Proteomic analysis of human sperm proteins in relation to sperm motility, morphology and energy metabolism

By

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Thesis presented in partial fulfilment of the requirements for the degree of

Master of Science in Medical Sciences (MScMedSci-Medical Physiology)

at the University of Stellenbosch

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December 2010

DECLARATION

I, the undersigned, hereby declare that the work in this thesis is my own original work that I have not previously in its entirety or in part submitted it at any other university for a degree.

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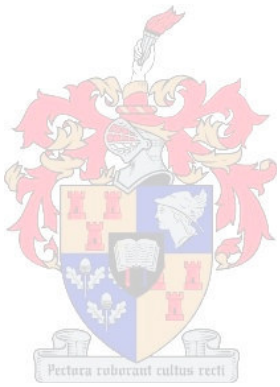

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ABSTRACT

Male infertility is often associated with impaired sperm motility and morphology (asthenoteratozoospermia) for which there is no specific therapeutic treatment. It has come to light that the modification and expression of human sperm proteins play a crucial role in sperm function. In the present study, we present proteomic data of human spermatozoa in the context of sperm dysfunction. Novel techniques have been used to successfully isolate and identify differences in protein expression on a cellular level associated with asthenoteratozoospermia.

In the first part of the study, differences in protein expression within the total sperm proteome were investigated between immature and mature sperm populations. Semen was collected from healthy donors (n=23) and separated into mature and immature sperm populations by 3-layer Percoll gradient centrifugation. Cells were washed and motility and morphology were measured by computer assisted sperm analysis (CASA). For the proteomic investigation cells were lysed and proteins separated by means of two-dimensional gel electrophoresis (2D electrophoresis). PD-Quest was used to identify the differentially expressed proteins. The protein spots of interest were excised and subjected to in-gel digestion. Peptides were separated by High Pressure Liquid Chromatography (HPLC) analysis and amino acid sequences determined by mass spectrophotometry. Proteins were identified by Mascot, using the Swiss Prot database.

The results show that the motility (immature; 26.1±1.75% total motile cells vs. mature; 60.93±3.24% total motile cells; $p<0.001$) and morphology parameters (immature; 64.1±2.75% normal head morphology vs. mature; 87.63±3.24% normal head morphology; $p<0.001$) of the two populations differed significantly. After 2D electrophoresis, 16 differentially expressed protein spots were identified within the total sperm proteome between the immature and mature sperm populations. 56% of the differentially expressed proteins were more abundant in the immature sperm population compared to the mature sperm population. Functions have been ascribed to these proteins of which only four proteins, namely Tubulin α-3C/D chain, Tubulin β-2C chain, Outer dense fibre protein 2 and A-Kinase anchoring protein 4 precursor, were directly related to sperm motility and morphology.

In the second part of the study the expression of nuclear proteins in human spermatozoa was investigated between immature and mature sperm populations. Semen was collected from healthy donors (n=156) and further separated from the seminal plasma by PureSperm® gradient centrifugation. The immature and mature sperm populations were retrieved and used during further analysis. For the proteomic analysis of nuclear proteins, cells were fractionated into four different subcellular protein fractions, instead of analyzing the whole sperm proteome. The results show that the motility (immature; 32.33±0.51% total motile cells vs. mature; 88.67±0.85% total motile cells; $p<0.0001$) and morphology parameters (immature; 13.51±0.87% normal head morphology vs. mature; 20.89±1.20% normal head morphology; $p<0.0001$) of the two

populations differ significantly. After 2D electrophoresis, 21 differentially expressed nuclear proteins were identified between the immature and mature sperm populations. 95% of the differentially expressed nuclear proteins were less abundant in the immature population compared to the mature population. Only one nuclear protein namely 78kDa Glucose regulated protein was more abundant in the immature population compared to the mature population. Functions ascribed to these individual proteins were directly related to sperm motility, morphology and energy metabolism.

In conclusion, in the current study novel techniques have been employed to investigate protein differences between immature and mature sperm populations. From these results it is evident that protein expression in the total sperm proteome and nuclear protein fraction is significantly different and incomplete in the immature population, compared to mature population. Based on these findings, it is recommended that further studies should be done on human spermatozoa to validate the role of the individual proteins in sperm function. Proteomics is an ideal tool to identify idiopathic causes of male infertility, as it can help to identify novel receptors (and signal transduction pathways) that can be used in the screening of drugs to alleviate sperm dysfunction.

OPSOMMING

Manlike infertiliteit word dikwels geassosieer met verlaagde sperm motiliteit en morfologie (asthenoteratozoospermia) waarvoor daar tot dusver nog geen spesifieke terapeutiese behandeling is nie. Dit het aan die lig gekom dat die modifisering en uitdrukking van menslike sperm proteïene ‘n belangrike rol speel in spermfunksie. In die huidige studie stel ons data voor van proteiene in menslike sperme in die konteks van abnormale spermfunksie. Unieke tegnieke was gebruik om verskille in proteïen uitdrukking op sellulêre vlak suksesvol te isoleer en identifiseer wat verband hou met asthenoteratozoospermia.

Tydens die eerste deel van die studie was verskille in proteïen uitdrukking binne die totale spermproteoom tussen onvolwasse en volwasse spermpopulasies ondersoek. Sperme van gesonde skenkers (n=23) is geskei in twee spermpopulasies (onvolwasse en volwasse sperme) deur middel van ‘n 3-laag Percoll gradiënt sentrifugasie tegniek. Selle is gewas en sperm motiliteit en morfologie is gemeet deur rekenaar geassisteerde sperm analise (CASA). Vir proteomiese analise is selle geliseer en proteïene geskei deur twee dimensionele gel elektroforese (2D-elektroforese). PD-Quest sagteware is gebruik om statisties beduidende proteïen verskille aan te dui. Die proteïene van belang is uitgesny en onderwerp aan in-gel vertering. Peptiede is geskei met behulp van hoë druk vloeistof chromatografie (HPLC) analise en aminosuurvolgordes is bepaal deur

massa spektrofotometrie. Proteïene is geïdentifiseer met behulp van Mascot deur van die Swiss Prot databasis gebruik te maak.

Die resultate toon dat die sperm motiliteit (onvolwasse; 26.1±1.75% totale motiele selle vs. volwasse; 60.93±3.24% totale motiele selle; p <0,001) en morfologiese parameters (onvolwasse; 64.1±2.75% normale kop morfologie vs. volwasse; 87.63±3.24% normale kop morfologie; p <0,001) tussen die twee populasies beduidend verskil. Na 2D-elektroforese is 16 proteïen kolle geïdentifiseer wat beduidend verskil het, tussen die totale sperm proteoom van onvolwasse spermpopulasies en volwasse spermpopulasies. 56% van die proteïene wat beduidend verskil het, was meer uitgedruk in die onvolwasse spermpopulasie ten opsigte van die volwasse sperm populasie. Funksies is toegeskryf aan hierdie proteïene waarvan net vier proteïene naamlik Tubulin α-3C/D ketting, Tubulin β-2C ketting, Buite digte vesel proteïen 2 en A-Kinase anker proteïen 4 voorloper direk verband hou met sperm motiliteit en morfologie.

In die tweede deel van die studie is die uitdrukking van nukluêre proteïene in menslike spermatozoa tussen onvolwasse en volwasse spermpopulasies ondersoek. Sperme was van gesonde skenkers (n=156) versamel en verder geskei van seminale plasma deur middel van ‘n PureSperm® gradiënt sentrifugasie tegniek. Vir die proteomiese analise van nukluêre proteïene is selle gefraksioneer in vier verskillende sub-sellulêre proteïen fraksies, in plaas van analise van die totale spermproteoom. Die resultate toon aan dat die sperm motiliteit (onvolwasse; 32.33±0.51% totale motiele selle vs.

volwasse; 88.67±0.85% totale motiele selle; p <0,001) en morfologiese parameters (onvolwasse; 13.51±0.87% normale kop morfologie vs. volwasse; 20.89±1.20% normale kop morfologie; p <0,001) tussen die twee populasies beduidend verskil. Na 2D-elektroforese is 21 kern proteïen kolle geïdentifiseer wat betekenisvol uitgedruk was tussen onvolwasse en volwasse spermpopulasies. 95% van die nukluêre proteïene wat beduidend verskil het, was minder uitgedruk in die onvolwasse spermpopulasie ten opsigte van die volwasse spermpopulasie. Slegs een kern proteïen naamlik 78kDa Glukose gereguleerde proteïen was meer uitgedruk in die onvolwasse spermpopulasie in vergelyking met die volwasse spermpopulasie. Funksies is toegeskryf aan hierdie proteïene wat direk verband hou met sperm motiliteit, morfologie en energie metabolisme.

Ten slotte, in die huidige studie is unieke tegnieke geïmplementeer om proteïen verskille tussen onvolwasse en volwasse spermpopulasies te ondersoek. Uit hierdie resultate is dit duidelik dat proteïen uitdrukking in die totale sperm proteoom en in die kern proteïen fraksie beduidend verskil en onvolledig is in die onvolwasse spermpopulasie ten opsigte van die volwasse spermpopulasie. Op grond van hierdie bevindinge word aanbeveel dat verdere studies op menslike sperme gedoen moet word ten einde die rol van individuele proteïene in sperm funksie te kan bepaal. Proteomika is ‘n ideale tegniek om die iodiopatiese oorsake van manlike infertiliteit te identifiseer, aangesien dit kan help in die identifisering van unieke reseptore (en seintransduksie

paaie) wat gebruik kan word om sperm disfunksie te verbeter deur farmaseutiese behandeling.

This dissertation is dedicated to

My parents and family

For their love, encouragement and support

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to the following persons for their assistance to the successful completion of this study:

Prof. S.S. du Plessis for his guidance support and positive inputs throughout the study

Mrs. S Hattingh for technical assistance and support

The **Harry Crossley foundation** for funding

The **University of Stellenbosch** for providing the research facilities

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ALPHABETICAL LIST OF ABBREVIATIONS

ADP = Adenine diphosphate

AKAP = A-Kinase Anchoring Protein

AR = Acrosome reaction

AP = Aminopeptidase

ASA = Antisperm antibodies

ATP = Adenosine triphosphate

ALH = Amplitude of lateral head displacement

BCF = Beat-cross frequency

BSA = Bovine serum albumin

BIP = Immunoglobulin heavy chain binding protein

cAMP = Adenosine-3', 5'-cyclic monophosphate

Ca/Ca^{2+} = Calcium

CaM = Calmodulin

CaMK = Calmodulin-dependent protein kinase

CaMp = Calmodulin dependent phosphatases

CASA = Computer assisted semen/sperm analysis

Co-A = Coenzyme A

CID = Collisionally induced decomposition

DDA = Data dependent acquisition

DNA = Deoxyribonucleic acid

ER = Endoplasmic Reticulum

ETC = Electron transport chain

EP-GP = Extraparotid glycoprotein

ESI Q TOF =Electro spray Quadrupole Time of Flight Mass Spectrometry

FA = Fatty acid

FAD = Flavin adenine dinucleotide

$FADH_2$ = Flavin adenine dinucleotide (fully reduced form)

FS = Fibrous sheath

GFP = Green fluorescent protein

GST = Glutathione transferase

GRP = Glucose regulated protein

GTP = Guanosine Triphosphate

GIFT = Gamete intrafallopian transfer

HCO_3^- = Bicarbonate

HSP = Heat shock protein

HPLC = High pressure liquid chromatography

ICSI = Intra-cytoplasmatic sperm injection

IEF = Iso-Electric Focusing

IgG = Immunoglobulin G

IUI = Intrauterine Insemination

IVF = In vitro fertilization

IVF-ET = In vitro fertilization and embryo transfer

IPG = Immobilised pH gradient

IRE's = Iron responsive elements

IRP's = Iron regulatory proteins

Kg = Kilogram

LIN = Linearity

LC-MS/MS = Liquid chromatography mass spectrometry/mass spectrometry

MALDI-TOF = Matrix assisted laser desorption ionization time of flight

mRNA = Messenger ribonucleic acid

MS = Mass spectrometry

m/z = Mass/charge ratio

Mr = Molecular weight

mQ = milli Q water/double distilled water

MHC = Major histocompatibility complex

NAD^+ = Nicotinamide adenine dinucleotide

NADH = Nicotinamide adenine dinucleotide (reduce form)

NADP = Nicotinamide adenine dinucleotide phosphate

NADPH = Nicotinamide adenine dinucleotide phosphate (reduce form)

ODF2 = Outer dense fibre protein 2

PAGE = Polyacrylamide gel electrophoresis

PBS = Phosphate buffered saline

PDC = Pyruvate dehydrogenase complex

PDI = Protein disulfide isomerase

PDK = Pyruvate dehydrogenase kinase

PDP = Pyruvate dehydrogenase phosphate

PGDH = D-3-phosphoglycerate dehydrogenase

Pi = Phosphate

pI = Iso-electric point

PIP = Prolactin inducible protein

PKA = Protein kinase-A

PTMs = Post translational protein modifications

PUFA = Polyunsaturated fatty acids

RC/DC = Reducing agent compatible as well as detergent compatible

ROS = Reactive oxygen species

rpm = Revolutions per minute

SAC = Soluble adenyl cyclise

SABP = Secretory actin binding protein

SDI = Sperm deformity index

SDH = Sacchorapine dehydrogenase

SEM = Standard error of the mean

Ser/Thr = Serine/Threonine phosphatases

SCA = Sperm Class Analyser

SOD = Superoxide dismutase

STR = Straightness

TCA-cycle = Tricarboxylic acid cycle

TPI = Triosephosphate isomerise

tmACS = Transmembrane adenylyl cyclase

TZI = Teratozoospermic index

VAP = Average path velocity

VCL = Curvilinear velocity

VSL = Straight-line velocity

WHO = World Health Organization

ZAG = Zinc a2-glycoprotein

ZIFT = Zygote Intrafallopian Transfer

ZP = Zona pellucida

CHAPTER 1

INTRODUCTION TO STUDY

1.1 Introduction

Male factor infertility accounts for 35% of all infertility cases worldwide [1-3]. Various factors contribute to this phenomenon such as environmental influences (e.g. toxins), radiation and poor semen quality [4]. It has been shown that the majority of infertile men suffers from infertility of unexplained aetiology [5]. However, the molecular details including the underlying mechanisms of male infertility is still unknown. Currently, there are no effective therapeutic treatments available for infertile men diagnosed with decreased sperm motility and morphology parameters.

The present study was designed to explore the molecular nature of sperm dysfunction associated with poor sperm motility and abnormal morphology by investigating human sperm proteins. In order to find suitable treatment options for men suffering from infertility, it is important to investigate the molecular causes behind it. This includes the identification of non-functional proteins associated with sperm dysfunction. These proteins can serve as potential targets for the development of biomarkers and the use of drugs to improve sperm function.

1.2 Background to study and problem statement

Infertility is a common problem affecting about 10-15% of couples of reproductive age worldwide [6]. Approximately 20% of these couples will have infertility of unexplained aetiology [7]. For some time it was thought that infertility was only caused by the female's inability to conceive, but it has been shown that male factor infertility is a major contributor of couple infertility globally [8].

Male infertility can be caused by various factors including hormonal disorders, infections and anatomical disorders [7]. Sperm parameters can also contribute to and are used to classify male infertility such as poor sperm motility, (asthenozoospermia), decreased sperm morphology (teratozoospermia) and low sperm concentration (oligozoospermia) or a combination of each [9]. However, it has been shown that most infertile men suffer from a combination of the two, decreased sperm motility and morphology (asthenoteratozoospermia) [10]. Currently, the only realistic treatment option for sub-fertile men with sperm dysfunction is in vitro fertilization (IVF) or intra-cytoplasmatic sperm injection (ICSI) [1]. These procedures are expensive, invasive, of limited success and not widely available [6].

Until recently, very little was known about the detailed clinical nature of sperm dysfunction. It is now known that a variety of other factors may contribute to sperm dysfunction such as increased reactive oxygen species (ROS) production, gene

mutations, deoxyribonucleic acid (DNA) damage and defects in metabolic enzymes [11]. These factors play crucial role in sperm function and are not always included in routine evaluations of human semen [12, 13]. On a molecular level, sperm dysfunction can also be caused by faulty spermatogenesis which often leads to increased production of immature/abnormal spermatozoa. Immature spermatozoa display a high content of DNA damage, including abnormal chromatin structure, DNA strand breaks and chromosomes with microdeletions [10].

Due to the complex nature of sperm dysfunction, a variety of other factors that have not yet been established, may contribute to fertilization failure [14-19]. This is most likely due to the relative lack of knowledge of the metabolic and biochemical pathways involved in the normal physiology of the mature spermatozoa [20].

1.3 Rationale / Relevance of study

Newly produced testicular spermatozoa are immature and incompetent of fertilization. During the process of spermiogenesis, mammalian spermatozoa undergo maturation in the epididymis prior to ejaculation. This is an indispensable step towards the acquisition of sperm motility and fertility. Currently, little is known about the proteins expressed in human sperm or whether defects in individual proteins are associated with male infertility.

It has come to light that the modification and expression of human sperm proteins plays a critical role in sperm function [21, 22]. These modifications are manifested during spermatogenesis and lead to differences in protein expression between fertile and infertile men [23]. It is postulated that spermatozoa with decreased motility and morphology have different protein profiles than functionally normal spermatozoa.

Several studies have been done on human spermatozoa in order to understand the role of sperm proteins in sperm function. For some time it was thought that mature spermatozoa are transcriptionally and translationally inactive until recently when Gur and co-worker (2006) has shown that mitochondrial-type ribosomes play an active role in translating nuclear encoded proteins[24]. In an attempt to elucidate the molecular nature of sperm dysfunction associated with asthenoteratozoospermia, spermatozoa are the ideal cells to study from a proteomic perspective. However, only a few studies have been done on the human sperm proteome in order to alert its the role sperm proteins play in male infertility [25]. Identification and quantification of proteins expressed in human spermatozoa may provide a clearer insight into the underlining pathology of male infertility. In addition to the importance of the study of sperm proteins to understand the fundamental aspects of reproduction, it is relevant towards the identification of idiopathic causes of male infertility.

1.4 Objectives and research strategy

The broad aim of this study was to determine if protein expression differs between immature and mature populations of spermatozoa, in order to establish if immature sperm may be used as an in vitro model for asthenoteratozoospermia.

1.4.1 Specific aims

The specific aims of this study were twofold:

I. To determine differences in protein expression within the total proteome of spermatozoa from immature and mature populations.

II. To determine differences in protein expression in the nuclear fraction of human spermatozoa from immature and mature populations.

1.4.2 Specific objectives

In order to satisfy these aims the following specific objectives were set:

I. Spermatozoa were separated into two sub-populations (i.e. mature and immature cells) by discontinuous density gradient centrifugation.

II. These populations were classified on the basis of motility and morphology according to Strict Criteria [26, 27]; normozoospermic (sperm parameters falls within the normal range for concentration, motility and morphology),

teratozoospermic (<14% normal morphology), asthenozoospermic (<50% total motility) and asthenoteratozoospermic (< 14% normal morphology plus < 50% total motility).

III. Differentially expressed proteins were isolated within the total sperm proteome.

IV. Nuclear protein fractions were isolated and differentially expressed nuclear proteins were identified.

V. Functions were attributed to these proteins.

1.5 Outline of study

Chapter 2 consists of a systematic literature review on proteomics in the context of sperm protein expression in relation to sperm dysfunction; decreased sperm motility, abnormal morphology and infertility. This serves as a background to the study while also outlining the lack of knowledge on sperm proteins that are essential in understanding the molecular and cellular function of the mature spermatozoa. Chapter 3 comprises the basic materials and methods employed in the study. This is followed by the results section (Chapter 4). The final chapter (Chapter 5) focuses on ascribing functions to all the proteins isolated and concludes with future perspectives on proteomics and its role in identifying idiopathic causes of male infertility.

1.6 Conclusion

In the current study, novel proteomics techniques have been employed which led to the successful identification of differences in protein expression between immature and mature sperm populations. Identifying and isolating proteins expressed in human spermatozoa provided clearer insight into the nature of pathologies involved in male infertility. Ascribing functions to these proteins is essential and of great value in the development of possible therapeutic options to infertile men, as well as research focussed on the discovering of male contraceptives and the identification of biomarkers in reproductive technologies.

CHAPTER 2

REVIEW OF THE LITERATURE

2.1 Introduction

Male factor infertility caused by abnormal semen parameters is the single most defined cause of infertility [1]. Approximately 1 in 15 men are subfertile and the condition is increasing in frequency [28]. Various factors contribute to male infertility including genetic factors such as aneuploidy (abnormal number of chromosomes), gene mutations and cancer [29, 30]. However, approximately 50% of infertile men have infertility of unexplained etiology [31]. This could be partly due to the limited knowledge regarding the molecular and cellular mechanisms involved in regulating sperm function [32, 33].

Many studies have been performed on human spermatozoa in order to investigate the causes of sperm dysfunction and fertilization failure [16, 34, 35]. In one such study done by Curi and co-workers (2003) it was shown that 81% of spermatozoa had defective motility while concentration and morphology were not affected [1]. Sperm parameters such as sperm motility and morphology are essential for normal fertilization and also play a crucial role in sperm function [36].

2.2 Functional parameters in human spermatozoa

The following section focuses on the role sperm parameters such as sperm motility and morphology plays in male fertility. It also highlights the lack of knowledge relating to the signaling pathways regulating human sperm function in the mature spermatozoa.

2.2.1 Sperm morphology

Sperm morphology – the size and shape of sperm - is considered to be the only consistent sperm parameter used to predict IVF success [37]. It is generally accepted that spermatozoa with normal sperm morphology have a greater chance of successful fertilization than abnormal spermatozoa. However, little is known about the role sperm morphology plays during the process of fertilization. Assessment of the percentage of normal spermatozoa with good morphology is a highly subjective issue and reproducibility among different laboratories throughout the world is almost impossible [38]. The ability to properly assess sperm morphological features mostly depends on personal experience and the protocol used in the particular laboratory. It is therefore important to improve international standardization of the technical methodology, and also find consensus on criteria in which sperm morphology is evaluated and interpreted. Currently, little is known about the detailed molecular nature of morphologically normal/abnormal spermatozoa or whether defects in individual genes or proteins could compromise sperm morphology.

2.2.1.1 Sperm morphology as predictor of male fertility

Semen parameters have been correlated with IVF success in a large number of studies [39-42]. Sperm morphology was the only parameter to consistently predict successful fertilization and is therefore of great importance in male infertility [37]. However, the main shortcomings of this parameter are the large number of classification systems used to describe what constitutes a morphologically normal/abnormal spermatozoon.

Universally, the most commonly accepted classification systems used for sperm morphology are the World Health Organization (WHO) Criteria and Strict Criteria [43, 44]. The present study will only focus on the Strict Criteria.

According to the Strict Criteria, a spermatozoon is considered normal when:

- A well defined acrosome comprises 40-70% of the sperm head.
- The length of a normal sperm head should be 5-6µm, and the diameter 2.5-3.5µm.
- Furthermore, there must be no visible defect of the neck, midpiece or tail.
- No cytoplasmic droplets of more than one-half of the sperm head size may be present. Borderline or slightly abnormal head forms are considered as abnormal.
- At least 100, but preferably 200 of tail-possessing spermatozoa should be analyzed [43].

The most important reason for using Strict Criteria is that the range of ‘normal variations’ is kept as small as possible to ensure reproducible assessments.

Several studies have shown that spermatozoa with normal morphology have greater chance of successful fertilization compared with abnormal spermatozoa [9, 19]. Nevertheless, it is not known what factors contribute to the formation of abnormal sperm. In addition, sperm morphology also correlates significantly with the spermatozoa's ability to bind to the zona which is a critical step in the process of fertilization [45, 46]. It has been shown that a strong correlation exists between normal sperm morphology and the inducibility of the acrosome reaction [47, 48].

2.2.2 Sperm maturation and motility development

After leaving the testis, mammalian spermatozoa from many species are morphologically differentiated but have acquired neither progressive motility nor the ability to fertilize a metaphase II-arrested oocyte [49].

During epididymal transit, sperm acquire the ability to move progressively, however, they are still incapable of fertilization. Most mammalian sperm display two types of physiological motility: activated motility as seen in freshly ejaculated sperm, and hyperactivated motility as is seen in most sperm recovered from the site of fertilization [50, 51]. Fertilization capacity is gained after residence in the female tract for a finite period of time.

Current evidence suggests that the role of activated motility is to aid in propelling the sperm through the female reproductive tract to the oviduct [52, 53]. In addition, the role of hyperactivated motility is to help sperm detach from the oviductal epithelium, reach the site of fertilization, and penetrate the cumulus and zona pellucida of the oocyte [54, 55]. Yet, little is known about the signaling pathways involved in the regulation of activated/hyperactivated motility. This is crucial for understanding the role that sperm motility plays in male infertility.

2.2.2.1 How is sperm motility regulated?

The adenosine-3', 5'-cyclic monophosphate (cAMP)/protein kinase A (PKA) and calcium (Ca^{2+}) signalling pathway (cascade) (see Figure 1) are generally recognised as the two signalling pathways that are most central to the regulation of mammalian sperm motility [56-59]. Heterotrimeric and small G-protein-mediated pathways, as well as pH changes, have also been implicated as important role players in sperm motility, although these mechanisms are not well characteristed in mature sperm [58, 60, 61].

A number of protein substrates for cAMP-dependent protein kinase have been identified in both membrane and soluble fractions of bovine and human sperm homogenates [62, 63]. It has been reported that Ca^{2+} and bicarbonate (HCO_3^-) can activate soluble adenyl cyclase (sAC) to generate cAMP from adenosine triphosphate (ATP) (see Figure 1).

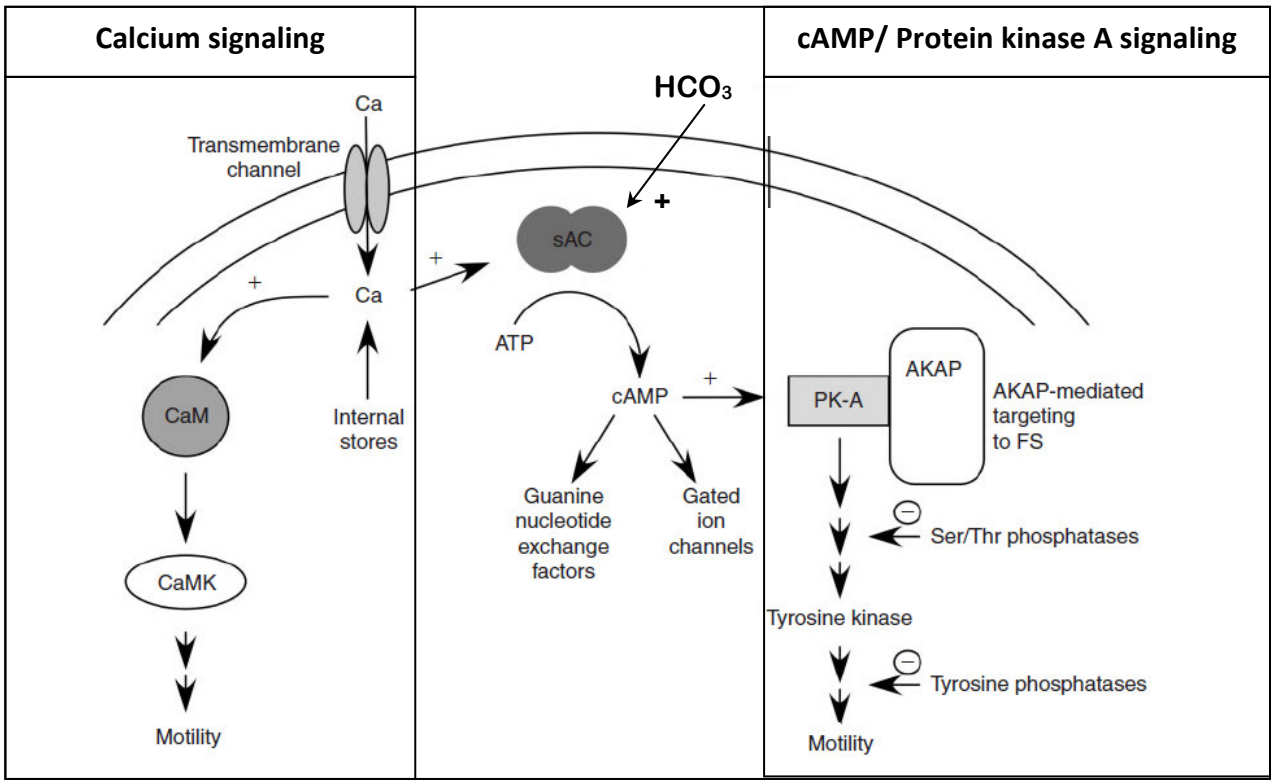

Figure 1. *Schematic representation of the signalling pathways (Calcium and cAMP/Protein Kinase A) known- or postulated of being involved in the regulation of mammalian sperm motility (ATP= Adenosine triphosphate, AKAP= A-kinase anchoring protein, HCO_3^-= Bicarbonate, Ca= Calcium, CaM= Calmodulin, CaMK= Calmodulin-dependent protein kinase, Ser/Thr= Serine and Threonine phosphatases, FS= Fibrous sheath, sAC= soluble adenyl cyclase, cAMP= Adenosine-3', 5'-cyclic monophosphate, PKA= Protein kinase A, (+)= activation of signal transduction pathways, (-)= inhibition of signal transduction pathways) [64].*

cAMP appears to activate cAMP-dependent kinase, which phosphorylates key proteins that are required for motility (see Figure 1). Deactivation occurs when these proteins are dephosphorylated by phosphoprotein phosphatase. However, studies to date concerning the control of protein phosphorylation by cAMP in sperm have been limited to homogenates and sub-cellular fractions where structural integrity and motility have been destroyed [65, 66]. This lack in knowledge is crucial as protein phosphorylation/dephosphorylation plays a critical role in sperm function.

It is known that a strong correlation exist between changes in cAMP content and motility in sperm [67]. More direct evidence that cAMP may actually regulate sperm motility was presented by Lindemann and co-workers (1983) who showed that the addition of cAMP to ATP-reactivated detergent lysed bovine sperm models produced measurable increases in beat frequency and percentage of motile cells [68].

The only known action of cAMP in eukaryotic cells is the stimulation of protein phosphorylation via activation of cAMP dependent protein kinase [68]. cAMP-dependent protein kinase has been shown to be present in sperm of a variety of species and represent a significant proportion of the proteins present in sperm extracts [68-70].

2.2.2.2 cAMP/protein kinase A signaling

The cAMP-dependant phosphorylation of flagellar proteins (see Figure 1) is at least partially responsible for the initiation and maintenance of activated motility in mammals [71, 72]. One of the phenotypes seen in mice with a target deletion of the sperm-specific isoform of the catalytic (C) subunit of cAMP-dependent serine/threonine kinase PKA is highly correlated with poor sperm motility [73].

Although PKA may work through multiple pathways to control flagellar function, one possible mechanism of action is that serine/threonine phosphorylation of PKA target proteins results in activation of a downstream, as yet unidentified tyrosine kinase (see Figure 1) or kinases whose targets are primarily located in the flagellum [74, 75]. Tyrosine phosphorylation of a specific subset of flagellar proteins then results in motility (see Figure 1). Until recently, only a few protein targets for PKA phosphorylation in sperm have been identified [76]. One of the targets is axonemal dynein protein and its phosphorylation is known to be a critical regulatory point of flagellar motility [66]. Serine/threonine phosphatases balance the actions of serine/threonine kinases (see Figure 1). The resulting net protein phosphorylation is one factor influencing the status of sperm motility [66, 77].

In addition to regulating the PKA pathway, cAMP may also activate other signalling pathways in sperm and testes, including a cyclic nucleotide-gated ion channel and cAMP–mediated guanine nucleotide exchange factors (see Figure 1) [78].

Tyrosine phosphorylation is also strongly associated with the onset of sperm motility and is likely to be downstream of serine/threonine phosphorylation in the motility regulation pathway [76]. Some specific protein targets of tyrosine phosphorylation in sperm have been suggested and phosphorylation of these proteins have been closely linked to the onset of motility in bovine sperm [79]. Tyrosine phosphorylation and dephosphorylation of flagellar proteins have also been linked to the onset and termination of hyperactivated sperm motility in primates and rodents, respectively [80, 81]. One of these phosphotyrosine containing proteins is an A-kinase anchoring protein (AKAP) known as AKAP4 (see Figure 1) [82]. The AKAPs are a family of proteins in specific subcellular locations of sperm flagellum that play important roles in sperm motility [83].

2.2.2.3 Calcium signaling

Extracellular Ca^{2+} is required for motility in most epididymal sperm samples and Ca^{2+} is known to regulate both activated and hyperactivated motility [56-58, 84-86]. One mechanism by which Ca^{2+} is directly linked to flagellar function (see Figure 1) is through its regulation of atypical sAC, which generates cAMP to activate PKA.

The sAC is required for sperm motility and is molecularly and biochemically distinct from transmembrane adenylyl cyclase (tmACS), in part because sAC is uniquely sensitive to both HCO_3^- and Ca^{2+} [87-92]. Thus, through sAC, Ca^{2+} signalling can be linked to PKA as part of a single pathway (Figure 1) by which it can have direct influence on sperm motility.

2.2.2.3.1 Downstream components of calcium signaling

There are two different calcium signaling pathways: one that is dependent of calmodulin (CaM) (e.g. sAC/PKA) and the other is not (Figure 1). Alternatively, calcium could have an effect at two different points within the same pathway (Figure 1). The effect of calcium on the flagellum is likely to be achieved through CaM, as inhibition of CaM decreases sperm motility [75, 93]. CaM is a ubiquitous, highly conserved protein that serves as a classical intracellular calcium receptor [94]. Calmodulin kinase (CaMK) is the downstream target of CaM [56, 95, 96]. Isoforms of CaMK are present in flagellum of mammalian sperm and CaMK-inbitors reduce sperm motility [93, 95]. A variety of different isoforms of CaMK could be present in sperm flagellum, therefore it can be postulated that the effects of CaM on motility can be achieved through one or more isoforms of CaMK in the flagellum.

2.2.2.4 Sperm motility as predictor of male fertility

Sperm motility is believed to be one of the most important sperm parameters for evaluating the fertility potential of ejaculated spermatozoa. Various objective methods for measuring sperm motility and velocity have been developed, of which computer-assisted sperm analysis (CASA) is well accepted in clinical evaluations of male fertility [97]. It is known that a strong correlation exists between sperm motility and IFV success, however, little is known about the molecular mechanism involved in regulating sperm motility during the process of fertilization [47, 98]. This lack of knowledge is crucial as sperm motility is essential for normal fertilization and male fertility.

2.2.3 Sperm energy metabolism

Energy metabolism is a key factor supporting sperm function (e.g. sperm motility) [67, 99]. Sustaining sperm motility and active protein modifications such as phosphorylation could be one of the reasons why sperm requires exceptionally more ATP than other cells [99]. Potential sources of ATP to support sperm motility are postulated to be compartmentalized in distinct regions along the length of the flagellum. Oxidative phosphorylation is thought to be confined to the proximal segment of the flagellum where the mitochondria are localized (midpiece).

In contrast, glycolysis appears to be restricted to the principal piece, which is distal to the midpiece and is the longest segment of the sperm flagellum [100-103]. Various studies have been done on spermatozoa in order to understand the role energy metabolism plays in sperm function [99, 104-106]. These approaches have identified critical metabolic pathways that support specific processes during germ cell development and fertilization.

In round spermatids, lactate and pyruvate are the preferred substrates for the acrosome reaction (AR) and ATP production by oxidative phosphorylation (mitochondria), while gamete fusion requires glucose to produce nicotinamide adenine dinucleotide phosphate (NADPH) by the pentose phosphate pathway [107]. Sperm motility appears to be supported by relatively low ATP levels, but high ATP levels are essential for tyrosine phosphorylation linked to hyperactivation [86]. Thus, each individual process and event requires a different substrate and metabolic pathway. Despite different preferences for energy substrates and metabolic pathways between species, analysis of knockout mice revealed that glycolysis is indispensable for mouse sperm function and that oxidative phosphorylation is not essential for male fertility [63]. This suggests that glycolysis could compensate for the lack of oxidative phosphorylation as it produces ATP independently of oxygen supply.

Knowledge of the metabolic pathways involved in the production of ATP that are required to assemble a functional flagellum, may allow us to eventually intentionally disrupt the normal function of crucial, sperm-specific proteins or metabolic enzymes, which could result in the development of a safe, effective male contraceptive.

2.3 Current treatment regimes for male infertility

Currently, the most popular techniques of assisted reproduction for the treatment of male subfertility and infertility are intrauterine insemination (IUI), IVF and ICSI [6, 13]. These techniques are expensive, invasive, and not readily available.

IVF initially gained popularity for the treatment of problems of oocyte and sperm transfer resulting from tubal obstruction or dysfunction [6, 108]. Since only a few thousand motile spermatozoa are required to obtain fertilization, IVF was used to treat longstanding infertility caused by oligoasthenoteratozoospermia (combination of decreased sperm concentration (oligo-), motility (astheno-) and morphology (terato-)) [109]. When oligoasthenoteratozoospermia is present, the fertilization rate is significantly decreased and a complete fertilization failure of all oocytes may occur in up to 1 out of 4 couples [110]. Current evidence suggests that IUI has proven to be a valid treatment for moderate oligoasthenozoospermia, however the outcome after conventional IVF is limited because of a high incidence of complete fertilization failure [12].

A recent randomized study done by Goverde and co-workers (2000) compared conventional IVF with IUI in patients with moderate oligozoospermia and duration of infertility of 1 year or longer [111]. Although the pregnancy rate per cycle was higher after IVF than after IUI, the cumulative pregnancy rate for IVF was not significantly better than that for IUI.

Moreover, couples undergoing IVF were more likely to give up treatment than those undergoing IUI, and the latter approach was more cost effective. Adjunctive measures such as raising the insemination sperm concentrations or use of metabolic stimulants such as pentoxyfiline have been proposed to increase fertilization [112, 113]. These measures generally have limited success. Several alternative techniques to in vitro fertilization and embryo transfer (IVF-ET) have been suggested for the treatment of male infertility, for example gamete intrafallopian transfer (GIFT) and zygote intrafallopian transfer (ZIFT). In these techniques, oocytes and spermatozoa (GIFT) or two-pronucleate oocytes (ZIFT) are transferred directly into the fallopian tube. However, meta-analysis shows that these alternative techniques fail to increase success rate in cases of male infertility since fertilization itself is the major bottleneck [114].

2.4 The value of proteomics in male fertility

Proteomics can be viewed as an experimental approach to explain the information contained in genomic sequences in terms of the structure, function, and control of biological processes and pathways. This technique provides fascinating insights into the pathology of cell dysfunction by systematic analysis of the proteins expressed in a cell or tissue. Since proteins and protein-protein interactions are responsible for cellular function, identification of protein changes in spermatozoa is crucial in understanding the molecular mechanisms involved in sperm dysfunction and associated subfertility. Currently, sperm proteomic analyses are largely conducted using 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

The human spermatozoon is a highly specialized cell with extremely marked compositional, morphological and functional differences, as compared to other somatic or germinal cells [35, 107, 115, 116]. For example, the sperm nucleus is condensed by protamines into a highly compact and hydrodynamic sperm head, and the cell is also equipped with a large flagellum to allow for motility [35, 107, 116, 117]. To date, efforts to identify sperm proteins have been directed towards the nuclear proteins [115, 118-121] in order to identify the proteins required for sperm motility [122, 123]. Most of the fundamental knowledge on the sperm protein composition has been gained using conventional protein purification and identification strategies [124-130].

Anomalies of the expression of the most abundant nuclear sperm proteins have been found to be associated with infertility [129, 131-135]. A previous case report has provided some evidence of the potential of proteomic tools to identify proteins involved in infertility [126]. More recently, PTMs during spermatogenesis in rat models have also been studied using fluorescent 2D-PAGE [136]. However, the majority of causes in male infertility are still unknown. Through the careful analysis of multiple replicated samples, Naaby-Hansen and co-workers (1997) established a comprehensive database of 1397 protein spots [137]. Within this data set, at least 98 protein spots were accessible to both I_{125} vectorial labeling and biotinylation, suggesting an association with the sperm surface. Furthermore, 22 protein spots were immunologically reactive to a phosphotyrosine antibody and clustered into five protein isoforms.

Until recently, higher resolution 2D-PAGE maps of human sperm proteins have been generated using a series of overlapping narrow pH ranges for the IEF step that contain 3872 different protein spots [138]. From this analysis, only 16 ‘landmark’ protein identities have been reported. In an attempt to understand which proteins might be important for contraceptive purposes, the distribution of immunodominant sperm antigens on 2D Western blots have been examined using anti-sperm antibodies from patients’ sera [139]. In total, 98 sperm auto- and iso-antigenic protein spots were recognized by sera derived from infertile patients but not from the fertile controls.

A similar study with a narrower focus on sperm-immobilizing antibodies, has identified four proteins as potential sperm antigens responsible for this activity [140]. Such 2D-PAGE-based studies have been valuable in generating data on the overall nature and complexity of the sperm proteome, at least in terms of molecular weight (M_r) and iso-electric point (pI).

2.5 Clinical applications of proteomics

Several studies have shown that a variety of PTMs take place in sperm structure, metabolism and motility as it matures in the epididymis in order for the mature sperm to be fully functional in its post-ejaculatory state. The molecular mechanisms underlining these events remain poorly understood, possibly due to the relative lack of knowledge of the proteins involved in the normal physiology of the mature spermatozoa [107, 116].

New advances in proteomics are having a major impact on our understanding of how spermatozoa acquire their capacity for fertilization and such information is strategically important for the development of novel approaches to fertility regulation [13, 25]. Spermatozoa are model cells for proteomic analysis in that they can be purified in large numbers and reliably and robustly driven into different functional states (non-capacitated, capacitated and acrosome reacted) using a variety of validated pharmacological manipulations.

In the apparent absence of contemporary gene transcription, the functionality of these cells is largely, if not solely, dependent on PTMs to their protein complement. Oocytes can also be obtained in highly purified form at different stages of maturation (e.g. germinal vesicle and metaphase II), but the restricted availability of material inevitably places limits on the proteomic characterization of this cell type which do not apply to spermatozoa.

Currently, the availability of 2D-PAGE techniques coupled to mass spectrometry (MS) offers the opportunity to compare the proteome maps of independent sperm samples. Anomalies in expression of sperm proteins have been found in patients using conventional protein purification and identification strategies [115, 117, 119, 141, 142]. A recent study has already provided some evidence of the potential of this proteomic approach in the identification of a variety of proteins involved in energy metabolism and sperm motility that were found to be down regulated in the asthenozoospermic group [143, 144]. Tandem MS (MS/MS) has also been used to identify proteins present in human spermatozoa. However, the full potential of the present proteomic tools to gain insight into fundamental aspects of the human sperm motility and the causes of asthenoteratozoospermia has not yet been fully explored [145, 146].

2.6 Summary and aim of study

Several studies have been done on spermatozoa in order to understand its role in sperm dysfunction. The present study aims to elucidate the molecular nature of asthenoteratozoospermia by investigating protein expression in human sperm. This includes proteins involved in sperm structure, flagellar assembly and metabolism. In order to find suitable treatment regimes (male contraceptives, drug treatment) for male factor infertility it is crucial to identify the non-functional genes and proteins associated with sperm dysfunction. This may eventually result in improved diagnostics and specific treatments for asthenoteratozoospermia and associated subfertility [147]

CHAPTER 3

MATERIAL AND METHODS

3.1 Introduction

This chapter will give an overview of all the material used in the present study, as well as detailed protocols of all the methods employed. A brief outline of the experimental procedure followed in this study is shown in Figure 2.

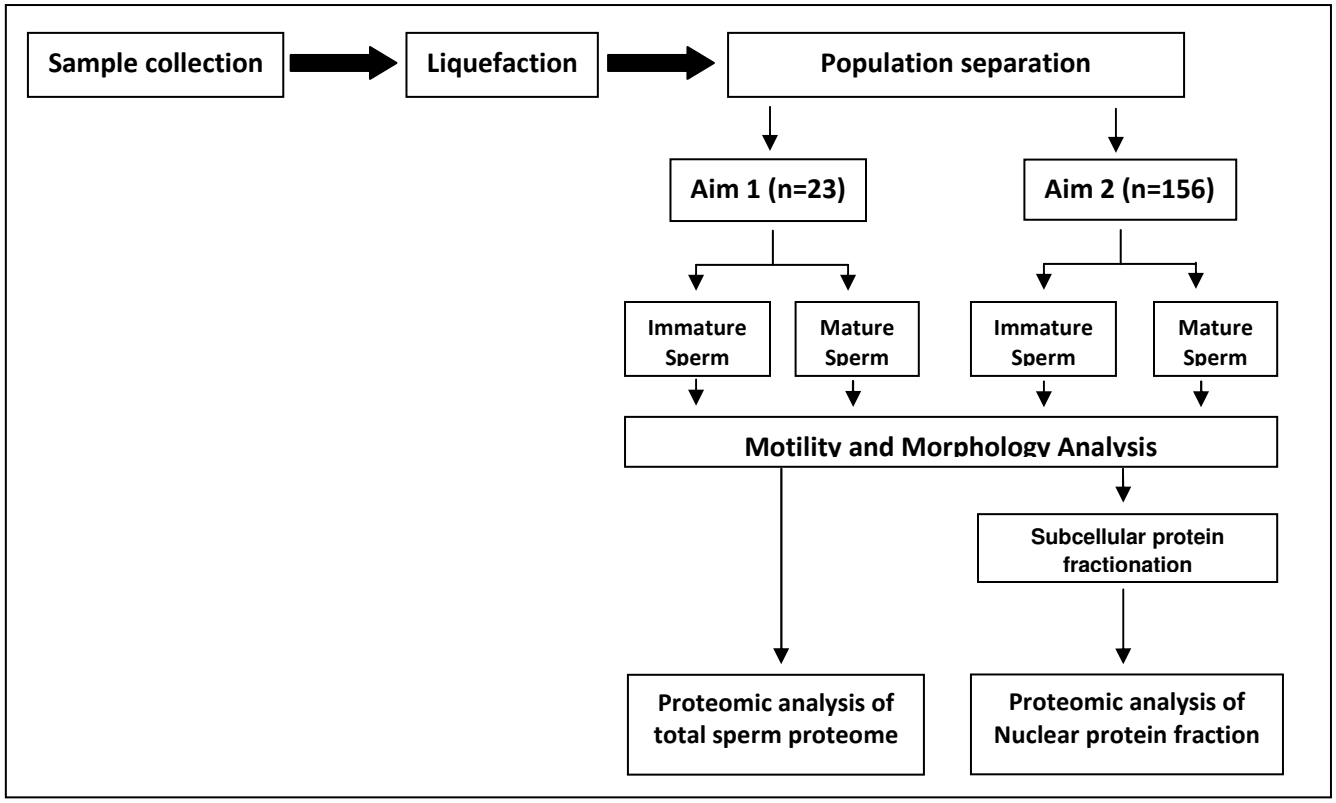

***Figure 2**. Flow chart showing the simplified experimental protocol used in the present study.*

3.2 Semen sampling

A total of 179 semen samples were obtained from healthy volunteers taking part in the sperm donor program at the Reproductive Research Laboratory at Stellenbosch University. All donors gave informed consent and were informed that their spermatozoa would purely be used for research purposes only and the excess discarded in an appropriate fashion. Ethical clearance for this study was granted by the Institutional Review Board (Ethics number: N09/05/159).

3.3 Semen collection

Semen was collected from healthy donors according to the WHO criteria [148]. In order to investigate Aim 1 (as set out in Chapter 1.4.1) semen was collected from 23 donors and for Aim 2 (see Chapter 1.4.1) semen was collected from 156 donors. The semen was allowed to undergo liquefaction at 37 $^{\circ}$C for 30 min before further analysis.

3.4 Discontinuous gradient centrifugation techniques

In order to isolate and separate a single semen sample into two sub-populations of mature and immature spermatozoa, two different discontinuous density gradient centrifugation methods were used. This was done to ensure sufficient separation of spermatozoa from seminal plasma and to exclude technical errors caused by different separation techniques. For Aim 1 a 3-layer Percoll (Sigma) gradient was used while for Aim 2 a double density gradient method (PureSperm®) was followed.

3.4.1 Percoll gradient centrifugation

A 90%, 60% and 40% solution of Percoll was prepared with Hams F-10 (Sigma, SA) containing 3% bovine serum albumin (BSA) (Roche, Scotland, U.K). Firstly, 2ml of the 90% solution was placed into a conical tube. Thereafter 2ml of the 60% and a further 2ml of the 40% solutions were carefully layered on top. Finally, 2ml of semen was placed on top of the three layered gradients, taking care not to cause mixing of the layers (see Figure 3). The tube was centrifuged for 20 min at 300 x g. This separated the semen into two distinct sperm populations (e.g. mature and immature cells). The mature cells migrated to the bottom of the tube and formed a pellet, whereas the immature cells migrated to the interface between the 60% and 90% Percoll layers. The seminal plasma and debris remained in the top layer.

The layers were removed carefully in order to prevent disturbance and mixing and placed into separate eppendorffs, washed with Hams F-10 (5 min, 300 x g) and resuspended in Hams F-10 in order to remove any Percoll.

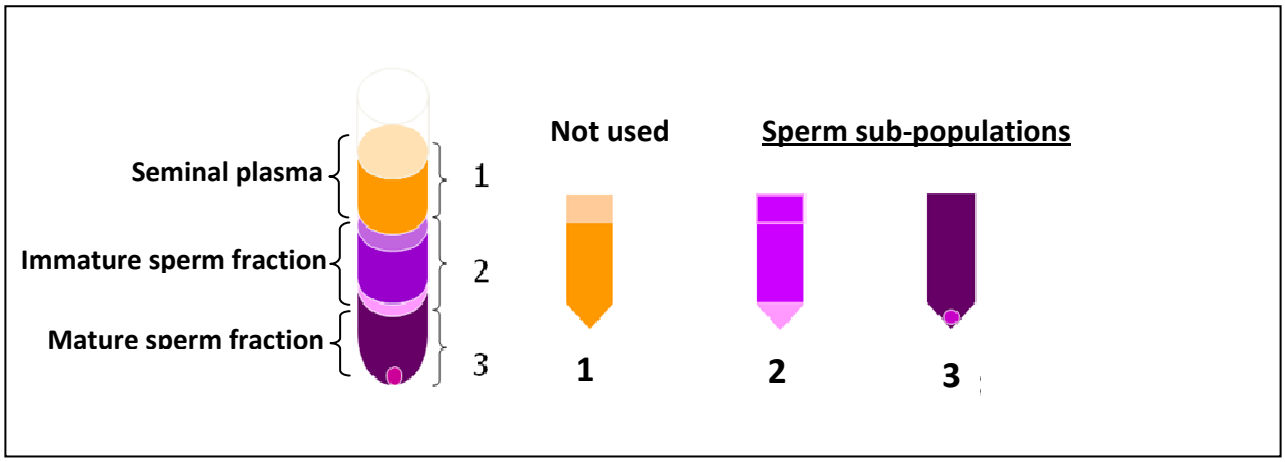

***Figure 3**. An illustration to show how the different sperm populations were obtained using the Percoll gradient centrifugation technique [149].*

3.4.2 PureSperm® gradient centrifugation

For Aim 2 an 80% and 40% discontinuous density gradient (PureSperm®, Nidacon, Hunter Scientific Limited, Saffron Walden, Essex, U.K) was used. Firstly 2ml of the 80% PureSperm® gradient solution was placed in the bottom of a conical tube. Subsequently 2ml of the 40% gradient was layered on top. Thereafter 2ml of the semen was placed on top of the 40% layer and the tube was centrifuged for 20 min at 300 x g (see Figure 4).

After centrifugation the different layers were carefully removed to prevent any disturbances and mixing. The immature cells were retrieved from the interface between the 40% and 80% gradient while the mature cells accumulated as a pellet at the bottom of the tube. The collected fractions were then washed with Hams F-10 (10 min at 300 xg) to remove the PureSperm® and resuspended in Hams F-10.

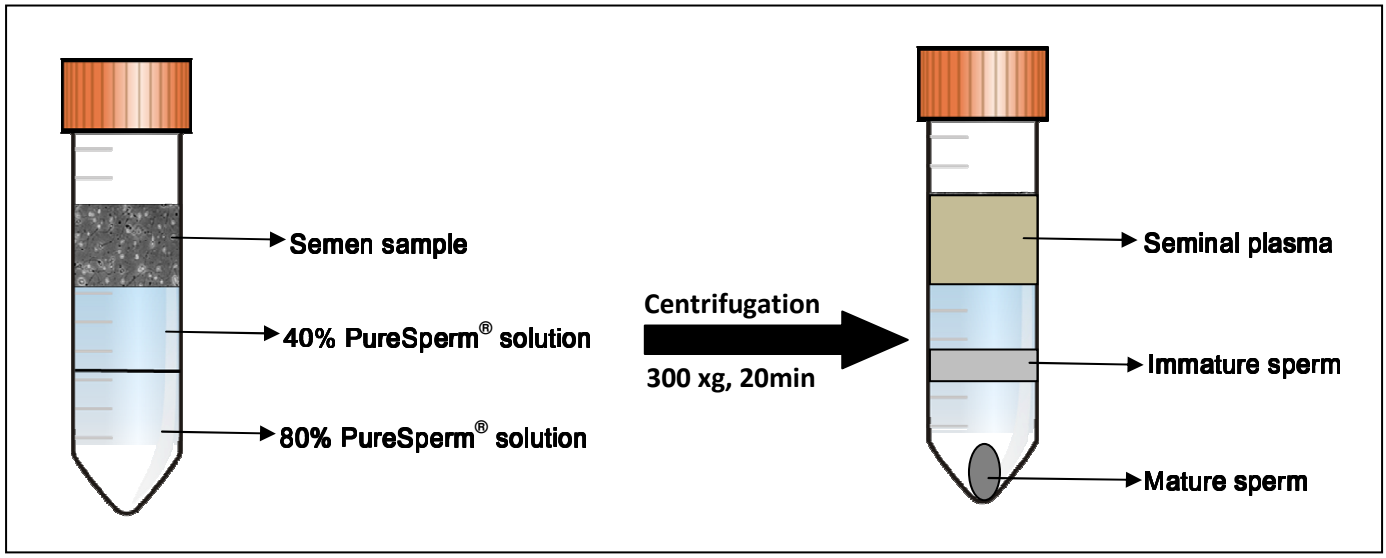

***Figure 4**. The PureSperm® double density gradient centrifugation technique [150].*

3.5 Semen analysis

3.5.1 Sperm motility

After the different sperm populations (i.e. immature and mature cells) were retrieved, sperm motility of each sample was measured by means of CASA using the Sperm Class Analyzer (SCA) (Microptic, Barcelona, Spain). The settings of the analyzer were as follows: optics, ph+; contrast, 435; brightness, 100; scale, 10x; chamber, Leja 20; capture, 50 images per second; curvilinear velocity (VCL); 10µm/s<slow<15µm/s; 15µm/s<medium<35µm/s; rapid<35µm/s; progressivity>80% of straightness (STR);

linearity (LIN), circular<50%; connectivity, 12; average path velocity (VAP) points, 5; and temperature, 37 ℃.

Several motility parameters were assessed as illustrated in Figure 5. These were:

- total motility (percentage of motile spermatozoa) will be used to report motility
- progressive motility (percentage of progressive motile cells)
- curvilinear velocity (VCL) (μm/s) (the time average velocity of sperm head along its actual curvilinear path, as perceived in two dimensions in the microscope)
- straight line velocity (VSL) (μm/s) (the time average velocity of sperm head along the straight line between its first detected position and its last)
- average path velocity (VAP) (μm/s) (the time average velocity of sperm head along its average path)
- amplitude of lateral head displacement (ALH) (μm/s) (the magnitude of lateral displacement of sperm head about its average path)
- linearity (LIN) (%) (the linearity of a curvilinear path)
- straightness (STR) (%) (linearity of the average path)
- beat-cross frequency (BCF); (beats/second) average rate at which the sperm's curvilinear path crosses its average path
- rapid cells; the percentage of rapid moving cells,
- static cells; percentage of static/motion-less cells.

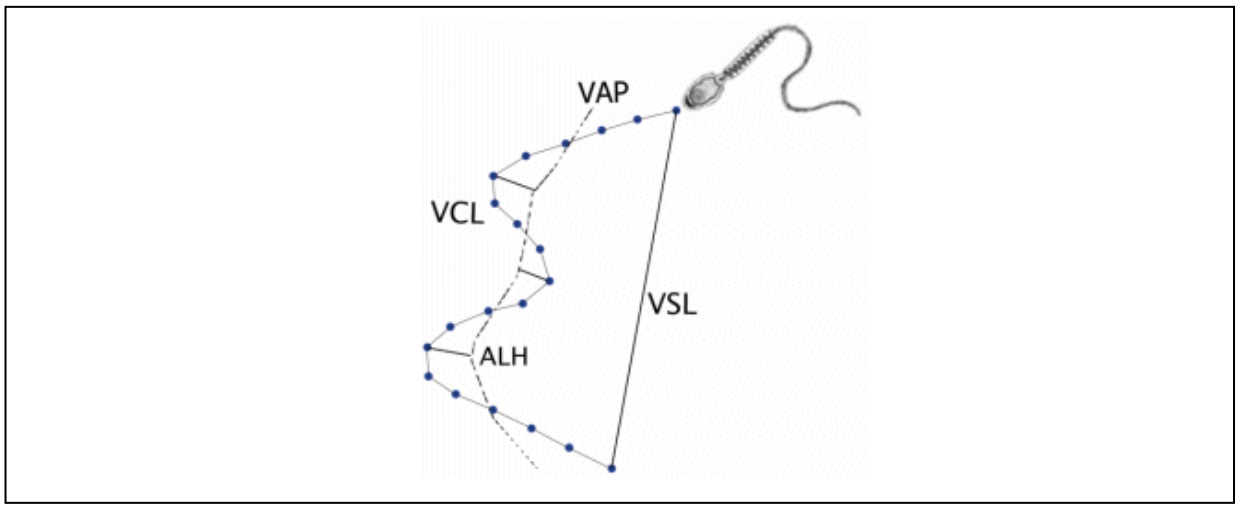

Figure 5. *An illustration of different sperm motility parameters measured using CASA (VCL= curvilinear velocity, VAP= average path velocity, ALH= amplitude of lateral head displacement, VSL= straight line velocity) [46].*

3.5.2 Sperm morphology

Sperm smears were made of immature and mature sperm populations. 10µl of each sample was spread evenly along the length of microscope slide and allowed to air dry for 20 min at room temperature. The resulting smear was fixed for 10 min in SpermBlue® fixative. Excess fixative was removed before staining spermatozoa with SpermBlue® stain for 15 min. In preparation for morphology analysis, stained microscope slides were mounted with DPX mounting medium. Sperm morphology analysis was done according to Strict Criteria using the SCA.

The settings of the analyzer were as follows; optics; ph+, contrast; 269, brightness; 591, scale; 100x, capture; 200 images were analysed per slide. Only head parameters were used to report morphology and it was expressed as percentage normal head morphology.

3.6 Division into groups

For Aim 1, individual semen samples were divided into two groups i.e. immature and mature sperm populations by discontinuous gradient centrifugation. To accomplish Aim 2 of the study, the retrieved fractions i.e. immature and mature sperm populations were further divided into two groups based on progressive motility. This was done to get a better separation of spermatozoa after discontinuous density gradient centrifugation. Only samples of which both the immature sperm population had a progressive motility of < 25% and the mature sperm population had a progressive motility of > 25% were included.

3.7 Sampling and storing

The retrieved fractions that adhered to the inclusion criteria were washed to reduce contamination caused by cell debri or separation media. This was done by adding phosphate buffered saline (PBS) (Gibco, Scotland, U.K) to the retrieved fractions up to a volume of 5ml. Fractions were centrifuged at 300 x g for 5 min.

The supernatant was removed, the pellet resuspended in PBS and cetrifuged again. After the final wash and centrifugation step, the supernatant was carefully removed from the spermatozoa and the tubes were labelled as mature or immature sperm population. The pellets of the immature and mature sperm were resuspended in PBS and frozen at -196 ℃ in liquid nitrogen until further analysis.

3.8 Sample preparation for 2D gel electrophoresis (2D-PAGE)

On the day of protein analysis, the spermatozoa were defrosted at room temperature. The pellets of the immature sperm were pooled as were the pellets of the mature sperm. Fractions were centrifuged at 300 x g for 5 min. The supernatant was carefully removed and pellets placed on ice. For Aim 1, a lysis buffer was prepared in order to homogenize cells for proteomic analysis.

Protocol for lysis buffer preparation:

- The following stock solutions were prepared and used in lysis buffer preparation:
- 1M TRIS (6.057g/50ml, pH 9),
- 10% DOC (10g/100ml + 5g/50ml),
- 500mM NaF (1.05g/50ml),
- 200mM ZnCl (136.3mg/50ml),
- 10mM $NaVO_3$ (18.35mg/10ml).

- Aprotinin, Leupeptin
- 5% Triton X-100, PMSF
- A 2ml lysis buffer was prepared with the mixture of the following solutions:
- 50µl TRIS,
- 100µl DOC,
- 100µl NaF,
- 100µl ZnCl,
- 100µl $NaVO_{3,}$
- 2µl Aprotinin,
- 2µl Leupeptin,
- 5µl Triton X-100,
- 5µl PMSF, 1536µl milli QH_2O to make up a total volume of 2000µl or 2ml
- 500µl of the lysis buffer was added to each sample i.e. immature and mature sperm.
- The lysis mixture was homogenized with a glass pestle set for 5min, followed by incubation on ice for 60 min.
- The homogenate was placed in a low protein binding eppendorf tube and centrifuged (4 ℃, 30 min, 14000 rpm).

For Aim 2 samples were subjected to subcellular protein fractionation using a commercially available ProteoExtract Sub cellular Proteome Extraction Kit (Merck, Calbiochem, S.A).

Protocol for subcellular protein extraction:

- The tubes containing the immature and mature sperm were centrifuged for 5 min at 500 x g to clear sperm from any contaminants.
- The supernatant was carefully discarded by inverting the tube on a paper towel.
- The samples were placed on ice in order to inhibit possible enzymatic activity.
- 500µl of extraction buffer I was added to each fraction separately i.e. immature and mature fraction, without touching the content of the tubes to minimize protein loss and reduce contamination.
- Solutions were gently mixed by pipetting the mixture up and down until a complete homogenate was formed.
- The solution was placed in a glass homogenizer.
- 2.5µl of inhibitor cocktail was added on top of the residual material/pellet of both fractions.
- A glass pestle was used to homogenize the cell solution mixture.
- The solution was incubated for 10 min at 4 ℃ under gentle agitation, followed by 10 min centrifugation at 1000 x g.
- The supernatant was transferred to a new tube (cytosolic fraction).
- 500µl of extraction buffer II was added to the residual material/pellet of the immature and mature fraction tube.
- 2.5µl of inhibitor cocktail was added on top of the residual material/pellet of both fractions.

- The solution was incubated for 30 min at 4 ℃ under gentle agitation, followed by 10 min centrifugation at 6000 x g.
- The supernatant was transferred to a new tube (membrane/organelle fraction).
- 250µl of extraction buffer III was added to the residual material/pellet of the immature and mature fraction tubes.
- 2.5µl of inhibitor cocktail was added on top of the residual material/pellet of both fractions.
- 1.5µl of Benzonase buffer was gently added to the residual material of both fractions.
- The solution was incubated for 30 min at 4 ℃ under gentle agitation, followed by 10 min centrifugation at 10 000 x g.
- The supernatant was transferred to a new tube (nucleic protein fraction).
- 250µl of extraction buffer IV was added to the residual material/pellet of the immature and mature fraction tubes.
- The pellets of the immature and mature fractions were resuspended in 250µl extraction buffer IV with 2.5µl inhibitor cocktail (cytoskeletal matrix protein fraction).

Only the nuclear protein fraction of the immature and mature samples were retrieved and used in further analysis (see Figure 6). It has been shown that anomalies of the expression of the most abundant nuclear sperm proteins have been found to be

associated with infertility, therefore the rational to focus on the nuclear protein fraction[134, 136, 137, 139, 140, 151].

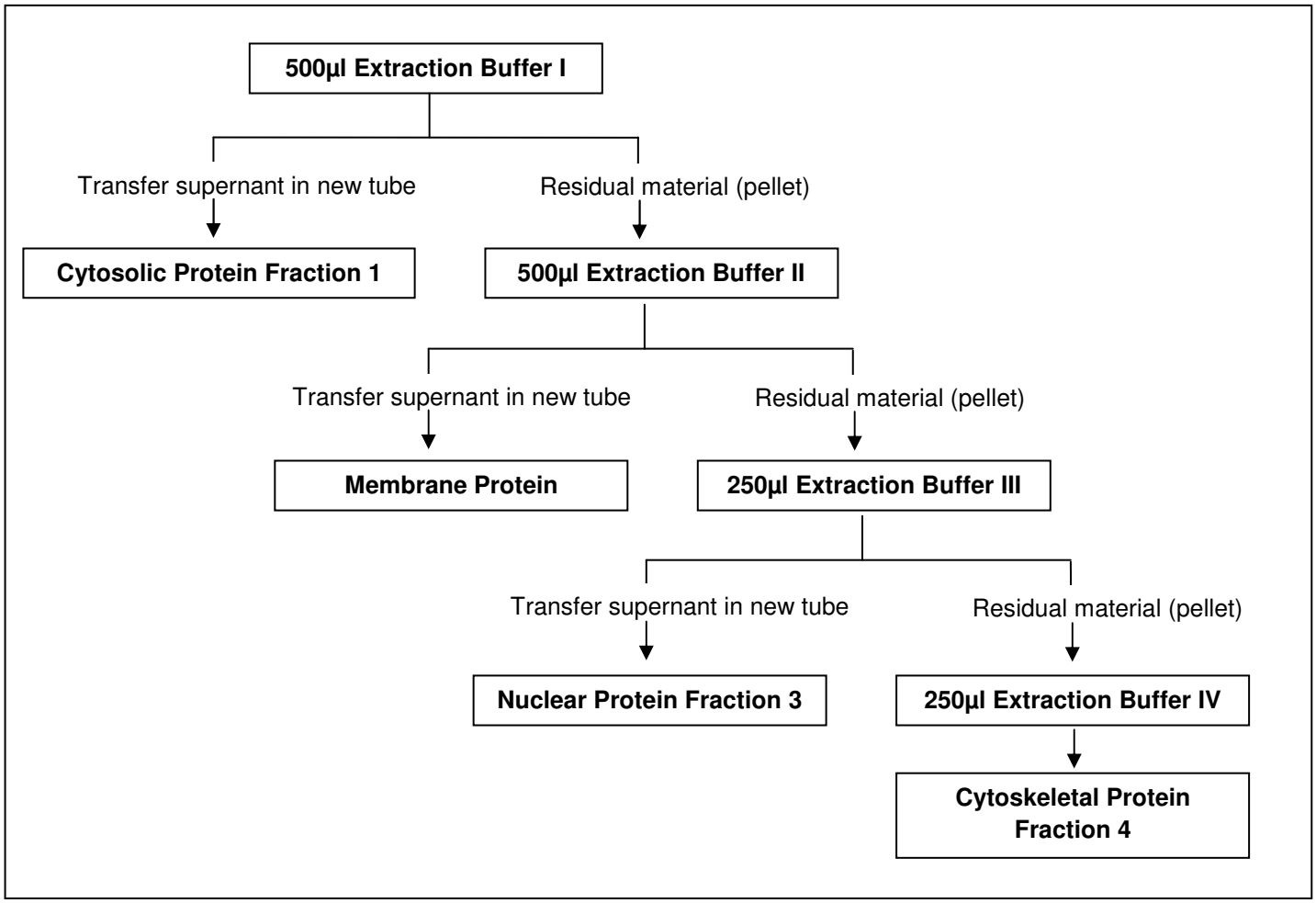

***Figure 6**. Flow chart showing the protocol used for subcellular protein extraction of human sperm proteins.*

A Bradford protein determination (Roche diagnostics, Mannheim, Germany) was subsequently performed on the lysate of Aim 1 and nuclear fraction of Aim 2. BSA fraction V was used as standards.

Standards for Bradford protein determination are summarized in Table 1. The tubes for the standard curve and samples were prepared in duplicate in microfuge tubes and incubated for 5 min, after addition of the Bradford Reagent. The absorbance of all the samples were measured at 595nm in a UV-visible spectrophotometer and the total protein content of each sample were determined.

Table 1. *Bradford protein determination protocol and preparation of tubes*

	BSA (2mg/ml stock) (µl)	**mQ H_2O (µl)**	**Bradford dye reagent (µl)**
Standards	0	100	900
	2.5	97.5	900
	5	95	900
	10	90	900
	20	80	900
Sample	5µl Sample (1:200)	95	900

3.9 Cleanup procedures

After the Bradford assay a cleanup was performed on the lysate of Aim 1 and nuclear fraction of Aim 2 using a commercially available ReadyPrep™ 2-D CleanUp Kit (Bio-Rad, Hercules, CA, U.S.A). This was done to reduce contamination and background staining; it also concentrates the proteins and removes all substances like ionic detergents, salts, lipids and nucleic acids.

CleanUp protocol:

- 1-500µg of protein in a total volume of 100µl was was transferred into a 1.5ml micro centrifuge tube. Note: sample quantities >500µg of protein per tube may reduce the efficiency of the clean up leading to poor quality IEF.
- 300µl precipitating agent 1 (Bio-Rad) was added to the protein sample and mixed well by vortexing and then incubated on ice for 15 min. Note: when adding the solution do not touch protein sample with the pipet tip. The protein may precipitate on the tip causing sample loss.
- 300µl precipitating agent 2 (Bio-Rad) was added to the mixture of protein and precipitating agent 1 and mixed carefully by vortexing.
- The tubes were centrifuged at a maximum speed of 18000 x g for 5 min at 4 ℃ to form a pellet.

- The tube was promptly removed once the centrifuge stopped so that the pellet did not disperse.
- Without disturbing the pellet, the supernatant was removed and discarded.
- The tube was positioned in the centrifuge as before (i.e. cap hinge of protein pellet facing outward) and centrifuged for 15-30 sec and any residual liquid was removed and discarded.
- After centrifugation step 40µl of wash reagent 1 was added on the pellet.
- The tube was positioned as before and centrifuge at 18000 x g for 5 min.
- After centrifugation step the wash reagent was removed and discarded.
- 25µl of Ready Prep proteomic grade water was added on top of the pellet.
- The tube was vortexed for 10-20 sec. Note: protein pellets may disperse but will not dissolve in water.
- 1ml of wash reagent 2 (precooled at -20 ℃ for at least 1 hour) and 5µl of wash additive was added to the tube then vortexed for 1 min. Note: protein pellet will not dissolve in wash reagent 2. If wash reagent 2 is not completely chilled, quantitave recovery may be affected.
- The tube was incubated at -20 ℃ for 30 min and vortexed for 30 sec every 10 min during the incubation period.
- After the incubation period the tube was centrifuged at top speed for 5 min to form a tight pellet.
- The supernatant was removed and discarded.

- The tube was briefly centrifuged for 15-30 sec and any remaining supernatant was removed and discarded.
- The pellet was air dried at room temperature for no more than 3 min. Note: Over dry pellets will be difficult to resuspend.
- 10µl of 0.2M NaOH was added to the pellet and incubated for 2 min at room temperature.
- Each pellet was resuspended by adding an appropriate volume of 2D-rehydration/sample buffer to the pellet (±345ml buffer).
- The tube was voltexed for at least 30 sec and then incubated at room temperature for 3-5 min.
- The tube was voltexed again for approximately 1 min or else the solution could be pipette up and down to fully resuspend the pellet.
- The tube was centrifuged at 18 000 x g for 2-5 min at room temperature to clarify the protein sample.
- The supernatant was removed and placed into a new microfuge tube.
- The pellets of the immature and mature sperm were re-suspended in 2D sample buffer (Bio-Rad, Hercules, CA, U.S.A) and used in further analysis. Any unused or remaining protein sample was stored in a clean microfuge tube at -80 ℃ for later analysis.

3.10 RCDC assay

A second protein determination was performed via RCDC protein assay to calculate the total protein content of the immature and mature sperm (Bio-Rad, Hercules, CA, U.S.A). This is a colorimetric assay for protein determination, but is slightly modified as it is based on the Lowry assay and is reducing agent compatible (RC) as well as detergent compatible (DC). Absorbance was read at 750nm in a spectrophotometer.

Protocol for RCDC assay:

- DC Reagent A and DC Reagent S was used to prepare Reagent S* :
- Reagent S* was prepared, by adding 5µl of DC Reagent S* to 250µl of DC reagent A.
- 127µl of reagent S* was needed for each standard and sample.
- Different dilutions a) to c) were made from the BSA stock solution in the following manner: (Standard curve)

    a) 1.5 mg/ml BSA solution:
        - 75µl BSA stock (2mg/ml) + 25µl H_2O

    b) 1.0 mg/ml BSA solution:
        - 60µl BSA stock (2mg/ml) + 60µl H_2O

c) 0.5 mg/ml BSA solution:

- 50µl of solution b plus 50µl H_2O

- 25µl of each stock solutions a) to c) were used in duplicated to set up the standard curve.
- 25µl of each sample i.e. immature and mature sperm was used in RCDC assay.
- 125µl RC reagent 1 (Bio-Rad) was added to all the tubes EXCEPT the blank.
- All tubes were then vortexed and incubated for 1 min at room temperature.
- 125µl RC reagent 2 (Bio-Rad) was then added to all the tubes EXCEPT the blank.
- All tubes were then centrifuged at 16 000 x g for 5 min at 18 °C.
- The supernatant was discarded by inverting the tubes on clean absorbent tissue paper. The liquid was allowed to drain completely from the tubes.
- 125µl RC reagent 1 was put into all the tubes EXCEPT the blank.
- All tubes were vortexed and incubated for 1min at room temperature.
- 40µl RC reagent 2 was then added to all the tubes EXCEPT the blank.
- All tubes were centrifuged at 16 000 x g for 5 min at 18 °C.
- The supernatant was then discarded by inverting the tubes on clean absorbent tissue paper. The liquid was allowed to drain completely from the tubes.
- 127µl of Reagent S* was added to each microfuge tube and vortexed including the blank.

- The tubes were incubated at room temperature for 5 min or until the precipitate was completely dissolved.
- After incubation step tubes were vortexed for second time.
- 1ml of DC Reagent B was then added to each tube and vortexed immediately. The tubes were then incubated at room temperature for 15 min.
- The absorbance were then read at 750nm in a spectrophotometer

Preparation of buffers after Iso-electric focusing (IEF)

The following solutions were used after IEF:

- 30% (v/v) glycerol,
- equilibration buffer I (Composition: 6M urea, 0.375M Tris-HCl, pH 8.8, 2% (Sodium Diodecyl Sulphate (SDS)),
- 20% glycerol,
- 20% (w/v) dithiothreitol (DTT),
- equilibration buffer II (Composition: 6M urea, 0.375M Tris-HCl, pH8.8, 20% (glycerol),
- 2.5% (w/v) iodoacetamide,

- MOBS running buffer and 2D rehydration sample buffer (Composition: 8M urea, 2% CHAPS, 50Mm DTT, O.2% Bio-Lyte 3/10 ampholytes, 0.0002% bromophenol blue).

- Equilibration buffer I was prepared as follows:

  a) 13.35ml of the 30% glycerol solution was added to lypholised equilibration buffer I powder and carefully dissolved until liquid becomes clear.

- Equilibration buffer II was prepared as follows:

  b) 500mg of iodoacetamide was dissolved in 13.35ml of the 30% glycerol solution and the mixture was added to lyophilised equilibration buffer II powder. Swirl buffer slowly to dissolve powder until liquid becomes clear.

- 3L of MOBS running buffer (XT MOPS 20X) were prediluted with 2850ml of milli-QH_2O.

- A total volume of 500ml of MOBS running buffer were used for each gel.

- The fixative solution was prepared with 240ml of methanol, 42ml of acetic acid and 318ml of milli-QH_2O.

- A total volume of 50ml fixative was used for each gel.

- The quick destain solution was prepared with 120ml of methanol, 30ml of acetic acid and 150ml of milli-QH_2O.

- A total volume of 50ml quick destain was used for each gel.
- The normal destain solution was prepared with 500ml of methanol and 1.5L of milli-QH_2O.
- A total volume of 100ml normal destain was used for each gel.
- The commasie blue stain was prepared with 240ml of Brilliant Blue G-Colloidal concentrate and 60ml methanol.
- A total volume of 50ml stain was used for each gel and 25% ammonium sulphate solution was prepared by dissolving 150g of ammonium sulphate in 600ml of milli-QH_2O. A total volume of 100ml ammonium sulphate was used per gel.

3.11 2D-PAGE

Prior to performing the 2D-PAGE on the collected samples from Aim 1 and 2, a test was done to determine the pH range in which most sperm proteins are expressed. IEF was first carried out on an 11 cm immobilised pH gradient (IPG) strip (Bio-Rad, Hercules, CA, U.S.A) of pH 3-10 (see Figure 7). From the test it was clear that the majority of proteins present in the total sperm proteome occurred in the pH 5-8 range (see Figure 7). Subsequently IPG strips with pH 5-8 were used for all 2D-PAGE experiments.

Protocol for 2D-PAGE:

- The IEF compartments of the running tray were loaded with the sample i.e. immature and mature sperm followed by the 2D sample buffer (total volume of 200µl). Note: IPG strips were kept in a freezer at -20 ℃.
- The IPG strips were placed into the compartment with the gel side down so that it is in contact with the sample. This was done carefully as to ensure that there was no air bubbles trapped as this would interfere with the separation process.
- The running tray was covered with a lid and IEF started.
- After one hour the IPG strips were covered with 2ml of mineral oil to avoid the protein from evaporating.
- The IPG strips were run for ±19 hours overnight at 8000V at 20 ℃.
- After the IEF was completed, IPG strips were removed carefully and the oil was allowed to drain from the strip.
- Once the oil has been removed, IPG strips were placed into the reducing tray with gel side up and incubated for 15 min at 20 revolutions per minute (rpm) with gentle shaking in 3ml equilibration buffer I (See appendix for preparation).
- The buffer was removed and the previous step was repeated with equilibration buffer II. After the incubation step with equilibration buffer II, IPG strips were rinsed with MOPS running buffer.
- Precast Criterion 2D gels were rinse with milli-Q H_2O.
- 5µl peqGold marker was loaded onto each gel.

- The IPG strips were placed on top of the the 2D gels.
- Low melting agarose was placed on top of the IPG strip and allowed to set ±5 min to secure the strip.
- The 2D gels ran at 200V for ±1 hour or until the front was near the end. This was done to transfer the proteins to the 2D gels and also separate the proteins in the 2nd dimension via their M_r.
- After the 2nd dimension run the IPG strips were removed carefully and discarded. The gels were immediately fixed (100ml/gel) and placed on a shaker at 40 rpm.
- After 1 hour the fixative was drained off and the stain was put on the gels (50ml/gel) overnight or 18 hours.
- The staining solution was drained off and quick destain (50ml/gel) was put onto the gels for 1 min.
- Normal destain was put onto the gels and removed every 2 hours and replaced by new destain to remove background stain until the protein spots become visible.

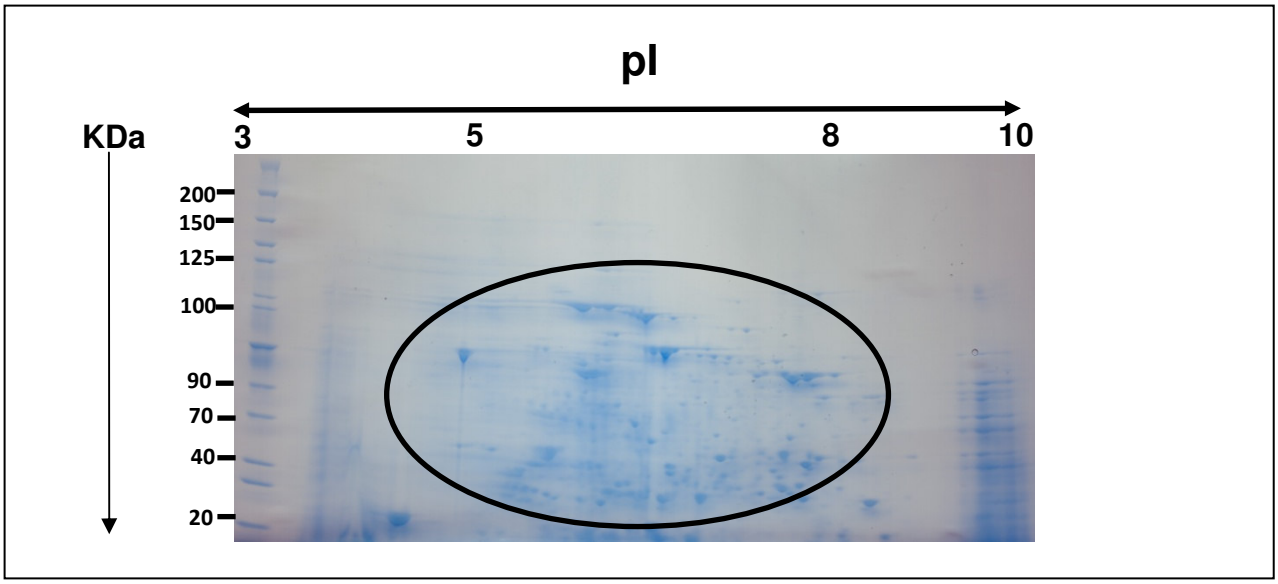

***Figure 7**. 2D gel image of human sperm proteins indicating the pH range (pI 5-8) of most abundant sperm protein expression (indicated with black circle).*

3.12 Data analysis for 2D-PAGE

When the gels were sufficiently de-stained, they were scanned, using the Quantity One-4.5.2 (Basic) software program (GS-800 Scanner, Bio-Rad) to create gel images. These gel images were analysed with the PD-Quest advanced software, version 8.0.1 (Bio-Rad, Hercules, CA, U.S.A). As the computer software program has a built-in statistical program, it was able to identify protein spots that differ significantly in intensity between the two sets of triplicate gels (i.e. 3x mature vs. 3x immature). It only took spots into consideration that were present on all gels.

This was done to exclude any possible technical error or artefacts on the gels. Only spots that differed significantly ($p<0.05$) between the mature and immature sperm fractions as identified by PD Quest were manually excised and prepared for liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis.

3.13 Protein identification using LC-MS/MS

Excised gel spots were subjected to in gel digestion with trypsin. The resulting peptides were extracted using the Micromass MassPrep Station (Walters Technologies, Manchester, U.K.) following the standard digestion protocol supplied by the manufacturer.

Protocol for LC-MS/MS:

- Peptides were retrieved and transferred to a cooled 96-well microtitre plate and if necessary, stored at -20 ℃.
- This 96-well microtitre plate containing the extracted peptides was transferred to a Micromass modular CapLC and auto sampler system (Walters Technologies, Manchester, U.K.).
- A 6.4µl aliquot of extract was mixed with 13.6µl of 0.1% formic acid and loaded onto a 0.5cm liquid chromatography (LC) Packings C18 5µm 100Å 300µm i.d µ-precolumn cartridge (Dionex Corporation, Sunnyvale, CA, U.S.A).
- Flushing the column with solution A (95% water, 4.9% acetonitrile, 0.1% formic acid) desalted the bound peptides before a linear gradient of solution B (4.9% water, 95% acetonitrile, 0.1% formic acid) at a flow rate of 200nl min^{-1} eluted the peptides for further resolution on a 15cm LC Packings C18 5µM 5Å 75µm i.d PepMap analytical column (Dionex Corporation, Sunnyvale, CA, U.S.A).
- Gradient separations were performed at 10 min (95%A: 5%B), 3 min (95%A: 5%B), 31 min (55%A: 45%B), 35 min (20%A: 80%B), 37 min (20%A: 80%B), 38 min (95%A: 5%B) and 47 min (95%A: 5%B).
- The eluted peptides were analyzed with a Micromass QT of Global Ultimate mass spectrometer (Walters Technologies, Manchester, U.K.) fitted with a nano-LC sprayer with an applied capillary voltage of 3.5kV.

- The instrument was calibrated against a collisionally induced decomposition (CID) spectrum of the doubly charged precursor ion of green fluorescent protein (GFP).
- A calibration was accepted when the error obtained on all subsequent acquisitions was <50ppm.
- Sensitivity was assessed by the detection of a 500fmol injection of GFP, with a base peak signal; noise ratio of <20:1 on the doubly charged ion.
- Both the sensitivity and calibration was checked at regular intervals during the analysis.
- The instrument was operated in data dependent acquisition (DDA) mode over the mass/charge (*m/z*) range of 50-2000.
- During the DDA analysis, both MS and tandem MS was performed on the most intense peptides as they eluted from the column.
- The uninterrupted MS/MS data was processed using the Micromass ProteinLynx software package, which converted the raw data into pkl files.
- These files can be used with the Mascot search engine (www.matrixscience.com).
- These data files were searched against the Swiss-Prot database by using the mascot search engine.
- Mammalia was selected and the following were selected; carbamidomethylated at cysteine residues, oxidized at mathionine residues, one missed trypsin

cleavage allowed a 0.2Da tolerance against the database-generated theoretical peptide and product ion masses and a minimum of 1 matched peptide.

- A positive identification was defined when the MOWSE scores (>40) were significant ($p<0.05$).
- All protein identifications were significant according to the probability-based MOWSE scores, which were reported as $-10^{*}Log_{10}(p)$, where p is the probability that the observed match is a random event.

3.14 Statistical analysis

Sperm motility and morphology data were analyzed using the independent Student t-test (GraphPad Prism, version 4.01). Motility and morphology data are presented as mean ± standard error of the mean (SEM). The significance level was set as $p<0.05$. Protein data was analyzed using PD-Quest Software program, version 8.0.1. This program has a built in Student t-test, enabling it to determine differences in protein spots on 2D-PAGE gels. Differences were considered significant if $p<0.05$. Proteomic data is presented in terms of fold decrease or fold increase. A fold decrease is indicated with negative e.g. -33.3 for protein X means that this protein is significantly 33.3 times less expressed in the immature fraction than mature fraction. On the contrary a fold increase is indicated with positive e.g. 2.45 for protein Y means that this protein is significantly 2.45 times more expressed in the immature fraction than the mature fraction.

CHAPTER 4

RESULTS

The results are presented in two parts corresponding to the two aims.

4.1 Results: Aim 1

4.1.1 Sperm motility

The motility of the immature and mature sperm populations retrieved during the Percoll separation method is shown in Figure 8. This figure illustrates that the immature fraction had a significantly lower percentage of motile cells than the mature fraction (immature; 26.1±1.75% vs. mature; 60.93±3.24%; $p<0.001$)

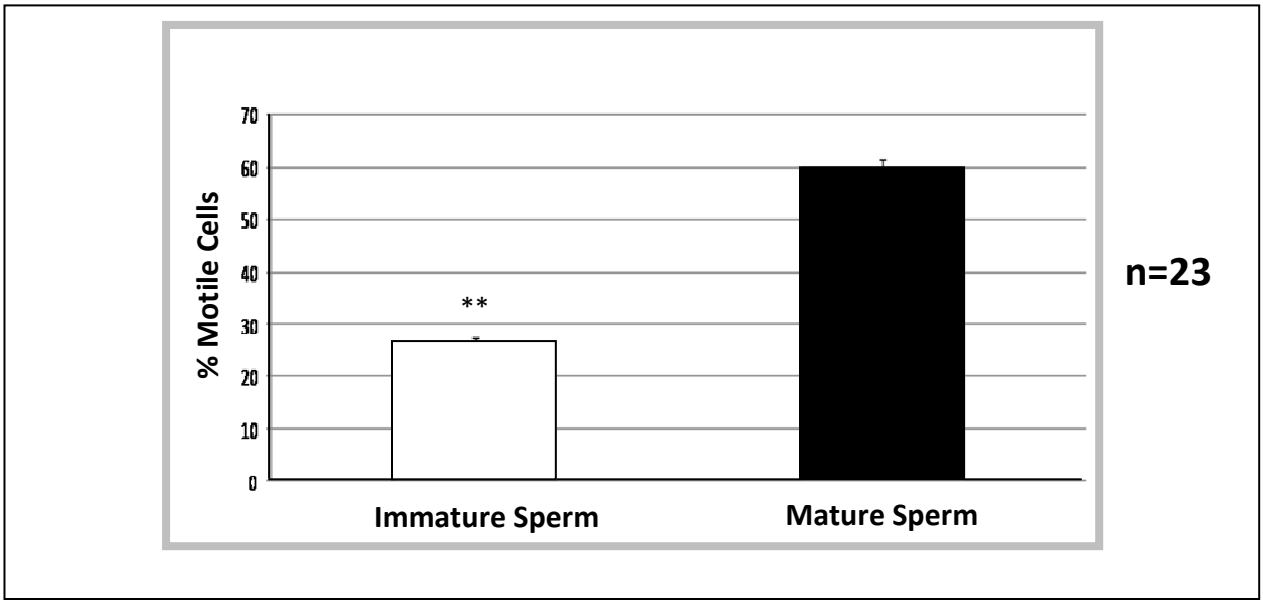

***Figure 8**. Comparison of the total motility of the immature vs. mature sperm populations as separated by Percoll gradient (**p<0.001 vs. mature sperm).*

4.1.2 Normal head morphology

The immature sperm population had a significantly lower percentage of cells with normal head morphology as compared to the mature population of spermatozoa separated by Percoll gradient centrifugation (immature; 64.1±2.75% vs mature; 87.63±3.24%; p<0.001) (see Figure 9).

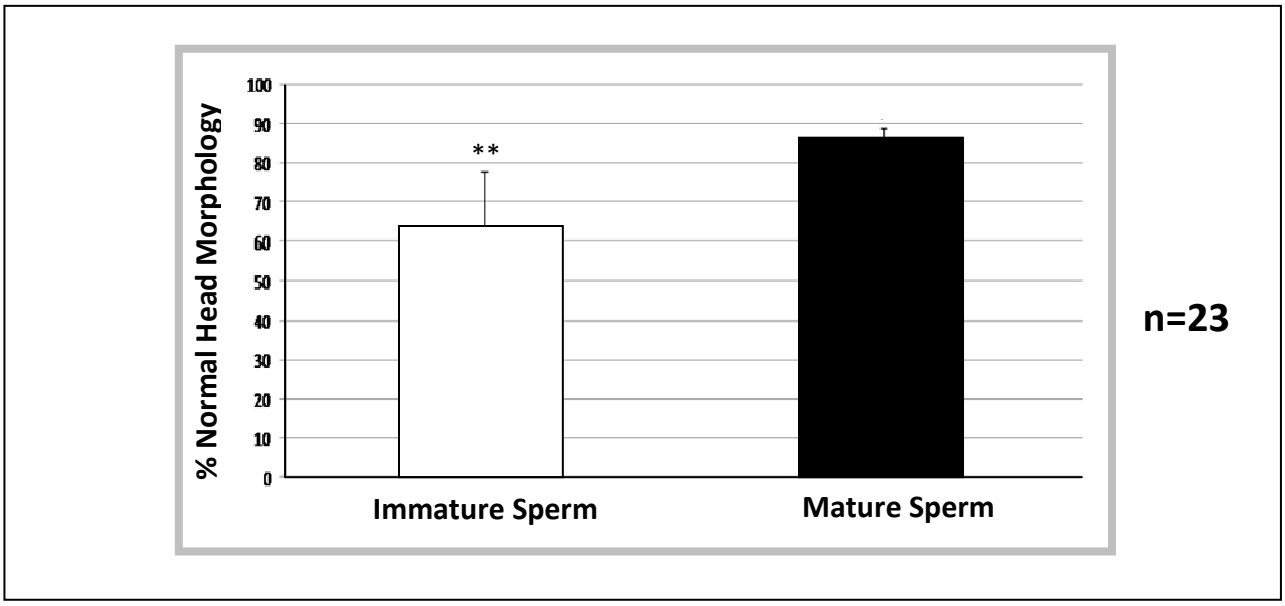

***Figure 9**. Comparison of normal head morphology of the immature vs. mature sperm populations as separated by Percoll gradient (**p<0.001 vs. mature sperm).*

4.1.3 Protein isolation and identification

The 2D gels of the separated proteins from both the immature and mature sperm populations are marked in Figure 10. Eighteen proteins were found to be differentially expressed between the two populations. Of these proteins only 16 could be identified by MS and Mascot. From these results it is evident that sperm protein expression differs within the total sperm proteome of immature and mature spermatozoa populations. Most abundant proteins in the proteome of the immature population were: A-Kinase anchoring protein 4 precursor, ATP synthase subunit d (mitochondrial), Sperm acrosome membrane associated protein 3, 78kDa glucose regulated protein,

Proteasome subunit β type 7, T-complex protein 1 subunit theta, Tubulin β-2C chain, α-enolase and β-enolase.

Less abundant proteins in the proteome of immature population were: Superoxide dismutase, Triosephosphate isomerase 1, D-3 phosphoglycerate dehydrogenase, ATP synthase subunit β (mitochondrial), Tubulin α-3C/D chain, Glutathione S-transferase Mu 3 and Outer dense fibre protein 2.

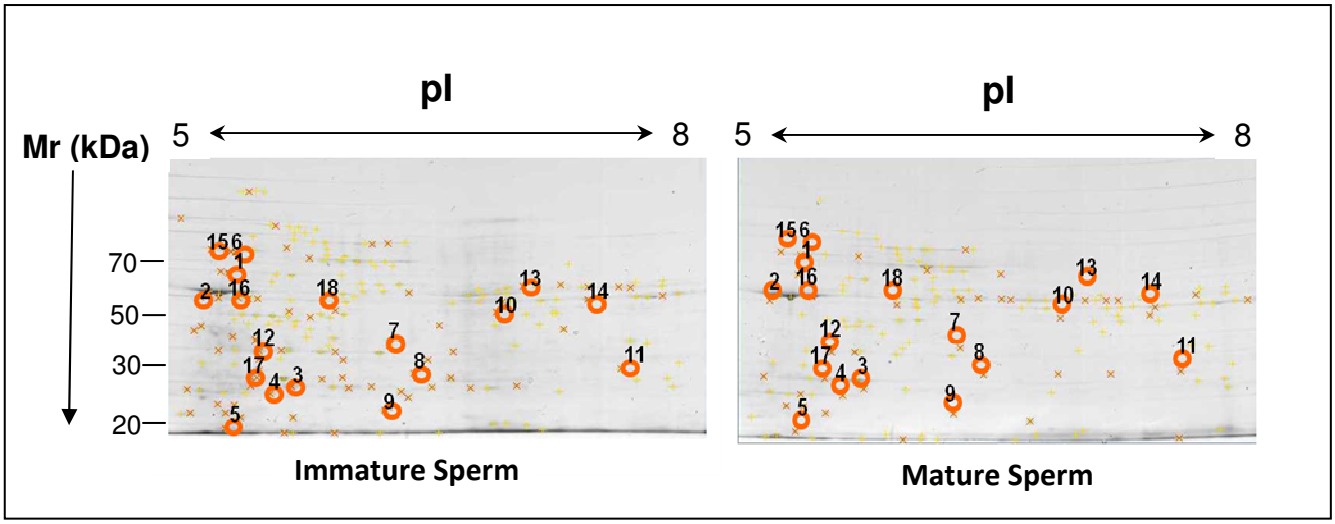

***Figure 10**. The 18 differentially expressed proteins (indicated with orange circles) on the 2D gels of the mature and immature sperm populations.*

Table 1 summarizes the proteins that were differentially expressed between the proteome of the immature and mature sperm populations. 56% of the differentially expressed protein spots were more abundant in the proteome of the immature population compared to the mature population.

Table 2. *Summary of sperm proteins differentially expressed in the mature and immature spermatozoa within the same semen sample as identified by Mascot ($p<0.05$).*

Protein spots	Protein names	PD Quest	Swiss Prot		Mascot score	Sequence coverage	Molecular mass	Iso-electric point	Fold change
		SSP nr.	Gene name	Accession number		(%)	(kDa)	(pI)	Immature: Mature
3	A-Kinase anchoring protein 4 precursor	3202	AKAP4	Q5JQC9	185	10%	95.8	6.56	3.453
4	ATP synthase subunit d, mitochondrial	3201	Atp5h	Q9DCX2	88	49%	18.5	5.21	1.903
5	Sperm acrosome membrane associated protein 3	1102	SPACA3	Q8IXA5	510	30%	24.1	5.07	1.990
6	78kDa glucose regulated protein	1702	GRP78	P11021	414	31%	72.4	5.07	2.031
8	Proteasome subunit β type 7	6301	PSMA7	O14818	91	14%	30.3	7.57	2.082
9	Superoxide dismutase (SOD)	5106	SOD1	P00441	98	25%	16.2	5.70	-1.650
11	Triosephosphate isomerase 1	8301	TPI1	P60174	369	63%	26.9	6.45	-1.786
12	T-complex protein1 subunit theta	2305	CCT8	P50990	77	11%	60.2	5.42	1.990

Table 2. *Continued*

Protein spots	Protein names	PD Quest	Swiss Prot		Mascot score	Sequence coverage	Molecular mass	Iso-electric point	Fold change
		SSP nr.	Gene name	Accession number		(%)	(kDa)	(pI)	Immature: Mature
12	Tubulin β-2C chain	2305	TUBB2C	P68371	204	19%	50.3	4.79	1.990
13	D-3 phosphoglycerate dehydrogenase	7702	PHGDH	O43175	193	21%	57.4	6.29	-1.921
14	α-enolase	8603	ENO1	P06733	278	47%	47.5	7.01	1.922
14	β-enolase	8603	ENO3	P13929	68	10%	47.2	7.59	1.922
16	ATP synthase subunit β, mitochondrial	1504	ATP5B	P06576	190	24%	56.5	5.26	-33.33
16	Tubulin α-3C/D chain	1504	TUBA3C	Q13748	175	30%	50.6	4.97	-33.33
17	Glutathione S-transferase Mu 3	2301	GSTM3	P21266	171	51%	27.0	5.37	-2.380
18	Outer dense fibre protein 2	4501	ODF2	Q5BJF6	161	13%	96.1	7.53	-1.893

4.2 Results: Aim 2

4.2.1 Sperm motility

The motility of the mature and immature spermatozoa retrieved during the PureSperm® separation method is shown in Figure 11. This figure clearly illustrates that the immature population had significantly less motile cells than the mature population (immature; 32.33±0.51% vs. mature; 88.67±0.85%; p<0.0001).

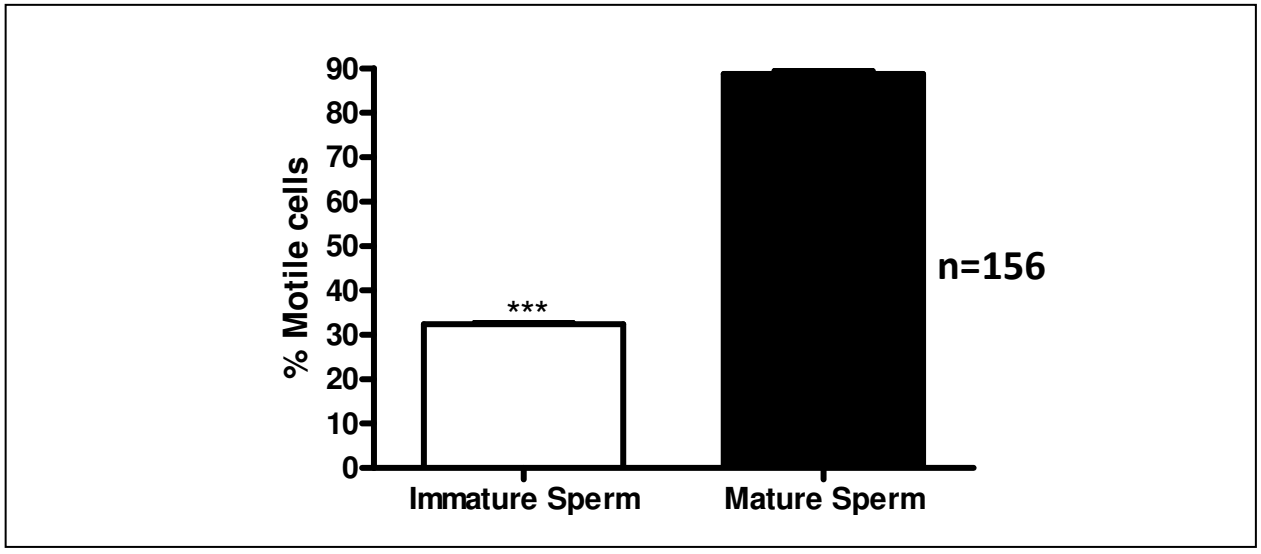

Figure 11**. Comparison of the total motility of the immature vs. mature sperm populations as separated by PureSperm® gradient (p<0.0001 vs. mature sperm).*

Table 3 summarizes the basic motility parameters of the mature and immature sperm populations. This table shows that the immature sperm fraction has a significant lower percentage of progressive (immature; 16.48±0.49% vs. mature; 69.81±0.88%; p<0.0001) and rapid motile cells (immature; 15.13±0.51% vs. mature; 55.92±1.79%; p<0.0001) compared to the mature sperm fraction. All other kinetic parameters were also significantly decreased in the immature population when compared the mature population apart from the percentage static and non progressive motile cells (Table 3).

Table 3*. Summary of basic motility parameters in the mature and immature sperm populations as measured by CASA*

Motility parameters	**Immature sperm**	**Mature sperm**
Sperm count ($X10^6$ sperm/ml)	31.20±1.54***	83.31±1.98
Motile cells (%)	32.33±0.51***	88.67±0.85
Rapid cells (%)	15.13±0.51***	55.92±1.79
Static cells (%)	30.78±1.52***	11.91±1.06
Progressive motility (%)	16.48±0.49***	69.81±0.88
Non progressive motility (%)	40.87±1.02***	29.76±1.00
Average path velocity (VAP) (µm/s)	20.66±0.69***	36.09±1.02
Straight line velocity (VSL) (µm/s)	10.63±0.49***	20.63±0.74
Curvilinear velocity (VCL) (µm/s)	36.92±1.06***	59.72±1.64
Amplitude of lateral head displacement (ALH) (µm/s)	1.31±0.05***	1.92±0.04
Beat cross frequency (BCF) (Hz)	7.07±0.38***	11.14±0.35
Straightness (STR) (%)	48.58±0.90***	54.16±0.75
Linearity (LIN) (%)	27.15±0.67***	33.03±0.71

***p< 0.0001 vs. mature sperm

4.2.2 Normal head morphology

Figure 12 shows that the immature sperm population had a significantly lower percentage of cells with normal head morphology compared to the mature sperm population (immature; 13.51±0.87% vs mature; 20.89±1.20%; p<0.0001).

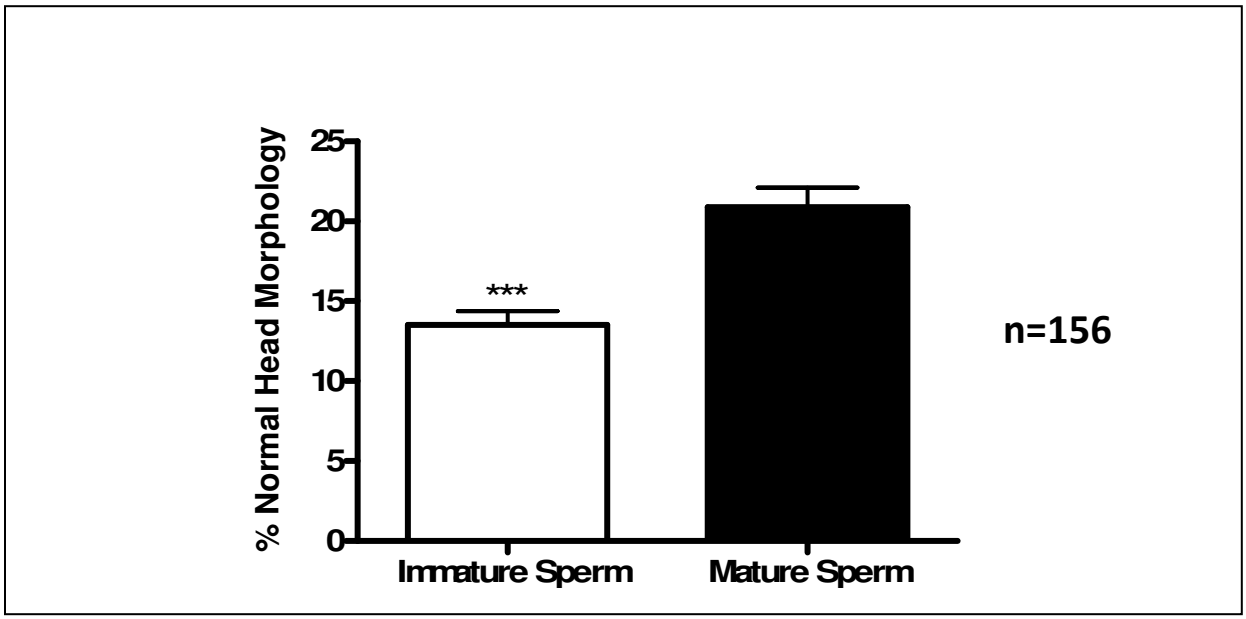

Figure 12. *Comparison of the normal head morphology of the immature vs. mature sperm populations as separated by PureSperm® gradient (***p<0.0001 vs. mature sperm).*

Table 4 summarize the basic morphology parameters of the mature and immature sperm populations. This table shows that the immature sperm population has a significant higher percentage cells with cytoplasmic droplets (3.52±0.26% vs.

2.04±0.20%; p<0.0001), head defects (84.72±0.94% vs. 65.17±1.81%; p<0.0001) and midpiece defects (35.97±1.52% vs. 25.37±1.25%; p<0.0001) compared to the mature sperm fraction. All other morphological parameters were also significantly decreased in the immature population when compared the mature population apart from the cytoplamic droplets, head defects and midpiece defects (see Table 4).

***Table 4**. Summary of basic morphology parameters between sperm sub-populations as measured by CASA*

Morphology parameters	**Immature sperm**	**Mature sperm**
Normal head morph (%)	13.51±0.87***	20.89±1.20
Teratozoospermic index (TZI) (%)	1.42±0.015***	0.91±0.024
Sperm deformity index (SDI) (%)	1.24±0.023***	0.93±0.024
Head defects (%)	84.72±0.94***	65.17±1.81
Midpiece defects (%)	35.97±1.52***	25.37±1.25
Cytoplasmic droplets (%)	3.52±0.26***	2.04±0.20
Multi defects (head&midpiece) (%)	34.42±1.54***	23.68±1.28
Single defects (head) (%)	50.30±1.11*	53.67±0.96
Single defects (midpiece) (%)	1.57±0.17	1.71±0.18
Normal Acrosome (%)	56.21±1.76***	63.45±1.99
Normal size (%)	31.95±1.23***	40.96±1.35
Sperm defects (micro) (%)	20.66±1.41***	19.19±1.37
Sperm defects (macro) (%)	46.87±2.05	39.85±1.92
Sperm defects (normal shape) (%)	39.02±1.46***	50.04±1.37

****p<0.0001 vs. mature sperm, *p<0.05 vs. mature sperm*

4.2.3 Nuclear protein isolation and identification

Numerous nuclear proteins were found to be differentially expressed (Figures 13 and 14) between the mature and immature sperm populations. These proteins are listed in Table 5. This table shows that 95% of the differentially expressed nuclear proteins were less abundant in the immature population compared to the mature population. From these results it is evident that sperm protein expression differs in the nuclear protein fraction of immature and mature populations of spermatozoa. The less abundant proteins in the immature sperm fraction were; Prolactin Inducible protein (PIP), Saccharopine dehydrogenase (SDH), Ferritin (mitochondrial), ATP synthase subunit β (mitochondrial), Pyruvate dehydrogenase E1 component subunit β (mitochondrial), Cytochrome b-c1 complex subunit 1, Tektin 2, Mitochondrial inner membrane protein, Dihydrolipoyl dehydrogenase, Cytosol aminopeptidase, 60kDa heat shock protein, Protein disulfide isomerase A3, Stress 70 protein, and Heat shock-related 70kDa protein 2. Apart from the less abundant proteins, one of the differentially expressed nuclear protein spots namely the 78kDa glucose regulated protein was more abundant in the immature population compared to the mature population (protein spot 10, Figure 15).

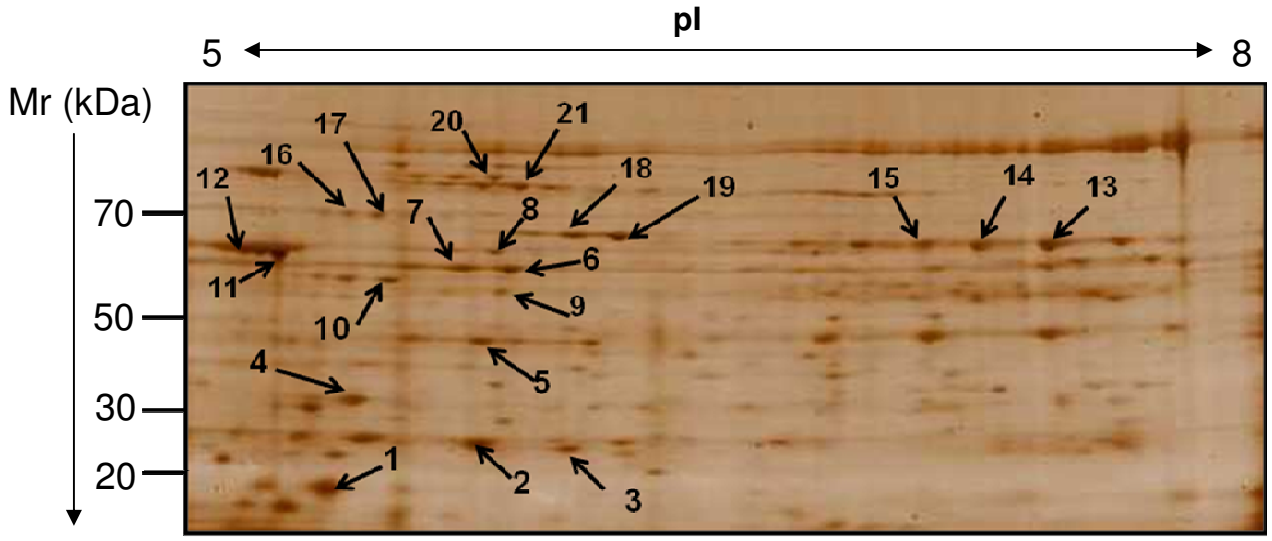

***Figure 13**. 2D gel image of the nuclear protein spots from the mature sperm population. The protein spots found to be differentially expressed by PD Quest are indicated by the numbered arrows differentially (1-21) (pH=5-8; Mr= 10-100kDa).*

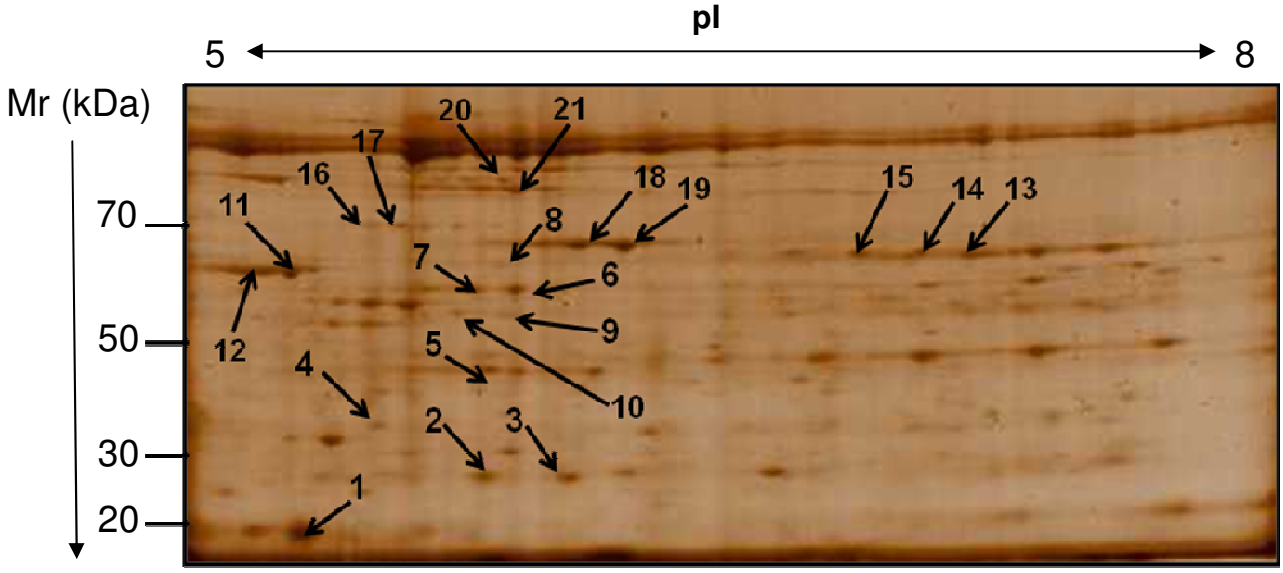

***Figure 14**. 2D gel image of the nuclear protein spots from the immature sperm population. The protein spots found to be differentially expressed by PD Quest are indicated by the numbered arrows differentially (1-21) (pH=5-8; Mr= 10-100kDa).*

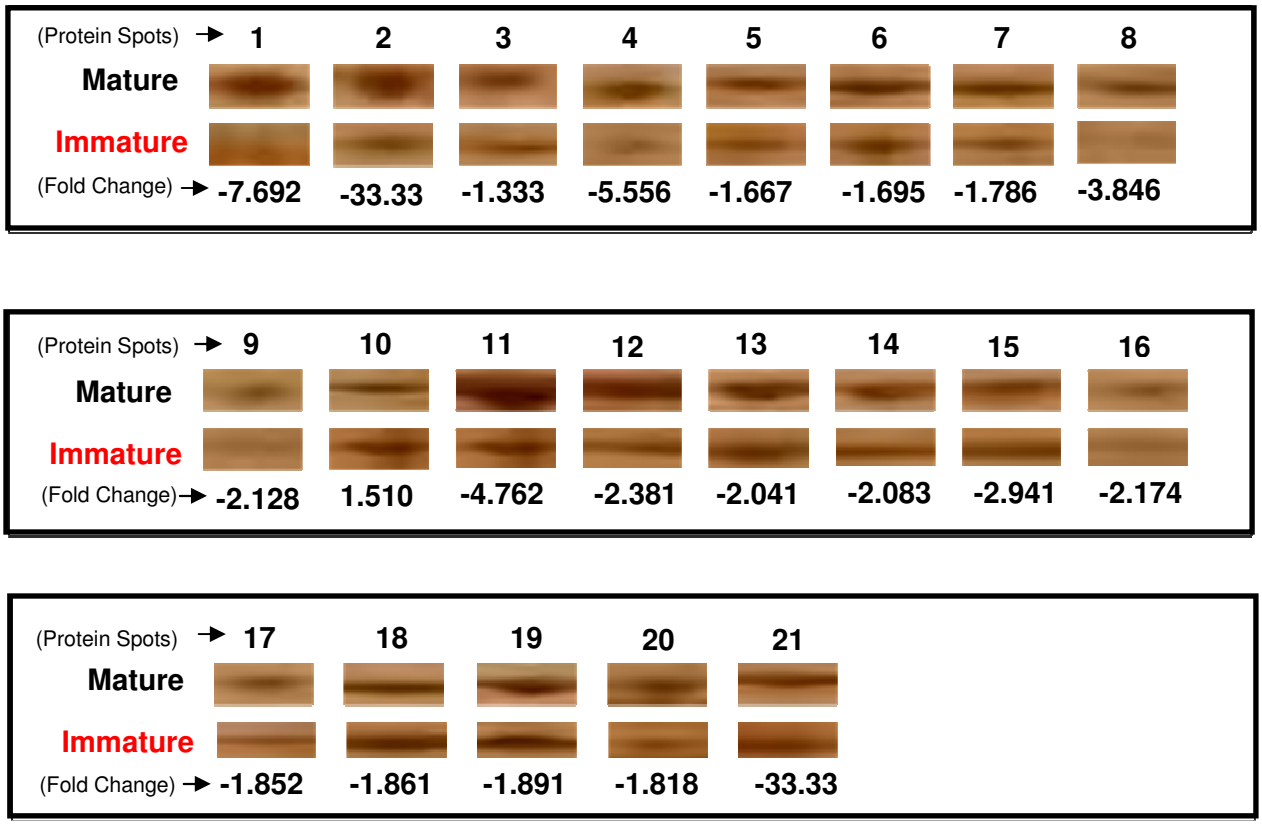

Figure 15. *Enlarged view of the individual nuclear protein spots differentially expressed between the mature and immature fractions respectively as isolated from the 2D gels. The difference in nuclear protein expression of the immature fraction in relation to the mature fraction is indicated by means of fold change. (Fold decrease; indicated with negative= nuclear proteins less abundant in immature vs. mature sperm, Fold increase; indicated with positive= nuclear proteins more abundant in immature vs. mature sperm).*

Table 5. *Summary of nuclear proteins, identified by Mascot (p<0.05), which are differentially expressed between mature and immature sperm populations*

Protein spots	Protein names	PD Quest	Swiss Prot		Mascot score	Sequence coverage	Molecular mass	Iso-electric point	Fold change
		SSP nr.	Gene name	Accession number		(%)	(kDa)	(pI)	Immature: Mature
1	Prolactin Inducible protein	1105	PIP	P12273	230	63%	16.8	8.26	-7.692
2	Saccharopine dehydrogenase	3101	SCCPDH	Q8NBXO	159	11%	47.5	9.24	-33.33
3	Ferritin, mitochondrial	4101	FTMT	Q8N4E7	95	16%	27.8	6.79	-1.333
4	ATP synthase subunit β, mitochondrial	1207	ATP5B	P06576	525	26%	56.5	5.26	-5.556
5	Pyruvate dehydrogenase E1 component, subunit β,mitochondrial	3301	PDHB	P11177	221	20%	39.6	6.20	-1.667
6	Cytochrome b-c1 complex subunit 1	3402	UQCRFS1	P47985	290	30%	53.3	5.94	-1.695
7	Cytochrome b-c1 complex subunit 1	2504	UQCRFS1	P47985	196	20%	53.3	5.94	-1.786
8	Tektin 2	3501	TEKT2	Q9UIF3	403	32%	50.2	5.39	-3.846

Table 5. *Continued*

Protein spots	Protein names	PD Quest	Swiss Prot		Mascot score	Sequence coverage	Molecular mass	Iso-electric point	Fold change
		SSP nr.	Gene name	Accession number		(%)	(kDa)	(pI)	Immature: Mature
9	Mitochondrial inner membrane protein	3401	TIMM9	Q9Y5J7	242	12%	84.0	6.08	-2.128
10	78kDa glucose regulated protein	2401	GRP78	P11021	510	30%	72.4	5.07	1.510
11	ATP synthase subunit β, mitochondrial	505	ATP5B	P06576	570	41%	56.5	5.26	-4.762
12	ATP synthase subunit β, mitochondrial	502	ATP5B	P06576	455	34%	56.5	5.26	-2.381
13	Dihydrolipoyl dehydrogenase	8601	DLD	P09622	363	21%	54.7	7.95	-2.041
14	Dihydrolipoyl dehydrogenase	3708	DLD	P09622	212	17%	54.7	7.95	-2.083
15	Cytosol aminopeptidase	6609	P28838	LAP3	244	17%	56.5	8.03	-2.941
16	60kDa heat shock protein	1602	HSPD1	P10809	522	33%	61.2	5.7	-2.174
17	60kDa heat shock protein	1603	HSPD1	P10809	450	33%	61.2	5.7	-1.852

Table 5. *Continued*

Protein spots	Protein names	PD Quest	Swiss Prot		Mascot score	Sequence coverage	Molecular mass	Iso-electric point	Fold change
		SSP nr.	Gene name	Accession number		(%)	(kDa)	(pI)	Immature: Mature
18	Protein disulfide isomerase A3	4601	PDIA3	P30101	501	39%	57.1	5.98	-1.861
19	Protein disulfide isomerase A3	4602	PDIA3	P30101	522	44%	57.1	5.98	-1.861
20	Stress 70kDa protein	3703	HSPA9	P38646	389	16%	73.9	5.87	-1.818
21	Heat shock-related 70kDa protein 2	3708	HSPA2	P54652	523	29%	70.3	5.56	-33.33

CHAPTER 5

DISCUSSION

This chapter is divided into two sections corresponding to the two study aims. The first part focuses on discussing results obtained in Aim 1, and is followed by a discussion of the results obtained in Aim 2. The last section of this chapter summarizes all the results and also elaborates on the novelty of the present study. A general conclusion is made at the end of Chapter 5.

5.1 Aim 1:

Determining differences in protein expression in the total proteome of spermatozoa between immature and mature sperm populations.

In the present study, we attempted to elucidate changes in protein expression responsible for differences in functional parameters (motility and morphology) between immature and mature sperm populations. Currently, it is known that defective spermatogenesis causes increased production of immature (abnormal) spermatozoa that is associated with sub-fertility. However, protein expression affected by defective spermatogenesis leads to differences in protein profiles between spermatozoa likely to fertilize and those unlikely to fertilize.

5.1.1 Differences in total sperm motility between the immature and mature sperm populations

Sperm motility is believed to be one of the most important parameters in evaluating the fertilizing ability of ejaculated sperm [152]. It is also known that a strong correlation exists between sperm motility and fertilization rates of human oocytes during IVF cycles [109, 153, 154]. Current evidence suggests that progressive motile spermatozoa have a greater chance of fertilizing the oocyte compared to spermatozoa with poor motility [155]. In the present study it has been shown that the immature population of spermatozoa contains a lower percentage motile cells compared to the mature population (immature; 26.1±1.75% vs. mature; 60.93±3.24%; $p<0.001$; Figure 8). The mature population of spermatozoa can therefore be compared to and used as a model of sperm with normal motility (fertilizing potential) while the immature population of spermatozoa can be compared to or used as a model of asthenozoospermia (non-fertilizing potential) in vitro.

It is known that higher percentages of immotile/dead spermatozoa may lead to increased production of ROS [156, 157]. The increased levels of ROS can cause oxidative stress and cellular damage by lipid peroxidation and decrease membrane integrity.

The reduction in membrane integrity can lead to a decrease in sperm motility. The decrease in sperm motility evident in the immature population might also be caused by increased lipid peroxidation as a result of excess cytoplasm or poor sperm morphology as most cells in this fraction are not fully matured. In addition, the Outer Dense Fibre Protein 2 (ODF 2) has been identified as a structural component of the sperm tail and plays a crucial role in sperm motility (Table 6). The down regulation of ODF 2 noted in the immature fraction might lead to the formation of dysfunctional flagella which could result in decreased sperm motility.

5.1.2 Differences in sperm morphology parameters between the immature and mature sperm populations

It is generally accepted that sperm morphology is the best indicator of male infertility [158]. Embryo quality is also known to be influenced by sperm morphology and especially by sperm head abnormalities, thereby suggesting an important role for sperm morphology in the early stages of embryogenesis [159]. In the present study it has been shown that the immature sperm population has a lower percentage of cells with normal head morphology compared to the mature sperm population (immature; 64.1±2.75% vs mature; 87.63±3.24%; $p<0.001$) (see Figure 9). From these results it can be deducted that the immature population had significantly more head morphology defects than the mature population.

As only head morphology and not midpiece or tail defects were taken into consideration it cannot be directly related to the cutt-off values of the Tygerberg Strict classification system.

Semen parameters have been correlated with IVF success by a large number of studies [160]. It has been shown by Parinaud and co-workers (1996) that the predictive value for IVF success is significantly higher in post-Percoll sperm preparations with normal morphology compared to neat semen [161]. Increased numbers of morphological normal spermatozoa after Percoll density gradient separation in the mature population of the present study is in argreement with these reports. Several studies have shown that Percoll is a superior medium for efficient density gradient isolation of spermatozoa free of contamination by other seminal constituents [161, 162]. In the present study it was found that the Percoll density gradient centrifugation technique successfully separates spermatozoa from seminal plasma, which is in agreement with these reports. However, it should also be kept in mind that the Percoll separation technique does not provide a completely homogenous populations of spermatozoa, as mature cells are found to be present in the immature populations and vice versa.

5.1.3 Differences in protein profiles within the total sperm proteome between the immature and mature sperm populations

The spermatozoon is a highly accessible cell which can be easily purified and therefore it is particularly well suited for proteomic analysis. It is also an extremely differentiated cell with very marked genetic, cellular, functional and chromatin changes compared to other cells, and have profound implications for fertility, embryo development and heredity.

Alterations in spermatogenic events result in the release of immature and abnormal spermatozoa in the ejaculate. Immature spermatozoa display a high content of DNA damage, nuclear alterations such as abnormal protamination and defective chromatin packaging [119, 163-166]. In order to understand the molecular nature of these abnormalities it is crucial to identify the proteins expressed in human spermatozoa. In the present study is has been shown that at least 18 proteins are differentially expressed between immature and mature sperm populations. Sixteen of these proteins could be identified by MS (see Table 2). From these results it is clear that sperm protein expression within the total sperm proteome differs between the immature and mature populations of spermatozoa. The role individual proteins play in sperm function and morphology is of great value in understanding the molecular nature of sperm dysfunction.

The proteins less abundant in the immature population compared to the mature population will be discussed followed by those proteins that are more abundant in the immature population compared to the mature population.

5.1.3.1 Proteins less abundant in the total sperm proteome of the immature sperm population

The present study demonstrated that about 44% of the differentially expressed proteins were less abundant in the immature sperm population compared to that of the mature population. Functions have been ascribed to these individual proteins in order to ascertain their role in sperm function (see Table 6). From these results it can be deduced that protein expression in the immature population is incomplete and could therefore have direct effects on sperm function (motility and morphology).

5.1.3.1.1 Proteins associated with the cytoskeleton, flagella and cell movement

Tubulin α-3C/D chain

Tubulin is the major structural component of microtubules, which are responsible for flagellum organization [167-169]. This protein has two subunits namely α and β subunits. In a study done by Gagnon and co workers (1996) on sea urchins, it was

demonstrated that sperm motility is negatively affected by low concentrations of α tubulin [170]. The α- and β-tubulins have also been reported to undergo post translational modifications, such as acetylation, tyrosine phosphorylation, polyglutamylation and polyglycylation [171]. Some of these modifications, such as polyglutamylation, are apparently participating factors in axonemal motility [170].

In the present study it has been shown that tubulin α-3C/D chain is -33.33 times less expressed in the immature fraction compared to the mature fraction (Table 2). Tubulin is known to play a crucial role in flagellum organization. The reduction of tubulin α-3C/D chain might lead to the formation of a defective sperm flagellum which can have a direct affect on sperm motility. The tubulin α-3C/D chain protein has also been shown to be linked to spermatogenesis and is increasingly expressed as the sperm reaches maturity [172]. Therefore tubulin expression in the immature fraction could be down regulated as most cells in this fraction are not fully matured.

Outer dense fibre protein 2

ODF 2 is a structural protein mainly situated in the tail of spermatozoa [173]. This protein plays a crucial role in stabilizing the flagella and is also one of the major proteins of the outer dense fibres (ODFs) [174].

The ODFs are sperm tail-specific cytoskeletal structures situated in the principle piece of the sperm tail [174]. To date, no active motility function has been assigned to the ODF.

It is generally accepted that the function of ODFs may be to maintain the passive elastic structures and elastic recoil of the sperm tail or to play a protective role against shearing forces encountered during epididymal transport and especially during ejaculation [173]. In the present study, it was shown that ODF 2 is -1.893 times less expressed in the immature population compared to the mature population (see Table 2). The reduction of this protein might result in decreased elasticity of sperm flagellum and could directly affect sperm motility. From these results it can be deduced that ODF 2 plays an important role in sperm function (motility and morphology).

5.1.3.1.2 Stress-related proteins

Superoxide dismutase

Superoxide dismutase (SOD) Cu-Zn is a highly specific scavenging enzyme for superoxide anions (O_2^-) and lipid peroxide produced by oxygen free radicals (ROS) in human seminal plasma and spermatozoa. SOD is known as the most important antioxidant enzyme as it plays a crucial role in ROS detoxification in human spermatozoa (see Figure 16).

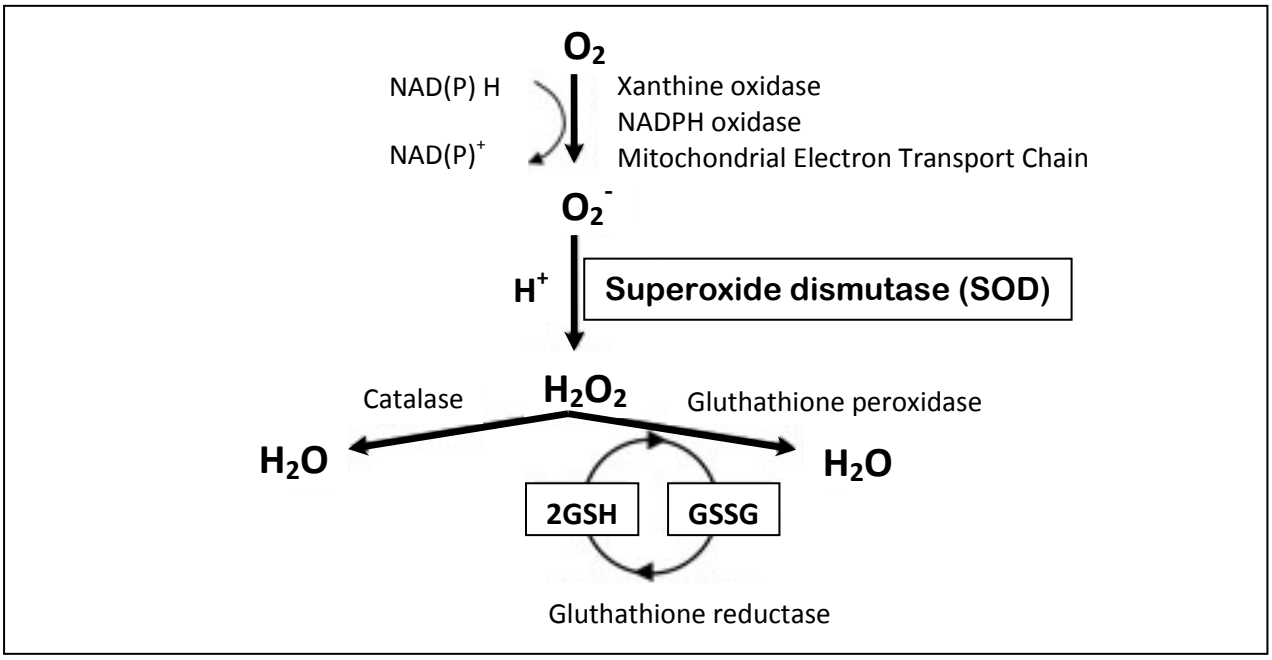

Figure 16. *Flow chart illustrating the role superoxide dismutase (SOD) plays in the breakdown of free radicals (O_2^-) caused by abnormal reactive oxygen species (ROS) production [175].*

Human spermatozoa exhibit a capacity to generate ROS and initiate peroxidation of the unsaturated fatty acids in the sperm plasma membrane, which plays a key role in the etiology of male infertility. The controlled generation of ROS in spermatozoa is associated with normal physiological functions. Uncontrolled and excessive production of ROS, however, seems to have a significant role as one of the major factors leading to an infertile status [147, 176-178]. Excessive ROS production causes oxidative stress, resulting in decreased sperm motility and increased midpiece defects [175, 179].

In this study it has been shown that SOD is -1.650 times less expressed in the immature population compared to the mature population. The reduction of SOD in the immature fraction might result in ROS build up and be responsible for the decreased motility and morphology (see Figures 8 & 9). Several studies have shown that 40%–88% of infertile patients have high levels of seminal ROS [177]. Immature spermatozoa are a possible source of abnormal ROS production in human sperm, for which SOD can possibly be used as a biomarker for the development of treatment regimes for male infertility.

Glutathione S-transferase Mu 3

Glutathione S-transferase Mu 3 has been identified as a enzyme involved in detoxification of carcinogens, drugs, toxins and products of oxidative stress [156]. This enzyme forms part of the mammalian glutathione transferase (GST) families, namely cytosolic, mitochondrial, and microsomal GST [180]. Besides detoxifying electrophilic xenobiotics, such as chemical carcinogens, environmental pollutants, and antitumor agents, these transferases inactivate endogenous *α*, *β*-unsaturated aldehydes, quinones, epoxides, and hydroperoxides formed as secondary metabolites during oxidative stress [181]. These enzymes are also intimately involved in the biosynthesis of leukotrienes, prostaglandins, testosterone, and progesterone, as well as the degradation of tyrosine [180, 181].

In this study it has been shown that Glutathione S-transferase Mu 3 is less abundant in the immature population. This could potentially result in the built up of toxins, carcinogens and ROS. High levels of ROS induce oxidative stress and have detrimental effects on spermatozoa e.g. apoptosis or sperm dysfunction. Under normal physiological conditions, ROS are cleared from the cell by the action of SOD, catalyse, or glutathione (GSH) peroxidase [182]. Therefore it can be postulated that the immotile/dead spermatozoa present in the immature fraction might be a result of the high levels of toxins, carcinogens and free radical built up due to the limited availability of Glutathione S-transferase Mu 3. In addition, immature sperm is often characterized by increased number of spermatozoa with abnormal structure e.g. cytoplasmic droplets that can act as a source of abnormal ROS production [182]. Glutathione S-transferase Mu 3 which is similar to SOD also exhibits protective properties against ROS and therefore plays a crucial in sperm function.

5.1.3.1.3 Metabolic enzymes

D-3 phosphoglycerate dehydrogenase

D-3-phosphoglycerate dehydrogenase (PGDH) has been investigated in a variety of organisms such as bacteria *Mycobacterium tuberculosis and Escherichia coli.* PGDH is part of a family of proteins classified as 2-hydroyacid dehydrogenase that are generally specific for substrates with a D-configuration [183].

PGDH plays a critical role in phosphorylated pathway of L-serine biosynthesis by converting D-3 phosphoglycerate to hydroxypyruvic acid phosphatase. It is known that PGDH exhibits electron carrier activity and plays a crucial role in amino-acid biosynthesis i.e. L-serine biosynthesis [184].

In the present study PGDH was isolated and identified in human sperm for the first time. PDGH was less abundant in the immature population compared to the mature population. The role PGDH plays in sperm is not known and needs to be investigated in further studies. This novel finding adds additional knowledge to current literature. The reduction of PGDH in the immature sperm might affect sperm energy metabolism and motility as this protein plays a crucial role in amino acid biosynthesis.

Triosephosphate isomerase 1

Triosephosphate isomerase 1 (TPI) is the glycolytic enzyme that catalyses the reversible inter conversion of glyceraldehydes 3-phosphate and dihydroxyacetone phosphate. TPI plays an important role in several metabolic pathways (i.e. glycolysis, gluconeogenesis, fatty acid biosynthesis and pentose phosphate shunt) and is essential for efficient energy production. It has also been shown that TPI is expressed in the Sertoli cells and plays an important role during spermatogenesis [185]. Several studies have shown that TPI is located specifically in the acrosome region surrounding the sperm head.

In addition TPI plays a role in mitosis and even the initiation of meiosis [186]. It has been shown that TPI plays a crucial role in the energy metabolism of the spermatozoa.

In this study TPI was less abundant in the immature population compared to the mature population. TPI plays a crucial role in energy production and therefore it can be postulated that the reduction of this protein can affect sperm energy metabolism and motility. It is known that TPI plays an important role during spermatogenesis and therefore decreased levels of TPI could have direct affects on sperm function (sperm motility and morphology).

5.1.3.1.4 Energy-related proteins

ATP synthase subunit β

ATP synthase subunit β (ATP synthase β) forms part of the ATP synthase complex (mitochondrial electron transport chain) of the sperm cell, and the β chain is the catalytic subunit. Although the function of this protein has not yet been analysed in spermatozoa, it has been shown to be present in other somatic cells. The ATP synthase complex consists out of a number of subunits namely α-, β-, ε- and γ- subunit. It has been shown that the β subunits all have a low affinity to bind to nucleotides. This could cause an inhibiting effect on the ATP synthase complex [25].

The β subunits also correspond to the different conformations of the ATP synthase, which could be either tight, loose, empty or open [187]. ATP synthase β was less abundant in the immature population compared to the mature population in the present study.

It has been shown that energy metabolism is a key factor supporting sperm function, in particular sperm motility [67, 99]. The decreased expression of ATP synthase β could directly affect energy production in sperm, as the ATP synthase β forms part of the ATP synthase complex. One of the main fuctions of the ATP synthase complex is to bind phosphate (Pi) to adenine diphosphate (ADP) to form ATP. The reduction in the ATP synthase β can result in a dysfunctional ATP synthase complex and therefore less ATP are produced which can lead to reduced sperm motility.

Table 6. *Functions of proteins found to be less abundant in the total proteome of immature spermatozoa compared to mature spermatozoa.*

Protein spots	Protein names	Fold change Immature: Mature	Function
	Cytoskeleton, flagella and cell movement		
16	Tubulin α 3C/D chain	-33.33	Tubulin is the major constituent of microtubules. It binds two molecules of GTP, one at an exchangeable site on the β chain and one at a non-exchangeable site on the α-chain.
18	Outer dense protein 2	-1.893	Component of the outer dense fibres of spermatozoa. May have a modulating influence on sperm motility.
	Stress-related proteins		
9	Superoxide dismutase	-1.650	Destroys radicals which are normally produced within the cells and which are toxic to biological systems.
17	Glutathione S-transferase Mu 3	-2.380	Belongs to the mu class of enzymes involved in the detoxification of carcinogens, drugs, toxins and products of oxidative stress.
	Metabolic enzymes		
13	D-3 phosphoglycerate dehydrogenase	-1.921	Amino-acid biosynthesis; L-serine biosynthesis; L-serine from 3-phospho-D-glycerate.
11	Triosephosphate isomerase 1	-1.786	Plays an important role in several metabolic pathways (i.e. glycolysis).
	Energy- related protein		
16	ATP synthase subunit β, mitochondrial	-33.33	Forms part of the mitochondrial ATP synthase complex, β chain is the catalytic subunit.

5.1.3.2 Proteins more abundant in the total sperm proteome of the immature sperm population

Increased protein expression in the immature population can have a direct effect on sperm function (motility and morphology). The majority of differentially expressed proteins (56%) were more abundant in the immature population compared to the mature population (see Table 2). Individual functions have been ascribed to these proteins in order to understand the possible role it plays in sperm function.

5.1.3.2.1 Proteins associated with the cytoskeleton, flagella and cell movement

A-kinase anchoring protein 4 precursor

A-kinase anchoring protein 4 (AKAP4) precursor has been shown to localize near specific targets in the fibrous sheath of the sperm flagellum [188]. Its target in the flagellum is AKAP4. When this precursor is processed, the mature AKAP4 can bind to AKAP3, which is needed for the cAMP cascade (see Figure 1). AKAP4 has a major role in completing the assembly of the fibrous sheath [189], making it essential for sperm structure and thus sperm morphology.

It is also an upstream activator of the cAMP cascade that is needed for initiation and maintenance of sperm motility [188] (see Figure 1). In the present study AKAP4 precursor is more abundant in the immature population compared to the mature population. It has been shown that AKAP4 precursor is only active in mature cells once it's bound to AKAP3. In order for AKAP4 precursor to become functional it needs to be modified by PTMs before it can bind to AKAP3. The high levels of AKAP4 precursor in the immature population could be a result of AKAP4 precursor not being modified into its functional form and therefore sperm motility is also affected. As AKAP4 precursor forms the essential part of the fibrous sheath, the structural component of sperm flagellum, it can therefore be postulated that high levels of AKAP4 precursor in the immature spermatozoa is to compensate for any abnormalities in the sperm flagellum due to the sperm not being fully matured.

Tubulin β-2C chain

Tubulin β-2C chain is a major component of microtubules. It is also involved in guanosine triphosphate (GTP) binding and major histocompatibilty complex (MHC) class 1 protein binding [170]. Tubulin binds two molecules of GTP. One binds to the β chain thus severing a part of sperm energy metabolism [190]. Although no reports have shown that Tubulin β-2C chain in human sperm is related to an increase in sperm motility, the role Tubulin plays in stabilizing sperm flagella makes it essential for sperm motility.

In the present study Tubulin β-2C chain was more abundant in the immature population compared to the mature population. Various factors can contribute to the increased expression of Tubulin β-2C chain, e.g. external conditions in the seminal plasma i.e. oxidative stress, pH changes and frictional forces exerted on spermatozoa during ejaculation. The up regulation of Tubulin β-2C chain in the immature fraction might be a cellular response to protect spermatozoa from damage caused by external stresses. Another possible explanation is that increased expression of Tubulin β-2C chain is in compensation for decreased sperm motility and morphology as tubulin plays an essential role in providing structural support to the sperm flagellum.

5.1.3.2.2 Stress-related proteins

78kDa Glucose regulated protein

78kDa Glucose regulated protein has been shown to play a crucial role in facilitating the assembly of multimeric protein complexes inside the endoplasmic reticulum (ER) [186]. In addition, 78kDa Glucose regulated protein also has the ability to bind ATP, nucleotides and proteins in the ER [191]. The metabolism of sperm requires certain energy substrates e.g. pyruvate or lactate in order for an increased energy build-up, therefore equipping sperm to swim the long distance required to the oocyte for fertilization. In the present study it has been shown that 78kDa Glucose regulated protein is more abundant in the immature population compared to the mature population. The majority of cells in the immature fraction are immotile/dead, illustrating

that 78kDa Glucose regulated protein might be expressed in high levels as a signal of cell stress. This is of great importance as protein-protein interaction activates signalling pathways responsible for initiating enzyme activity e.g. apoptosis. From these results it can be deduced that high levels of 78kDa regulating protein play a role in early events of cell apoptosis by initiating cell response to environmental stress e.g. oxidative stress.

5.1.3.2.3 Metabolic enzymes

α-enolase

The multi-functional enzyme α-enolase plays a few roles in sperm metabolism. It has a role in glycolysis and various other processes such as hypoxia tolerance and the allergic responses. This protein has been isolated in both the tail and nucleus of mature spermatozoa. Although this protein has only been described in rats, it can be extrapolated that it would have similar actions in humans. A nucleotide-dependant association of α-enolase with microtubules in spermatozoa regulates the enzyme activity by ensuring balanced energy production and utilization [192].

In the present study α-enolase was more abundant in the immature fraction suggesting it might play a critical role in sperm motility as it forms part of the microtubules (functional units of sperm flagellum). α-enolase was found to be functional only in mature spermatozoa (rats) [187].

The up regulation of α enolase could therefore be postulated to have no effect on sperm metabolism or motility as this protein is apparently only active in mature spermatozoa [187]. High levels of α-enolase in the immature population could be related to sperm energy metabolism, as this protein plays a crucial role in glycolysis. The majority of cells in the immature population are not fully matured, therefore their metabolism are not fully developed that can also affect sperm motility. As pyruvate and lactate are the preferred energy substrates for immature sperm, high levels of α-enolase might be to compensate for insufficient sperm energy metabolism.

β-enolase

β-enolase exhibits the same multi-functionality as the previous protein (α-enolase) but is found in the cytoplasm of the sperm. It has been shown to be significantly correlated to the increase in the percentage of normal morphology in spermatozoa [63, 192]. However, the function of β-enolase is dependant to that of α-enolase. Therefore it can be postulated that β-enolase could also have no effect on sperm metabolism or motility as this protein could be non-functional.

5.1.3.2.4 Energy-related proteins

ATP synthase D chain

ATPase synthase D chain plays a role in the production of ATP from ADP in the presence of a proton gradient across the mitochondrial inner membrane. The D chain specifically exhibits hydrolase activity and metal ion binding while it also act an H^+ transmembrane transporter in the mitochondrion of somatic cells [193].

It is thought to have similar functions in sperm, which will cause an increase in energy processed and thus lead to an increase in motility in mature spermatozoa [25, 74]. However, in the present study, ATP synthase D chain was up-regulated in the immature fraction, suggesting it might be involved in sperm motility.

5.1.3.2.5 Protein associated with sperm-egg interaction and cell recognition

Sperm acrosome associated protein 3

Despite spermatozoa being motile and morphologically normal, if they do not possess certain proteins crucial for sperm-oocyte interaction, the spermatozoa will not be able to fertilize the oocyte.

Therefore proteins like sperm acrosome membrane associated protein 3 are important to sperm. This protein has been found to be located on the sperm surface membrane and may be involved in the sperm-oocyte plasma membrane adhesion and fusion during fertilization. As this protein also exhibits binding capacity it could be a potential receptor for the oocyte oligosaccharide residue N-acetyl glucosamine.

In this study, sperm acrosome associated protein 3 was more abundant in the immature population. The increased expression of sperm acrosome associated protein 3 in immature fraction suggests it might be an important protein in sperm as it matures.

5.1.3.2.6 Protein possibly involved in signal transduction

T-complex protein 1 subunit theta

A molecular chaperone called T-complex protein 1 subunit theta assists protein folding after ATP hydrolysis and is known to be involved in folding actin and tubulin. This protein is found in both the centrosome and the microtubules of the manchette which is unique to sperm germ cells. This protein has also been implicated by Souès and co-workers (2003) to help remodel and repair the heterochromatin of the sperm [194]. In addition, T-complex protein 1 subunit theta also keeps DNA in the correct conformation, ensuring that the genetic data needed for fertilization is intact, making this an essential component to the spermatozoa.

However, T-complex protein 1 subunit theta was more abundant in the immature fraction, thus illustrating that this protein plays a crucial role as sperm matures. When sperm are released into the ejaculate, it loses the nurturing role provided to it by the epididymis. The up regulation of T-complex protein 1 subunit theta could play a protective role. Therefore this protein helps to remodel and repair possible damage (e.g. to heterochromatin) as most spermatozoa in this fraction are immature and do not have the ability to repair their own heterochromatin.

5.1.3.2.7 Proteins associated with protein turnover

Proteasome subunit β type 7 precursor

Proteasome subunit β type 7 precursor is shown to have catalytic functions in its β-subunits [195]. This protein is also involved in events upstream of the plateau phase of Ca^{2+} influx during exocytosis of the acrosome, thereby facilitating the acrosome reaction [196]. Imported proteins must first be ubiquinated and denatured before the protein can enter the complex and be degraded [197]. This protein is therefore multi-functional. It also allows for the catalysing of the breakdown of certain proteins in order to weaken the acrosome membrane and thus allow capacitation and for the acrosome reaction to occur [19]. However, in the present study, proteasome subunit β was found to be up-regulated in the immature fraction, suggesting that this protein might be crucial as sperm matures.

5.1.3.3 Summary of results: Aim 1

In the first part of this study 16 differentially expressed protein spots were successfully isolated and identified within the proteome of the immature and mature sperm populations. The majority (9 out of 16 proteins in total or 56%) of these proteins were more abundant in the immature sperm population compared to the mature sperm population. These findings illustrates that protein expression within the total sperm proteome of the immature population is incomplete.

Functions have been ascribed to these proteins of which only four proteins namely Tubulin α-3C/D chain, Tubulin β-2C chain, Outer dense fibre protein 2 and A-Kinase anchoring protein 4 precursor were directly related to sperm motility and morphology. Interestingly, Tubulin α-3C/D chain and Outer dense fibre protein 2 were less abundant in the immature population, while Tubulin β-2C chain and A-Kinase anchoring protein 4 precursor were more abundant in the immature population compared to the mature population. In addition, stress-related proteins i.e SOD and Gluthathione transferase Mu3 were less abundant in the immature population compared to the mature population. Low levels of SOD and Gluthathione transferase Mu3 in the immature population can lead to an increase in ROS production, as these proteins play a crucial in ROS detoxification in human spermatozoa. High levels of ROS can cause oxidative stress in human sperm that can affect sperm function (sperm morphology and motility).

Energy metabolism is the driving force of sperm function and plays a crucial role in male fertility. In the present study different metabolic enzymes i.e D-3 phosphosglycerate dehydrogenase and Triosephosphate isomerase were less abundant in the immature population compared to the mature population. The majority of cells in the immature population are not fully developed and therefore its metabolism is also not fully functional, as a result less energy is produced that can directly affect sperm motility.

Most cells in the immature population are not fully matured and therefore have decreased sperm motility and morphology. Proteins directly related with sperm motility and morphology would be expected to be less abundant in the immature population compared to the mature population, as most mRNA in these cells might not be transcriped to protein. Another explanation for specific proteins to be less abundant in the immature population could be due to failure of PTMs to take place and therefore the expression of certain proteins is affected.

This shows that proteins cannot be classified strictly into two categories of morphology and motility as the one will have an effect on the other. For example if the spermatozoa have a very low percentage of normal morphology, the motility will also be compromised. As a sperm without a tail, for example, will not be able to swim it does not matter how fast the metabolism is working. Therefore an increased motility would have to go hand in hand with an increased normal morphology and with increased ability to fertilize the oocyte.

Another possible explanation for the difference in protein expression between the two sperm populations is that it is purely due to chance, although this probability is very small.

Table 7 summarizes the functions of the proteins that were more abundant in the immature population. Figure 17 illustrates the different roles individual proteins play that was found to be differentially expressed within the total sperm proteome between immature and mature population. Proteins more abundant in the immature population are indicated (↑) arrows while the less abundant proteins are indicated with (↓) arrows.

Table 7. *Functions of the proteins found to be more abundant in the total proteome of immature spermatozoa compared to mature spermatozoa*

Protein spots	Protein name	Fold change Immature:Mature	Function
	Cytoskeleton, flagella and cell movement		
3	A-Kinase anchoring protein 4 precursor	3.453	Major structural component of sperm fibrous sheath. Plays a role in sperm motility.
12	Tubulin β-2C chain	1.990	Tubulin is the major constituent of microtubules. It binds two molecules of GTP, one at an exchangeable site on the β-chain and one at a non-exchangeable site on the α-chain.
	Stress-related proteins		
6	78kDa glucose regulated protein	2.031	Involved in spermatid development and male meiosis.
	Metabolic enzymes		
14	α enolase	1.922	Component of the outer dense fibres of spermatozoa. May have a modulating influence on sperm motility.
14	β enolase	1.922	Component of the outer dense fibres of spermatozoa. May have a modulating influence on sperm motility.
	Energy-related proteins		
4	ATP synthase subunit d, mitochondrial	1.903	Mitochondrial ATP synthase. This gene encodes d subunit of F0 complex.

Table 7. *Continued*

Protein spots	Protein name	Fold change Immature: Mature	Function
	Sperm- egg interaction and cell recognition		
5	Sperm acrosome membrane associated protein 3	1.990	May be involved in the sperm-egg plasma membrane adhesion and fusion during fertilization.
	Signal transduction		
12	T-complex protein1 subunit theta	1.990	Phosphoprotein binding, Kinase activity (PI3 kinase complex).
	Protein turnover		
8	Proteasome subunit β type 7	2.082	Subunit β of the proteasome with a potential regulatory effect.

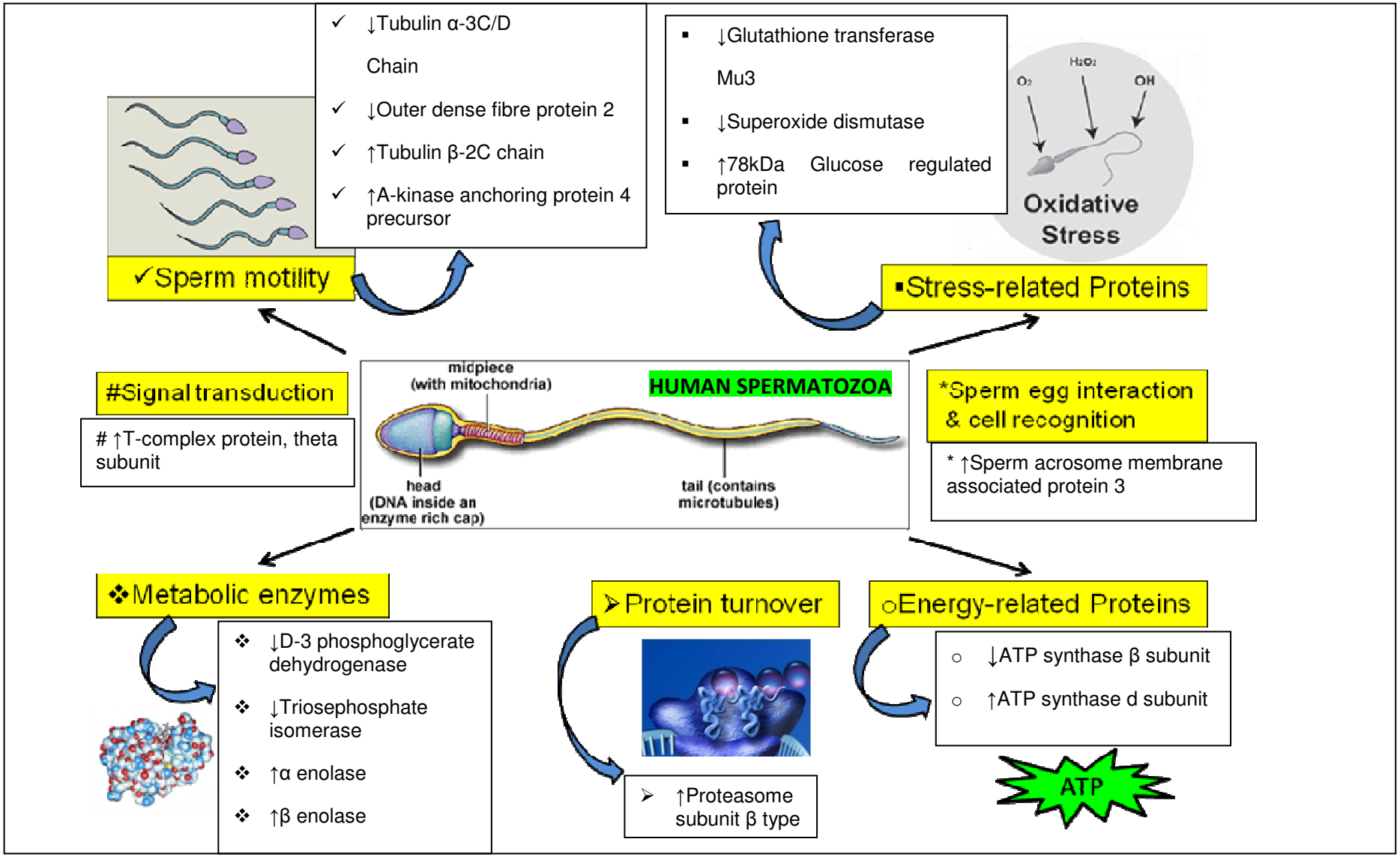

Figure 17*. Summary of differentially expressed proteins isolated in study aim 1*

5.2 Aim 2:

Determining differences in protein expression from the nuclear fraction of human spermatozoa from mature and immature sperm populations

Current evidence suggests that DNA damage in human spermatozoa is linked with a variety of adverse clinical outcomes i.e. impaired fertility and increased risk of miscarriage [198]. Many factors such as apoptosis, incomplete chromatin remodelling, environmental influences (e.g. radiation and oxidative stress) can contribute to DNA damage. A major cause of oxidative stress appears to be the high rate of ROS generation associated with the retention of excess residual cytoplasm in the midpiece of immature spermatozoa. Faulty spermatogenesis caused by environmental influences (e.g. toxins) often lead to increased production of immature / abnormal spermatozoa [199].

In addition, oxidative stress also induces peroxidative damage in the sperm plasma membrane and DNA in both the mitochondrial and nuclear genome [147]. Nuclear DNA damage of parental spermatozoa may be associated with pathologies in the offspring, including childhood cancer and infertility. In the present study, we present data on nuclear protein changes in human sperm linked with decreased sperm motility and normal morphology. These results might provide clearer insight into the molecular nature of DNA damage and associated sub-fertility (asthenoteratozoospermia).

5.2.1 Differences in the total sperm motility between the immature and mature sperm populations

It is generally accepted that normal sperm motility is a central component of male fertility [200]. Individuals with poor sperm motility or immotile sperm are typically infertile or sterile unless ART are used [4]. Most infertile men suffer from asthenozoospermia [1].

Several studies have reported sperm immobility as a good predictor of male infertility [28]. It is known that a strong correlation exist between progressively motile spermatozoa and IVF success [201]. In addition, an extremely low proportion of rapid progressive motility in fresh semen indicates a high risk of complete fertilization failure with conventional IVF [202].

It has been shown in the present study that the immature sperm populations have a lower percentage of rapid and progressively motile cells compared to the mature population (Tables 3). The immature population of spermatozoa can therefore be classified as asthenozoospermic according to the strict criteria and can thus be used as an in vitro model of spermatozoa unable to fertilize, while the mature population adhered to all the Strict Criteria parameters for fertile sperm. From these results it's evident that by separating the sperm from the seminal plasma with the discontinuous gradient system, two distinctly different sperm populations (immature and mature sperm populations) free from contaminants can be retrieved purely based on motility.

5.2.2 Differences in sperm morphology parameters between immature and mature sperm populations

Semen parameters have been correlated to fertilization ability of spermatozoa in several studies of which sperm morphology was the only consistent predictor of male infertility [4, 19, 28]. It is known that low levels of morphologically normal sperm in the ejaculate can be an indicator of male factor infertility [203].

Separating sperm from seminal plasma by a discontinuous density gradient does not only select sperm based on their motility, but also on the grounds of their morphology. In the present study it has been shown that heterogeneous sperm populations (immature vs mature sperm) present within the same semen samples have different morphological features (Table 4). From the results of the present study it is evident that the immature sperm population contains the majority of cells with decreased sperm morphology characteristics compared to the mature sperm fraction (Table 4). Based on the significant decrease in morphological parameters (Table 4) in the immature population compared to the mature population, it can be postulated that the mature population contains better quality sperm than the immature population.
However, the immature population can not be classified as teratozoospermic as only the percentage cells with normal head morphology was measured in the current study.

Several studies have reported that the presence of excess residual cytoplasm (cytoplasmic droplets) on spermatozoa is associated with poor sperm function [4, 156,

204]. The retention of such a droplet is related to a shorter axoneme [205], abnormal head, midpiece morphology [205-207] and a greater extent of chromatin breaks and DNA damage [199]. In the present study it has been shown that the immature population had a significantly higher percentage of cells with cytoplasmic droplets compared to the mature population (Table 4). In addition most of the morphological parameters were significantly decreased in the immature population compared the mature population that is in agreement with these reports.

5.2.3 Differences in nuclear protein profiles between the immature and mature sperm populations

Various factors (DNA integrity, abnormal gene products, genetic disorders, etc.) may contribute to poor semen quality of which sperm DNA integrity has been suggested as a more objective marker of sperm function than the standard semen analysis [208]. It has been shown that spermatozoa from most infertile men contain various nuclear alterations including abnormal chromatin structure, chromosome with micro deletions and DNA strand breaks [11]. Therefore the main focus of Aim 2 was to identify possible differences in nuclear protein expression associated with sperm dysfunction. Results from the present study show that numerous nuclear proteins are down regulated in the immature sperm population compared to the mature sperm population. These proteins play crucial roles in sperm function (motility and morphology) and energy metabolism in human spermatozoa (see Table 8).

From these results it is clear that sperm protein expression in the nuclear protein fraction differs between the immature and mature populations of spermatozoa. The nuclear proteins that were less abundant in the immature sperm populations were associated with six different functional groups: cytoskeleton, flagella and cell movement, metabolic enzymes; transport proteins; protein turnover; stress related proteins and energy related proteins (see Table 8). In addition only one nuclear protein (78kDa glucose regulated protein) which can be classified as a stress-related protein was more abundant in the immature sperm population (see Table 9). Nuclear proteins less abundant in the immature population will be discussed in the next section followed by a discussion of the nuclear proteins which are more abundant in the immature population compared to the mature sperm population.

5.2.3.1 Nuclear proteins less abundant in the immature sperm population

Sperm DNA integrity is a novel marker for male fertility potential [199]. DNA fragmentation affects abnormal spermatozoa in the semen of subjects with low sperm motility [1, 199].

It can also prevent fertilization, embryo development and increase the risk of genetic defects in the offspring. The molecular nature of DNA fragmentation lies within the gene products (proteins) expressed within the sperm nucleus. In the present study it has been found that the majority of differentially expressed nuclear proteins were less abundant in the immature population vs. the mature population (see Table 5). From these results it can be deduced that nuclear protein expression in the immature fraction is incomplete and can have a direct influence on sperm function (motility and morphology). The following section focuses on the role individual proteins plays in sperm function that were found to be down regulated in the immature fraction.

5.2.3.1.1 Proteins associated with cytoskeleton, flagella and cell movement

Tektin 2

Tektin 2 is involved in stabilizing tubulin protofilaments [209]. It also forms the attachment points between α and β-tubules of ciliary / flagellar doublet microtubules. Tektin 2 forms central attachments with the C-tubules of centrioles in order to generate long-range patterns of binding sets for axonemal components, such as nexins, radial spokes and dynein arms [209].

Increased expression of testicular Tektins contributes to the formation of sperm flagella in the adult testis. It is a known fact that Tektin 2 protein is localized specifically in the flagellum of the elongating spermatid up to the mature sperm stage [209]. This suggests that Tektin 2 plays an important role in the formation of the sperm flagellum and has direct influence on sperm motility. Tektin 2 plays a critical role in protecting sperm against the shear forces exerted on sperm flagellum during ejaculation. The down regulation of tektin 2 in the immature fraction can directly affect sperm motility as shear forces can cause damage to the sperm flagellum. Damage to the flagellum due to shear forces can therefore be ascribed to decreased expression of tektin 2 and subsequently decreased motility.

5.2.3.1.2 Stress-related proteins

Prolactin inducible protein

Prolactin inducible protein (PIP) is reported to be a 17-kDa glycoprotein present in human seminal plasma and known by various names such as secretory actin binding protein (SABP); extraparotid glycoprotein (EP-GP) and glycoprotein 17 (GP 17) due to its versatile nature and function in reproductive and immunological systems [210, 211]. It has been shown that PIP is expressed in several exocrine tissues such as the lacrimal, salivary and sweat glands [212]. Due to its over-expression in metastatic breast and prostate cancer, PIP is presently considered as a prognostic marker for cancer [213, 214]. PIP has been found in the post-acrosomal zone, and it remains bound to the sperm surface after capacitation [215]. Interestingly, the expression of PIP is up regulated by prolactin and androgens, and down regulated by estrogen [216, 217].

Although PIP is a small protein, it has unique features and has multiple important functions in biological systems. Its ability to bind potentially with CD4-T cell receptor, immunoglobulin G (IgG), actin, zinc a2-glycoprotein (ZAG), fibronectin and enamel pellicle, reveals its important role in biological functions [218, 219].

Several studies have reported that seminal plasma has immune modulating properties that are mediated by prostaglandins [220], complement inhibitors [221], cytokines [204], and proteins capable of binding IgG via the F_C fragment [222].

These IgG binding proteins are $F_{C\gamma}$ receptor-like proteins and can be regarded as antibody-binding proteins. PIP has strong affinity for the F_C fragment of immunoglobulin G (IgG), and it could bind with antisperm antibodies (ASA), which are meant to protect spermatozoa from IgG damage (Figure 18). A possible mechanism of protection is that PIP binds to circulating IgG that is bound to ASA and thereby protecting sperm from damage caused by IgG (Figure 18). It must also be taken into consideration that IgG is only produced when there is a disruption in the blood testis barrier or during an infection (bacterial or viral).

In the present study it has been shown that PIP is down regulated in the immature population. The reduction of PIP could result in an increase in apoptosis as most cells in the immature population are immotile/dead [223, 224]. Previous studies have found that PIP specifically degrades the fibronectin molecule that is one of the major protein constituents of the seminal coagulum and constitutes at least 1% of seminal plasma proteins [225-227]. This suggests that PIP could contribute to fibronectin cleavage during liquefaction. It has been previously reported that the beat frequency of the flagellum is inversely related to the viscosity of the surrounding medium [228]. Thus, the decreased intensity of PIP in the immature population detected in the present study might lead to incomplete liquefaction of the ejaculate. The more viscous seminal vesicular fluid becomes the higher the restriction of movement of the spermatozoa. Intense research efforts continue in the biochemical characterization of this protein but its exact function is not well understood.

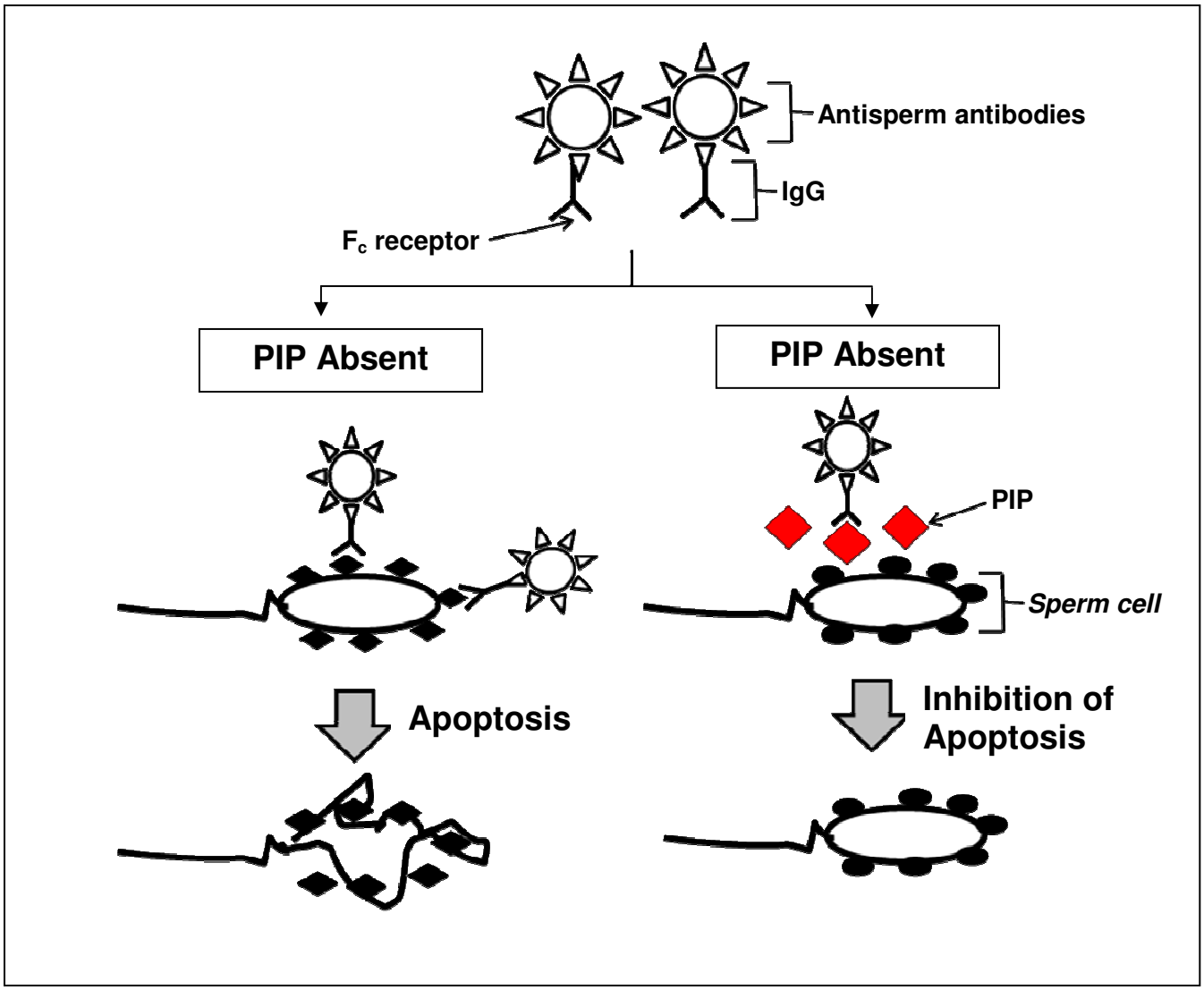

Figure 18. *Postulated mechanism of protection by which PIP can possibly inhibit apoptosis when ASA and IgG are produced after infection/blood-testis barrier disruption (PIP=Prolactin Inducible Protein, IgG=* immunoglobulin G, ASA= Antisperm antibodies) [222].

60kDa heat shock protein

The 60kDa heat shock protein forms part of the family of mammalian heat shock proteins (HSPs). These proteins are evolutionarily highly conserved molecular chaperones, which appear to have been derived from prokaryotic ancestors that originally evolved to solve problems in protein folding [229]. The 60kDa heat shock protein (hsp60) is present in mammalian cells and is produced in response to various stresses, e.g. elevated temperature, infection, inflammation and toxic chemicals [230]. Its function appears to be to prevent protein denaturation or inappropriate polypeptide aggregation during the period of stress, thereby allowing the cell to survive and resume normal functions after the stress is alleviated [231, 232].

The down regulation of hsp60 in the present study could inhibit protection against environmental stress. Another explanation for decreased expression of hsp60 might be due to sperm not being fully matured as this protein is more abundantly expressed in the mature sperm population.

Stress 70kDa protein

Stress 70kDa protein has been reported to be involved in the control of cell proliferation and cellular aging. Typically, the regulation of stress 70kDa protein expression is considered to occur at a transcriptional level. Misfolded proteins can directly activate the expression of 70kDa stress related protein genes [233].

In the present study stress, 70kDa protein was less abundant in the immature population compared to the mature population. Since the immature fraction contains mostly immature/abnormal spermatozoa, one can postulate that there would be a variety of misfolded proteins present as sperm are not fully matured. When proteins are misfolded they might also be inactive and therefore not functional. The inactive state of certain proteins could be related to the maturation of sperm as only specific proteins are activated as the sperm develop. More research needs to be done on stress related proteins to understand their role in sperm function.

Heat shock related 70kDa protein 2

Heat shock related 70kDa protein 2 forms part of family of mammalian HSP's. HSP's are classified into families according to their molecular weights and serve two major functions. One of those is as a molecular chaperone involved in facilitating protein folding and assembly, while the other is as a stabilizer of damaged proteins involved in the prevention of aggregation, following an opportunity for repair of degradation in cells experiencing cellular stress [234].

Recently the ability to inhibit apoptosis has become widely recognized as a function of HSP's since this contributes to their protective effect in cells [235]. Although the mechanisms are unclear, recent evidence suggests an important role for HSP's in fertilization [236, 237]. The sperm-oocyte interaction involves several events that culminate in fertilization.

These include recognition of acrosome-intact spermatozoa, attachment to the zona pellucida, induction of the acrosome reaction, exocytosis of the outer acrosomal membrane, penetration of the zona pellucoda, fusion of the oollema, and finally, activation of nuclear events leading to zygote formation [58]. Many of these events are mediated by protein molecules found on the sperm plasma membrane and zona pellucida [238].

A recent study has shown that HSP70 is present on the acrosome of ejaculated bovine spermatozoa and undergoes re-localization to the equatorial segment during induced capacitation and the acrosome reaction [239]. This finding is of particular significance since other surface proteins that are redistributed during membrane remodelling, such as, pH-20, fertilin, CE9, SP-10 and 2B1, play important roles in the sperm-oocyte interaction during IVF [201]. Although it is possible that HSP70 serves as a stress protector for spermatozoa prior to fertilization [240], the distribution and re-localization of HSP70 on bovine spermatozoa suggest that HSP70 play a role in gamete interaction. The down regulation of HSP70 in the present study could therefore affect the fertilizing ability of spermatozoa. This provides preliminary evidence on the possible role sperm protein expression plays during fertilization.

5.2.3.1.3 Metabolic enzymes

Pyruvate dehydrogenase E1, component, subunit β

Pyruvate dehydrogenase E1 component, subunit β forms part of the pyruvate dehydrogenase complex (PDC) and is known to be involved in glycolysis (Figure 19). The mechanisms that control mammalian PDC activity include its phosphorylation (inactivation) by a family of pyruvate dehydrogenase kinases (PDKs 1–4) and its dephosphorylation (activation) by the pyruvate dehydrogenase phosphate phosphatases (PDPs 1 and 2). Isoform specific differences in kinetic parameters, regulation, and phosphorylation site specificity of the PDKs introduce variations in the regulation of PDC activity in differing endocrine and metabolic states. Pyruvate dehydrogenase E_1 component subunit β plays an important role in glycolysis when puruvate is broken down to acetyl CoA and further utilize during oxidative phosphorylation.

All α-keto acid dehydrogenase complexes are constructed from three protein components: E1, E2, and E3 (Figure 19). The identities of E1, E2, and E3 depend on the particular class of enzyme complex, but usually the E1 component is constructed with both α and β subunit and functions as a substrate decarboxylase. The E2 component forms the core of the complex and acts as a dihydrolipoamide acetyltransferase. The E3 component is a dihydrolipoamide dehydrogenase.

The mitochondrial pyruvate dehydrogenase complex (PDC) catalyzes the oxidative decarboxylation of pyruvate, linking glycolysis to the tricarboxylic acid cycle and fatty acid (FA) synthesis (Figure 19).

Knowledge of the mechanisms that regulate PDC activity is important, because PDC inactivation is crucial for glucose conservation when glucose is scarce, whereas adequate PDC activity is required to allow both ATP and FA production from glucose.

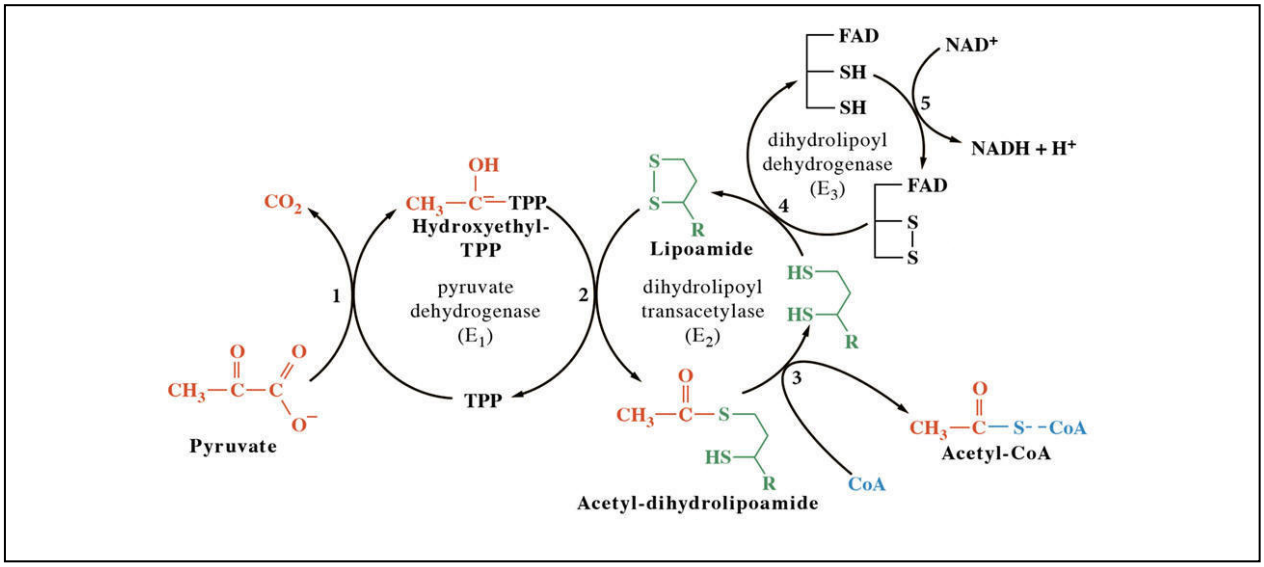

Figure 19. *Diagram of the Pyruvate dehydrogenase complex (PDC) and its different enzymatic subunits (E1= pyruvate dehydrogenase, E2= dihydrolipoyl transacetylase and E3= dihydrolipoyl dehydrogenase) [241].*

In the present study pyruvate dehydrogenase E_1 was down regulated in the immature fraction which could be related to sperm energy metabolism. It is known that lactate or pyruvate is the preferred energy sources for motility in sperm cells. Due to the role the enzyme pyruvate dehydrogenase plays during glycolysis, it can be postulated that pyruvate might be an essential energy source for sperm.

Therefore the reduction in pyruvate dehydrogenase E1 component might affect PDC function and thereby cause disruption in sperm energy metabolism. Any irregularities in sperm metabolism have a direct effect on sperm motility as ATP production plays crucial role in sperm function.

Dihydrolipoyl dehydrogenase

Dihydrolipoyl dehydrogenase forms part of the pyruvate dehydrogenase complex (PDC) and is also known as enzyme subunit E_3. This protein plays a crucial role in glycolysis and could play an important role in the energy metabolism in spermatozoa. Pyruvate and α-ketoglutarate, substrates of dihydrolipoyl dehydrogenase containing enzymes, promote pronounced ROS release in permeabilized wild-type mitochondria. The vast majority of studies on mitochondrial ROS generation have used mitochondria and respiratory chain inhibitors as tools to maximize ROS production and to identify potential sites of ROS generation [156]. However, little is known about the mechanism by which dihydrolipoyl dehydrogenase enzyme contributes to ROS production in human spermatozoa.

In the present study it has been shown that dihydrolipoyl dehydrogenase is less abundant in the immature fraction. As this protein is found in the mitochondria and plays a role in glycolysis, it can be postulated that it might be linked to oxidative phosphorylation via the tricaboxylic acid (TCA) cycle. Low levels of dihydrolipoyl dehydrogenase in the immature population could result in NAD^+ accumulation. High levels of NAD^+ could contribute to ROS production as this molecule is highly reactive and unstable. However, it is not clear what the specific role dihydrolipoyl dehydrogenase plays in sperm, but its reduction in the immature population might contribute to decrease sperm motility.

Protein disulfide isomerase A3

Protein disulfide isomerase A3 (PDI) is known to play an active role in peptide folding [242]. It also promotes the renaturation of reduced Fab by catalyzing the formation of native disulfide bonds [242]. PDI stimulates the folding of the peptide chain and reduced disulfide-containing proteins in vitro [243].

PDI-related proteins are residents of the lumen of the ER. They have various functions, including redox and chaperone activities, regulation of calcium homeostasis, and regulation of protein export from the ER for degradation. These functions are essential for maintaining a productive folding environment for many secretory proteins within the ER [243-246].

The chaperone function of these proteins, and probably to varying extents redox activity as well, relies on their ability to interact non-covalently with specific peptide sequences or epitopes in substrate proteins [244]. In the present study it has been shown that PDI is less abundant in the immature sperm and might be related to sperm motility as this protein plays a critical role in calcium homeostasis. Decreased expression of PDI can result in calcium imbalance that could directly affect sperm motility (Figure 1). However, the specific role PDI plays in human sperm is unknown.

Saccharopine dehydrogenase

Saccharopine dehydrogenase (SDH) is the last enzyme in the α-aminoadipate pathway for L-Lysine catabolism. Saccharopine dehydrogenase catalyzes the reversible oxidative deamination of α-ketoglutarate (α-Kg) and saccharopine to produce glutamine and α aminoadioate 6-semialdehyde using NAD^+ as the oxidant (Figure 20). On the basis of detailed kinetic studies of SDH, an ordered sequential kinetic mechanism was proposed [247] with NAD^+ as the first substrate to bind to form an NAD^+ binary complex in the physiologic reaction direction. Upon binding of saccharopine to the binary complex, the reaction proceeds through a series of intermediates to give products. In the reverse reaction direction, NADH adds to the enzyme first, followed by random addition of α-Kg and lysine (Figure 20).

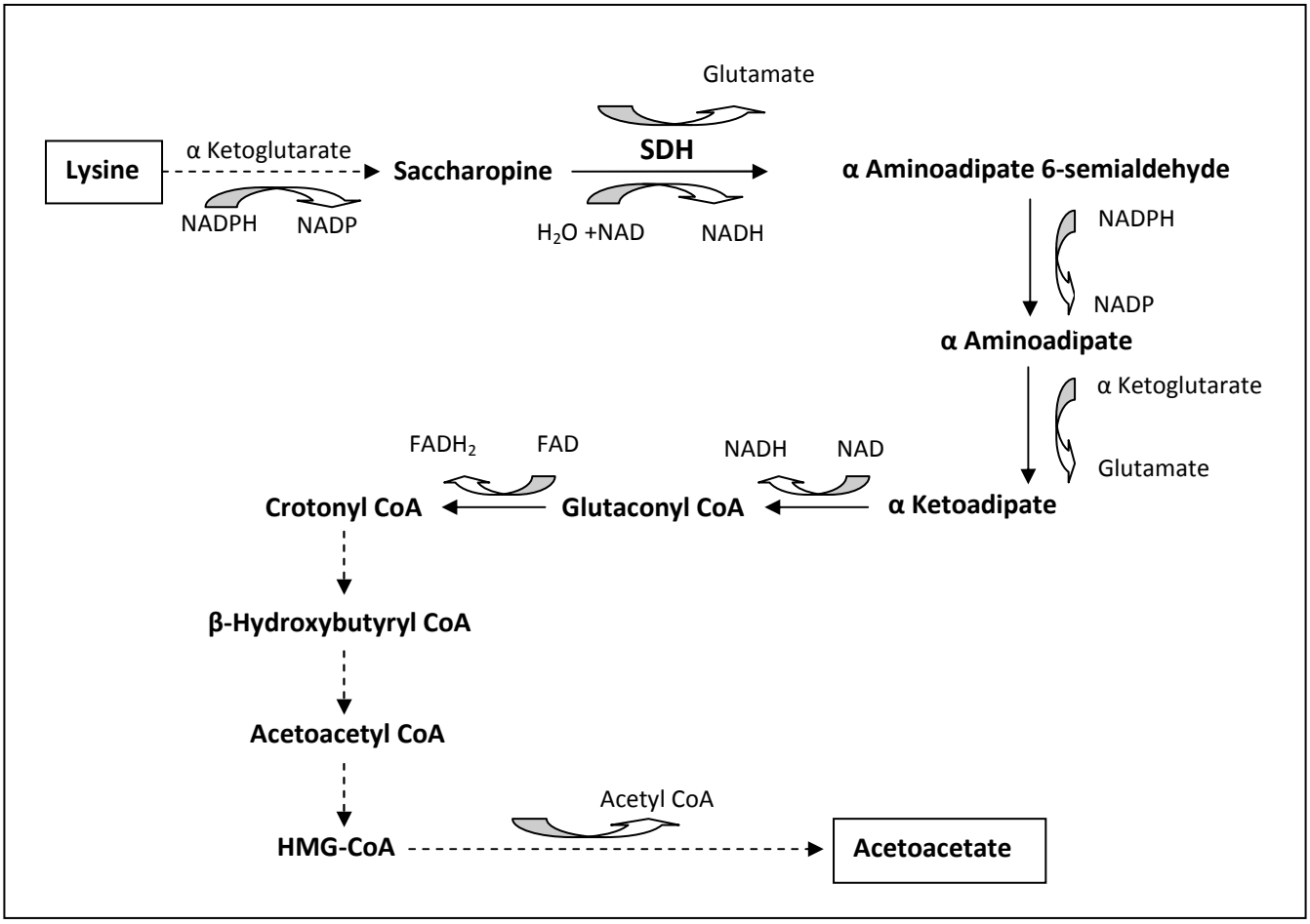

Figure 20. *Schematic diagram illustrating the crucial role enzyme Saccharopine dehydrogenase (SDH) play in lysine catabolism (NADPH= nicotinamide adenine dinucleotide phosphate (reduced form), NADP= nicotinamide adenine dinucleotide phosphate, NAD= nicotinamide adenine dinucleotide, NADH= nicotinamide adenine dinucleotide (reduced form), FAD= flavin adenine dinucleotide, $FADH_2$= flavin adenine dinucleotide (fully reduced form) and HMG-CoA= hydroxymethylglutaryl coenzyme A [248].*

Detailed inhibition studies suggest that oxaloacetate, glutarate, and lysine can bind to the free enzyme form in deadend fashion [247]. Lysine catabolism is also an important mechanism for the control of the concentration of soluble lysine in maize endosperm [248, 249]. The first two steps of lysine degradation in plants and mammals are catalyzed by the bifunctional enzyme protein lysine 2-oxoglutarate reductase-saccharopine dehydrogenase (LOR-SDH) (also known as lysine *α*-ketoglutarate reductasesaccharopine dehydrogenase LKR-SDH) [249-251].

In the present study SDH was less abundant in the immature fraction. From the results it is evident that the mature spermatozoa have an increased motility when compared to the immature population. As SDH is less expressed in the immature population it might be a contributing factor to decreased motility of these cells, because lysine cannot serve as an additional/alternative source of energy. The postulated mechanism by which lysine can serve as an additional energy source in sperm metabolism is illustrated in Figure 21.

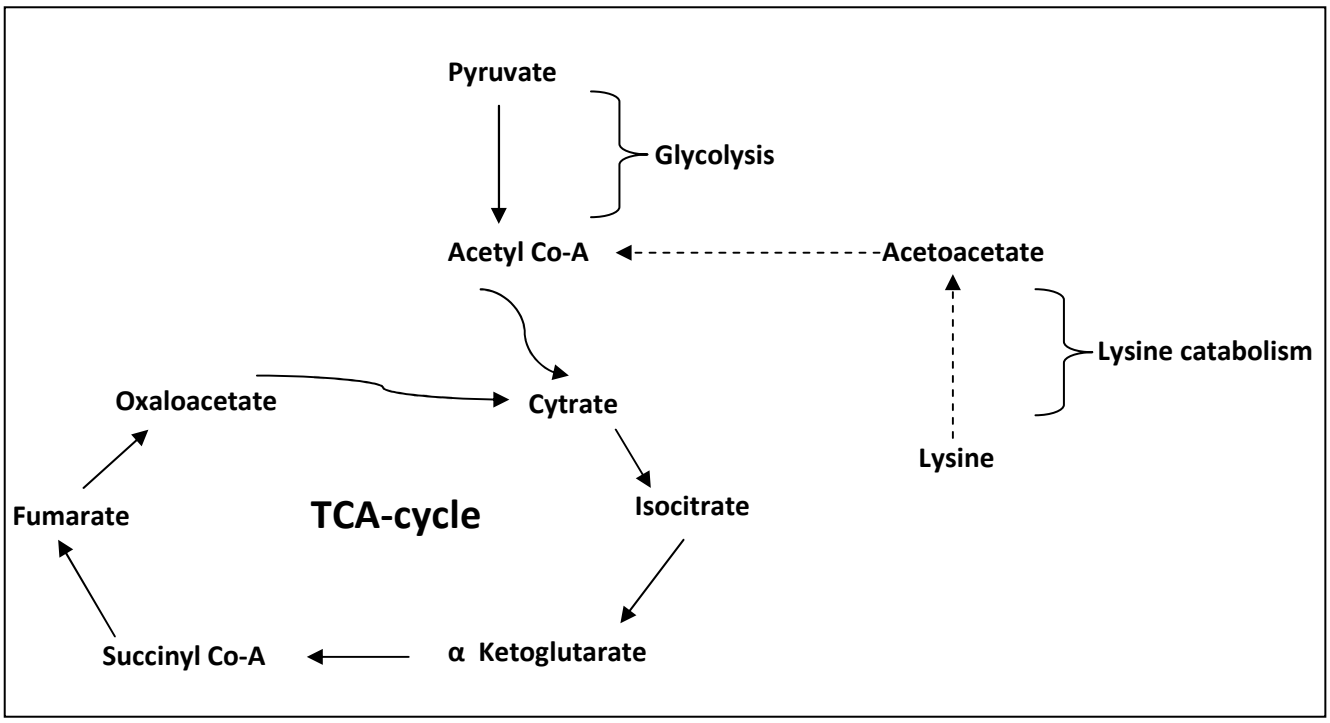

Figure 21*. Postulated mechanism (indicated with dot arrows) in which Lysine catabolism can be linked to oxidative phosphoralytion when substrate availability is limited (TCA-cycle=Tricaboxylic acid cycle, Acetyl Co-A=Acetyl coenzyme A, Succinyl Co-A=Succinyl coenzyme A)* [249-251].

5.2.3.1.4 Energy related-proteins

ATP synthase subunit β

ATP synthase β subunit forms part of the ATP synthase complex. This complex is made up of a soluble component linked to a membrane sector. The soluble part (F1), which contains the catalytic activity for ATP synthesis, is composed of five non-identical subunits. The two largest subunits α and β are present in three copies per ATP synthase complex. There is a high degree of sequence conservation among the β subunits of the bacterial, chloroplast and mitochondrial complexes [252]. The β subunit plays a central role in ATP synthesis since it contains amino acids involved in substrate binding at the catalytic site [2].

The α and β-subunits of membrane-bound ATP synthase complex bind ATP and ADP: β contributes to catalytic sites and α may be involved in regulation of ATP synthase activity. The sequences of β subunits are highly conserved in Escherichia coli and bovine mitochondria. Furthermore α and β subunits are weakly homologous to each other throughout most of their amino acid sequences, suggesting that they have common functions in catalysis. Related sequences in both α and β subunits and in other enzymes that bind ATP or ADP in catalysis, notably myosin, phosphofructokinase, and adenylate kinase, help to identify regions contributing to an adenine nucleotide binding fold in both ATP synthase subunits.

In the present study ATP synthase subunit β was less abundant in the immature population compared to the mature population, thus influencing the function of ATP synthase complex. This could potentially results in decrease ATP production, as the β subunit is the catalytic subunit (ATP synthase complex) that is directly involve in energy production. Reduce energy production can directly affect sperm motility in the immature population.

Cytochrome b-c1 complex subunit 1

The cytochrome b-c1 complex subunit 1 also known as complex III is an oligomeric membrane protein complex which is a component of the mitochondrial respiratory chain (ETC). The cytochrome b-c1 complex contains three electron transfer proteins; cytochrome b, cytochrome c1 and iron- sulfur protein. Complex III is the third complex in the ETC, playing a critical role in biochemical generation of ATP (oxidative phosphorylation). It has been shown that Complex III is present in the mitochondria of all animals and all aerobic eukaryotes and the inner membranes of most eubacteria.

In the present study cytochrome b-c1 complex subunit 1 was less abundant in the immature population compared to the mature population. The reduction of cytochrome b-c1 complex subunit 1 can directly affect energy production as this protein plays a critical role in the biochemical generation of ATP (oxidative phosphorylation). This could lead to a decrease energy production that can directly affect on sperm motility [99].

5.2.3.1.5 Proteins associated with protein turnover

Cytosol aminopeptidase

Cytosol aminopeptidase (Cytosolic AP) has been reported to be involved in the processing and regular turnover on intracellular proteins. Aminopeptidase (AP) is found in many different organisms including mammals, yeast, and bacteria. Mammalian AP was first identified in porcine kidney [253]. It is now known that there are at least two distinct forms of mammalian AP, a membrane-bound form and a cytosolic form.

The cytosolic form of AP has been purified from human leukocytes [254], human platelets [255], rat brain [256], and guinea pig brain [257]. Cytosolic AP also hydrolyzes bradykinin and other peptides with a penultimate proline residue and is inhibited by chelating agents [254]. Cytosolic AP functions as a single Mn-dependent enzyme. Both proteins were reported to have subunits that separated on SDS-PAGE with a molecular mass of 71 kDa. The cDNA encoding a putative cytosolic form of AP has been isolated from a human lymphocytic cDNA library [258], although the expression and subsequent functional characterization of the protein has not been reported. In the present study Cytosolic AP was isolated and identified in human sperm for the first time. In addition it has been shown that Cytosolic AP was less abundant in the immature population compared to the mature population. This novel finding needs to be investigated further to identify the role Cytosolic AP plays in sperm function.

Mitochondrial inner membrane protein

It has been reported that mitochondrial inner membrane protein is required for protein import into the mitochondrial matrix. Most proteins of the mitochondrial matrix are synthesized on cytosolic ribosomes and must therefore be imported across the outer and inner mitochondrial membranes. Translocation across the inner membrane occurs through the inner membrane channel and is driven by the membrane potential and an import motor [259-261]. Three critical components of this motor are the major Hsp70 molecular chaperone of mitochondria, the peripheral inner membrane protein Tim44 and the nucleotide release factor. Multiple cycles of Ssc1 binding to and release from translocating polypeptide, driving the import process, are required for import of proteins into the mitochondrial matrix.

Mitochondrial inner membrane protein was less abundant in the immature populations compared to the mature population in the current study. The reduction of this protein might be associated with protein build up as mitochondrial inner membrane protein plays a critical role in protein import. Therefore it can be postulated that proteins (e.g. structural protein) that are not in the correct functional conformation might be inactive thus affecting sperm structure and morphology and thus directly affect sperm motility.

5.2.3.1.6 Iron transport protein

Ferritin

Ferritin is an iron transporting protein and plays a crucial role in detoxifying potentially harmful free ferrous iron [262]. Iron represents a paradox for living systems by being essential for a wide variety of metabolic processes (oxygen transport, electron transport, DNA synthesis, etc), but also having the potential to cause deleterious effects. However, in aqueous media at physiological pH, free iron spontaneously forms precipitates of ferric (Fe^{3+}) hydroxide that are not biologically useful [263]. Because of iron's virtual insolubility and potential toxicity under physiological conditions, specialized molecules for the acquisition, transport, and storage of iron in a soluble, nontoxic form have evolved to meet cellular and organism iron requirements.

Intricate biological systems have developed for the transport, cellular uptake and storage of iron in a useful, nontoxic state [264]. Molecular components of these systems include the soluble iron transport protein transferrin, the cell membrane transferrin receptor, and the cytoplasmic storage protein ferritin. Ferritins originated early in phylogenesis and are present in archeobacteria, eubacteria, plants, invertebrates, and mammals [265].

Although excess iron is stored primarily in the cytoplasm, most of the metabolically active iron in cells is processed in mitochondria. Ferritin is a ubiquitous protein that plays a critical role in regulating intracellular iron homoeostasis by storing iron inside its multimeric shell. It also plays an important role in detoxifying potentially harmful free ferrous iron to the less soluble ferric iron by virtue of the ferroxidase activity of the H subunit. In addition, it is abundant in the iron-loaded mitochondria of erythroblasts of patients with sideroblastic anaemia. The characterization of recombinant and transfected mitochondrial ferritin indicated that this protein has a role in protecting mitochondria from iron-induced damage.

Physiologically, the majority of cells in the organism acquire iron from a well-characterized plasma glycoprotein, transferrin. Iron uptake from transferrin is reasonably well understood, and involves the binding of transferrin to the transferrin receptor, internalization of transferrin within an endocytotic vesicle by receptor-mediated endocytosis, and the release of iron from the protein by a decrease in endosomal pH (Figure 22). Most of the transferrin-bound iron is used for the synthesis of haemoglobin by developing erythroid cells.

In vitro evidence indicates that relatively soluble ferrous iron can enter or be released from ferritin. However, little is known about the exchange of iron with ferritin in intact cells, and some evidence indicates that the degradation of the ferritin protein may be an important mechanism for the release of iron within the cell.

Cellular iron uptake and storage are co-ordinately regulated through a feedback control mechanism mediated at the post-transcriptional level by cytoplasmic factors known as iron-regulatory proteins (IRPs) 1 and 2. These proteins "sense" levels of iron in the transit pool and, when iron in this pool is scarce, they bind to stem-loop structures known as iron-responsive elements (IRE's) on the 5' untranslated region of the ferritin messenger ribonucleic acid (mRNA) and 3' untranslated region of the transferrin mRNA [266] (Figure 22) . Such binding inhibits translation of ferritin mRNA and stabilizes the mRNA for transferrin receptors. The opposite scenario develops when iron in the transit pool is abundant (Figure 22). This remarkable regulatory mechanism prevents the expansion of a catalytically active intracellular iron pool, while maintaining sufficient concentrations of the metal for metabolic needs. Ferritin expression is modulated by a variety of conditions associated with oxidative stress that act either directly on gene expression or indirectly via the modification of IRPs [267].

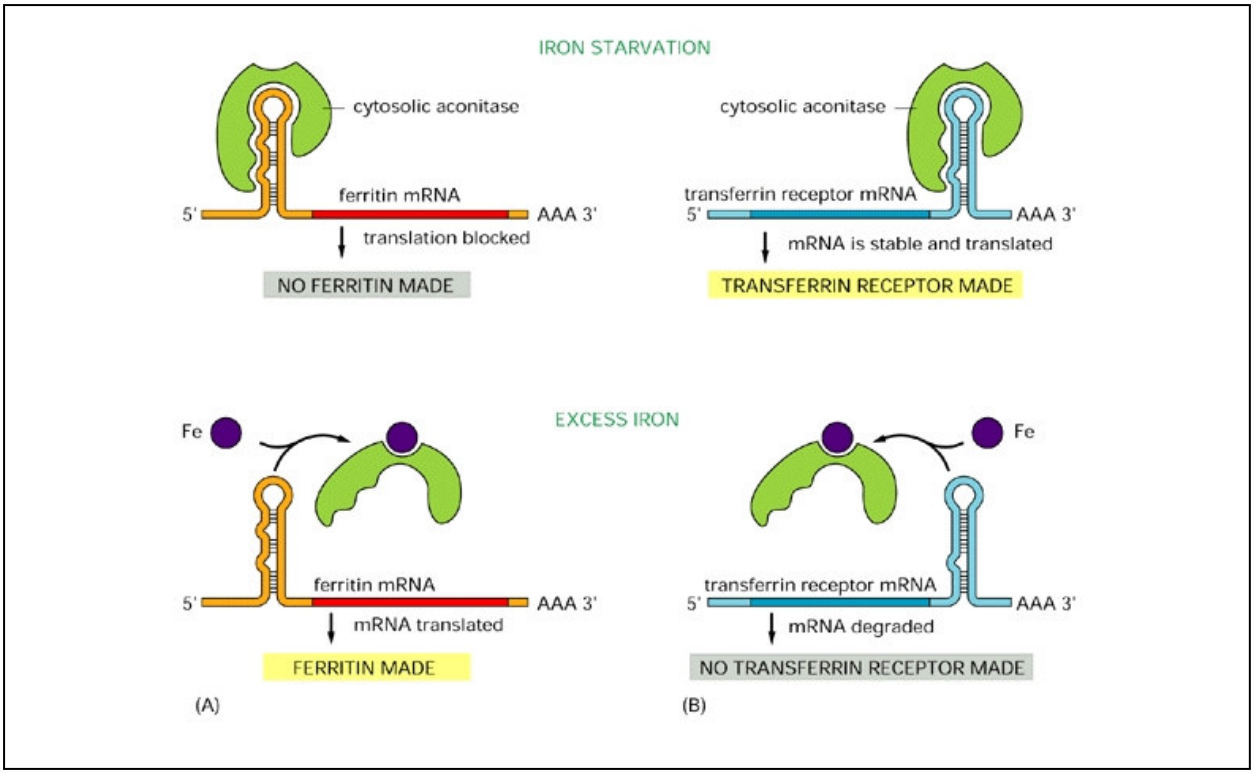

Figure 22. ***Two post translation controls mediated by iron****: In response to an increase in iron concentration in the cytosol, a cell increases its synthesis of ferritin in order to bind the extra iron (A) and decreases its synthesis of transferring receptors in order to import less iron (B). Both responses are mediated by the same iron-responsive regulatory protein (aconitase) which recognizes common features in a stem-and-loop structure in mRNAs encoding ferritin and transferring receptor. Aconitase dissociates from the mRNA when it binds iron. Because the transferrin receptor and ferritin are regulated by different types of mechanisms, their levels respond oppositely to iron concentrations even though they are regulated by the same iron responsive regulatory protein [266, 268].*

In association with the increase in levels of testicular Fe^{2+}, is the accumulation of ferritin, where Fe^{2+} is safely stored within Leydig cells. A novel ferritin type specifically targeted to mitochondria has been recently found in human and mouse. It is structurally and functionally similar to the cytosolic ferritins. These cytosolic ferritins are well-characterized molecules found in most living systems which are designed to store and detoxify cellular iron [264]. Cytosolic ferritins in mammals are ubiquitous while mitochondrial ferritin expression is restricted mainly to the testis, neuronal cells and islets of Langerhans [263].

In the present study Ferritin was found to be less abundant in the immature population compared to the mature population. Decrease expression of ferritin can result in increased concentration of ferric iron (Fe^{3+}). Free ferric ions can spontaneously forms participates of ferric hydroxide that are not biologically useful and can directly affect sperm function (sperm motility and morphology). It can also be postulated that decrease expression of Ferritin in the immature population can be associated with abnormal ROS production and oxidative stress as both SOD and Glutathione responsible for the reduction ROS is also less abundant in the immature population. Therefore Ferritin would be an ideal target for the development of biomarkers in male infertility associated with oxidative stress.

Table 8. *Functions of proteins less abundant in the nuclear fraction of immature population compared to the mature population*

Protein spots	Protein name	Fold change Immatue:Mature	Function
	Cytoskeleton, flagella and cell movement		
8	Tektin 2	-3.846	Contribute to the formation of sperm flagella and plays critical role in sperm motility.
	Stress-related proteins		
1	Prolactin Inducible protein	-7.692	Involve in some kind of actin binding function but not yet clear.
16	60kDa heat shock protein	-2.174	May facilitate the correct folding of imported proteins.
17	60kDa heat shock protein	-1.852	May facilitate the correct folding of imported proteins.
20	Stress 70kDa protein	-1.818	Implicated in the control of cell proliferation and cellular aging. May also act as a chaperone.
21	Heat shock- related 70kDa protein 2	-33.33	Involved male meiosis and spermatid development.
	Metabolic enzymes		
13	Dihydrolipoyl dehydrogenase	-2.041	Oxidoreductase,FAD binding, protein binding, electron carrier activity.
14	Dihydrolipoyl dehydrogenase	-2.083	Oxidoreductase,FAD binding, protein binding, electron carrier activity.

Table 8. *Continued*

Protein spots	Protein name	Fold change Immature:Mature	Function
	Metabolic enzymes		
18	Protein disulfide isomerase A3	-1.861	Cysteine-type endopeptidase activity and Phospholipase activity.
19	Protein disulfide isomerase A3	-1.861	Cysteine-type endopeptidase activity and Phospholipase activity.
5	Pyruvate dehydrogenase E1 component, subunit β,mitochodrial	-1.861	The pyruvate dehydrogenase complex catalyzes the overall conversion of pyruvate to acetyl-CoA.
2	Saccharopine dehydrogenase	-33.33	Plays a role in Amino acid metabolism specifically lysine biosynthesis.
	Energy-related proteins		
4	ATP synthase subunit β, mitochondrial	-5.556	Mitochondrial ATP synthase, β chain is the catalytic subunit.
11	ATP synthase subunit β, mitochondrial	-4.762	Mitochondrial ATP synthase, β chain is the catalytic subunit.
12	ATP synthase subunit β, mitochondrial	-2.381	Mitochondrial ATP synthase, β chain is the catalytic subunit.
6	Cytochrome b-c1 complex subunit 1	-1.695	Plays a critical role in the biochemical generation of ATP. Electron transport chain (mitochondrial).
7	Cytochrome b-c1 complex subunit 1	-1.786	Plays a critical role in the biochemical generation of ATP. Electron transport chain (mitochondrial).
	Protein turnover		
15	Cytosol aminopeptidase	-2.941	Presumably involved in the processing and regular turnover of intracellular proteins.

Table 8. *Continued*

Protein spots	Protein name	Fold change Immature: Mature	Function
	Protein turnover		
9	Mitochondrial inner membrane protein	-2.128	Required for protein import into the mitochondrial
	Transport proteins		
3	Ferritin, mitochondrial	-1.333	Plays a role in detoxifying potentially harmful free ferrous iron

5.2.3.2 Nuclear proteins more abundant in the immature sperm population

The mature population was used as the in vitro model of fertile sperm in the present study, as this population contains most mature sperm with increased motility parameters and higher normal morphology. One nuclear protein has been identified to be expressed more in the nuclear protein fraction of immature spermatozoa. The protein has been identified as the 78 kDa glucose-related protein.

5.2.3.2.1 Stress-related protein

78kDa glucose regulated protein

78kDa glucose regulated protein (GRP78) is also known as immunoglobulin heavy chain binding protein (BIP'). GRP78 is a stress protein belonging to the Hsp70 multigene family. GRP78 acts as a ''molecular chaperone,'' assisting the correct folding of naïve secretory proteins and preventing the export of damaged proteins from the ER lumen [269]. GRP78 is an inducible protein. Its rate of synthesis increases many fold over the constitutive level in cells subjected to glucose starvation and also in cells exposed to a variety of specific inducers, including both glycosylation inhibitors (like tunicamycin) and agents that perturb intracellular calcium homeostasis.

In the present study GRP78 was more abundant in the immature population compared to the mature population (see Table 9).

The increased expression of GRP78 in the immature population could be related to sperm energy metabolism. The metabolism of cells in the immature fraction is not completely developed therefore external stress such glucose starvation can be detrimental to sperm. It can be postulated that increased expression of GRP78 in the immature population could be a cell response triggered by external stress in which GRP78 plays a protective role in the immature spermatozoa.

Table 9. *Functions of proteins most abundant in the nuclear fraction of immature population compared to the mature population.*

Protein spots	Protein name	Fold change Immature:Mature	Function
	Stress-related protein		
10	78kDa glucose regulated protein	1.510	Probably plays a role in facilitating the assembly of multimeric protein complexes inside the ER

5.2.4 Summary of results: Aim 2

In the second part of this study 21 differentially expressed nuclear proteins were successfully isolated and identified between the immature and mature sperm populations. The majority (95%) of these proteins were less abundant in the immature sperm population compared to the mature sperm population (Table 5). These findings illustrates that nuclear protein expression in the immature sperm population is incomplete.

Functions have been ascribed to these proteins of which only one protein e.g. Tektin 2 was directly related to sperm motility and morphology. The majority of the less abundant nuclear proteins in the immature sperm population were typically stress-related and energy-related proteins. From these findings it can be deduced that the energy metabolism in immature spermatozoa are not fully developed. Therefore the energy metabolism of immature spermatozoa is incomplete and can potentially lead to a decrease in motility and an increase in abnormal morphology.

Figure 23 illustrates the different roles individual nuclear proteins play that was found to be differentially expressed between the immature population and mature populations. Proteins more abundant in the immature population are indicated with (↑) arrows while less abundant proteins are indicated with (↓) arrows.

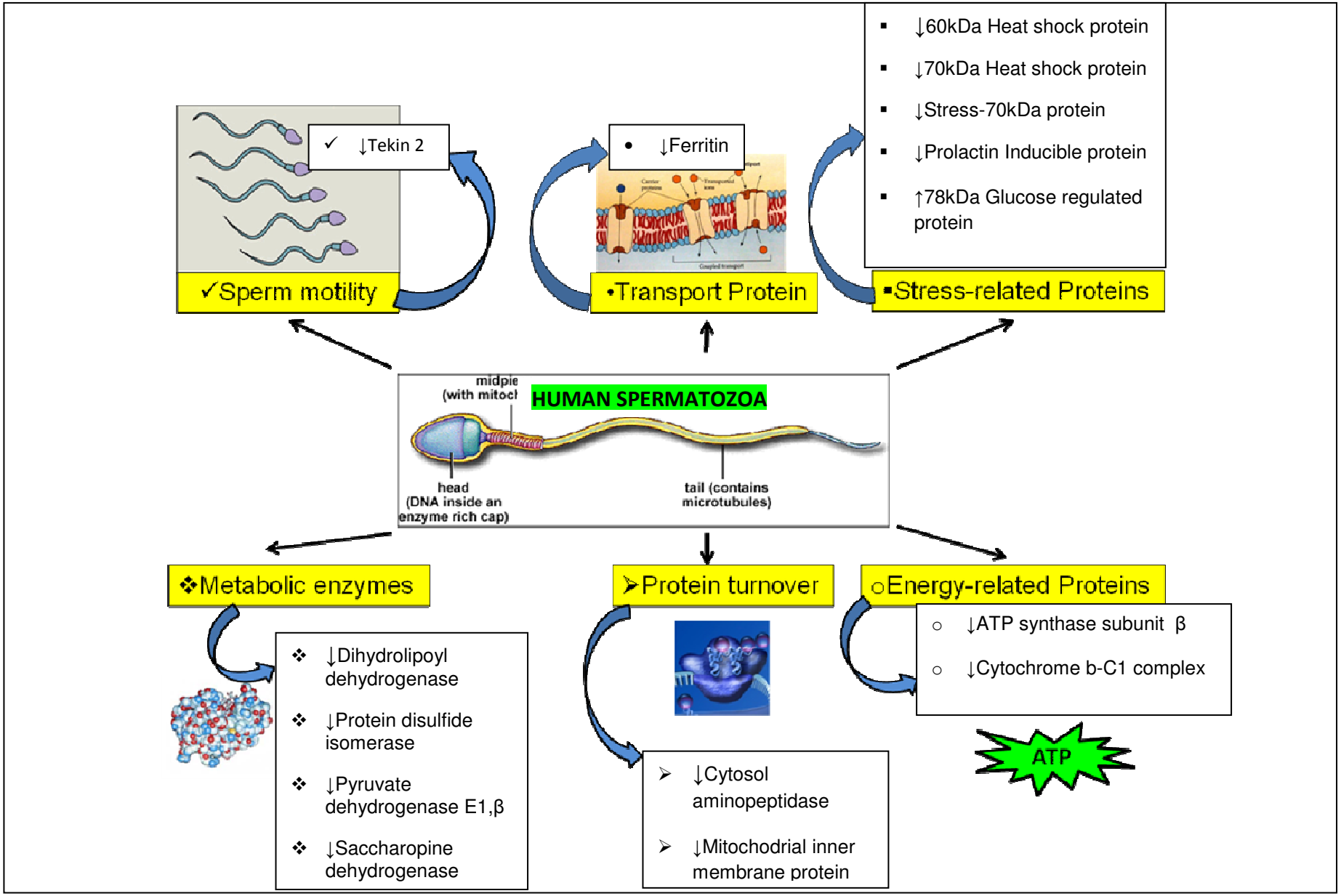

***Figure 23**. Summary of differentially expressed nuclear proteins isolated in study aim 2.*

5.3 Conclusion

It has been shown in this study that there are differences in protein expression between subpopulations of spermatozoa of the same ejaculate, retrieved and grouped according to their motility. The separation grouped the cells into a mature and immature population. The overall morphology characteristics of the mature group were better than that of the immature. In addition to this, differences in nuclear protein expression have been identified between immature populations and mature populations. Specific proteins (e.g. Tektin 2, Outer dense fibre protein 2, Tubulin β-2C chain, AKAP4 and Tubulin α-2C chain) that can potentially lead to a decrease in motility and an increase in abnormal morphology were identified in the immature sperm population.

In conclusion, in the present study, we have successfully isolated functional proteins within the total sperm proteome as well in the nuclear protein fraction of human sperm. The majority of differentially expressed nuclear proteins were found to be less abundant in the immature population compared to the mature population. From these results it can be deducted that nuclear protein expression is incomplete in the immature sperm population, which could have direct influence on sperm motility and morphology. Some of the individual proteins identified play important roles in sperm motility, morphology and energy metabolism and could be crucial in understanding the molecular mechanism involved in sperm dysfunction. Identifying and isolating functional proteins is important for both the management of male infertility and the development of novel approaches to male contraception.

5.4 Future perspectives/Future research

These novel findings can help to not only explain the possible differences that might exist at protein level between fertile and infertile males, but also potentially help target these proteins pharmacologically for the development of a therapy to treat male infertility or act as a basis for the development of a male contraceptive. The isolated proteins i.e. Ferritin, SOD and Glutathione S-transferase can therefore serve as potential bio-markers in the screening of drugs to improve sperm dysfunction. Proteomics is therefore an ideal tool to identify idiopathic causes of male infertility.

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