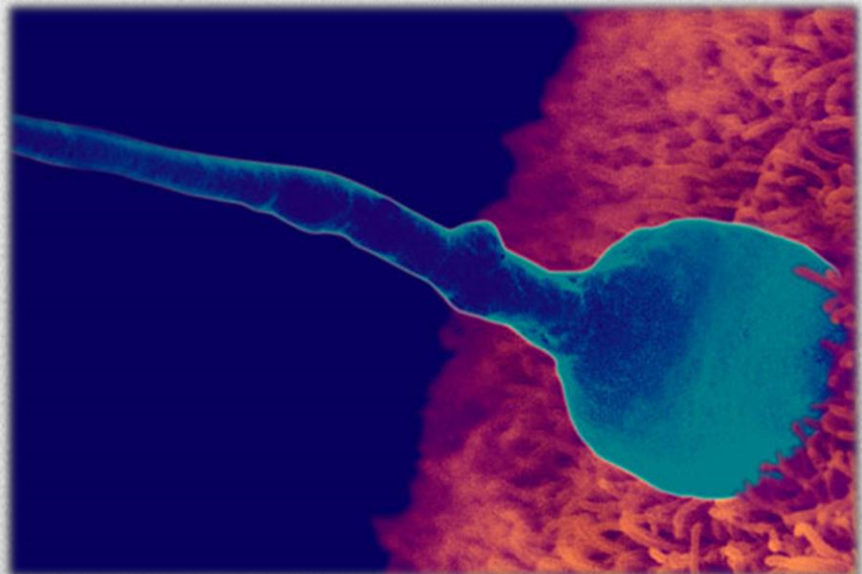
indeed there are multiple populations, have different roles and molecular sizes. The human and mouse polyclonal antibodies used by Grasa *et al.* (2008), Young *et al.* (2009) and Heytens *et al.* (2009) were re-purified in this study. Following characterisation of the antibodies through fluorescent immunocytochemistry on human and mouse sperm, identical localisation patterns were evident as those observed by Grasa *et al.* (2008) and Young *et al.* (2009). These antibodies were therefore utilised throughout this project. This antibody appears to be highly specific when used in fluorescent immunocytochemistry and immunohistochemistry; but does not produce a clean signal in immunoblotting techniques, as discussed in Chapter 4.

In order to produce a monoclonal antibody against human PLC ζ , a purified human PLC ζ recombinant protein would be required to act as the antigen to which an antibody could be produced. Many attempts were made herein to purify a human PLC ζ protein using a bacterial expression system, but the efficiency was low and no protein was made. The importance of such a tool however warrants that further attempts are made in order to produce this antibody. Indeed with the recent advancements in the successful purification of human recombinant PLC ζ protein by Kashir *et al.* (2012), Yoon *et al.* (2012) and Nomikos *et al.* (2013), the latter two who also used a bacterial expression system, and Kashir *et al.* (2012) through a mammalian expression system, or indeed the protein synthesised in Chapter 4 should a positive identification be confirmed, the production of a monoclonal anti-PLC ζ antibody is certainly the next calling.

5.4.4 Summary of findings

- It is potentially possible to determine an indication of oocyte activation ability of sperm derived from azoospermic patients by TESE by determining the presence/absence of PLC ζ
- Developmental expression of PLC ζ in human spermatogenesis is first recognised at the round spermatid stage
- There is great potential for the quantitative analysis of sperm PLC ζ using flow cytometry as a fertility biomarker
- Production of a monoclonal PLC ζ antibody failed in this study but the quest for its attainment must be continued.

Chapter 7 | GENERAL DISCUSSION



(Dr David M. Phillips / Visuals Unlimited)

6.1 Aims of the current study

It is clear that PLC ζ plays a fundamental role in the activation of mammalian oocytes, and that any molecular, biochemical or genetic perturbation is strongly linked to OAD and male factor infertility. Consequently, there is huge scope and potential for PLC ζ for its translation into the ART clinic in the form of a diagnostic/prognostic biomarker as well as providing therapeutic options for male factor infertility. However, for it to accomplish this potential, many fundamental issues which remain unresolved need to be addressed. It was the aim of this project to address some of these key matters in order that PLC ζ may achieve its full potential in reproductive medicine.

This project aimed firstly to determine the developmental expression of PLC ζ during spermatogenesis and epididymal maturation using a mouse model, and determine the effect of male age upon the total PLC ζ expression and its localisation during the sperm maturation process; secondly, to investigate the effect of male age on total PLC ζ expression and its localisation in human sperm; thirdly, attempt to synthesise a purified active recombinant PLC ζ protein to develop its therapeutic potential, and try to further the understanding of the mechanism of action of PLC ζ in mammals using a human cell line model; fourthly, assess and develop tools for PLC ζ in research and the clinic through the development of a fertility biomarker, development of PLC ζ antibodies, and finally with the accessibility of human testicular biopsies, determine the developmental expression of human PLC ζ as well as the potential of ascertaining the potential fertility outcome of sperm retrieved from biopsies.

6.2 Developments in our molecular and biochemical understanding of PLC ζ

Despite all the advances in the field of PLC ζ , much remains unclear about the mechanism of action of PLC ζ and its functional role during fertilisation. With the detection of specific localisations of PLC ζ within the sperm head of different species including humans, mice, monkeys, boars, hamsters, equine and bulls (Cox *et al.*, 2002; Saunders *et al.*, 2002; Yoneda *et al.*, 2006; Grasa *et al.*, 2008; Young *et al.*, 2009; Cooney *et al.*, 2010; Bedford-Guaus *et al.*, 2011; Kaewmala *et al.*, 2012), it is still not established whether the different localisations serve specific differential functions, although hypotheses do exist. Findings obtained in this study, together with all the localisation studies published thus far, support previous suggestions of the involvement of the post-acrosomal localisation in oocyte activation. A post-acrosomal localisation of PLC ζ has been identified in sperm of human, mice, hamster, bull, pig and boar sperm (Fujimoto *et al.*, 2004; Yoon and Fissore, 2007; Grasa *et al.*, 2008; Young *et al.*, 2009; Kaewmala *et al.*, 2011; Nakai *et al.*, 2011). Findings herein see a development of a predominant post-acrosomal PLC ζ localisation with sperm maturity in the mouse, with 81% of sperm in the cauda epididymus possessing this localisation pattern. Furthermore, the immunoanalysis of fertile human sperm indicate a predominant post-acrosomal and equatorial localisation of PLC ζ . This supports the theory that localisation of the endogenous oocyte activation factor occurs in the region that fuses with the oocyte (Sutovsky *et al.*, 2003). Contrary to the suggestion of a recent study that the localisation of PLC ζ may not be important for its oocyte activation ability (Kashir *et al.*, 2012), the present study advocates that the localisation of PLC ζ that is predominant in mature/fertile sperm in both human and mouse sperm does seem to be in the region of the sperm cell that fuses with the oocyte, strongly suggesting that this population plays a

functional role in oocyte activation. Confirmation of this suggestion can only occur with the manual micro-dissection of this population and its subsequent micro-injection into oocytes to detect whether oocyte activation occurs, as well as using fluorescent PLC ζ markers and through time-lapse imaging, determine the immunolocalisation of PLC ζ during sperm-oocyte fusion in IVF.

Establishing the developmental expression of PLC ζ as sperm develop full fertility capacity would further our understanding on the aetiology of male factor infertility as well as shed light on therapeutic options of ICSI using testis- or epididymal-retrieved sperm. For ethical and accessibility reasons it would not be possible to determine the expression of PLC ζ during epididymal progression in humans, so this study employed a mouse model to ascertain the change in total PLC ζ levels with epididymal maturation. Interestingly, the level of total PLC ζ did not change with epididymal maturation in mouse sperm, despite the change in the localisation patterns. This lack of change in levels suggests that sperm derived from any stage of epididymal sperm development has fertility potential if injected directly into an oocyte. It also suggests that PLC ζ protein within the sperm head undergoes translocation during maturation to attain the position most apt for its role at full maturity.

Determining the stage at which PLC ζ is first expressed in spermatogenesis is important for understanding the mechanism of generation of PLC ζ in the testis. No previous work has demonstrated the spermatogenic developmental protein expression of PLC ζ , although Saunders *et al.* (2002) and Yoneda *et al.* (2006) have shown the expression of PLC ζ mRNA from the round spermatid stage, and Yoneda *et al.* (2006) suggest its translation in the elongated spermatid. This study however identified PLC ζ in the round spermatid stage of mouse spermatogenesis suggesting that translation does occur at this stage.

This detection may have practical applications for the development of clinical techniques such as ICSI using round spermatids (ROSI). Although a previous study showed a lack of activation of mouse round spermatids when injected into oocytes (Yazawa *et al.*, 2001), which suggests that despite the identification of PLC ζ in mouse cells of this developmental level, it may not be in the form that renders it active. The present study also nullifies a recent study which suggests that PLC ζ is present at the round spermatid stage, but then diminishes in the elongated spermatid (Aarabi *et al.*, 2012). Findings herein demonstrate very strong positive signals in the acrosome at the elongated spermatid stage, and that positive signals are observed during all stages of sperm development onwards from the round spermatid stage. Indeed, Aarabi *et al.* (2012) further claimed that PLC ζ is excreted from the epididymal epithelial cells, but this claim is abolished herein with the demonstration of an immunising peptide block experiment indicating background staining of the epididymal lining, with specific positive staining in the sperm head.

The developmental expression of PLC ζ in human testis has not been demonstrated previously, but this study shows for the first time that PLC ζ is first expressed at the round spermatid stage of human spermatogenesis. Previous clinical studies have demonstrated that ICSI using round spermatids have a dramatically low success rate, with 21.8% fertilisation rate, and a 2.8% clinical pregnancy rate (Sousa *et al.*, 2002). Indeed, no clinical pregnancies have been reported following ROSI since 2002 (Vloeberghs *et al.*, 2013). This may again suggest that despite the presence of PLC ζ in the round spermatids, they may not be at sufficient levels to produce a physiological response, or they are not in a functional state, and since ICSI using elongated spermatid has a much higher success rate than ROSI: 48.4% fertilisation rate, and a 28.9% pregnancy rate (Sousa *et al.*, 2002) which are

comparable to success rates using testicular spermatozoa (Vloeberghs *et al.*, 2013), suggests that PLCζ undergoes some sort of PTM during chromatin remodelling and final condensation that occur in spermiogenesis, and that these PTMs enable PLCζ to acquire functional capacity. Indeed such a PTM occurs during spermiogenesis with the chromatin components of the sperm cell, where histone variants are modified into transition proteins and protamines (Govin *et al.*, 2004, 2007, 2012). PLCζ may not only play a role in fertilisation, but during spermatogenesis too since knockout mice appear unable to complete spermatogenesis, with spermatocytes failing to proceed beyond elongation (Ito *et al.*, 2010, 2011).

The effect of male age on oocyte activation ability has not been previously studied although many studies have demonstrated the effect of increasing male age on semen quality and genetic integrity (Sloter *et al.*, 2006; Wyrobek *et al.*, 2006; Silva *et al.*, 2012). With PLCζ established as the oocyte activation factor, it would be conceivable to expect its decline or aberration with male age to correlate with the reduced fecundity. However, the present study did not observe any decline in total PLCζ levels with increasing male age in either human and mouse sperm. Similarly, there was no significant change in the localisation patterns observed with increasing age during epididymal maturation in the mouse. Little evidence exists regarding the success rate of fertilisation with advanced paternal age, although advanced paternal age appears to be associated with an increased risk of spontaneous abortion and increased frequency of some autosomal dominant conditions (Reproductive Endocrinology and Infertility Committee, 2011). This suggests that the reduced fecundity, reduction in pregnancy rates and increased time to attain a successful pregnancy are due to factors unrelated to the level of the oocyte activation

factor, but are more related to the genetic integrity and quality of the sperm. Indeed, many authors now suggest that genetic screening becomes routinely incorporated into fertility testing (Evenson *et al.*, 1980; Barratt *et al.*, 2010; McLachlan and O'Bryan, 2010; De Jonge, 2012; Barratt and Mansell, 2013).

Until recently, it was unclear whether the PIP_2 substrate that sperm $\text{PLC}\zeta$ binds to was in the oolemma or in an internal organelle of the oocyte, and precisely where this $\text{PLC}\zeta$ is localised whilst stimulating Ca^{2+} release. A recent study suggested that whilst $\text{PLC}\delta$ acts by hydrolysing PIP_2 in the oolemma, $\text{PLC}\zeta$ hydrolyses an intracellular membranous source of PIP_2 on distinct vesicular structures within the oocyte cortex (Yu *et al.*, 2011). Moreover, another recent study injected fluorescently-tagged $\text{PLC}\zeta$ into a human cell line as a model system for activating oocytes, and findings indicate that $\text{PLC}\zeta$ is localised to discrete regions within the cell cytoplasm adjacent to the nuclear envelope (Kashir *et al.*, 2011). The present study followed on from the latter study and also through the use of a fluorescently-tagged $\text{PLC}\zeta$ and a human cell line as a model, identified these regions to be the ER. In accordance with these findings, $\text{PLC}\zeta$ remains in an inactive state and fail to induce Ca^{2+} oscillations in the CHO cell line, which exhibit an absence of PIP_2 in its organelles (Phillips *et al.*, 2011). As well as providing evidence for $\text{PLC}\zeta$'s Ca^{2+} releasing target, this also suggests that specific factors within the ooplasm may be required for $\text{PLC}\zeta$ -mediated Ca^{2+} release (Phillips *et al.*, 2011).

Thus far, the structure-function relationship of $\text{PLC}\zeta$ has been dubious, particularly since $\text{PLC}\zeta$ lacks the PH domain which binds to the substrate PIP_2 in other PLC isoforms. The present study through laboratory-designed mutations determined that the EF hands in the $\text{PLC}\zeta$ structure are responsible for ER translocation, and an absence of this domain through

another laboratory-designed truncated mutation, allowed translocation to nuclear as well as ER regions. Indeed previous studies have shown that deletion of the PLC ζ EF hand domains leads to a loss of *in-vitro* catalytic activity (Kouchi *et al.*, 2005; Bi *et al.*, 2009) and injection of high concentrations of cRNA expressing high levels of this truncated form fails to trigger Ca²⁺ oscillations in mouse oocytes (Kouchi *et al.*, 2005; Nomikos *et al.*, 2005; Kurokawa *et al.*, 2007). An endogenous form of this truncated version of PLC ζ has been identified in human sperm by Bi *et al.* (2009) which was found to antagonise PLC ζ , further implying that the EF hands play an essential role in oocyte activation.

6.3 Developments in the diagnostic application of PLC ζ

With the determination of a potential localisation pattern associated with fertile capacity of sperm, and its presence within the sperm head (Grasa *et al.*, 2008; Yoon *et al.*, 2008), it was conceivable that a biomarker based upon the quantification of PLC ζ can be established. The present study, as well as a recently published study (Kashir *et al.*, 2012), demonstrate significant variability in the total levels of PLC ζ in fertile controls, and even between fertile controls and OAD samples, where in some cases total PLC ζ levels were comparable. This not only casts considerable doubt, but nullifies the usefulness of quantitating PLC ζ total protein levels as a biomarker of potential oocyte activation ability of a sperm sample. However, considerable differences were observed when the proportion of sperm exhibiting PLC ζ were compared, and the preliminary study undertaken herein comparing fertile controls and OAD samples, although employing a single OAD sample,

demonstrated a clear significant reduction in the proportion of sperm exhibiting PLC ζ in the OAD sample compared with fertile controls. This was demonstrated using a larger sample group in the study by Kashir *et al.* (2012).

The fact that it is the proportion of sperm exhibiting PLC ζ in a sample rather than the total protein expression, hence the mere existence of PLC ζ , bequeaths additional credibility for the potential usefulness, efficacy and convenience of using a technique such as flow cytometry as a biomarker of fertility potential. The present study was not able to optimise a fertility assessment assay using flow cytometry due to a problem in the samples that were present and time restrictions in awaiting further samples. However, preliminary experiments did indicate strong potential of this technique for clinical application, and further studies using new consistently-prepared samples is imperative. The relative expression of PLC ζ mRNA using microarray analysis also poses as a strong option as a credible biomarker of the fertilising potential of a sperm sample, as mentioned in Chapter 5.

Further to determining the developmental expression of PLC ζ in human testes, the analysis of the testicular biopsies which were made accessible for this study, provided an indication of how the analysis of biopsies obtained by TESE can provide potential fertility outcome of the sample following ICSI by analysing the presence of PLC ζ . This was not confirmed as time restrictions did not allow for the acquirement of clinical data associated with the biopsies analysed, but should they relate with the analyses drawn, it may serve as a tool for azoospermic patients. However, the analysis of spermatogenic markers may provide a less invasive first step for these patients as discussed in Chapter 5, which may save some patients from unnecessary emotional and financial ramifications.

Interestingly, a recent study has shown that by assessing the rhythmical cytoplasmic movements triggered by Ca^{2+} oscillations at fertilisation, using particle image velocimetry (PIV), it was possible to predict the developmental potential of the zygote (Ajduk *et al.*, 2011), thus offering a potential viability index for oocytes fertilised *in-vitro*. Furthermore, Swann *et al.* (2012) have validated this idea through their finding that the frequency and occurrence of PLC ζ -induced cytoplasmic Ca^{2+} oscillations and the cytoplasmic movements within aged ICSI-failed human oocytes were synchronous in nature. The authors also used PIV to monitor cytoplasmic movements suggesting the potential for this technique as a non-invasive clinical method for monitoring Ca^{2+} oscillation patterns in aged human oocytes, providing an early and effective indication of zygote viability following IVF (Ajduk *et al.*, 2011; Swann *et al.*, 2012).

In parallel with determination of the levels of PLC ζ in a sperm sample as a marker of fertility, a study by Kashir *et al.* (2011) investigated the effects of routinely used techniques in infertility treatment on the levels of PLC ζ in fertile male subjects (Kashir *et al.*, 2011). The authors suggest that cryopreservation, a very fundamental technique, both for infertile patients and patients undergoing radio/chemotherapy or surgery (Zribi *et al.*, 2010), has a significant detrimental effect on the levels of PLC ζ compared with fresh sperm (Kashir *et al.*, 2011). Furthermore, Nakai *et al.* (2011) have also shown that sperm pre-treatment reduces the oocyte-activating ability of sperm by showing a significant decrease in total PLC ζ levels of pre-treated pig sperm compared with whole untreated sperm. Interestingly, on a tangent, the latter authors found a PLC ζ population in the tail of pig sperm and found that the removal of the tail through pre-treatment reduced oocyte-activation ability, indicating

the possible requirement of the injection of whole sperm in ICSI procedures in pigs (Nakai *et al.*, 2011).

Further to the current UK PLC ζ screening service now set up by Dr Kevin Coward in the Coward laboratory (Oxford University), a recent collaboration with Professor Karl Swann (Cardiff University) aims to develop a new screening service – the calcium oscillation activity test (COAT) – involving initially the current service of PLC ζ screening using immunofluorescence, as well as a PLC ζ mutation screen, following on from the identification of the genetic mutations in the patient with the H233L and H398P mutations discussed in Chapter 4. Should a positive result yield from the initial PLC ζ screening, the sample would undergo cryopreservation and be shipped to the Swann laboratory for micro-injection into mouse oocytes and Ca²⁺ imaging. This new COAT service has been approved by the Human Fertilisation and Embryology Authority (HFEA) and Research Ethics Committee (LREC), and is pending Material Transfer Agreement (MTA).

6.4 Developments in the therapeutic application of PLC ζ

Cases of oocyte activation failure can currently be treated by AOA methods (Eldar-Geva *et al.*, 2003; Heindryckx *et al.*, 2008; Yanagida *et al.*, 2008; Taylor *et al.*, 2010). However, given the significant concern of the detrimental effects to embryo viability and future health (Ozil, 1990; Ozil *et al.*, 2006; Nasr-Esfahani *et al.*, 2010), and potential repercussions on epigenetic processes (Ciapa and Arnoult, 2011) from the use of these chemical methods, solicits the introduction of a more physiological therapeutic option given

the incidence of ICSI failure. There is significant hope for PLC ζ to function as this physiological therapeutic option, to substitute or augment endogenous PLC ζ activity.

Recent advancements in the successful development of a purified active recombinant PLC ζ protein provides great hope and may revolutionise the prospects of male factor infertility. The recent breakthrough by Kashir *et al.* (2011), Yoon *et al.* (2012) and Nomikos *et al.* (2013) in accomplishing the challenge of the past decade of synthesising a recombinant human PLC ζ protein that is both pure and instigates oocyte activation when microinjected into oocytes provides great excitement at the potential prospects. This is more so with the demonstration of application in human oocytes, where failed activation in human oocytes which express dysfunctional PLC ζ were rescued through the co-injection of this recombinant PLC ζ protein, with development up to blastocyst stage (Nomikos *et al.* 2013).

Following on from the successful synthesis of an active PLC ζ protein, there are several critical steps before PLC ζ can be introduced into the clinic. Firstly, considering that the potency of human PLC ζ is a hundred-fold greater than mouse PLC ζ , activity assays must therefore be carried out on human oocytes using the synthesised recombinant PLC ζ which has proved active on micro-injection into mouse oocytes. Secondly, the mode of application must be considered, whether the route of administration would be in the form of a tablet or direct injection, which also relates to the form in which the therapeutic is stored such that the activity is not affected. Only after these stages are surpassed can the PLC ζ therapeutic be considered for a clinical trial, before being deemed as effective and approved for clinical use.

With the establishment of PLC ζ 's role in oocyte activation, it is plausible to deliberate how this knowledge can assist with the generation of a novel non-hormonal male contraceptive technique for fertile males by blocking the activity of PLC ζ . An enhanced understanding is required of the precise mechanism of action of PLC ζ and what is the activating component in the oocyte that induces PLC ζ to cause Ca²⁺ release and ensuing oocyte activation. Moreover, the determination of the three-dimensional structure by crystallography, and a further understanding of how the mutations identified cause failure of activation is required in order that their action can be mimicked to bring about the inactivation of PLC ζ . Inactivation of the protein is theoretically possible, but in the case of inactivating PLC ζ for contraceptive reasons levies hesitancy, as it must be a safe, reversible and effective inactivation. Furthermore, PLC ζ RNA has been identified in ejaculated sperm (Aghajanian *et al.*, 2011), suggesting that even if PLC ζ protein is blocked within epididymal sperm, RNA is still present and able to induce oocyte activation (Rogers *et al.*, 2004; Ross *et al.*, 2008; Yoon *et al.*, 2008; Kashir *et al.*, 2011). Moreover, the identification of PLC ζ in the ovary and brain of pufferfish (Coward *et al.* 2011) may present a cause of hesitancy; albeit a narrow possibility that PLC ζ may be expressed in the human brain or other tissues considering the differences in the species, but no such data is yet available.

6.5 Future directions

The scope for clinically applying the knowledge we possess on oocyte activation and the role of PLC ζ is vast and warrants further research. Particular areas require specific scrutiny due to their great potential in becoming an important tool as a therapeutic for male-factor infertility, and as a biomarker for infertility. A purified active recombinant PLC ζ

protein promises to bring much hope to couples unable to conceive due to a defect of the male sperm, particularly after the latest developments in obtaining a purified active recombinant protein (Kashir *et al.*, 2011; Yoon *et al.*, 2012; Nomikos *et al.*, 2013). This protein would be injected alongside ICSI, to trigger oocyte activation and embryo development. A concern which must undergo investigation and assessment would be whether the defect in the sperm may introduce epigenetic anomalies due to their increased likelihood of defective nuclear content; such as globozoospermic sperm, which possess a high percentage of sperm DNA fragmentation (Taylor *et al.*, 2010; Avendaño and Oehninger, 2011; Ciapa and Arnoult, 2011). Indeed, DNA-damaged sperm for ICSI was associated with pathological changes, such as a higher incidence of cancer and premature aging of resultant mouse adult offspring (Fernandez-Gonzalez *et al.*, 2008; Barratt *et al.*, 2009). Moreover, there is accumulating evidence suggesting that ART, particularly ICSI, can result in long-lasting transcriptome disturbances, or epigenetic asymmetry, due to many confounding factors such as age, parental subfertility and genetic variations (Jenkins and Carrell, 2012; van Montfoort *et al.*, 2012; Weiner-Megnazi *et al.*, 2012; Kohda and Ishino, 2013).

It may be possible to avoid DNA-damaged or chromosomally-unbalanced sperm by selecting ICSI sperm by motility or by maturation markers, or via novel non-invasive high-magnification imaging techniques to morphologically select sperm such as IMSI (intracytoplasmic morphologically-selected sperm injection) (Antinori *et al.*, 2008; Balaban *et al.*, 2011; Ebner *et al.*, 2011; Pacey, 2012) or MSOME (motile sperm organelle morphology examination) (Bartoov *et al.*, 2002; Ebner *et al.*, 2011; Sermondade *et al.*, 2011; Silva *et al.*, 2012). Indeed a relationship between DNA damage and nuclear vacuoles has been demonstrated, with MSOME offering closer evaluation for sperm selection (Silva *et al.*,

2012). Moreover, MSOME has been shown to select for PLC ζ -containing sperm cells in a severely globozoospermic patient (Kashir *et al.*, 2012). These possibilities and others warrant urgent investigation to be used alongside the purified recombinant PLC ζ protein. Using a recombinant PLC ζ protein would offer a much more physiological AOA than the use of other current AOAs such as Ca²⁺ ionophores or even PLC ζ RNA, as aforementioned. A recent study showing the successful pregnancy using MSOME without the use of an AOA, questions the need for the use of an oocyte activator at all in fact (Sermondade *et al.*, 2011), more so after the demonstration of similar fertilisation rates in AOA-treated and non-treated oocytes.

Correspondingly, of particular importance is optimising a tool to assess the fertilisation potential of a semen sample. The limited clinical value of the basic semen assessment has now been recognised (Barratt *et al.*, 2011; Pacey, 2012), and other assays are necessary. Recent work has provided great advancements in attaining this tool, particularly through the localisation of PLC ζ protein expression in a sperm sample (Heytens *et al.*, 2009; Kashir *et al.*, 2011), and quantitatively analysing the level of PLC ζ mRNA (Aghajanour *et al.*, 2011), relating to fertile controls. Important aspects to consider when obtaining a fertility biomarker is ease, safety, accuracy and speed of analysis. Furthermore, the existence of mRNA in sperm is unexplained thus far, and what it functions in the zygote remains to be ascertained. Future work is required to further understand the role of PLC ζ in fertilisation and embryo development.

Furthermore, to provide credible support for the translational applications of PLC ζ , it is important to understand the mechanism of action behind PLC ζ 's functional roles and elucidate the precise structure-function relationships. More and more evidence is surfacing

regarding the importance of oocyte factors in the fertilising potential of an oocyte. Already, evidence has shown that the oocyte undergoes cellular arrangement and modifications during maturation to optimise the internal environment for maximal Ca^{2+} responses on sperm fusion (Wakai *et al.*, 2012), and the dysfunction of this arrangement, e.g. with aging of the oocyte, leads to an impedance of the fertilising process (Zhang *et al.*, 2011). An advanced understanding may allow the assessment of Ca^{2+} dynamics within the oocyte for oocyte viability (Ajduk *et al.*, 2011; Swann *et al.*, 2012). Studies demonstrating expression within a cell line or Ca^{2+} release on injection of PLC ζ into a cell line but not another (Kashir *et al.*, 2011; Phillips *et al.*, 2011), indicate that there may be an oocyte component that is necessary for the activation process. Elucidation of what these oocyte factors are may explain how PLC ζ is maintained in an inactive state in the sperm but is activated on release into the oocyte. The suggestion that oocyte quality plays a role in fertility is further strengthened by a recent study reporting that the process of vitrification in ART affects the distribution of IP_3Rs and subsequently impairs Ca^{2+} oscillatory activity and embryo development, thus explaining low rates of blastocyst formation in ICSI-experiments using vitrified oocytes compared with fresh oocytes (Kim *et al.*, 2011).

Furthermore, there is evidence suggesting that PLC ζ , as well as interacting with oocyte factors, may be undergoing some form of PTM before attaining the ability to induce Ca^{2+} release. Cooney *et al.* (2010) suggest that this is a key requirement for the development of PLC ζ and its endogenous activity (Wu and Chu, 2008; Cooney *et al.*, 2010). Kashir *et al.* (2011), Yoon *et al.* (2012) and Nomikos *et al.* (2013), who successfully synthesised an active PLC ζ recombinant protein, all found that there was an apparent delay between injection of recombinant PLC ζ protein and the onset of Ca^{2+} oscillations, strongly

suggesting that this may be the case. Moreover, mounting evidence suggests that PLC ζ undergoes proteolytic cleavage with smaller-sized PLC ζ fragments identified in porcine and mouse sperm (Kurokawa *et al.*, 2005, 2007), which despite cleavage, retain their Ca²⁺-inducing activity. Future studies must aim to elucidate whether PTMs and proteolytic cleavage, are important for the functional role of human PLC ζ .

It has been postulated by previous studies that the different populations of PLC ζ identified in sperm of different species, may play differential roles. It is debated whether the population of PLC ζ in the acrosomal region plays a role in the acrosome reaction as some studies suggest (Grasa *et al.*, 2008; Bi *et al.*, 2009; Young *et al.*, 2009), more so after recent developments have indicated through assessment of the correlation between the percentage of sperm with a small acrosome and the relative expression of PLC ζ , found no significant difference between these parameters (Aghajanpour *et al.*, 2011), and the reduction of PLC ζ mRNA levels found in globozoospermic sperm, was not specifically due to the absence of the acrosome. Aghajanpour *et al.* (2011) suggest that the localisation of PLC ζ to the acrosome may be independent of PLC ζ mRNA expression, which suggests that the reduced expression of PLC ζ mRNA in globozoospermic sperm may be related to other factors and not the absence of the acrosome. However, it is generally more accepted that the post-acrosomal PLC ζ population is responsible for oocyte activation as discussed previously, but it still remains to be confirmed. It is indeed plausible that the acrosomal population may play a role in events pre-fertilisation, and likewise the post-acrosomal population may play other roles. Understanding the precise localisation patterns as well as the roles of the differential populations is required for manufacture of therapeutic options, thus requires further research.

Whilst many studies argue and provide credible evidence that PLC ζ is indeed the putative oocyte activation sperm factor, a recent study by Coward *et al.* (2011) identified the expression of PLC ζ in the ovary and brain of pufferfish species, not in the testis. Moreover, micro-injection of its encoding cRNA into mouse oocytes did not elicit Ca²⁺ oscillations, although a PLC ζ cRNA with a sequence highly homologous to the pufferfish, that of the medaka fish, initiated Ca²⁺ oscillations in these oocytes (Coward *et al.*, 2011). The evidence, despite being strong in mammalian studies of PLC ζ being the putative oocyte activation sperm factor, cannot be confirmed until an unfertile knock-out male mouse is produced, which would provide unequivocal evidence, but thus far, its production has been unsuccessful (Ito *et al.*, 2010, 2011). Nevertheless, all in all, existing mammalian studies emphasise PLC ζ 's role in fertilisation and advocate the association of certain types of infertility with defects in PLC ζ .

In this same study by Coward *et al.* (2011), the authors highlighted the interesting correlation between the position of the *PLC ζ* gene and the actin-capping protein gene *CAPZA3*. PLC ζ has been found to share its bi-directional promoter region with *CAPZA3* (Coward *et al.*, 2011), and interestingly a defect in this promoter has been linked to the absence or defective PLC ζ and globozoospermia (Geyer *et al.*, 2009; Coward *et al.*, 2011; Kashir *et al.*, 2012). Indeed a recent study has shown a correlation between genetic variants which occur as a result of changes in the promoter region of PLC ζ exerting an effect on the expression of PLC ζ affecting its physiological function as well as semen quality in Chinese Holstein bulls (Pan *et al.*, 2013). It would thus be interesting for future studies to ascertain whether such alterations in the promoter region shared by *CAPZA3* and *PLC ζ* have any

associations with PLC ζ expression levels and localisation patterning in human sperm, or in cases of abnormal sperm morphology such as globozoospermia.

Revolutionary advances in stem cell research have not left the field of andrology uninfluenced. To be able to grow sperm as well as recreate human spermatogenesis *in-vitro* would allow not only the study of spermatogenesis using a characterised model, which would have a huge impact on our understanding of the physiological and genetic pathways of male reproduction, but allow also furthering the developments in the quest for treatment of male infertility. Currently, there are both *in-vivo* and *in-vitro* model systems involving transplantation of spermatogonial stem cells and pluripotent embryonic stem cells respectively (Lo and Domes, 2012). There is significant scope with primordial germ cell and stem cell research, but thus far *in-vitro* culturing and maturation of these germ cells have proved challenging (Lo and Domes, 2012), although a recent study has developed a technique of directly differentiating human embryonic stem cells or induced pluripotent stem cells directly into advanced male germ cell lineages, but not spermatogonia, spermatocytes or spermatids (Easley *et al.*, 2012). These offer a novel strategy for studying spermatogenesis *in-vitro*, but more translational and clinical research is still required before these *in-vivo* and *in-vitro* acquired germ cells can be put to clinical use.

An important criterion to be met before *in-vitro* derived sperm can be considered clinically viable is their ability to initiate successful oocyte activation and embryogenesis. An effective sperm viability biomarker is thus required. Since ICSI would be used to deliver these artificial gametes into the oocytes, a marker that assesses sperm function downstream to gamete fusion would be thus required (Kashir *et al.*, 2012). PLC ζ would herein

represent a critical biomarker for the oocyte activation ability of the artificially-derived sperm cells (Kashir *et al.*, 2012).

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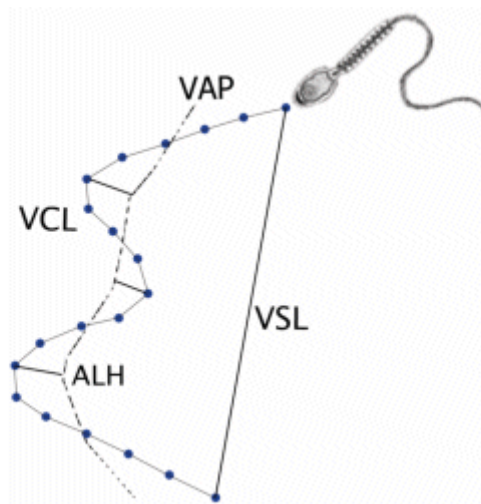
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APPENDICES

APPENDIX 1 | Computer-assisted sperm analysis (CASA)

The Hamilton Thorne CASA system was used in the mouse studies to provide quantitative data on the concentration and motility of sperm from each region of the epididymis. This was used as a standardised reproducible test for sperm concentration and motility. CASA attempts to quantify sperm movement using complex mathematics. Automated sperm movement measurements or *kinematics* include straight line velocity, curvilinear velocity and mean angular displacement. The Animal Motility Software on the Hamilton Thorne CASA system may be used to analyse the sperm of almost any species with unique parameters required to analyse sperm of different species stored in memory and accessed by selecting the species of the sample under analysis.

To perform CASA, 25µl of sperm suspension is placed into a specialised slide chamber on a heated stage. A video camera then records moving images, at 45 frames in less than a second, and digitises them. The digital images are composed of pixels whose locations are recorded frame by frame. The changing locations of the sperm are recorded by the computer and trajectories are determined. Motile sperm analyses is performed by following the sperm trajectory and determines the velocity. According to the WHO, 200 sperm are required for analysis. Five fields were captured for each sample to enable the analysis of as much sperm as possible, and in order that enough sperm are present in lower concentrated samples. Data for concentration and motility grade was acquired from this.



Parameters obtained from CASA to determine motility grade. [VAP – average velocity of sperm head along average trajectory; VCL – curvilinear velocity; ALH – amplitude of lateral head displacement; VSL – straight-line velocity]

The motility of sperm are divided into 4 grades:

- **Grade a:** Sperm with progressive motility. These are the strongest and swim fast in a straight line.
- **Grade b:** These also move forward but tend to travel in a curved or crooked motion (non-linear motility).
- **Grade c:** These have non-progressive motility because they do not move forward despite the fact that they move their tails.
- **Grade d:** These are immotile and fail to move at all.

APPENDIX 2 | Quantitative immunofluorescence

Images from a fluorescent microscope are formed by detecting light, and these small amounts of light can be identified in terms of photons. Photons are emitted from fluorescent molecules within the sample being imaged. These photon-emitting molecules are generally in the region of interest in our study. The image can thus allow us to interpret whether our molecule of interest is present or not, its size, shape and concentration. By using the region of interest (ROI) manager on Image J, it is possible to manually draw around our ROI, in this case a sperm head, and measure the number of photons detectable in that ROI. This would indicate the concentration of our molecule of interest, in this case PLC ζ .

The sperm heads of intact sperm (100 sperm cells) are randomly chosen in the dark field, so as to not give any preferences in choosing for example those sperm cells with positive fluorescence staining, and from different fields of view, and then transferred to its corresponding fluorescent image. The average fluorescence of the 100 ROI points are obtained for each sample, indicating the average amount of expression of our molecule of interest in our chosen sample. Since all parameters are kept constant on the microscope and computer imaging settings, it is possible to compare the relative expression in different samples by detecting the average fluorescence of 100 ROI points from each sample.

APPENDIX 3 | Principle of an immunogenic peptide block

Non-specific binding of an antibody to proteins other than the antigen can sometimes occur. This occurs more commonly with polyclonal antibodies. To determine which staining is specific, an immunising peptide blocking experiment can be performed. Before proceeding with the staining protocol, the antibody is neutralised by incubating it with an excess of peptide(s) that corresponds to the epitope(s) recognised by the antibody. The antibody that is bound to the blocking peptide is no longer available to bind to the epitope present in the protein in the cell. Samples are incubated in neutralised antibody side-by-side with samples incubated in the antibody alone, and the results are compared. By comparing the staining from the blocked antibody versus the antibody alone, it is possible to determine which staining is specific: this staining will be absent from the immunostaining performed with the neutralised antibody, but present in the immunostaining performed with the antibody alone.

Determining the specificity of immunostaining by an antibody, particularly a polyclonal antibody such as the anti-PLC ζ antibody used herein, is important to confirm specificity of the antibody and the validity of the positive staining and its observed localisation.

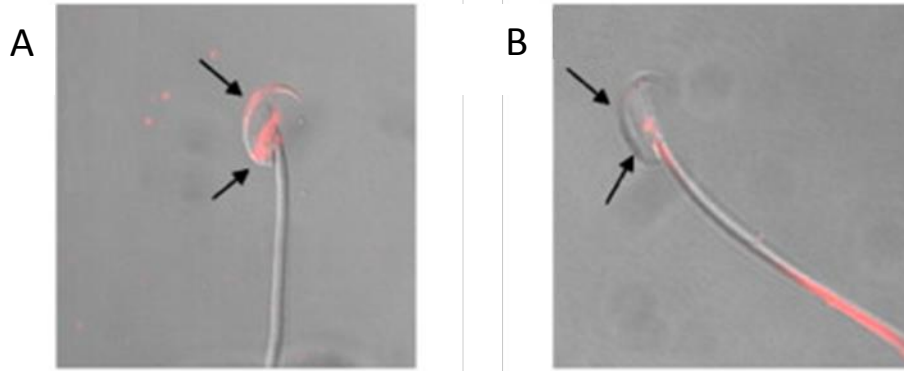
APPENDIX 4 | Statistical analysis

In chapters 2, 3 and 4, the one-way analysis of variance (one-way ANOVA) was the chosen statistical analysis technique used since this technique allows the comparison of the means of two or more samples of one independent variable. The different age groups, or different regions of sperm development, present different groups with which to compare the variance of the means. Should the mean of only two groups be compared, a student's *t*-test would be employed. The Bonferroni *post hoc* correction is also applied with the one-way ANOVA to control the probability of false positives. It controls for the error rate and observes where significant differences lie.

APPENDIX 5 | Fixatives used for immunohistochemical analysis of the testis.

Paper	Tissue	Fixative used	Counterstain	Section thickness (µm)	Additional notes
Bedford-Guaus <i>et al.</i> (2011)	Horse testis	4% PFA	Haematoxylin	n-a	Permeabilised in 0.05% PBS-T
Heytens <i>et al.</i> (2010)	Mouse testis	4% PFA		10	
Kaewmala <i>et al.</i> (2011)	Boar testis			8	Immunofluorescence
Kim <i>et al.</i> (2009) Histochem Cell Biol 131:501-507	Mouse testis	4% PFA	Haematoxylin	5	PBS-T in diluent of Abs
Contuk <i>et al.</i> (2012) Acta Histochemica 114:244-251	Rat testis	4% PFA	Haematoxylin	5	Used scoring (+++ = intense; ++ = dense; + = weak; +/- = very weak; - = no staining)
Contuk <i>et al.</i> (2012) Acta Histochemica 114:244-251	Rat testis	Bouins fluid	Haematoxylin and oesin	5	General morphology analysis
Tafari <i>et al.</i> (2010) Regulatory peptides 164:53-57	Rat testis	Bouins fluid	Haematoxylin	6	
Giampietri <i>et al.</i> (2003) Cell death & differentiation 10:175-184	Mouse testis & epididymis	Bouins fluid		3	
Kuijk <i>et al.</i> (2010) PLOS one 5(6):e10987	Mouse/canine/pig testis	4% PFA or Bouins	Haematoxylin	5-7	
Tokuda <i>et al.</i> (2007) Biol of Reprod 76(1):130-141	Mouse testis	10% neutral buffered formalin containing 10% methanol		8	
Bluthman <i>et al.</i> (1982) The EMBO J 1(4):479-484	Mouse testis	Carnoy's fluid or Bouins fluid	Haematoxylin and oesin	5	Both fixatives gave similar results with respect to preservation of morphology and antigenicity of tissue

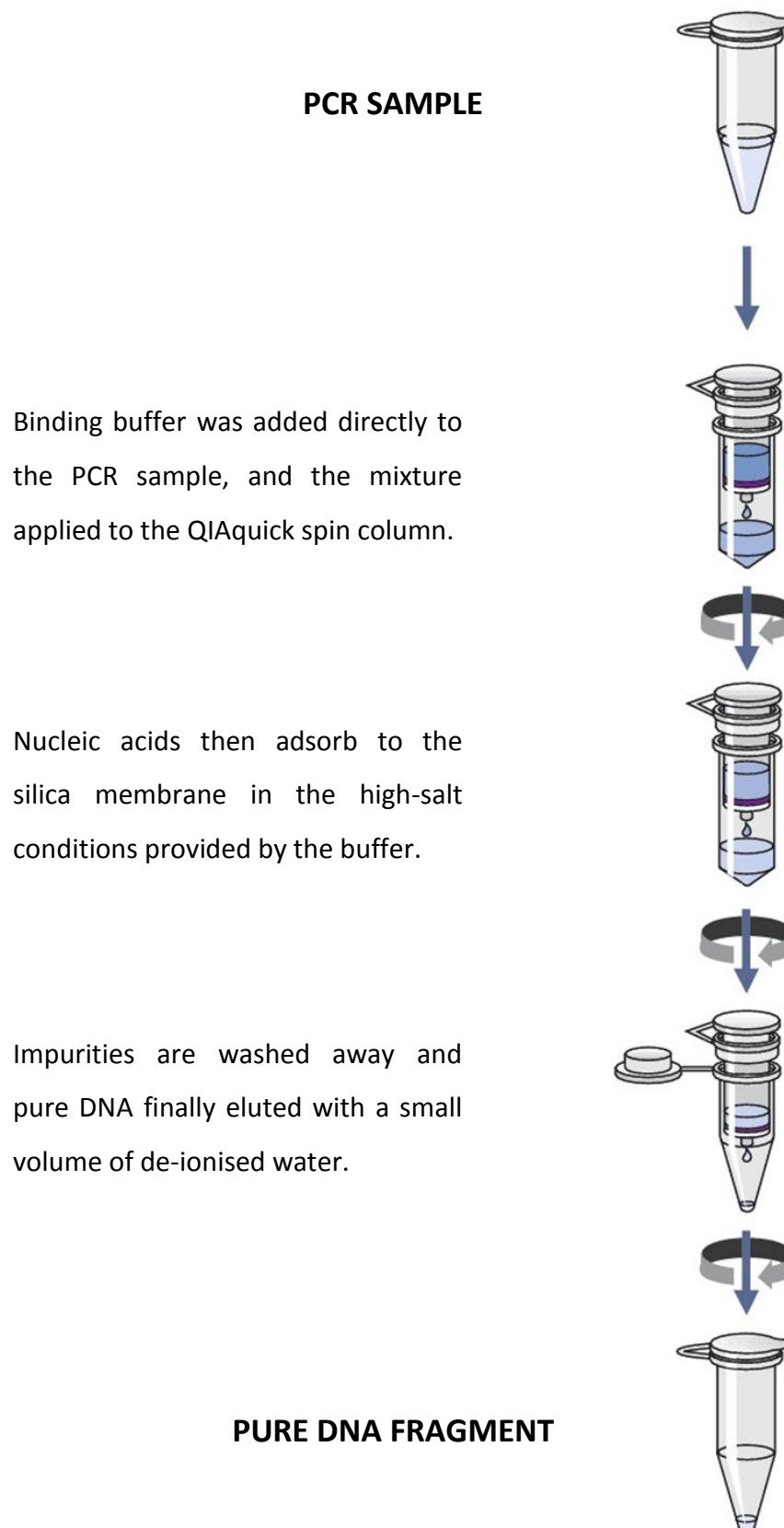
APPENDIX 6 | Immunofluorescence peptide block originally reported by Young *et al.* (2009).



Merged fluorescent and brightfield PLC ζ immunolocalisation, in (A) absence and (B) presence of immunogenic peptide. Arrows indicate position of positive PLC ζ immunoreactivity.

Young et al. (2009)

APPENDIX 7 | Schematic flowchart summarising the QIAquick PCR purification protocol (Qiagen, UK) in accordance with the manufacturer's instructions.



APPENDIX 8 | TESE procedure.

In brief, the testicular sperm extraction (TESE) procedure was carried out under general anaesthesia, with a median scrotal incision made to expose both testicles. A transverse incision was then made into a relatively avascular region of the tunica albuginea present on the anterior surface of the testis after which specimens of testicular tissue approximately 3-5mm in size were excised from each testis with the aid of an operating microscope (Micro TESE) using sharp scissors. Specimens were then transferred into a petri-dish containing IVF culture medium. Immediately following the surgical procedure, the testicular specimens were mechanically sectioned using sterile scalpel blades after which they were gently macerated further using sterile needles. Following testicular maceration, the resulting testicular suspension was transferred into a conical tube containing fresh IVF culture medium and centrifuged at 1200g for 10 minutes. The supernatant was discarded and the pellet resuspended in 1ml of fresh IVF culture medium and vortexed for 2 minutes. Using an inverted microscope at 400x magnification, the testicular suspension was then checked for the presence of mature sperm for use in ICSI. After each patient's ICSI cycle, the testicular biopsy suspensions were transferred into cryogenic straws which were then frozen in liquid nitrogen, and stored at -80°C.

[Professor Petra de Sutter / Dr Bjorn Heindryckx, Personal Communication]

APPENDIX 9 | Research output: Publications and presentations.

- **Articles in international peer-reviewed journals |**

Walaa M. Ramadan, Junaid Kashir, Celine Jones and Kevin Coward. 2012. Oocyte activation and phospholipase C zeta (PLCζ): diagnostic and therapeutic implications for assisted reproductive technology. *Cell Communication & Signaling*, **10**(12).

Junaid Kashir, Celine Jones, Ginny Mounce, **Walaa M. Ramadan**, Bernadette Lemmon, Bjorn Heindryckx, Petra De Sutter, John Parrington, Karen Turner, Tim Child, Enda McVeigh, and Kevin Coward. 2013. Variance in total levels of phospholipase C zeta (PLCζ) in human sperm may limit the applicability of quantitative immunofluorescent analysis as a diagnostic indicator of oocyte activation capability. *Fertility & Sterility*, **99**(1): 107-117.

- **Popular science articles |**

Junaid Kashir, Celine Jones, **Walaa Ramadan**, Youn-Jung Kang, Janet Carver, Tracey Griffiths, Karen Turner and Kevin Coward. March 2012 Magnifying human fertility: microscopy and assisted reproductive technology. *Infocus Magazine, Royal Microscopy Society*. **25**: p4-23.

- **Manuscripts in preparation |**

Walaa M. Ramadan, Junaid Kashir, Celine Jones, Carmen Navarro, Ginny Mounce, Karen Turner, Lucy Coleman, Tim Child, Enda McVeigh and Kevin Coward. Male age does not affect the expression or localisation pattern of the oocyte activation factor phospholipase C zeta (PLCζ) in human sperm. [Original Research Article]

Walaa M. Ramadan, Celine Jones, Junaid Kashir, Bjorn Heindryckx, Petra De Sutter and Kevin Coward. Immunohistochemical analysis of the oocyte activation factor phospholipase C zeta (PLCζ) in human testicular biopsies acquired by testicular sperm extraction (TESE) may provide an indicator of the oocyte activation ability of recovered sperm for subsequent intracytoplasmic sperm injection (ICSI). [Original Research Article]

Walaa M. Ramadan, Celine Jones, Junaid Kashir, John Parrington and Kevin Coward. The effect of age and epididymal maturation upon levels and localisation patterns of the oocyte activation factor phospholipase C zeta (PLCζ) in mouse sperm. [Original Research Article]

- **Conference Abstracts** |

W.M. Ramadan, C. Jones, E. Thurston, J. Kashir, J. Parrington and K. Coward. The effect of age and epididymal maturation upon levels of the oocyte activation factor phospholipase C zeta (PLCζ) in mouse sperm. 2011. *Medical Sciences Division DPhil Day, Oxford, UK* [Poster presentation presented by WMR]

W.M. Ramadan, C. Jones, J. Birks, E. Thurston, J. Kashir, J. Parrington and K. Coward. The effect of age and epididymal maturation upon levels and localisation patterns of the oocyte activation factor phospholipase C zeta (PLCζ) in mouse sperm. 2011. *British Andrology Society (BAS) Annual Meeting 2011, Birmingham, UK* [Poster presentation presented by WMR]

J. Kashir, C. Jones, G. Mounce, **W. M. Ramadan**, B. Lemmon, B. Heindryckx, P. De Sutter, J. Parrington, K. Turner, T. Child, E. McVeigh, K. Coward. Proportional immunofluorescent analysis of phospholipase C zeta (PLCζ) is a promising indicator of oocyte activation capability in human sperm. 2012. *European Society for Human Reproduction and Endocrinology (ESHRE), Istanbul, Turkey* [Poster presentation presented by JK]

W.M. Ramadan, C. Jones, J. Kashir, C. Navarro, J.C. Díaz, L. Coleman, K. Coward. Levels of the oocyte activator PLC ζ may be linked to male age. 2012. *Middle East Fertility Society (MEFS) Annual Meeting, Dubai, UAE [Oral presentation presented by WMR]*

W.M. Ramadan, C. Jones, J. Kashir, C. Navarro, J.C. Díaz, L. Coleman, K. Coward. Quantitative immunofluorescent analysis of fertile human sperm suggests that levels of the oocyte activation factor, phospholipase C zeta (PLC ζ), may be linked to male age. *Fertility* 2013, *Liverpool, UK [Accepted for oral presentation by WMR]*