Gamete proteomic profile of male patients suffering from sexually transmitted infections

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Dissertation presented for the degree of
Doctor of Philosophy (Medical Physiology) in the
Faculty of Medicine and Health Sciences at Stellenbosch University

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March 2016

Declaration

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Abstract

The aim of this study was to provide better insight into the effects of *Neisseria gonorrhoea*, *Chlamydia trachomatis*, and *Trichomonas vaginalis* on semen characteristics. In addition, a crucial focus was also to determine the protein profile of spermatozoa isolated from these infected semen samples. Identification of semen samples positive for bacterial colonisation of *N. gonorrhoea* and *T. vaginalis* were done through the use of differential isolation mediums. For the detection of samples positive for *C. trachomatis*, the ImmunoComb® was employed, which is a quantitative indirect enzyme immunoassay. Macro- and microscopic semen and spermatozoa parameters were assessed, including: volume, pH, viscosity, concentration, motility, morphology, viability, acrosome reaction, leukocyte count, and PMN-elastase concentration. To assess prostate and seminal vesicle functioning, the seminal concentrations of citric acid and fructose were photometrically quantified. The quantification of the level of reactive oxygen species (ROS) production was determined by means of flow cytometry and the DNA fragmentation was detected using a commercially available assay. For proteomic analysis, samples were iTRAQ (isobaric tags for relative and absolute quantification) labelled and underwent liquid chromatography-mass spectrometry, followed by data analysis, protein identification, and quantification.

Results of the study showed that amongst the three microorganisms, the most prevalent occurrence rate in the population tested was that of *T. vaginalis*. The effects of the studied sexually transmitted infections (STIs) on the spermatozoa parameters demonstrate the negative impact of the microorganisms on the fertility outcome of the male partner. The study has shown the relationship between leukocyte-derived ROS and DNA fragmentation, which can significantly impair the fertility outcome. Additionally, the findings of decreased concentrations of fructose in the ejaculates positive for *N. gonorrhoea*, demonstrates the effect of an STI on the glandular functioning of the secretory activity of the seminal vesicles. The observed negative correlation between ROS and DNA fragmentation in samples positive for *N. gonorrhoea* demonstrates the impact of a pro-oxidant overload on spermatozoa DNA integrity. A considerable number of 178 differentially expressed proteins (DEPs) were identified in the STI positive sample groups. The study subsequently focused on

specific proteins according to their role in male fertility. Insight into the role DEPs may play in spermatozoa metabolism and the impact on the motility was provided. Amongst structural related proteins, the down-regulation of outer dense fibre 2, in samples positive for *C. trachomatis*, was evaluated. Oxidative stress related proteins included the up-regulated superoxide dismutase 1 in the ejaculates positive for *N. gonorrhoea* and down-regulated peroxiredoxin 5 in the *C. trachomatis* positive group. From the proteins involved in the response to physiological stress, heat shock proteins was discussed, with the down-regulation of the 70-kDa heat shock protein and the up-regulated HSP 90-kDa-beta member 1, both identified in samples positive for *N. gonorrhoea*. Among immune response proteins, prolactin-inducible protein (*C. trachomatis*) was found to be down-regulated, while azurocidin (*N. gonorrhoea*) and filamin-B (*T. vaginalis*) were up-regulated. Proteins involved in DNA condensation included down-regulated protamine-2 (*C. trachomatis*).

The identification of proteins, which are differentially expressed between spermatozoa from samples positive for STIs, can provide crucial insight into their possible influence on male fertility and role as potential biomarkers for further research. Research on a proteomic and molecular level could allow for the prevention of the long-term obstacles facing partners experiencing compromised fertility. Despite the ongoing research focusing on the sperm proteome, it can be stated with reasonable certainty, that this is the first study into the proteomic profile and corresponding parameters of spermatozoa isolated from semen samples positive for STIs.

Opsomming

Die doel van hierdie studie was om ‘n beter insig te kry rakende die effekte van *N. gonorree*, *C. trachomatis* en *T. vaginalis* op semen eienskappe. ’n Bykomende fokus was ook om die proteïen profiel van hierdie spermatozoa, wat geïsoleer is vanuit die besmette semen monsters, te bepaal. Semen monsters positief vir bakteriële kolonisasie van *N. gonorree* en *T.vaginalis* is geïdentifiseer met behulp van verskillende differensiële isolasie mediums. ‘n Kwantitatiewe indirekte ensiem immunoassay, die ImmunoComb®, was gebruik vir die identifikasie van monsters positief vir *C. trachomatis*. Makro- en mikroskopiese semen en spermatozoa parameters was geassesseer, insluitend: volume, pH, viskositeit, konsentrasie, motiliteit, morfologie, vitaliteit, akrosoom reaksie, leukosiet telling en PMN-elastase konsentrasie. Seminale vesikel en prostaat funksies is deur middel van fotometriese kwantifikasie van die seminale konsentrasies van sitroensuur en fruktose bepaal. Reaktiewe suurstof spesies (ROS) vlakke is met behulp van vloeisitometrie gekwantifiseer en ‘n kommersiële beskikbare toets was gebruik vir die deteksie van DNA fragmentasie. Vir proteomiese analise doeleindes is monsters met iTRAQ (isobariese merkers vir relatiewe en absolute kwantifikasie) gemerk, waarna dit vloeistofkromatografie- massa spektrometrie ondergaan het. Dit is daarna opgevolg deur data analise and proteïen identifikasie asook kwantifikasie.

Die resultate van die studie toon aan dat *T. vaginalis* die mees algemeenste van die drie mikro-organismes was wat voorgekom het in die populasie wat getoets is. Die effek van die bestudeerde seksueel oordraagbare infeksies op spermatosoa parameters dui die negatiewe impak van hierdie mikro-organismes op die fertiliteit van die man aan. Die studie dui ook die verhouding aan tussen leukosiet afkomstige ROS en DNA fragmentasie, wat vrugbaarheid aansienlik kan benadeel. Daarbenewens dui die bevinding van verlaagde fruktose konsentrasies in die ejakulate positief vir *N. gonorree* ook daarop dat hierdie seksueel oordraagbare siekte die sekretoriese aktiwiteit van die seminale vesikels beïnvloed. Die waargenome negatiewe korrelasie tussen ROS en DNA fragmentering, in monsters positief vir *N. gonorree* toon ook die impak van 'n pro-oksidant oorlading op die DNA integriteit van spermatosoa. Die aansienlike getal van 178 differensieel uitgedrukte proteïene (DEPs) is in die STI positiewe groepe geïdentifiseer. Hierdie studie het gevolglik

gefokus op spesifieke proteïene volgens die rol wat hul vervul in manlike vrugbaarheid. Insig in die rol wat DEPs speel in spermatosoë metabolisme en die impak daarvan op motiliteit is gebied. Onder die strukturele verwante proteïene is die af-regulering van buitenste digte vesel 2, in monsters positief vir *C. trachomatis*, bespreek. Oksidatiewe stres verwante proteïene sluit in die op-gereguleerde superoksied dismutase 1 in die ejakulate positief vir *N. gonorree* en die af-gereguleerde peroxiredoxin 5 in die *C. trachomatis* positiewe groep. Van die proteïene wat betrokke is by die reaksie op fisiologiese stres is die volgende hitte-skok proteïene (HSP) geïdentifiseer in monsters positief vir *N. gonorrhee*: die 70-kDa HSP is af-gereguleer terwyl die HSP 90-kDa-beta lid 1 op-reguleer was. Onder die immuunrespons proteïene is die prolaktien-induseerbare proteïen (*C. trachomatis*) bevind om af-gereguleerd te wees, terwyl azurocidin (*N. gonorree*) en filamin-B (*T. vaginalis*) op-gereguleerd was. Proteïene betrokke by DNA kondensasie sluit in die af-gereguleerde protamien-2 (*C. trachomatis*).

Die identifikasie van proteïene wat differensieel uitgedruk word tussen spermatosoa van monsters positief vir seksueel oordraagbare siektes kan noodsaaklike insig verskaf rakende hul moontlike invloed op manlike vrugbaarheid en kan potensieel gebruik word as biomerkers. Navorsing op 'n proteomiese en molekulêre vlak kan voorsiening maak vir die voorkoming van die langtermyn-struikelblokke vir lewensmaats wat vrugbaarheidsprobleme ervaar. Ten spyte van die voortdurende navorsing wat fokus op die sperm proteoom, kan dit met redelike sekerheid gestel word dat dit die heel eerste omvattende studie is met betrekking tot die proteomiese profiel en ooreenstemmende parameters van spermatosoa geïsoleerd vanuit semen monsters positief vir seksueel oordraagbare siektes.

Dedication

To my parents Keith and Angela, for their constant support and immense kindness. I am incredibly privileged to have such wonderful parents!

To Dr Florian Boehlandt, an exceptional man. For his patience and laughter when much needed.

Acknowledgements

Professor Stefan Du Plessis and Emeritus Professor Gerhard van der Horst, for their academic insight, consistent support and greatly appreciated optimism.

Division of Medical Physiology, in particular the Stellenbosch University Reproductive Research Group, for all the assistance and many happy memories.

The NRF for my Academic Scholarship and the Harry Crossly Foundation for the financial assistance towards the research expenses.

Dr Marè Vlok, (Central Analytical Facility) and Dr Suzelle Hattingh (Division of Medical Physiology) for their expertise in proteomics. Professor Kidd for his statistical assistance.

South Africa-Netherlands (SANPAD) PhD program (2012-2013), for the guidance and leadership.

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Abbreviations:

AIDS	Acquired immune deficiency syndrome
AKAP3	A-kinase anchor protein 3
ALH	Amplitude of lateral head displacement
AR	Acrosome reaction
ART	Assisted Reproductive Technique
ASA	Antisperm antibody
ASG	Accessory sex gland
AT	Agglutination test
ATP	Adenosine-5'-triphosphate
BCF	Beat-cross frequency
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$C0_2$	Carbon dioxide
CABYR	Calcium-binding tyrosine phosphorylation-regulated protein
cAMP	Adenosine-3', 5'-cyclic monophosphate
CASA	Computer assisted sperm analysis
CID	Collision-induced dissociation
CFU	Colony forming units
cP	Centipoise

C. trachomatis	*Chlamydia trachomatis*
DCF	Dichlorofluorescein
DCFH-DA	2, 7 Dichlorofluorescein diacetate
DEP	Differentially expressed protein
DFA	Direct fluorescent antibody assay
dH_20	Distilled water
DHE	Dihydroethide
DIBA	Dot-immunobinding assay
DIGE	Differential in-gel electrophoresis
DNA	Deoxyribonucleic acid
EB	Elementary body
EDTA	Ethylene diaminetetra acetic acid
ELISA	Enzyme-linked immunoabsorbant assay
F(ab')	Antibody fragment
FDR	False-discovery-rate
FITC	Fluorescein isothiocyanate
GST	Glutathione S-transferase
H^+	Hydrogen
H_20_2	Hydrogen peroxide
HAMS	Human albumin serum

HCl^-	Hydrochloric acid
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSP	Heat shock protein
ICAT	Isotope-coded affinity tags
IEF	Isoelectric focusing
Ig	Immunoglobulin
iTRAC	Isobaric tags for relative and absolute quantification
IVF	In-vitro fertilisation
kDa	Kilodalton
LC	Liquid chromatography
LIN	Linearity index
MACS	Magnetic activated cell sorting
MAGI	Male accessory gland infection
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
mg	Milligram
MGT	Male genital tract
ml	Millilitre
MMTS	Methylthiosulphonate
Mr	Molecular weight

mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS	Mass survey
m/z	Mass/charge ratio
N^+	Nitrogen
NAA	Nucleic acid amplification
$NaCl^-$	Sodium chloride
NADH	Nicotinamide adenine dinucleotide-dependant oxidoreductase
NADPH	Nicotinamide adenine dinucleotide phosphate-dependant oxidase system
ng	Nanogram
N. gonorrhoea	*Neisseria gonorrhoea*
NGU	Nongonococcal urethritis
nm	Nanometre
0_2^-	Superoxide anion
OD	Optical density
ODF2	Outer dense fiber 2
OH^-	Hydroxyl radical
OS	Oxidative stress
P	Protamines
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
pI	Isoelectric point
PI	Propidium iodide
PKA	Protein kinase A
PMN	Polymorphonuclear
pmol	Picomole
PS	Phosphatidylserine
PSA	Pisum sativum agglutinin
PSA	Prostate specific antigen
PTM	Post-translational modification
PUFA	Polyunsaturated fatty acid
r	Correlation coefficient
RBU	Reproductive Biology Unit
RNA	Ribonucleic acid
$ROO^{\cdot}$	Peroxyl radical
ROS	Reactive oxygen species
rpm	Revolutions per minute
SCA®	Sperm Class Analyser
SDS	Sodium dodecyl sulphate

SEM	Standard error of the mean
SPE	Solid-phase extraction
STI	Sexually transmitted infection
STR	Straightness index
SURRG	Stellenbosch University Reproductive Research Group
TBH	Tygerberg Hospital
TCA	Tricarboxylic acid cycle
TCEP	Tris-(2-carboxyethyl) phosphine
TdT	Terminal deoxynucleotidyl transferase
TEAB	Triethylammonium bicarbonate
TFA	Trifluoroacetic acid
TMB	Tetramethyl-benzidine
TUNEL	Terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end labeling
T. vaginalis	*Trichomonas vaginalis*
UTI	Urinary tract infection
V	Voltage
VAP	Average path velocity
VCL	Curvilinear velocity

VSL	Straight line velocity
WBC	White blood cell
WHO	World Health Organisation
WOB	Oscillation index

Chapter 1: Introduction

1.1 Background

Infertility, which is regarded as "the inability to achieve pregnancy after one year of regular, unprotected sexual exposure" (Kruger and Van der Merwe, 2010) is a growing concern in the field of reproductive physiology. Various factors contribute towards sub-fertility and failed fertilisation rates can be attributed to numerous conditions in both partners. However, it has been estimated that the male partner is an important determinant in decreasing the possibility of a successful pregnancy. Within the male genital tract (MGT), the presence and subsequent infection from microorganisms such as *Neisseria gonorrhoea*, *Chlamydia trachomatis*, and *Trichomonas vaginalis* can result in high concentrations of leukocytes, a condition frequently observed in fertility clinics. One of the predominant contributing factors to idiopathic male infertility has been shown to be the imbalance arising between oxidants and antioxidants, in favour of the former, resulting in oxidative stress (OS), as the levels of reactive oxygen species (ROS) highly exceeds the concentration required for normal physiological functions. Spermatozoa are particularly susceptible to OS, primarily due to the plasma membrane's high concentrations of polyunsaturated fatty acids (PUFAs), which have a subsequent negative impact on the male fertility status through compromised spermatozoa parameters and DNA damage.

In the field of proteomics, studies which are focusing on the human spermatozoa proteome is a recent avenue of research. This particular cell type is highly compatible to proteomic analysis as it is abundant and can be isolated and purified with relative ease. The identification of proteins allows for the subsequent assignment of each protein to a specific biological pathway, utilising a bioinformatics tool. This enables for proteins to be grouped with regards to their corresponding functional pathways, such as; metabolism, immune regulation and DNA condensation among others

1.2 Motivation of the study

Annually, a significant number of males seeking fertility assessment attend the Reproductive Biology Unit (RBU) at Tygerberg Hospital (TBH) for primary, secondary, or idiopathic infertility. Amongst a demographic profile that faces a broad spectrum of social challenges such as unemployment and poor access to health care services, there is a need for a comprehensive study into the effects of sexually transmitted pathogens on the male reproductive system. Regardless of previous male fertility research having been conducted at the RBU, and within the South African context, it appears that no previous studies have been focused on the detection of proteins of spermatozoa, which are differentially expressed amongst this particular subset of male subjects presenting with sexually transmitted infections (STIs).

1.3 Outline of the study

Chapter 2 will provide a background of the three microorganisms that were investigated in the study i.e. *N. gonorrhoea, C. trachomatis* and *T. vaginalis*. A comprehensive literature review will focus on the effects of the presence of the aforementioned bacteria within the MGT, with a description of the effects on the male reproductive status. A background into the field of proteomics shall be described and its application in fertility based studies. Chapter 3 includes the protocols employed in the experimental approaches. Chapter 4 encompasses the statistical analysis of the results, with the corresponding graphs and tables. Chapter 5 is the discussion of the study's findings, with a focus on the interpretation and explanation of the results and a description of the study's proteins according to their cellular function. The study is concluded in the sixth chapter. The appendix contains addenda of specific buffers and reagents, the results of the correlation analysis, as well as a summary table of the differentially expressed proteins (DEPs).

1.4 Aims and objectives

The aim of this study was to provide better insight into the effects of *N. gonorrhoea, C. trachomatis,* and *T. vaginalis* on semen characteristics. In addition, a crucial focus was to determine the protein profile of

spermatozoa isolated from the infected semen samples. Specifically, the objectives of this study are the following:

Objective 1:

Determine the prevalence of N. gonorrhoea, C. trachomatis, and T. vaginalis isolated from semen samples.

Objective 2:

Examine the effects of N. gonorrhoea, C. trachomatis and T. vaginalis, in comparison to the control group, on the standard semen parameters which includes; pH, volume, leukocyte count, viscosity and polymorphonunclear elastase concentration, and the spermatozoa parameters including; motility, concentration, morphology, viability and acrosome reaction.

Objective 3:

Investigate the impact of infection on the generation of ROS and the possible impact on the spermatozoa's DNA.

Objective 4:

Investigate the effect of N. gonorrhoea, C. trachomatis and T. vaginalis on the secretory function of the prostate and seminal vesicles in comparison to the control, determined through the quantification of seminal plasma citric acid and fructose concentrations.

Objective 5:

Determine the differentially expressed proteins in the spermatozoa isolated from samples positive for N. gonorrhoea, C. trachomatis, and T. vaginalis.

1.5 Conclusion

The substantial occurrence of STIs of within Sub-Saharan Africa is a growing concern and with the increasing number of couple's experiencing compromised fertility, there is an important need for research into the effect of infection of the male carrier. A comprehensive study into the effects that common STIs such as *N. gonorrhoea, C. trachomatis* and *T. vaginalis* may elicit on the spermatozoa's parameters, will allow for a more detailed understanding of the impact of an infection on the male gamete's functional capacity. A comprehensive assessment of the levels of ROS in the ejaculates positive for STI pathogens can allow for an insight in to the possible impact on the spermatozoa's DNA integrity. Additionally, an evaluation of the secretory products of the prostate and seminal vesicles, the predominant contributors to the ejaculate, offers insight into the effects of bacteria on the male ASGs. Proteomics offers an avenue of insight into the field of male reproductive biology beyond the standard investigations into the causative factors for the increasing number of men experiencing compromised fertility. The proteomic analysis of spermatozoa isolated from semen samples that are positive for an STI, offers the possibility of crucial understanding into the sperm-specific proteome, which is a novel and compelling field of research. The identification and discussion of the proteins, which are differentially expressed amongst *N. gonorrhoea, C. trachomatis* and *T. vaginalis* can allow for a more comprehensive understanding of the complex array of differences, which contribute to compromised fertility.

Chapter 2: Literature Review

2.1 Sexually transmitted infections

The World Health Organisation (WHO) published a report in 1999 which stated that globally, an estimated 340 million new cases of curable STIs were reported per annum (WHO, 1999), which was followed by an estimated 448 million new cases in 2005 (WHO, 2005). Amongst the viral and bacterial microorganisms behind the considerable quota of infection rates, *N. gonorrhoea, C. trachomatis,* and *T. vaginalis* were recognised in the top five. In developing countries such as South Africa, there are additional factors, which can lead to an increased rate of transmission of STIs amongst partners. Examples of such have been recognised, which include: multiple sexual partners (Helms *et al*., 2008), a lack of accessible healthcare facilities (Sena *et al*., 2007), as well as financial limitations experienced by a high portion of the population, which limits the accessibility to medical assistance (Pham-Kanter *et al*., 1996). Despite literature reporting a wide range of values when the prevalence of STIs is examined, the properties of the studied populations must be taken into consideration. In addition, the lack of laboratory resources, which can allow for the identification of STIs in resource-poor regions, can exacerbate the impact of the infection on the patient (Sena *et al*., 2007).

The predominant pathogens behind occurrences of STIs amongst the male partner can be summarised as the following: bacteria (*N. gonorrhoea, C. trachomatis, Treponema pallidum);* viruses (human immunodeficiency virus, mumps virus, human papillomavirus); protozoa's (*T. vaginalis*) and yeasts (*Candida albicans*) (Ochsendorf *et al*., 2008). Research into the possible causative effects of certain bacteria species present in the MGT on the fertility status of the subject has resulted in conflicting opinions (Fraczek *et al*., 2004), despite studies which have shown that 60% of infertile men presented semen samples with bacterial strains (Lacroix *et al*., 1996). Furthermore, this finding was substantiated, whereby it was shown that seminal cultures positive for specific bacterial species contained spermatozoa with decreased concentration, motility, and abnormal morphology (Gardner *et al*., 1996; Nunez-Calonge *et al*., 1998). This opened an avenue of study into the negative effects of microorganisms on spermatozoa parameters, which

have been found to be the following: reduced motility; contrasting morphology and decreased acrosomal reactions (Gdoura *et al*., 2007; Golshani *et al*., 2007).

2.1.1. *Neisseria gonorrhoea*

N. gonorrhoea, a Gram-negative diplococci, is an obligate human pathogen (Haizlip *et al*., 1995). This bacterium is the most prevalent microorganism behind acute urethritis (Pham-Kanter *et al*., 1996), causing urethral secretions, and agonising dysuria (Bar-Chama and Fisch 1993). Amongst sexually active men, the infection rates are more prevalent in emerging population groups (Taylor-Robinson *et al*., 2001). A study published in 2007, which concentrated on the prevalence of co-infection between *N. gonorrhoea, T. vaginalis* and *C. trachomatis* amongst a population group of 253 men in the Pretoria region, showed that 110 (43.5%) of the subjects presenting with symptoms of urinary tract infections (UTI's) were suffering from *N. gonorrhoea* (De Jongh *et al*. 2007).

Within South Africa, the frequency of gonococcal urethritis has been found to be prominently different between caucasian and black men (Pham-Kanter *et al*., 1996). When considering the significant difference between certain racial groups and the prevalence of *N. gonorrhoea*, an important component to consider is the socioeconomic status. Within the South Africa context, the predominant racial group of black and coloured citizens also represent the sector of the population that are constrained by financial and other limitations which do not always promote adequate access to health facilities (Pham-Kanter *et al*., 1996). In 1998, research conducted by the Centre for Epidemiological Research at the South African Medical Research Council found that within STI clinics in the Cape Town area, *N. gonorrhoea* was the most reported infection amongst the 171 male subjects included in the study (Mathews *et al*., 1998).

In 1964, there was a highlighted need for the introduction of an improved laboratory approach for the diagnosis of *N. gonorrhoea*, which culminated in the development of the selective Thayer-Martin agar, which allowed for a high degree of specificity when used to isolate *N. gonorrhoea* (Thayer and Martin,

1964). The medium consists of a Mueller-Hinton agar, enriched with 5% chocolate blood agar, as well as the antibiotic cocktail consisting of the following compounds: vancomycin, colistin and nystatin, allowing for the specific isolation of *N. gonorrhoea* growth.

2.1.2 *Chlamydia trachomatis*

C. trachomatis has been recognised for many years as the most prevalent bacterial pathogen and a substantial causative factor behind compromised fertility in both partners (Gallegos *et al*., 2008). The spherical obligate Gram-negative bacterium is predominantly asymptomatic in nature, which heightens the rate of transmission between partners, as between 50-80% of men and women suffering from the condition present asymptomatically (Zimmerman *et al*., 1990; Marrazzo *et al*., 1998). In 1978, the damaging effect of *C. trachomatis* on the male reproductive potential was introduced when a study showed the bacteria to be a common causative factor of infection of the ASGs (Berger *et al*., 1978). It is now established that *C. trachomatis* accounts for approximately 50% of all aetiological cases of urethritis (Oriel *et al*., 1992) and can result in prostatitis and/or epididymitis (Sakka *et al*., 2005; Gallegos *et al*., 2008). Within the field of male reproductive biology, research has been conducted on *C. trachomatis* and the subsequent effects elicited on the fertility status of both sexes. The obligate parasite, capable of replicating within eukaryotic cells (Satta *et al*., 2006) requires a host cell to reproduce and is characterised as infectious by an elementary body (EB) which binds to receptors within the host cell (Cevenini *et al*., 2002). The pathogen has been referred to as a "bacterial hitchhiker" owing to its ability to attach to spermatozoa, which allows for *C. trachomatis* to be simply transferred to the female reproductive tract following ejaculation during sexual intercourse (Eggert-Kruse *et al*., 1996). *C. trachomatis*, in conjunction with *N. gonorrhoea*, has been isolated as a causative factor behind the following: opthalmia neonatorum, a condition of conjunctivitis, contracted by the newborn when passing through the birth canal, as well as neonatal pneumonia (Peeling *et al*., 2005). Amongst women, the bacteria are known to cause pelvic inflammatory disease and tubal damage, as well as increase the risk of ectopic pregnancies (Close *et al*., 1987; Peeling *et al*., 2005), which has been suggested to be as a result of the female immunoreaction (Eggert-Kruse *et al*., 1996).

2.1.3 *Trichomonas vaginalis*

The site specific flagellated protozoan *T. vaginalis* ranges between 2-14 µm in width and 10-20 µm in length and is localised to the genitourinary system, whereby it predominantly infects the squamous epithelium (Swygard *et al*., 2004). In the infected male subject, the STI can cause prostatitis, urethritis, as well as testicular sensitivity and painful ejaculation (Bar-Charma and Fisch 1993). In addition, *T. vaginalis* has been recognised as a contributor to male factor infertility though the inflammatory reaction of the MGT (Lloyd *et al*., 2003). An overwhelming incidence of approximately 174 million new cases of *T. vaginalis* has been reported annually (WHO, 2001), of which 154 million cases were localised to resource-limited settings (Johnson *et al*., 2008). Amongst sexually active couples, the infected male partner has been considered "important as vectors for transmission of the disease" (Krieger *et al*., 2003), due to the predominantly asymptomatic presentation in men. A study in 2006 (Hobbs *et al*., 2006) which investigated the semen and urine samples from the partners of woman suffering from *T. vaginalis*, found that 72% of the men whom were positive for the STI remained asymptomatic.

For the female partner, *T. vaginalis* cytopathogenicity can result in pelvic inflammatory disease, as well as affecting the pregnancy outcome as the STI has been found to cause preterm delivery (Pastorek *et al*., 1996; Cotch *et al*., 1997; Minkoff *et al*., 1999). The techniques available for the routine diagnosis of *T. vaginalis* infection have progressed significantly over the past decades. A study conducted in 1976 at hospitals and family planning clinics in Johannesburg, Gauteng, South Africa, utilised only staining methods such as Giemsa and Papanicolaou for the detection of *T. vaginalis* (Mason *et al*., 1976). However, a considerable number of techniques are now available to detect the organism, which includes chocolate agar, isolation mediums, enzyme linked immunoabsorbant assays (ELISA) and PCR (Patel *et al*., 2000), thereby allowing for a more comprehensive diagnosis.

2.2 Leukocytospermia

Symptomatic and asymptomatic leukocytospermia ($\geq 10^6$ white blood cells (WBCs)/ml) is a condition frequently observed in infertility clinics (Arata de Bellabarba *et al*., 2000; Kaleli *et al*., 2007). The condition, also termed pyosaemia, leukospermia or pyospermia (WHO, 2010), can be indicative of infection within the MGT (Kokab *et al*., 2010). This negative effect on the male's fertilizing potential can be a result of the direct correlation which is found between increased concentrations of leukocytes and chromatin alterations and morphological abnormalities (Alvarez *et al*., 2002), all of which can result in a degree of compromised male fertility (Aitken *et al*., 1992; De Lamirande *et al*., 1995). A trend was observed amongst 162 patients attending the RBU for fertility assessments with 31% of the subjects suffering from WHO-defined leukocytospermia (Flint, *et al*., 2013). Subsequent to infection is the production and activation of granulocytes known as polymorphonuclear (PMN) leukocytes, which release a particular protease termed elastase (Eggert-Kruse *et al*., 2009). The infiltration and degranulation of PMN leukocytes can be utilised in studies to quantitatively determine the concentrations of elastase as a marker for infection and leukocytospermia (Ricci *et al*., 2000).

2.2.1 Generation of ROS

The action of metabolising oxygen results in the formation of highly reactive agents, known as ROS (Cocuzza *et al*., 2003). The group of free radicals, which can be described as "short-lived atoms or molecules that contain one or more electrons with unpaired spin" (Ochsendorf *et al*., 1999), are natural by-products of physiological actions (Cocuzza *et al*., 2007; Makker *et al*., 2009). Within the context of molecular reproductive biology, the following are the recognised predominant molecules: peroxyl radical (ROO^-), hydrogen peroxide (H_20_2), superoxide anion (0_2^-) and the hydroxyl radical (OH^-) (Maneesh and Jayalekshmi, 2006). With ROS being regulated at a stable concentration with continuous inactivation (Saleh *et al*., 2002; Cocuzza *et al*., 2007), the free radicals act as a bio-positive influence, however, upon an increase in the oxidising agents and the absence of inactivation, ROS can result in the removal of hydrogen (H^+) (Fariello *et al*., 2009). This can cause damage to a wide range of biomolecules and elicit a state of OS (Saleh *et al*., 2002a; Cocuzza *et al*., 2007). The compounding factor for the increased risk of OS is the wide variety of

cellular components which are sensitive to supra-physiological concentrations of ROS. This includes; proteins, nucleic acids and lipids. Various conditions can result in an increase in cellular ROS, such as; a decrease in antioxidants, elevated cellular metabolism, as well as inflammation (Ochsendorf *et al*., 1999).

Spermatozoa were the first cell type in which the production of ROS was studied and described (MacLeod J, 1943). Numerous states of infertility in the male reproductive system have been attributed to influences of OS, such as prostatitis, spinal cord injury and varicocele (Sharma *et al*., 2001). The resultant state of OS can result in a variety of impacts on spermatozoa functioning, for example; motility, sperm hyperactivation, fusion with the oocyte, capacitation and the acrosome reaction (Athayde *et al*., 2007). Research which compared the concentrations of ROS between four sample cohorts, found teratospermic, oligospermic and asthenospermic subject groups to have significantly increased seminal ROS levels when compared to the normozoospermic cohort (Sharma *et al*., 1996). The high concentration of polyunsaturated fatty acids (PUFAs), primarily docosahexaenoic acid, within the sperm plasma membrane, renders spermatozoa to be more susceptible to lipid peroxidation by the aforementioned free radicals (Ochsendorf *et al*., 1999; Agarwal *et al*., 2005; Aydemir *et al*., 2008). One such free radical, H_20_2, has been isolated as the compound with the most toxicity towards spermatozoa (Agarwal *et al*., 2004; Aitken *et al*., 2007; Makker *et al*., 2009). The resultant peroxidative damage can cause the sperm plasma membrane to permeabilise, which compromises the spermatozoa's functional capacity for fertilisation (Aitken *et al*., 1992; Saleh *et al*., 2002a; Agarwal *et al*., 2004).

2.2.2 Oxidative insult on spermatozoa DNA

When assessing the reproductive potential of the male partner, the analysis of sperm DNA integrity offers a comprehensive insight beyond the parameters established by the WHO (Shamsi *et al*., 2011). More than a quarter of men who are experiencing compromised fertility, are suffering from idiopathic infertility (Sharma *et al*., 2001) and it has been estimated that sperm DNA fragmentation was a causative factor in 20 percent of these cases (Nakada *et al*., 2006). Despite spermatozoa DNA being remarkably resilient against denaturation

from chemical or physical influences, strand breaks within DNA is indicative of a decline in the functional capacity (Philpott *et al*., 1992) and even levels of OS that are undetectable in semen can be responsible for altered condensation of spermatozoa DNA (De Lamirande *et al*., 1997). The isolation of the specific point whereby sperm DNA damage is induced is challenging, as it can occur throughout the development process of spermatogonial germ cells to the sperm that are ejaculated (Shamsi *et al*., 2011).

Mitochondrial DNA damage, as a result of OS, is a comprehensively studied occurrence in all aerobic cells (Mannesh *et al*., 2006). In comparison to the somatic cell, the male gamete's nuclear volume is 40 times less, due to the high compaction of this genetic material (Ward *et al*., 1991). This dense arrangement of DNA within spermatozoa offers a degree of protection against oxidative insult (Mannesh *et al*., 2006). However, OS can result in an excessive occurrence of single and double strand DNA breaks (Twigg *et al*., 1998a; Aitken *et al*., 2001), chromosomal rearrangements (Duru *et al*., 2000), chromatin cross linkage and DNA base oxidation (Kullisaar *et al*., 2007). Although ROS can be produced in minimal quantities by spermatozoa upon capacitation, the amount generated by PMN granulocytes during phagocytosis, is 1000 times higher (De Lamirande *et al*., 1997).

2.2.3 Result of infection on the accessory sex glands

Bacterial colonisation of the MGT can result in chronic infection of the ASGs, which includes the seminal vesicles and prostate, with the latter being recognized as the ASG which is primarily targeted by inflammatory conditions (Wolff *et al*., 1998). The assessment of the concentrations of the components released from the ASGs can allow for the identification of a possible infection (Lewis S, 2007). The predominant compound released by the prostate is citric acid and upon infection, the levels of the acid decrease in the ejaculate (Comhaire *et al*., 1999). Inflammation of the prostate can be categorized into five conditions: asymptomatic- inflammatory-, acute bacterial-, chronic bacterial- and achronic-prostatitis (Nieschlag and Behre, 2000). In order for the assessment of prostate functioning, spectrophotometric

quantification of the levels of seminal citric acid in the semen can be measured and the normal physiological level is considered 52 µmol (9.36 mg) or more per ejaculate (WHO, 2010).

The second most prevalent contributor, are the secretory products of the seminal vesicles, which includes; ascorbic acid, prostaglandins and fructose. The latter being the predominant compound, which is released from the secretory cells lining the epithelium (Heath and Young, 2000). In the state of an infection, the onset of inflammation can result in a state of atrophy of the epithelial layer and a resultant decrease of the concentration of fructose is observed in the ejaculate (Lu *et al*., 2007). Subsequently, spermatozoa motility can be compromised, as the monosaccharide is a significant reservoir for glycolytic energy (WHO, 1992). Per ejaculate, 13 µmol (2.34 mg) or more is considered by the WHO as the normal reference value based on the research by Cooper *et al*. (Cooper *et al*., 1991).

2.3 Proteomics

Proteomics was termed by Marc Wilkins following his research allowing for a method of identifying the expression of individual proteins (Wilkins *et al*., 1996). The broad definition of proteomics can be stated as "the study of the proteome: the collection of all the proteins expressed from the genome in all isoforms, polymorphisms and post-translational modifications (PTM's)" (Graham *et al*., 2005). Proteins found in the seminal plasma were first published in 1888, whereby a study utilising urine as a specimen, isolated the compound propetene, which is a combination of compounds resulting from proteolytic digestion. The protein was traced back to the contamination of the urine sample with semen. The fact that hundreds of proteins can be detected simultaneously, allows for proteomics being a highly sensitive and specific means of analysing modified protein profiles (Fung *et al*., 2004). Research into the quantitative and qualitative protein profile of cells has advanced significantly over the past three decades (Du Plessis *et al*., 2011), with two fields of focus in reproductive biology having been isolated: deciphering the cause behind male infertility and developing a comprehensive framework in understanding human reproduction (De Mateo *et al*., 2007). An example of proteomics within the context of the male reproductive system was the isolation of the prostate-specific

antigen (PSA) from semen (Rao *et al*., 2008). Cancer of the prostate has fast become a growing concern in the medical community due to both the increasing incidence of diagnosis, as well as the mortality rate in men over the age of fifty years (Pienta and Esper, 1993). PSA is phenotypically expressed by the luminal secretory cells of the prostate into the seminal fluid (Lalani *et al*. 1997). PSA plays a role in the degradation of the coagulated semen following ejaculation and acts as a thorough indicator of malignant prostate tissue (Drabovich *et al*., 2014). In comparison to levels found in the blood serum (1.2 mg/ml), the concentration of PSA isolated in the seminal plasma is significantly higher (4 ng/ml), a 300,000-fold increase (Catalona *et al*., 1991; Wang *et al*., 1998).

2.3.1 Proteomic approaches

Within the field of proteomics, there are several techniques available for identifying proteins, as shown summarised in Table 1. All of which differ in their technical approach and include the following: mass spectrometry (MS), differential in gel electrophoresis (DIGE), matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and 2D polyacrylamide gel electrophoresis (2D-PAGE) (Du Plessis *et al*., 2011). The latter procedure was introduced in the 1970's (O'Farrell, 1975) and has become important in the study of proteomics, as it offers a high degree of efficacy in assessing both the molecular weight (Mr) and the charge of the proteins when isolated from the surrounding matrix (Xu *et al*., 1994). A comprehensive study of sperm proteins was published in 1997 (Naaby-Hansen *et al*., 1997), whereby 2D-PAGE analysis created a database of 1397 protein spots. Varying techniques are used in compiling the protein profile of both sexes' reproductive organs, tissues and secretions, as well as the blastocyst *(Table 1).*

Table 1: Proteomic tools used in the study of reproductive samples in male and female tissues

Proteomic technique	Male	Female
2-DE	Testis; Epididymis; Sperm	Uterine fluid
DIGE	Testis; Epididymis; Sperm	Endometrium
MALDI-TOF	Testis; Sperm	Endometrium
LC-MS	Testis; Epididymis; Sperm	Uterine fluid; Endometrium

2.3.2 Proteomic investigations in male fertility

Within the rapidly evolving field of proteomics, the establishment of extensive databases of thousands of proteins specific to the spermatozoa can allow for a research into male fertility to extend to scenarios such as diagnostics and the subsequent treatment modality (Oliva *et al*., 2009). Research, which has focused on the sperm specific protein profile, has constructed the extensive list of housekeeping proteins, which contribute to glycolysis and oxidative phosphorylation, which are crucial metabolic pathways (Johnston *et al*., 2005). In addition to the housekeeping proteins, past studies have elucidated intricate network of receptor molecules, which are localised on the surface of the spermatozoa, for example; tyrosine kinase receptors (Baker *et al*., 2007).

A significant component of studying spermatozoa proteomics is the establishment of a protein profile of the normal physiological state. This can allow for determining whether compromised spermatozoa are because of post-translational processing of proteins unique to the cell type (Aitken and Baker 2007). The development of spermatozoa to reach the functional capacity required for fertilisation requires the transcription and translation of specific proteins. Despite spermatozoa being highly specialised cells, they lack an extensive system to compensate for protein damage that may result from influences in their microenvironment (Duncan and Thompson 2007), as well as extensive exchange they undergo when passing

through both the male and female genital tracts during ejaculation (Oxenham *et al*., 2010). With advancements into proteomic research focusing on the sperm-specific proteome, there is the possibility of crucial understanding into spermatozoa in varying states such as OS (Du Plessis *et al*., 2011) and the correlations of certain proteins with factors such as spermatozoa DNA integrity (De Mateo *et al*., 2007). Amongst the set, protamine's (P), P1 and P2 remain the predominantly investigated nucleoproteins in mature sperm (Balhorn *et al*., 2007). An altered expression of the P1/P2 ratio has been found to be a signature of infertility in past studies (De Yebra *et al*., 1993; Mengual *et al*., 2003). A substantial study into protamine concentrations (P1, P2 and total protamine composition) revealed that sperm with diminished levels of the nuclear proteins have increased DNA fragmentation (Aoki *et al*., 2005). The findings of the study initiated the protective role that the proteins could elicit of the DNA integrity in the spermatozoa cells.

Chapter 3: Materials and Methods

3.1 Introduction

The following chapter will describe the experimental methods and protocols used in this study. Appendix A provides a list of the solutions and buffers.

3.2 Semen collection

Prior to the commencement of the study, ethical approval was obtained from the Health Research Ethics Committee (HREC) of the Faculty of Medicine and Health Sciences, Stellenbosch University (S12/11/307). The semen samples originated from two sample cohorts (n=120). The first sample group consists of patients referred to the RBU at TBH for a routine spermiogram to assess male factor fertility (n=100), according to WHO guidelines (WHO, 2010). The second included volunteer donors aged 18-26, taking part in the sperm donor program at the Stellenbosch University Reproductive Research Group (SURRG) (n=20).The volunteer donors were included in the study in order to observe the possible rate of STI occurrence rates amongst the cohort.

From this total number of samples (n=120), the four sample groups were identified following microbiological assessment and divided according to the following cohorts: *N. gonorrhoea* positive (n=19); *T. vaginalis* positive (n=25); *C. trachomatis* positive (n=11); and the control group (n=65). The control group consisted of samples from both cohorts, which were negative for the three aforementioned STIs. An exclusion criterion for the control group was a sample that was positive for leukocytospermia ($>1x10^6$ WBC/ml) (WHO, 2010). All samples that were identified as polymicrobial (WHO, 2005) thus positive for two or more microorganisms under investigation were omitted from the study as an exclusion criterion. Samples were collected in accordance to the WHO guidelines (WHO, 2010), by means of masturbation into a sterile wide mouth plastic container following a 2-3 day period of sexual abstinence and were left in an incubator (Heal Force® Smart Cell $C0_2$, Nison™, Shanghai, China), to undergo liquefaction (37°C, 5% $C0_2$, 30 min) prior to further analysis.

Objective 1: *Determine the prevalence of N. gonorrhoea, C. trachomatis and T. vaginalis isolated from semen samples.*

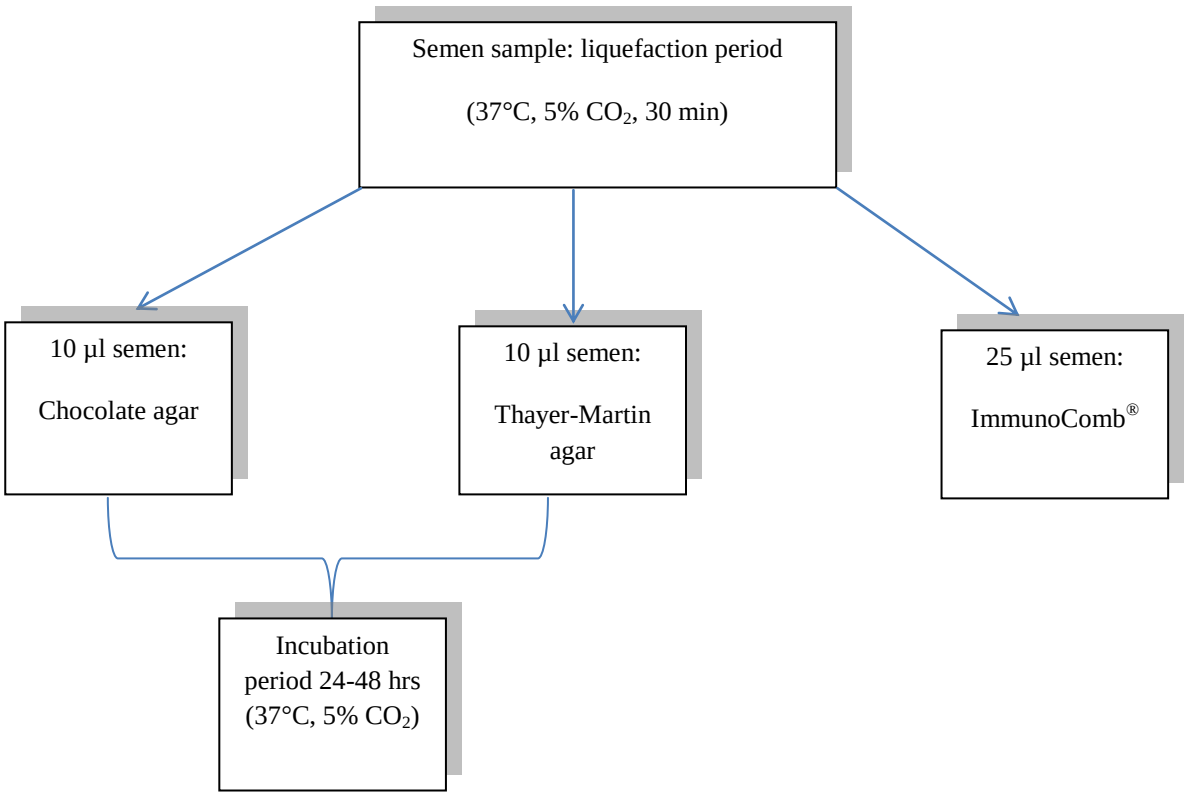


Figure 1: Flow chart of the simplified experimental procedure for objective 1

3.3 Differential isolation media

Classification of semen samples positive for bacterial colonisation of *N. gonorrhoea* and *T. vaginalis* was implemented through the use of the following differential isolation media: Thayer-Martin agar (MediaMage™, Gauteng, South Africa) and chocolate agar (Bio-Rad™ Laboratories, California, USA). Following liquefaction (37°C, 5% $C0_2$, 30 min), a portion of the semen sample was plated using a disposable plastic loop (10 µl) (Nunc™, Thermo Scientific, Denmark). The plates were then placed in an incubator (37°C, 5% $C0_2$) and left to culture for a 24-48 hour period. Following the incubation period, the media was examined macroscopically. The classification of semen samples as positive for bacterial species was defined with a culture of >1 x 10^3 /ml (colony-forming units (CFU)/ml), which is equivalent to one observed colony (WHO, 2013).

3.4 Enzyme immunoassay

For the identification of samples positive for *C. trachomatis*, the ImmunoComb® (Orgenics, Yvane, Israel) was used, which is a quantitative indirect enzyme immunoassay for the qualitative determination of IgA antibodies to *C. trachomatis* in semen. Anti-*C. trachomatis* IgA antibodies detect the active status in acute chronic and recurrent *C. trachomatis* infection (Clad *et al*., 1994). This particular assay includes a card with projections, each of which is sensitized with two reactive goat antibodies to human IgA (internal control) and inactivated antigens of *C. trachomatis*, as well as containing a developing plate consisting of 6 rows. The developing plate was left for an incubation period (37°C, 5% CO_2, 20 min), after which 25 µl of semen was pipetted into row A (antigen-antibody reaction row) of the developing plate and left for 40 minutes at room temperature. After the reaction period of 40 minutes, the projections were left for 2 minutes in row B (first wash), followed by insertion into row C (alkaline phosphatase-labeled goat anti-human IgA antibody solution) to allow for the binding of the conjugate. After 20 minutes, the comb was immersed for 2 minutes in row D followed by row E, both of which are washing solutions. The projections were then inserted and left for 10 minutes into row F (colour reaction) which consists of a chromogenic substrate solution containing 5-bromo -4-chloro-3-indoyl phosphate and nitro blue tetrazolium. After the reaction period, the comb was inserted into row E for 1 minute to halt the reaction.

To analyse the result, the intensity of the colour reaction on each projection was observed in comparison to the positive control and samples positive for *C. trachomatis* were identified by the developed blue stain marking.

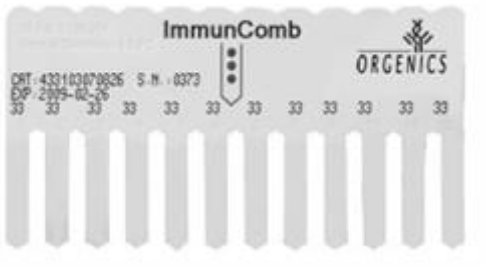


Figure 2: ImmunoComb® quantitative indirect enzyme immunoassay for the detection of *C. trachomatis*

Objective 2:

Examine the effects of N. gonorrhoea, C. trachomatis and T. vaginalis, in comparison to the control group, on the standard semen parameters which includes; volume, pH, viscosity, leukocyte count and polymorphonunclear elastase concentration, and the spermatozoa parameters including; motility, concentration, morphology, viability and acrosome reaction.

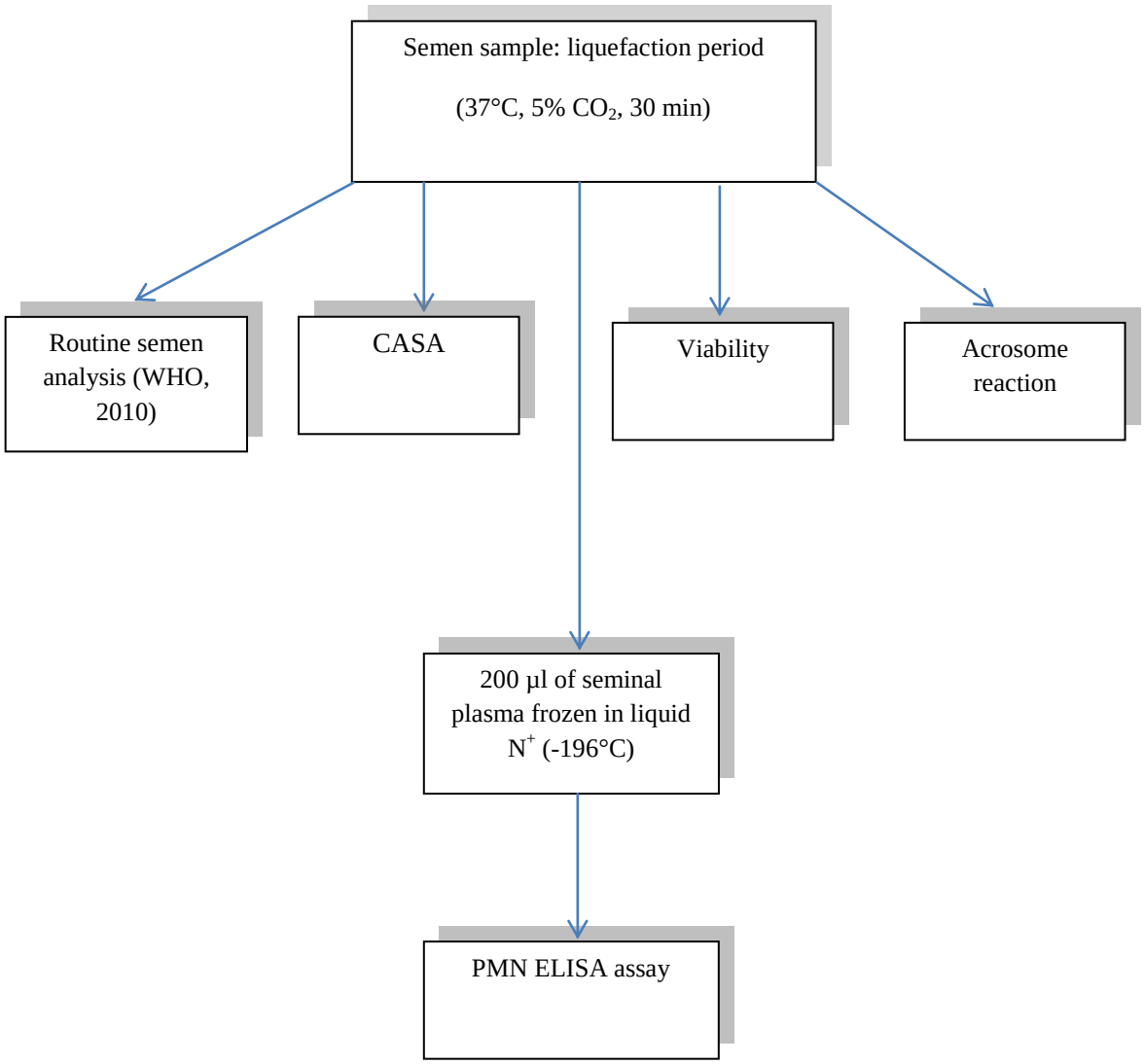


Figure 3: Flow chart of the simplified experimental procedure for objective 2

3.5 Semen sample analysis

The parameters were assessed in terms of the guidelines outlined by the WHO (WHO, 2010) and the protocols shall be discussed in detail below.

3.5.1 Volume

Following the incubation period (37°C, 5% CO_2, 30 min) to allow for the liquefaction of the ejaculate, semen samples were directly decanted from the plastic collection container into a graduated 15 ml plastic Falcon tube. The volume was recorded in millilitres.

3.5.2 pH

pH was assessed by immersing litmus paper in the semen samples and graded according to the pH scale.

3.5.3 Viscosity

An Eppendorf micropipette was used to place 2 µl of semen into the filling area of a single chamber in a Leja® disposable 4 chamber slide (depth, 20 µm; length, 21 mm; width, 6 mm) (Leja® Products B.V., Nieuw-Vennep, The Netherlands). The filling time of each semen sample was measured twice, and the average of these times was recorded as the result. The result was then quantified according to the regression line equation below, according to Rijnders *et al*. (2007) and expressed in the unit Centipoise (cP):

$$y = 0.34(x) + 1.34$$

(x = filling time in seconds)

3.5.4 Leukocyte count

For the identification and quantification of leukocytes in the semen samples, a leukocyte peroxidase test was performed using Leukoscreen (FertiPro; Belgium). A wet mount was initially prepared with 20 µl of mixed semen on a covered slide in order to examine the presence of non-sperm cells under a phase-contrast light

microscope (x10 objective). Prior to performing the test, a working solution was prepared with 30 µl of reagent 2 (30% H_20_2) mixed with 1 ml of reagent 1 (benzidine, cyanosine and methanol), which is the stain. Subsequent to the preparation of the working solution, 100 µl of the neat semen sample was mixed with 100 µl of the prepared working solution in an aliquot, mixed thoroughly with a vortex and left for five minutes at room temperature on the laboratory bench.

Following the five minute reaction period, 10 µl was placed on a slide and covered immediately after mixing to avoid the formation of air bubbles that can interfere with the interpretation of the results. A count of the peroxidase positive cells was performed in a similar manner as the standard spermatozoa count, with the aid of a Neubauer haemocytometer (depth 100µm) (Marienfeld, Germany). Upon examination of the slides, read under the bright-field objective (magnification 400x), the following was considered: yellow to brown stained cells can be regarded as peroxidase positive cells, thus neutrophilic polymorphous leukocytes, while the pink and unstained cells can be regarded as peroxidase negative cells. For statistical purposes, the number of leukocytes present in each ejaculate can be quantified and expressed as the number of peroxidase positive cells ($x10^6$/ml).

3.5.5 PMN elastase

An ELISA kit (Merck, Darmstadt, Germany) was used for the quantitative detection of extracellular PMN-elastase. The frozen seminal plasma was thawed at room temperature. Once the seminal plasma has completely thawed, it was diluted (1:100) with the sample diluent and 100 µl of sample diluent mixture of seminal plasma and reagents were added in duplicate to each of the blank wells, which are coated with the PMN elastase antibody. The wells were then covered with a plate cover and incubated at room temperature for 1 hour on a rotator and then washed with 300 µl of wash-buffer according to manufacturer's instructions. Following the wash, 150 µl of Horseradish Peroxidase conjugate was added to each of the wells and incubated at room temperature for 1 hour.

Once the incubation period was completed, the wells were washed 4 times as described above. Next, 200 µl of the Tetramethyl-benzidine substrate solution was added to all the wells, including the blanks, and the plate was incubated at room temperature for 20 minutes on a rotator, avoiding direct exposure to intense light. After the incubation period, the enzyme reaction was suspended by pipetting 50 µl of the stop solution into each well. After the reader had been blanked with the previously prepared blank wells, the absorbance of each microwell was read at 450 nm using a Microplate reader (FLUOstar™ Omega, BMG Labtech, Germany). The results were quantified in ng/ml *(Figure 4).*

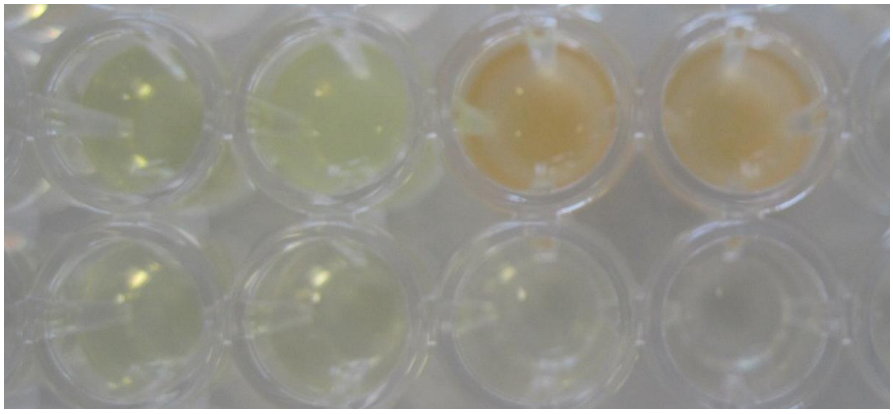


Figure 4: ELISA assay microtitre plate for the photometric quantification of the enzymatic activity of elastase in seminal plasma (450 nm)

3.6 Microscopic spermatozoa parameters

3.6.1 Sperm preparation

In order to obtain the most isolated subpopulation of spermatozoa from the ejaculate, an allocated volume of each ejaculate underwent a double-wash (WHO, 2010), whereby semen was placed in a 15 ml plastic Falcon tube, in which a volume of pre-warmed (37°C) HAMS-F10 medium (Sigma Chemicals Co., St Louis, MO, USA) twice the volume of the semen was placed. The samples underwent centrifugation (x300 *g*, 10 min), followed by the careful aspiration and disposal of the supernatant. The isolated pellet was resuspended in 1.5 ml of HAMS-F10, and the centrifugation process was repeated (x300 *g*, 10 min). Following the second centrifugation, the final pellet containing the isolated spermatozoa population was then resuspsended in 1.5

ml of HAMS-F10, supplemented with preheated (37°C) 3% bovine serum albumin (BSA) (Sigma, Cape Town, South Africa).

3.6.2 Computer aided sperm analysis

SCA® motility and concentration

The sperm concentration and motility parameters were assessed using computer aided sperm analysis (CASA) and the Sperm Class Analyzer (SCA®) (Microptic, S.L., Barcelona, Spain, Version 4.2) at a frame rate of 50 frames per second. From each sample 2 µl was pipetted into a single chamber of a 20 µm deep disposable Leja® slide (Leja® Products B.V., Nieuw-Vennep, The Netherlands). The SCA® is equipped with a Basler A312fc digital colour camera (Microptic, S.L., Barcelona, Spain), mounted on a Nikon E200 Microscope (IMP, Cape Town, South Africa) and a stage warmer (Omron™, Kyoto, Japan) which was heated to 37°C. The SCA® settings for the automated analysis were as follows: positive phase contrast observation setting; objective, 10x; green filter; brightness 160 and contrast 400. WHO reference values adopted in this study are as follows: total motility >40%; progressive motility >32%; normal morphology >4% (WHO, 2010).

The following motility parameters were recorded: total motility (percentage of type A, B and C level of spermatozoa); progressive motility (percentage of type A+B), as well as the total percentage of immotile spermatozoa (type D). Figure 4 represents the SCA® motility colour paths, which are as follows: Type A (red); B (green); type C (blue) and type D (yellow) *(Figure 5)*. In addition, the SCA® records the kinematic and velocity parameters of spermatozoa such as; curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH) and beat cross-frequency (BCF). The percentages of rapid-, medium- and slow-swimming spermatozoa were recorded. The concentration of spermatozoa per ejaculate and per millilitre, of each sample was recorded in both the neat, as well as the sample fraction having undergone the double-wash as previously described. A minimum of five fields per a chamber was recorded.

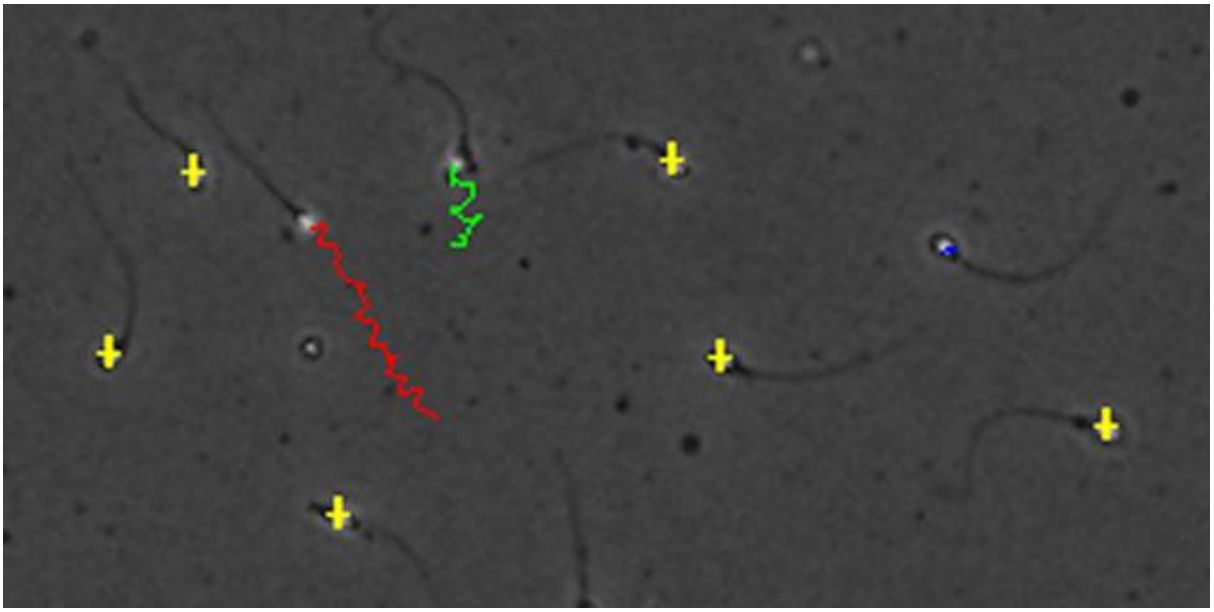


Figure 5: SCA® analysis displaying the various motility colour paths. Type A (red); B (green); type C (blue) and type D (yellow)

3.6.3 Morphology

Evaluation of the morphology of the spermatozoa was performed with the preparation of 10 µl of semen placed on a microscopic slide (76 x 26 mm) and spread over the surface with a second slide to make an evenly spread smear. Each slide was left for an average period of 15 minutes to air dry at room temperature on the lab bench. Following the drying period, the slide was immersed vertically in a Coplin jar containing the fixative for a 10-minute period (SpermBlue®, Microptic, S.L., Barcelona. Spain). Following fixation, the slides were placed at an angle, allowing contact with filter paper to drain the excess fixative. Next, the slides were immersed in the stain solution for 15 minutes, followed by a period of air-drying. Once the slides were fully dried, they were prepared for analysis by the placement of a coverslip, using DPX mounting medium (Dako, CA, USA). Morphological analysis was performed under oil immersion, using the SCA® on the following settings: filter, blue; objective, 100x; brightness, 314; contrast, 100. Spermatozoa that were found to be overlapping were not counted. 100 spermatozoa were recorded and analysed according to WHO criteria (WHO, 2010).

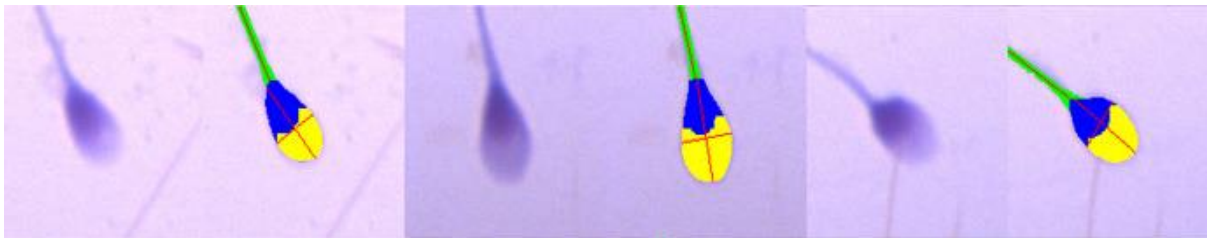


Figure 6: Morphology SCA® analysis of spermatozoa using SpermBlue™. To the right is the SCA® analysis of the stained spermatozoa showing the following: mid-piece (green); head (blue) and acrosome (yellow)

3.6.4 Viability

The assessment of the percentage of viable cells in each sample was determined using a dye exclusion technique. The membrane-impermeant Eosin-Nigrosin stain (Sigma-Aldrich, St Louis, MO, USA) allows for the classification of the viability status, which is based on the microscopic visualization of dye that has passed through the compromised plasma membrane. A mixture of 10 µl of $5x10^6$ spermatozoa/ml, 10 µl Eosin and 10 µl Nigrosin was smeared across the length of a microscope slide and allowed to air dry at room temperature. The slide was then mounted with non-aqueous DPX mounting medium (Dako, CA, USA) and a coverslip was placed on the slide. 100-200 spermatozoa were assessed under oil-immersion light microscopy (100x) for the visual analysis of the viability of the cells and scored by the investigator according to the technique set out by Bjorndahl *et al*. (Bjorndahl *et al*. 2003). The WHO reference value of >58% viable cells was adopted.

3.6.5 Acrosome reaction

The acrosomal status of each sample was assessed using fluorescently labelled lectins to allow for visualisation of the spermatozoa's acrosome under fluorescent microscopy. Following an incubation period (37°C, 5% CO_2, 180 min), a glass slide smear of 10 µl of a $5x10^6$ spermatozoa/ml concentration was prepared and after air drying on the laboratory bench at room temperature, was fixed with 70% ethanol (4°C,

30 min) (WHO, 1999). Following the fixation period, the slides were stained and left to dry in a darkened environment with a mixture of 50 µl fluorescein isothiocyanate-labelled pisum sativum agglutinin (FITC-PSA) (Sigma Chemicals Co., St Louis, MO, USA) and 450 µl phosphate buffered saline (PBS) (Gibco, Scotland, UK). Each slide was then prepared for microscopic analysis (Zeiss LSM 780, Heidelberg, Germany) by being immersed several times in dH_20 to remove any excess probe. After the rinsing period, DACO Antifade mounting medium (Glostrup, Denmark) was placed on the center of each slide with a coverslip was added. Under green fluorescence (100x oil immersion; 510-560 nm), 200 spermatozoa were evaluated on the level of fluorescence emitted from the acrosomal region, with a heightened degree of fluorescence indicating an intact acrosome (Esteves *et al*., 2007). All slides were scored by the principal investigator.

Objective 3:

Investigate the impact of infection on the generation of ROS and the possible impact on the spermatozoa's DNA.

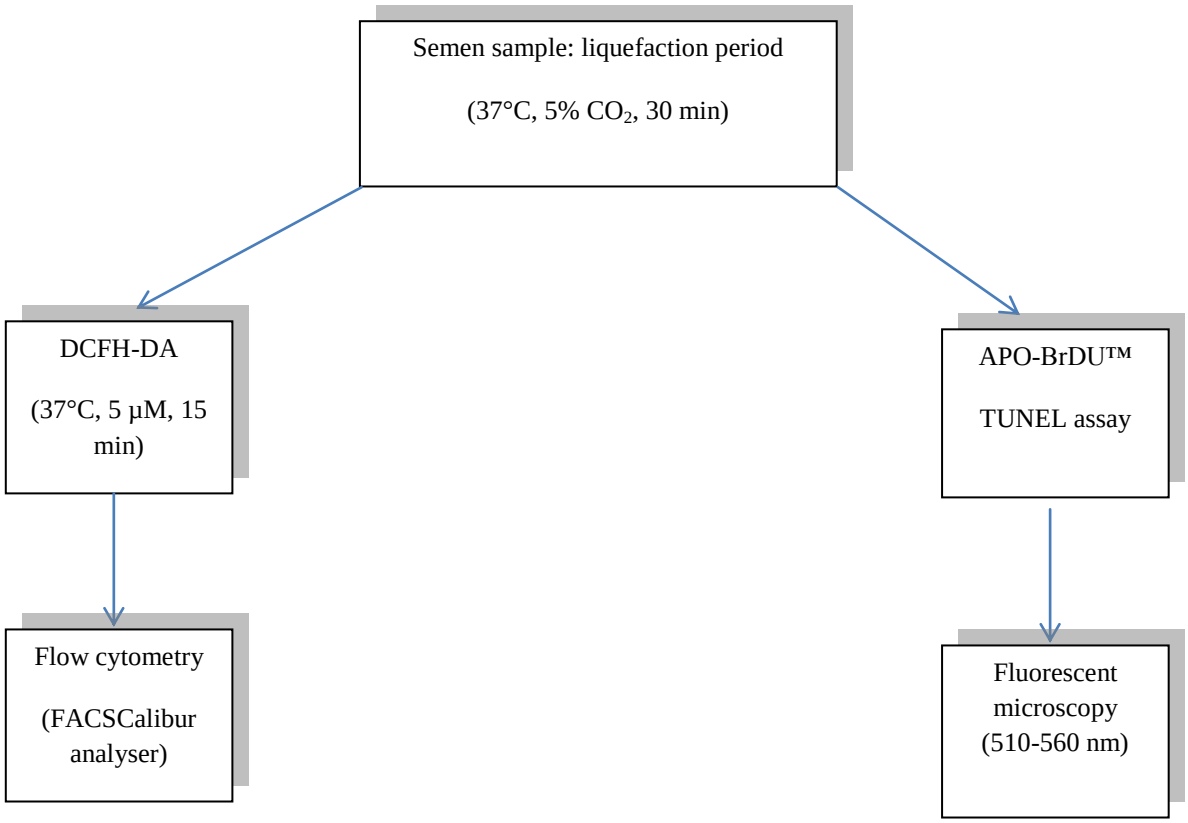


Figure 7: Flow chart of the simplified experimental procedure for objective 3

3.6.6 ROS

The quantification of the level of ROS production was determined by means of flow cytometry, whereby a suspension of $5x10^6$ spermatozoa/ml was treated with 2,7-dichlorofluroscein diacetate (DCFH-DA) (37°C, 5 µM, 15 min) (Sigma Chemicals Co., St Louis, MO, USA) in a darkened environment to circumvent the probe's sensitivity to light. The cell permeable probe is used to detect intracellular ROS, in particular H_20_2. Following the incubation period as previously described, the aliquot of semen was washed twice (x300 *g*, 5 min) and resuspended in 1.5 ml of PBS (Gibco, Scotland, UK).

Flow cytometry (Becton Dickinson FACSCalibur analyser) was used to quantify the fluorescence emitted at a single cell level (excitation wavelength 488 nm; emission wavelength 530 nm). Forward and side scatter light signals allowed for the mean fluorescence intensity to be determined. In total, 10 000 sperm cells were captured and their fluorescence was recorded on a frequency histogram using logarithmic amplifiers calculated by the software WinMDI (Windows Multiple Document Interface Flow Cytometry Application) *(Figure 8)*.

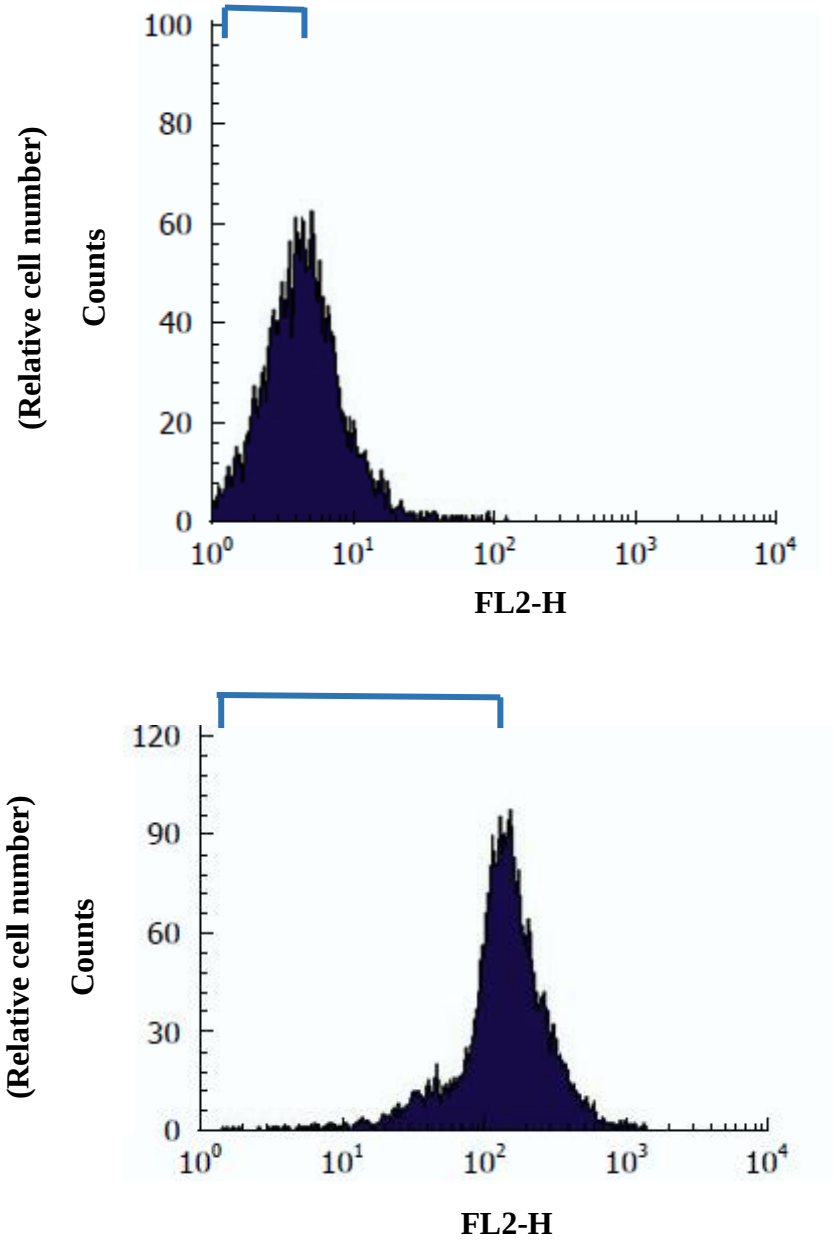


Figure 8: a) Flow cytometric frequency histograms with baseline fluorescence (x-axis) (log; FL2-H) and cell count (y-axis) b) demonstrating the shift in DCFH indicating an increased intensity

3.6.7 DNA fragmentation

The DNA fragmentation of apoptotic cells were detected using the APO-BrDU™ TUNEL assay kit (Molecular Probes™, Invitrogen). The initial step involves cell preparation and fixation of each semen sample, whereby $2x10^6$ spermatozoa/ml in 0.5 ml of 1% paraformaldehyde was suspended in PBS (Gibco, Scotland, UK) and left on ice for 15 minutes. The cells underwent a double-wash, in 5 ml of PBS and the retrieved pellet was resuspended in 0.5 ml PBS. The spermatozoa were adjusted to a final concentration of $1x10^6$ spermatozoa/ml in 70% ice-cold ethanol. For the staining, samples, underwent centrifugation (x300 *g*, 5 min) after which the supernatant was aspirated and the pellet resuspended in 1 ml of the wash buffer and centrifugation was repeated (x300 *g*, 5 min). Following the centrifugation period, the pellet was resuspended in 50 µl of the staining solution and left to incubate (37°C, 5% CO_2, 60 min).

Upon completion of the incubation, the cells were washed twice in 1 ml of the rinse buffer (x300 *g*, 5 min), the pellet was retrieved and resuspended in 0.5 ml of the PI/RNase staining buffer and left to incubate for 30 minutes in a darkened environment. Each slide was prepared for microscopic analysis by several rounds of immersion in dH_20 to rinse the slides, followed by the addition of DACO Antifade mounting medium (Glostrup, Denmark) onto the surface of each slide. A coverslip was then placed on the surface and adhered to the slide. Under green fluorescence (Zeiss LSM 780, Heidelberg, Germany) (100x oil immersion; 510-560 nm), 100-200 spermatozoa were assessed on the level of fluorescence emitted, with raised fluorescence being indicative of DNA damage.

Objective 4:

Investigate the effect of N. gonorrhoea, C. trachomatis and T. vaginalis on the secretory function of the prostate and seminal vesicles in comparison to the control, determined through the quantification of seminal plasma citric acid and fructose concentrations.

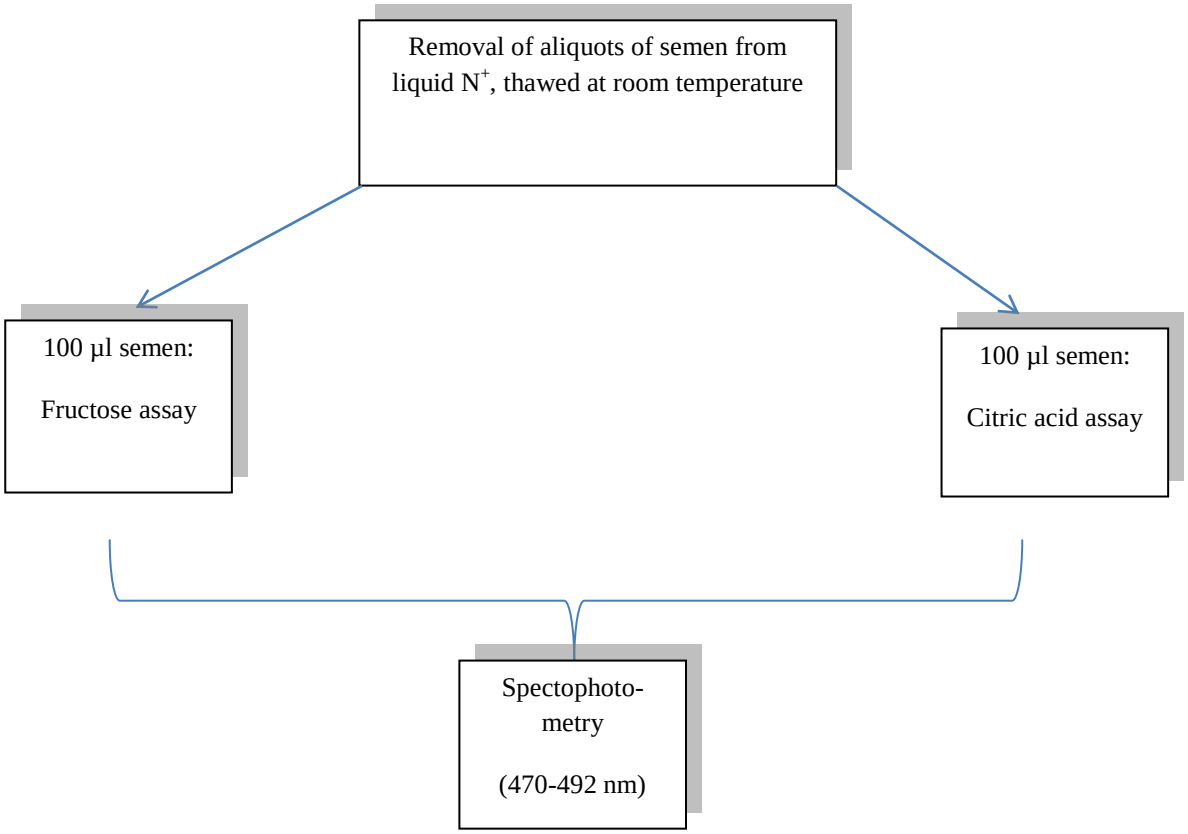


Figure 9: Flow chart of the simplified experimental procedure for objective 4

3.7 Sample preparation

For the assessment of the biochemical parameters, the semen samples were centrifuged (x1000 *g*, 10 min), after which, the seminal plasma was aspirated and stored in a labelled cryopreservation tube and placed in liquid N^+ (-196°C) until analyses.

3.7.1 Fructose

The test for quantifying fructose in the semen was done by means of a photometric kit (FertiPro, Belgium). In preparation for the test, the seminal plasma was removed from the liquid N^+ and thawed at room temperature. During this time, a standard curve with differing fructose concentrations was calculated according to the set instructions. In order to obtain reliable results, each of the standards, samples and blanks were prepared in duplicate. Following the necessary thawing period, the seminal plasma was thoroughly mixed on a vortex mixer and 100 µl was pipetted into a test tube. Next, 100 µl of the fructose standards and an aliquot of 0.5 ml of reagent 1, a Trichloroacetic solution, was added and the mixture was centrifuged (x1000 *g*, 10 min). Following centrifugation, 20 µl of the resultant supernatant that formed was pipetted into an Eppendorf tube. To each of the tubes, 200 µl of reagent 2 (concentrated HCl^-) and 20 µl of reagent 3 (indole) was added and incubated (37°C, 5% CO_2, 60 min).

After the incubation period, the colour reaction was inhibited by the addition of 200 µl of reagent 4 ($NaOH^-$) and 200 µl of the sample was read by means of a spectrophotometer at 470-492 nm in a plate reader (FLUOstar™ Omega, BMG Labtech, Offenburg, Germany). Once all the tubes had been read, the measured optical density (OD) values of the samples were plotted against the standard curve using the standard values. To obtain the total fructose concentration for each sample, the results were multiplied with the total volume of the semen sample. Normal values based on past studies is 13 µmol (2.34 mg) or more per ejaculate (Cooper *et al*., 1991).

3.7.2 Citric acid

To be able to quantify the citric acid concentration in the semen sample and therefore assess prostate functioning, a photometric test was carried out using a Citric Acid Test (FertiPro, Belgium). In preparation for the test, the seminal plasma was removed from the liquid N^+ and thawed at room temperature. Once completely thawed, it was mixed well with a vortex mixer. A standard was first prepared by mixing 100 µl of reagent 3 (citric acid standard) with 100 µl of reagent 2 (isopropanol and sulphuric acid). Thereafter, 100 µl of semen from each sample being tested was mixed with 100 µl of reagent 2 in an Eppendorf tube. Following centrifugation (x1000 *g*, 10 min), 25 µl of the supernatant was carefully pipetted into an empty well and 200 µl of reagent 1 (iron (III) chloride) and sulphuric acid was added. For the purposes of reliability, the samples and standards were prepared in duplicate in the plate reader and the mean of the results were calculated. The OD of the sample was read by means of a spectrophotometer (FLUOstar™ Omega, BMG Labtech, Offenburg, Germany) at 405 nm and was used to calculate the total citric acid concentration using a given equation provided in the manufacturer's guidelines (FertiPro, Belgium). Readings were assessed according to the WHO guidelines (WHO, 2010), whereby the reference level for normal citrate concentrations is 52 µmol (9.36 mg) or more per ejaculate.

3.8 Sample storage

Upon completion of the described protocols of objectives 1-4, the remaining volume of each sample was suspended in PBS (Gibco, Scotland, UK) to form a volume of 5 ml and centrifuged (x300 *g*, 5 min). Following the 5 minute period and retrieval of the spermatozoa pellet from the supernatant, each sample was resuspended in PBS and the centrifugation process was repeated (x300 *g*, 5 min). Once the remaining supernatant was carefully removed by aspiration, the pellets were placed in labelled cryopreservation tubes and stored at -196°C in liquid N^+ until further analysis in objective 5.

Objective 5:

Determine the differentially expressed proteins in the spermatozoa isolated from samples positive for N. gonorrhoea, C. trachomatis and T. vaginalis.

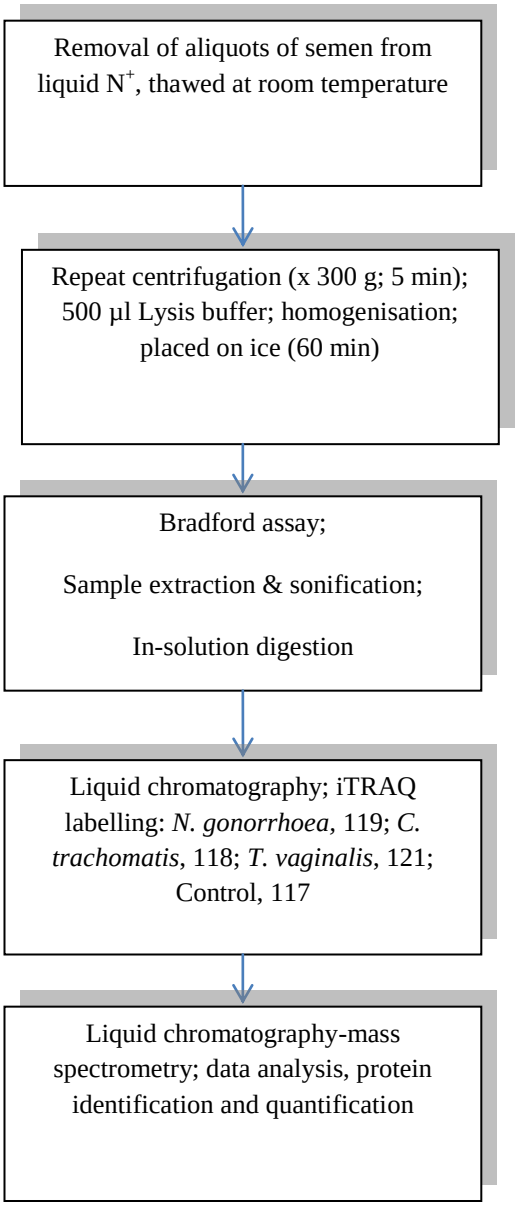


Figure 10: Flow chart of the simplified experimental procedure for objective 5

3.9 Proteomics

3.9.1 Sample preparation

Samples were removed from the liquid N^+ and thawed at room temperature and were prepared for proteomic analysis by first being pooled in the respective groups according to the identified STI. Eight samples constituted each sample cohort in order to achieve consistency in the results. Following centrifugation (x300 *g*, 5 min), the seminal plasma supernatants of the pooled ejaculates were aspirated and the sperm pellets were resuspended in 2 ml of PBS. Each separated fraction underwent 3 repeat cycles of centrifugation (x300 *g*, 5 min), to ensure the most isolated spermatozoa pellet followed by homogenisation, whereby 500 µl of the prepared lysis buffer (Appendix A) was added to the 4 pooled groups and homogenised in a low protein binding Eppendorf for 5 minutes using a glass pestle. The resultant homogenate was placed on ice for 60 minutes to inhibit enzymatic activity, followed by centrifugation (4°C, 30 min, 14000 *rpm*).

3.9.2 Bradford assay

Prior to the proteomic analysis using the Orbitrap™, a Bradford protein determination (Bradford, 1976) was carried out on the spermatozoa groups in order to determine the total protein concentration of each of the 4 groups (*N. gonorrhoea C. trachomatis*, *T. vaginalis* and the control). A 4-15% Criterion™ TGX™ precast gel (Bio-Rad™ Laboratories, California, USA) was loaded with 10 µl of spermatozoa. The gel was run on a shaker plate for 60 minutes, followed by an hour fixation period (40% methanol, 7% acetic acid) and was left overnight to stain on a shaker plate with Brilliant Blue G Colloidal Concentrate (Sigma Chemicals Co., St Louis, MO, USA). Thereafter, a destaining process was performed (25% Methanol), to obtain visualisation of the wells.

3.9.3 Sample extraction

Spermatozoa were extracted in an extraction buffer (100 mM Tetraethylammonium bromide (TEAB), 100 mM $NaCl^-$, 2 mM EDTA, 1% OGP, 1% Rapigest, 5 mM triscarboxyethyl phosphine (TCEP)) by vortex and sonication for 1 minute each. The procedure was repeated three times.

3.9.4 Sample preparation and digestion

Samples were reduced by adding 50 mM TCEP (Fluka™, Sigma-Aldrich, MO, USA) in 100 mM TEAB, to achieve a final concentration of 5mM TCEP, after which it was left for 30 minutes at room temperature to allow for a reduction period. Following reduction, cysteine residues were modified to methylthio using 200 mM methane methylthiosulphonate (MMTS; Sigma-Aldrich, MO, USA) in 100 mM TEAB (final concentration 20 mM) for 30 minutes. After modification, samples underwent dilution to 98 µl with 100 mM TEAB.

Proteins were digested by adding 10 µl trypsin (Promega, WI, USA) solution (1 µg/µl), a serine protease that facilitates protein cleavage on the carboxyl side of arginine and lysine residues (Olsen *et al*., 2004) and incubated (37°C) overnight. The samples were resuspended in 30 µl of 2% acetonitrile: water; 0.1% formic acid (Sigma-Aldrich, MO, USA).

3.9.5 Desalting

Residual digest reagents were removed from the samples, using an in-house manufactured C_{18} stage tip (Empore™ Octadecyl C_{18} extraction discs, Supelco, PA, USA). The samples were loaded onto the stage tip after activating the C_{18} membrane with 30 µl methanol (Sigma-Aldrich, MO, USA) and equilibration with 30 µl 2% acetonitrile: water; 0.05% trifluoroacetic acid (TFA). The bound sample was washed with 30 µl 2% acetonitrile: water; 0.05% TFA before elution with 30 µl 50% acetonitrile: water 0.05% TFA for LC-MS analysis.

3.9.6 Liquid chromatography

3.9.6.1 Sample preparation and digestion

Samples were reduced in a solution comprising of 50 mM triscarboxyethyl phosphine (TCEP; Fluka; Sigma-Aldrich, MO, USA) in 100 mM TEAB (final concentration 5 mM TCEP) for 30 minutes at room temperature. Following reduction, cysteine residues were modified to methylthio using 200 mM methane

methylthiosulphonate (MMTS; Sigma-Aldrich, MO, USA) in 100 mM TEAB (final concentration 20 mM) for 30 minutes. After modification, the samples underwent dilution to 98 µl with 100 mM TEAB. Proteins were digested by adding 10 µl trypsin (Promega, WI, USA) solution (1 µg/µl) and incubated (37°C) overnight.

3.9.7 iTRAQ labelling

The digested samples were resuspended in 20 µl of 300 mM TEAB. 70 µl of 2-propanol was added to each of the isobaric labels, before combining it with the respective samples. Following protein digestion, isobaric labelling involves the covalent labelling of a peptide's side chain amine and the N-terminus, with tags of varying masses. Labelling was performed 2 hours before the excess labels were hydrolysed by the addition of 100 µl analytical grade H_20.

3.9.8 Liquid chromatography

3.9.8.1 First dimension liquid chromatography

First dimension chromatography was undertaken using batch-wise elution from an in-house manufactured C_{18} SPE device as specified above. The sample was dissolved in 2% acetonitrile/100mM TEAB, (pH=8) and eluted with 2.5%, 5%, 7.5%, 10%, 12.5%, 15% 17.5%, 20%, 25%, 30% and 50% acetonitrile in 100 mM TEAB.

3.9.8.2 Second dimension liquid chromatography

Liquid chromatography was performed on a Thermo Scientific™ Ultimate 3000 RSLC (Thermo Scientific™ Inc, USA), equipped with a 2 cm x 100 µm C_{18} trap column and a 35 cm x 75 µm C18 (Luna C18, 5 µm; Phenomenex®, CA, USA) analytical column. The solvent system used was loaded with: 2% acetonitrile: water; 0.1% FA; Solvent A: 2% acetonitrile: water; 0.1% FA and Solvent B: 100% acetonitrile: water. The samples were loaded onto the trap column using loading solvent at a flow rate of 5 µl/min from a temperature controlled auto sampler set at 7°C. Loading was undertaken 10 minutes prior to the sample

being fixed onto the analytical column. Flow rate was set to 350 nl/minute and the gradient generated as follows: (i) 2.0% A (5 min) (ii) 2%-35% B (5-70 min) using Chromeleon non-linear gradient 7 (iii) 35%-50% (70-75 min). Thereafter, the column was washed for 10 minutes with 80% B followed by equilibration. Chromatography was run at 50°C and the outflow delivered to the mass spectrometer through a stainless steel nano-bore emitter.

3.9.9 Mass spectrometry (MS)

MS was performed using a Thermo Scientific™ Fusion mass spectrometer (Thermo Scientific™ Inc, USA) equipped with a Nanospray Flex™ ionization source. A volume of each sample, calculated by MS software, was introduced through a stainless steel emitter and data was collected in positive mode, with spray voltage set to 1.9 kV and ion transfer capillary set to 275°C. Spectra were internally calibrated using polysiloxane ions at m/z = 445.12003 and 371.10024. Mass Survey (MS) MS^1 scans were performed using the Orbitrap™ detector set at 12 000 resolution over the scan range 350-1650 with automatic gain control (AGC) target at 2 E5 and maximum injection time of 40 ms. Data was acquired in profile mode and the MS^2 acquisitions were performed using monoisotopic precursor selection for ion with charges of +2 - +6 with an error tolerance set to +/- 10 ppm. Precursor ions were excluded from fragmentation once for a period of 30 seconds and were selected for fragmentation in Collision-induced dissociation (CID) mode using the quadrupole mass analyser with CID energy set to 35%. Fragment ions were detected in the ion trap mass analyser using rapid scan rate. The AGC target was set to 1E4 and the maximum injection time to 35 ms. The data was acquired in centroid mode. MS^3 acquisitions were performed on MS^2 fragments over the range m/z = 100-500 with detection in the Orbitrap™ mass analyser at 30 000 resolution. Fragmentation mode was set to HCD at 65% with a maximum fill time of 100 ms.

3.9.10 Data analysis

The raw files generated by the mass spectrometers were imported to Proteome Discoverer™ (Version 1.4, Thermo Scientific) and processed using the Mascot MOWSE-algorithm (Matrix Science, MA, USA) (http://www.matrixscience.com/search_form select.html), in addition to the SequestHT algorithm included in

Proteome Discoverer™. Mascot's database consists of all known proteins against which the analyte sample originates from, thereby allowing a database to be constructed of the theoretical spermatozoa peptides and their corresponding masses. An algorithm was employed to generate the Mascot scores, which were calculated according to $-10*\text{Log}_{10}(P)$, where P accounts for the probability that the observed match between experimental data and a protein sequence is a random event and is calculated for each protein in the sequence database (Perkins *et al*., 1999).

Data analysis was structured to allow for methylthio as fixed modification as well as NQ deamidation (NQ), oxidation (M) and N-terminal acetylation. Peptide validation was completed using the percolator node set to search against a decoy database with a strict false-discovery-rate (FDR) of 1%. Database interrogation was performed against the Uniprot *Saccharomyces cerevisiaee* database (2015 edition) with trypsin cleavage allowing for two missed cleavages. Quantitation was assessed in the MS^3 and no deviations were recorded. A positive identification was defined when the MOWSE scores exceeded 40 and were considered significant when $p<0.05$.

For the quantification of the DEPs, the fold changes of the proteins was determined using the Proteome Discoverer™, whereby a cut-off value based on the $-10*\text{Log}_{10}(P)$, distribution of the proteins was calculated. The *N. gonorrhoea*, *C. trachomatis* and *T. vaginalis* samples were assessed against the control group as up-regulated with a ratio of >1.5 (more abundant proteins), and down-regulated <0.77 (less abundant proteins) (Jung *et al*., 2009; Karp *et al*., 2010; Breitwieser *et al*., 2011). Pathway analyses were assessed using the Reactome database (http://www.reactome.org). Reactome allows for a detailed evaluation of each protein uploaded to the database and is cross-referenced with highly reputable databases such as UniProt, to the protein's individual gene accession numbers (Haw *et al*., 2011).

3.10 Statistical analysis

Statistica® (Version 12) was used to evaluate the results for the study's objectives 2, 3 and 4, whereby the data was analysed using Kruskal-Wallis one-way analysis of variance (ANOVA) with a Least Significant Difference posthoc test. Statistical significance was considered when $p<0.05$. Results in the graphs *(Figure 16 - Figure 29)* are plotted as the mean with vertical bars representing the 95% confidence intervals. It must be noted that despite the data points on the graphs being joined, the measured variables are independent of each other.

The red lines marking the graphs represent the WHO (WHO, 2010) reference values.

Correlations between the parameters were determined using correlation studies and are represented by the Spearman rank correlation coefficient (r). Results that were shown to have significant correlations ($p<0.05$) are represented in linear regression graphs *(Figure 33 - Figure 36)* with a residual plot and 95% confidence band.

Chapter 4: Results

4.1 Objective 1:

Determine the prevalence of N. gonorrhoea, C. trachomatis and T. vaginalis isolated from semen samples.

Following the incubation period (37°C, 5% $C0_2$), the selective agar plates were examined for culture 24 - 48 hours after plating, for the identification of *N. gonorrhoea* and *T. vaginalis* colonies. Ejaculates were classified according to the WHO guidelines of >1 x 10^3 /ml (CFU/ml) (WHO, 2013). Figure 14 shows the colony growth on the Thayer-Martin agar, from a sample plated on both medias, with the sample exhibiting a positive result for *N. gonorrhoea.*

From the total 120 ejaculates, 15.8% (n=19) were found to be positive for the presence of the bacterium *N. gonorrhoea*, whilst 20.8% (n=25) were positive for *T. vaginalis*, as detected using chocolate agar media. The quantitative indirect enzyme immunoassay ImmunoComb® detected 9.2% (n=11) of the samples positive for *C. trachomatis*.

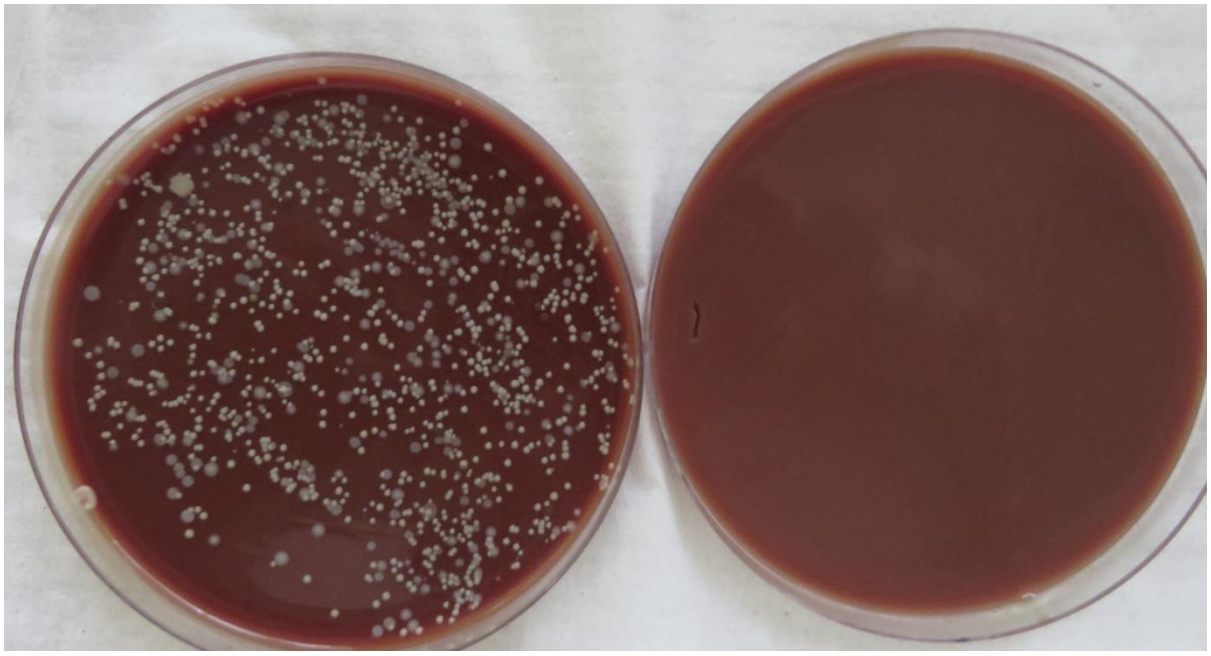


Figure 11: Thayer-Martin agar (left) positive for *N. gonorrhoea* and a chocolate agar plate (right) after incubation plated with the same ejaculate

4.2 Objective 2:

Examine the effects of N. gonorrhoea, C. trachomatis and T. vaginalis, in comparison to the control group, on the standard semen parameters which includes; pH, volume, leukocyte count, viscosity and polymorphonunclear elastase concentration, and the spermatozoa parameters including; motility, concentration, morphology, viability and acrosome reaction.

Table 2 shows a summary of the semen and spermatozoa parameters assessed in this study. The Kruskal-Wallis ANOVA results between the three STI positive groups investigated in the study and the control group will be represented in Figures 12-26.

Table 2: Summary of the macro- and microscopic semen and spermatozoa parameters (mean ± SEM)

Parameter	*N. gonorrhoea* (n=25)	*C. trachomatis* (n=19)	*T. vaginalis* (n=11)	Control (n=65)
pH	7.76 ± 0.09	7.61 ± 0.07	7.69 ± 0.09	7.60 ± 0.04
Volume (ml)	2.49 ± 0.64	2.90 ± 0.51	3.66 ± 0.52	3.06 ± 0.11
Leukocyte count ($x10^6$/ml)	1.20 ± 0.11^{a}	1.28 ± 0.15^{a}	1.38 ± 0.17^{a}	0.34 ± 0.09^{b}
Viscosity (cP)	7.81 ± 0.71^{cb}	9.06 ± 0.52^{ab}	10.96 ± 0.99^{a}	5.98 ± 0.46^{c}
PMN elastase (ng/ml)	582.38 ± 7.72^{a}	603.25 ± 20.00^{a}	538.25 ± 27.50^{a}	217.63 ± 16.34^{b}
Total motility (%)[1]	37.10 ± 3.18^{a}	37.76 ± 4.23^{a}	47.60 ± 2.15^{a}	82.14 ± 1.06^{b}
Progressive motility (%)[2]	13.40 ± 1.93^{a}	13.63 ± 2.34^{a}	15.28 ± 1.97^{a}	51.79 ± 2.51^{b}
Normal morphology (%)	1.50 ± 0.38^{a}	2.13 ± 0.52^{a}	3.13 ± 0.67^{a}	13.25 ± 1.95^{b}
Concentration ($x10^6$/ml)	21.39 ± 3.39^{a}	19.45 ± 2.49^{a}	17.43 ± 3.45^{a}	84.64 ± 3.07^{b}
Total sperm count ($x10^6$/ejaculate)	53.26 ± 2.17^{a}	56.41 ± 1.27^{a}	63.79 ± 1.79^{a}	258.99 ± 0.34^{b}
Viable cells (%)	47.34 ± 5.91^{a}	58.13 ± 5.72	63.83 ± 5.49	69.08 ± 4.88^{b}
Acrosome intact cells (%)	69.67 ± 1.07^{a}	70.03 ± 0.79^{a}	70.32 ± 0.75^{a}	51.18 ± 1.27^{b}
DCFH-DA fluorescence (%)	95.41 ± 1.64^{a}	92.80 ± 4.44^{a}	93.06 ± 4.12^{a}	69.50 ± 13.64^{b}
TUNEL positive cells (%)	26.99 ± 1.91^{a}	29.73 ± 1.99^{a}	26.90 ± 2.13^{a}	11.98 ± 0.76^{b}

[1]=Percentage of progressive and non-progressive motile spermatozoa; [2]= percentage of type A+B; different letters in superscript following means in the same row indicate statistical significance ($p<0.05$)

Table 3: Representative summary of the differences observed amongst the macro- and microscopic semen and spermatozoa parameters

Parameter	*N. gonorrhoea* (n=25)	*C. trachomatis* (n=19)	*T. vaginalis* (n=11)	Control (n=65)
pH	↑	↓	↑	↓
Volume (ml)	↓	↓	↑	↑
Leukocyte count ($x10^6$/ml)	↑^{a}	↑^{a}	↑^{a}	↓^{b}
Viscosity (cP)	↑^{cb}	↑^{ab}	↑^{a}	↓^{c}
PMN elastase (ng/ml)	↑^{a}	↑^{a}	↑^{a}	↓^{b}
Total motility (%)[1]	↓^{a}	↓^{a}	↓^{a}	↑^{b}
Progressive motility (%)[2]	↓^{a}	↓^{a}	↓^{a}	↑^{b}
Normal morphology (%)	↓^{a}	↓^{a}	↓^{a}	↑^{b}
Concentration ($x10^6$/ml)	↓^{a}	↓^{a}	↓^{a}	↑^{b}
Total sperm count ($x10^6$/ejaculate)	↓^{a}	↓^{a}	↓^{a}	↑^{b}
Viable cells (%)	↓^{a}	↓	↓	↑^{b}
Acrosome intact cells (%)	↑^{a}	↑^{a}	↑^{a}	↓^{b}
DCFH-DA fluorescence (%)	↑^{a}	↑^{a}	↑^{a}	↓^{b}
TUNEL positive cells (%)	↑^{a}	↑^{a}	↑^{a}	↓^{b}

Different letters in superscript following means in the same row indicate statistical significance ($p<0.05$)

Table 4: Summary of the SCA® motility parameters as measured by CASA (mean ± SEM)

Motility parameter	*N. gonorrhoea* (n=25)	*C. trachomatis* (n=19)	*T. vaginalis* (n=11)	Control (n=65)
Total (%)[1]	37.10 ± 3.18[a]	37.76 ± 4.23[a]	47.60 ± 2.15[a]	82.14 ± 1.06[b]
Progressive (%)[2]	13.40 ± 1.93[a]	13.63 ± 2.34[a]	15.28 ± 1.97[a]	51.79 ± 2.51[b]
Non-progressive (%)	23.70 ± 1.25	24.13 ± 1.89	32.32 ± 0.18	30.35 ± 1.45
Rapid (%)[3]	6.36 ± 3.18	13.96 ± 7.08	15.89 ±2.97	42.41 ± 6.09
Medium (%)[4]	12.29 ± 2.21	11.13 ± 7.42	20.33 ± 1.93	29.49 ± 4.17
Slow (%)[5]	9.18 ± 2.92	15.78 ± 6.12	18.64 ± 3.32	13.36 ± 2.49
VCL (µm/s)[6]	20.59 ± 5.11[a]	22.85 ± 14.92[a]	25.44 ± 18.47	42.39 ± 27.76[b]
VSL (µm/s)[7]	9.23 ± 3.77[a]	10.39 ± 2.48	11.43 ± 1.82	19.11 ± 1.95[b]
VAP (µm/s)[8]	10.50 ± 2.62[a]	12.18 ± 1.87[a]	18.68 ± 1.82[a]	28.58 ± 3.66[b]
LIN (%)[9]	25.70 ± 4.55	31.03 ± 4.12	39.54 ± 1.49	48.46 ± 6.27
STR (%)[10]	47.34 ± 5.91	58.13 ± 5.72	63.83 ± 1.94	69.08 ± 4.88
WOB (%)[11]	52.49 ± 6.03	62.70 ± 3.97	59.71 ± 1.32	69.03 ± 3.72

[1]=Total motility (percentage of progressive and non-progressive motile spermatozoa), [2]=Progressive motility (percentage of type A+B), [3]=Rapid swimming sperm, [4]=Medium swimming sperm, [5]=Slow swimming sperm [6]=VCL Curvilinear velocity, [7]=VSL Straight line velocity, [8]=VAP Average path velocity, [9]=LIN Linearity index, [10]=STR Straightness index, [11]=WOB Oscillation index; different letters in superscript following means in the same row indicate statistical significance ($p<0.05$)

Table 5: Summary of the SCA® morphology parameters as measured by CASA (mean ± SEM)

Morphology parameter	*N. gonorrhoea* (n=25)	*C. trachomatis* (n=19)	*T. vaginalis* (n=11)	Control (n=65)
Normal (%)	1.50 ± 0.38[a]	2.13 ± 0.52[a]	3.13 ± 0.67[a]	13.25 ± 1.95[b]
Head defects (%)	95.41 ± 0.58[a]	92.85 ± 1.57	93.06 ± 1.46	69.50 ± 1.49[b]
Midpiece defects	47.71 ± 6.04[a]	47.80 ± 5.69[a]	35.63 ± 3.84	21.75 ± 3.69[b]
Cytoplasmic	3.94 ± 1.88[a]	3.25 ± 1.25	4.00 ± 1.00[a]	1.75 ± 0.59[b]
Teratozoospermy	1.51 ± 0.06	1.52 ± 0.05	1.40 ± 0.05	1.26 ± 0.04
Deformity index	1.47 ± 0.07	1.44 ± 0.06	1.33 ± 0.04	0.93 ± 0.07
Single defects	49.29 ± 5.63	47.44 ± 5.41	58.90 ± 4.22	51.25 ± 3.46
Single defects	1.59 ± 0.52	2.38 ± 0.59	1.48 ± 0.61[a]	3.50 ± 1.45[b]
Multiple defects	46.13 ± 5.69[a]	45.41 ± 6.06[a]	34.15 ± 4.35	18.25 ± 3.69[b]
Defects (normal)	42.81 ± 8.10[a]	55.55 ± 4.94[a]	58.36 ± 9.57	84.01 ± 6.98[b]

Different letters in superscript following means in the same row indicate statistical significance ($p<0.05$)

4.2.1 pH

The pH values of the four groups is shown in Figure 12. The samples positive for *N. gonorrhoea* (7.76 ± 0.09) had the highest pH level in comparison to the *C. trachomatis* (7.61 ± 0.07) and *T. vaginalis* (7.69 ± 0.09) samples. Whereas the control sample group was only slightly more acidic (7.60 ± 0.04). However, there was no significant difference found among the pH values recorded in this study (p=0.29).

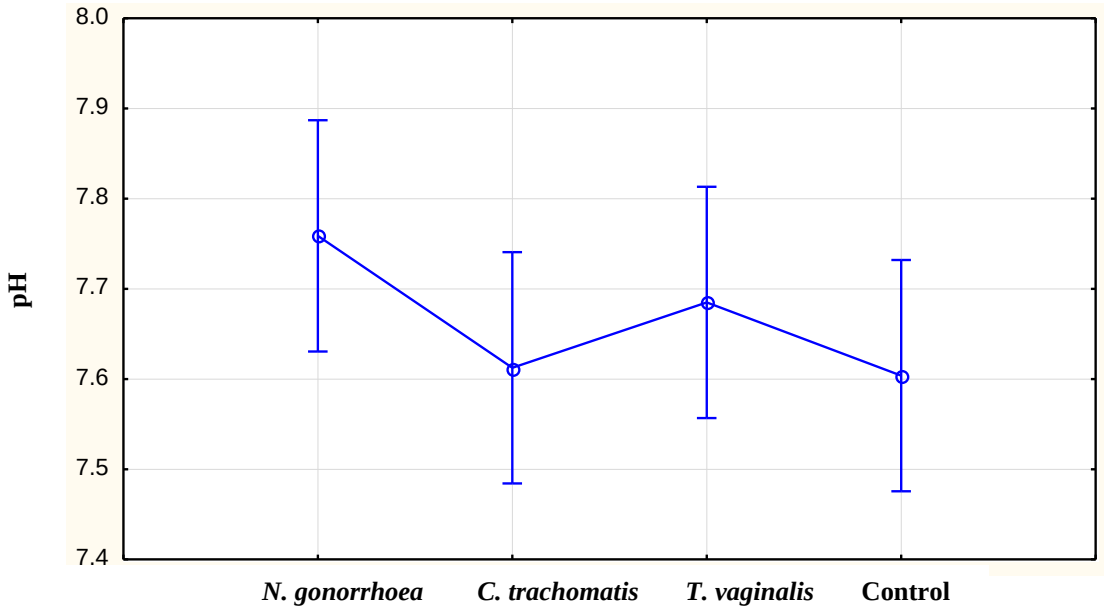


Figure 12: pH of the four sample groups

4.2.2 Volume

No statistically significant difference in volume was found between the four sample groups (p=0.16), as depicted in Figure 13. The sample population positive for *N. gonorrhoea* (2.49 ± 0.64 ml) had the lowest mean volume of ejaculate. However, all four of the sample groups exceeded the WHO reference value of 1.5 ml (WHO, 2010) with the following recorded: *C. trachomatis* (2.90 ± 0.53 ml), *T. vaginalis* (3.66 ± 0.52 ml) and the control group (3.06 ± 2.79 ml)

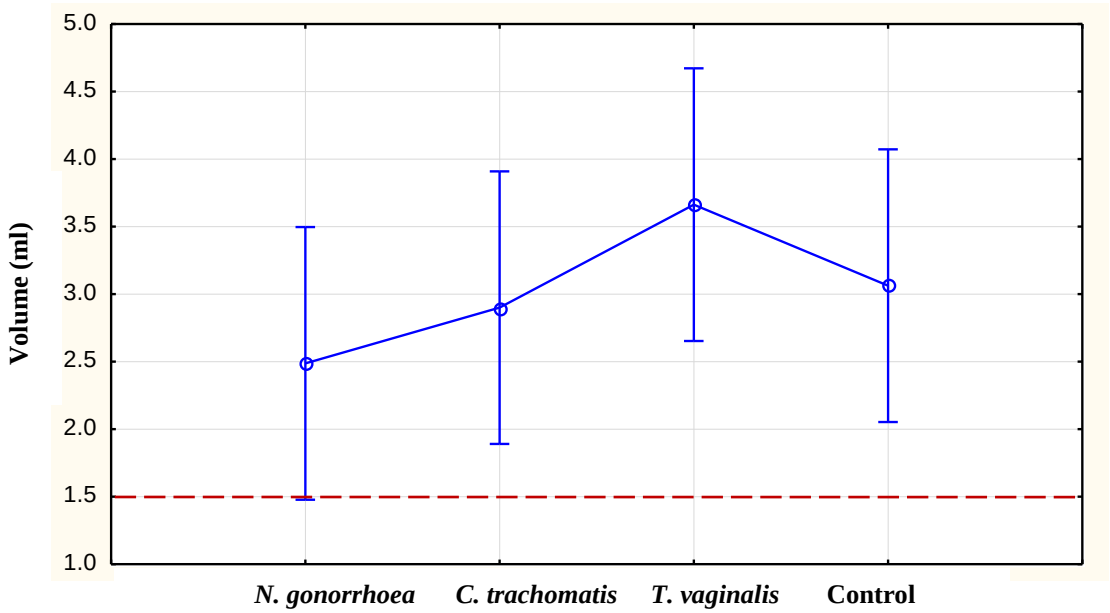
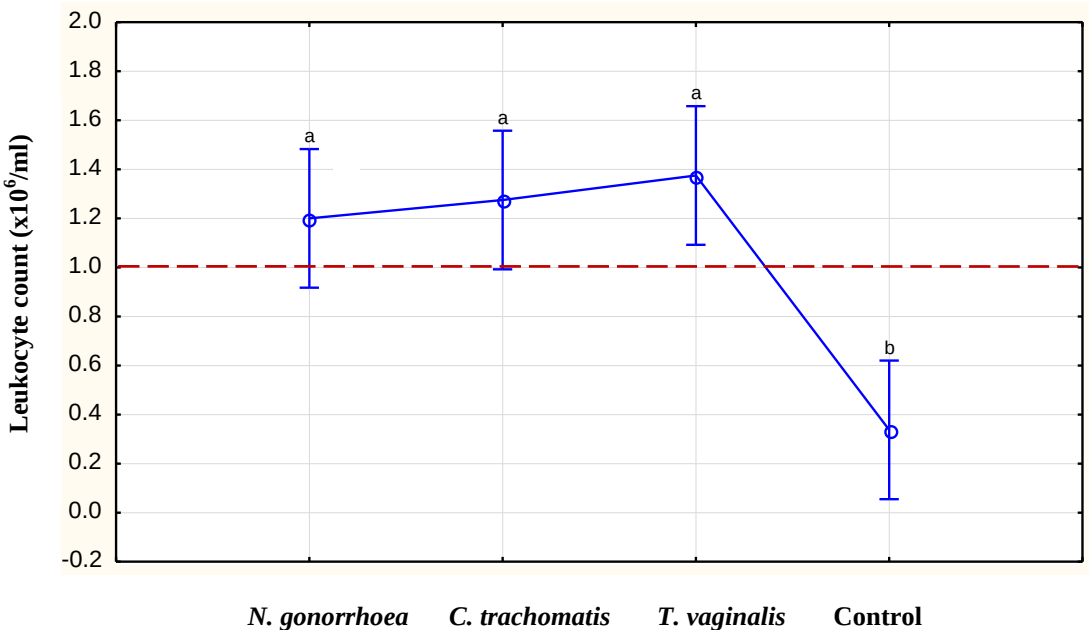


Figure 13: Volume (ml) of the four sample groups

4.2.3 Leukocyte count (x10^6/ml)

Leukocytospermia, as detected by the number of peroxidase positive cells, is the condition of more than 10^6 WBCs per milliliter of semen (WHO, 2010). The ejaculates that presented with the bacterium *T. vaginalis* had the highest concentration of peroxidase positive cells (1.38 ± 0.17 x10^6/ml), which is shown in Figure 14, followed by *N. gonorrhoea* (1.20 ± 0.01 x10^6/ml) and *C. trachomatis* (1.28 ± 0.15 x10^6/ml). The leukocyte count of the control group (0.34 ± 0.09 x10^6/ml) (p=0.032) was statistically significantly (p<0.05) lower in comparison to the three STI positive sample populations and falls below the criteria for leukocytospermia.

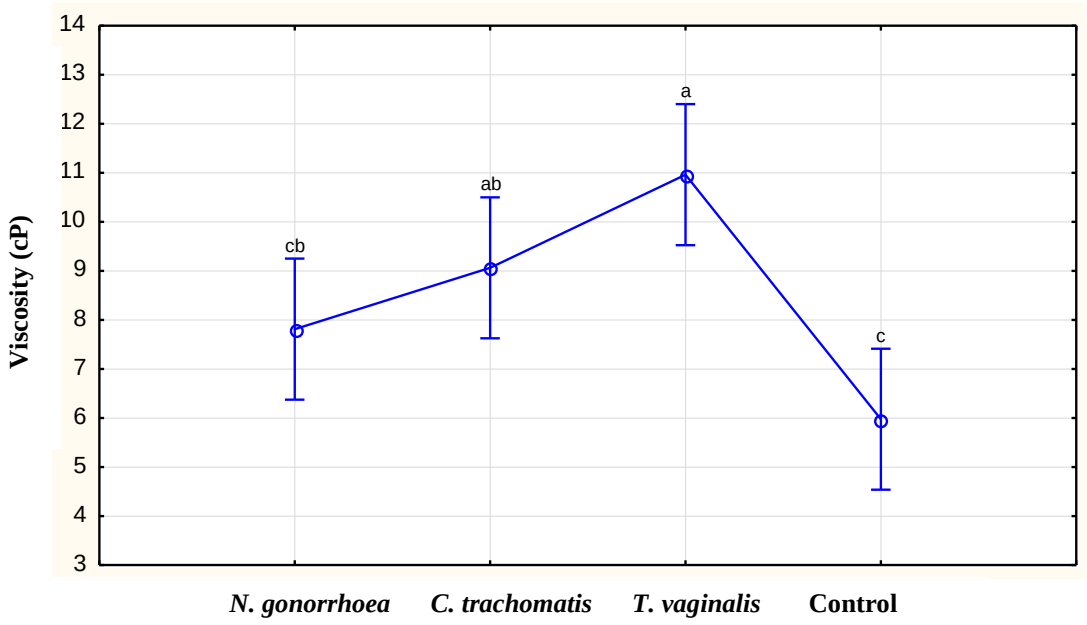


Means with different letters denote significance (p<0.05)

Figure 14: Leukocyte count (x10^6/ml) of the four sample groups

4.2.4 Viscosity (cP)

Figure 15 shows the different viscosity values, quantified in centipoise. Based on a past study (Mendeluk *et al*., 2000); semen with a viscosity of 4.3 ± 0.2 cP can be regarded as normal consistency. The filling times of the lamellar capillary of the Leja® chamber-slide and the calculated viscosity thereof, was the highest in samples identified as positive for *T. vaginalis* (10.96 ± 0.99 cP), which was statistically significantly different ($p<0.05$) in comparison to the control group (5.98 ± 0.46 cP) ($p=0.033$). In addition, the samples positive for *N. gonorrhoea* (7.81 ± 0.71 cP) were statistically significantly ($p<0.05$) lower in comparion to the samples positive for *T. vaginalis* ($p=0.048$). The samples positive for *C. trachomatis* (9.06 ± 0.52 cP) displayed significantly higher ($p<0.05$) levels of viscosity in comparison to the control group ($p=0.038$).

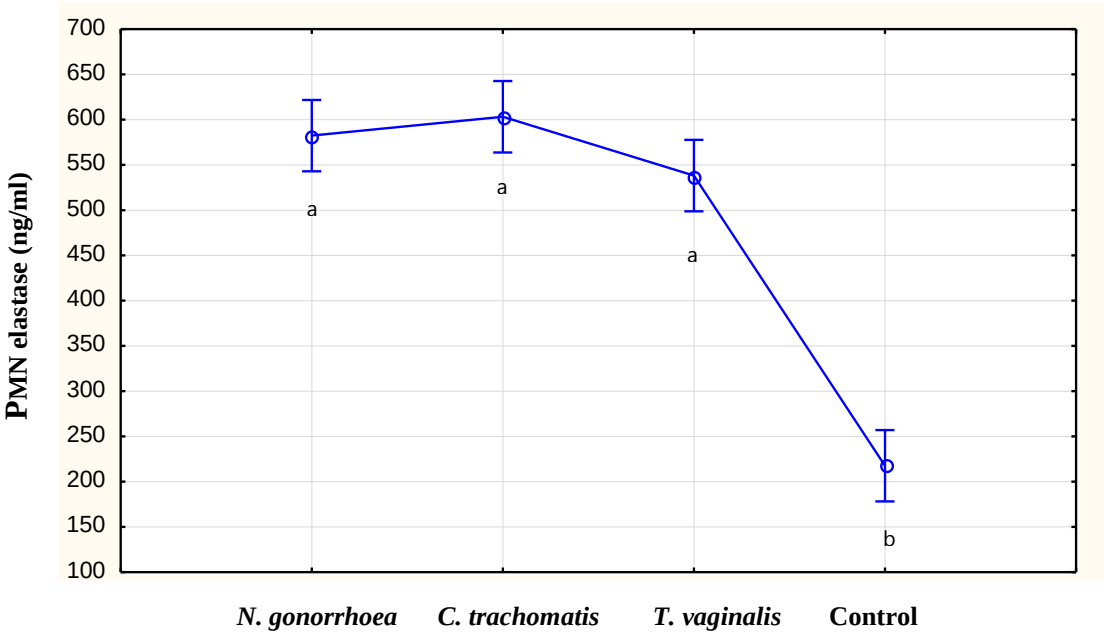


Means with different letters denote significance ($p<0.05$)

Figure 15: Viscosity (cP) of the four sample groups

4.2.5 PMN elastase

Figure 16 represents the concentrations of PMN elastase as detected by the ELISA test. The *C. trachomatis* sample group showed the highest concentration of PMN elastase (603.25 ± 20.00 ng/ml), followed by the sample group positive for *N. gonorrhoea* (582.38 ± 7.72 ng/ml) and *T. vaginalis* (538.25 ± 27.50 ng/ml). The control group (217.63 ± 16.34 ng/ml) falls below the threshold level of 280 ng/ml, the concentration considered as positive for leukocytospermia (Ludwig *et al*., 2003). The mean concentrations of PMN elastase in the STI positive samples were statistically significantly ($p<0.05$) higher than the control group ($p=0.024$).

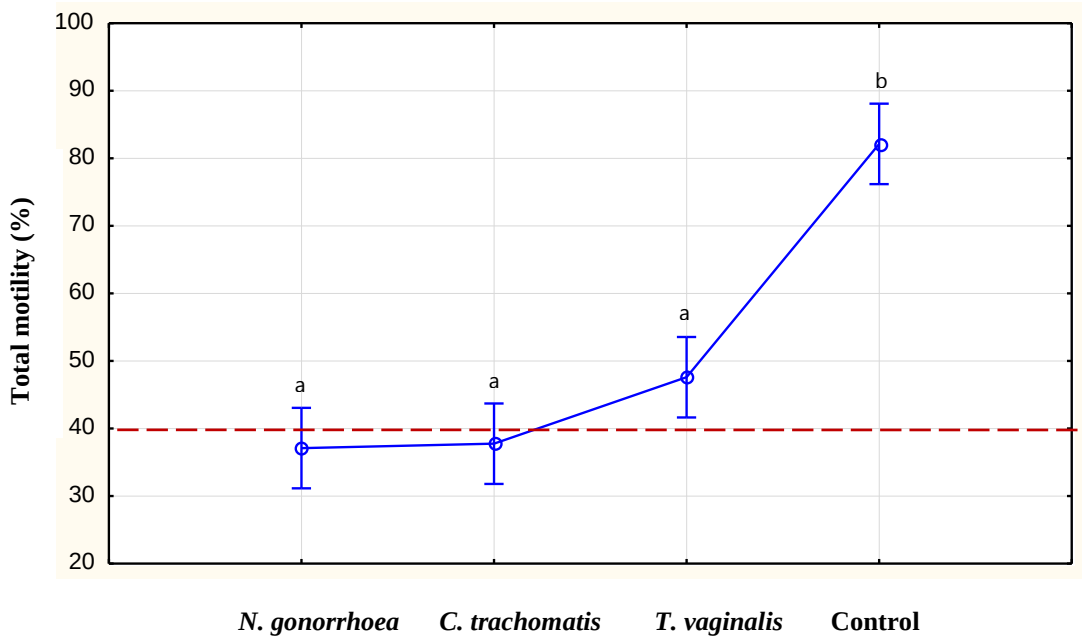


Means with different letters denote significance (p<0.05)

Figure 16: PMN Elastase (ng/ml) of the four sample groups

4.2.6 Motility

The CASA results of total motility (%) are shown in Figure 17. Among the STI positive sample populations, only the semen samples identified as positive for *T. vaginalis* (47.60 ± 2.15 %) had spermatozoa which exceeded the WHO reference value for total motility of 40% (WHO, 2010). *N. gonorrhoea* (37.10 ± 3.18 %) and *C. trachomatis* (37.76 ± 4.23 %) were recorded below this reference value. The total motility of all three STI positive groups were statistically significantly ($p<0.05$) lower in comparison to the control group (82.14 ± 1.06 %) ($p=0.016$).

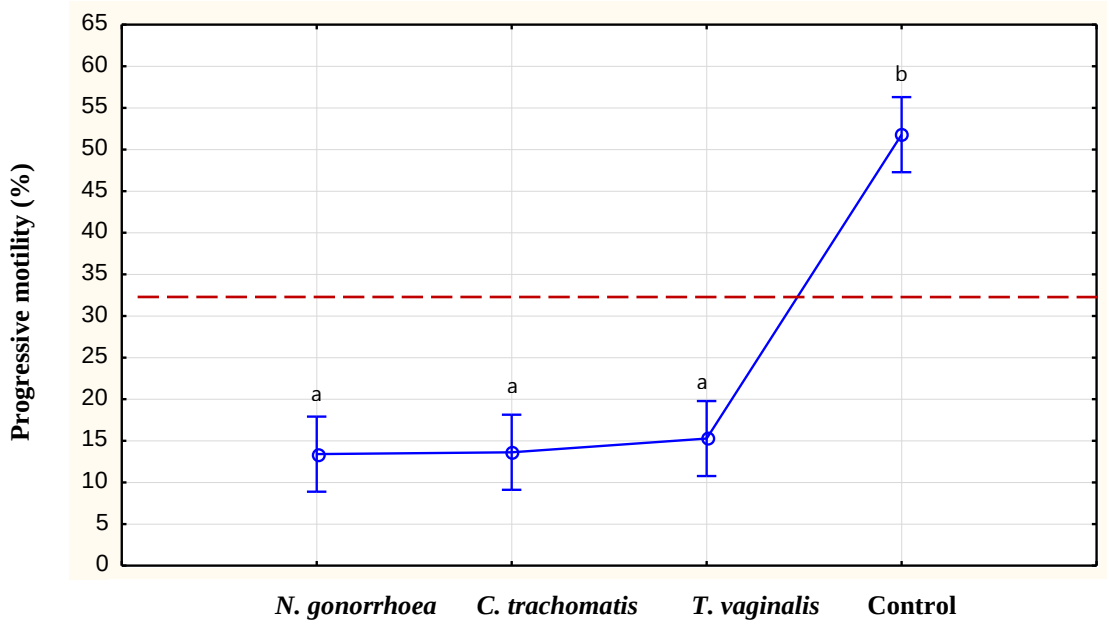


Means with different letters denote significance ($p<0.05$)

Figure 17: Total motility (%) of the four sample groups

Progressive motility

Figure 18 represents the percentage of progressively motile spermatozoa obtained from the four sample groups. The substantial decrease in this parameter amongst the three STI positive sample groups is highlighted as the mean progressive motility of the samples positive for *N. gonorrhoea* (13.40 ± 1.93 %), *C. trachomatis* (13.63 ± 2.34 %) and *T. vaginalis* (15.28 ± 1.97 %) fall below the WHO reference value of 32% (WHO, 2010). The control group's (51.79 ± 2.51 %) mean percentage of progressively motile spermatozoa was statistically significantly ($p<0.05$) higher than the STI positive groups ($p=0.0016$).

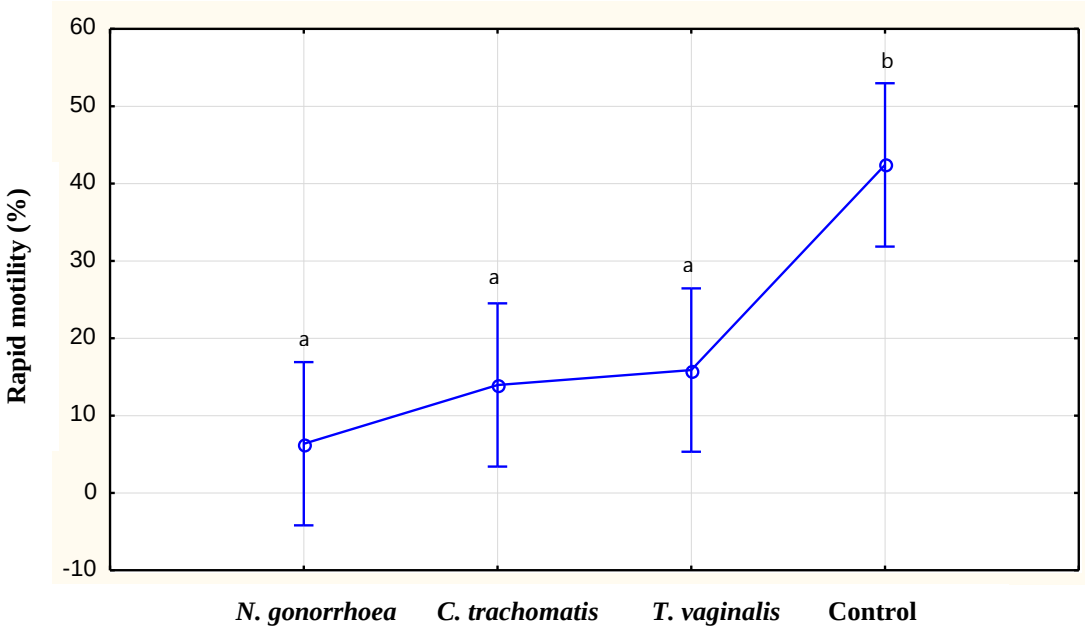


Means with different letters denote significance ($p<0.05$)

Figure 18: Progressive motility (%) of the four sample groups

Rapid swimming spermatozoa

CASA results of the percentage of rapidly motile spermatozoa are shown in Figure 19. Samples positive for *N. gonorrhoea* had the lowest mean (6.36 ± 3.18 %), followed by *C. trachomatis* (13.96 ± 7.08 %) and *T. vaginalis* (15.89 ± 2.97 %). The three STI sample groups had statistically significantly ($p<0.05$) lower rapid swimming spermatozoa in comparison to the control group (42.41 ± 6.09 %) ($p=0.0011$).

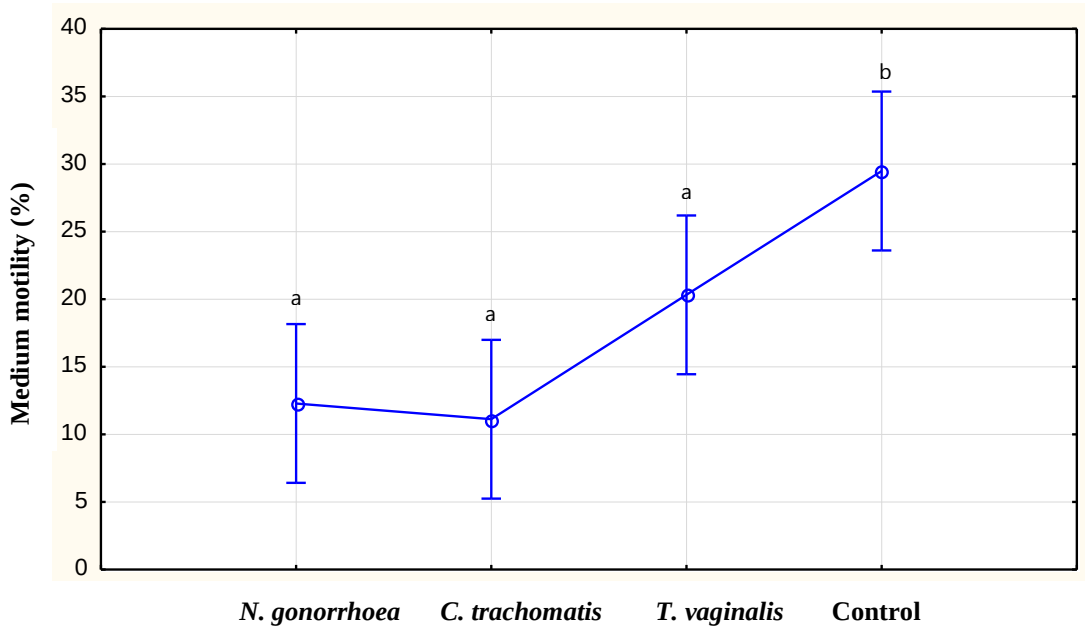


Means with different letters denote significance ($p<0.05$)

Figure 19: Rapid swimming spermatozoa (%) of the four sample groups

Medium swimming spermatozoa

The percentages of medium swimming spermatozoa are represented in Figure 20, with the sample group positive for *C. trachomatis* (11.13 ± 7.42 %) displaying the lowest mean percentage. In conjunction with the *C. trachomatis* group, *N. gonorrhoea* (12.29 ± 2.21 %) and *T. vaginalis* (20.33 ± 1.93 %) sample groups had statistically significant ($p<0.05$) lower percentages of medium swimming spermatozoa in comparison to the control group (29.49 ± 4.17 %) ($p=0.031$).



Means with different letters denote significance ($p<0.05$)

Figure 20: Medium swimming spermatozoa (%) of the four sample groups

Slow swimming spermatozoa

Figure 21 represents the mean percentages of slow swimming spermatozoa. *N. gonorrhoea* positive samples had the lowest percentage of slow swimming spermatozoa (9.18 ± 2.92 %), whilst samples positive for *T. vaginalis* had the highest percentage (18.64 ± 3.32 %) followed by *C. trachomatis* (15.78 ± 6.12 %) and the control group (13.36 ± 2.49 %). No statistical significance was observed (p=0.10).

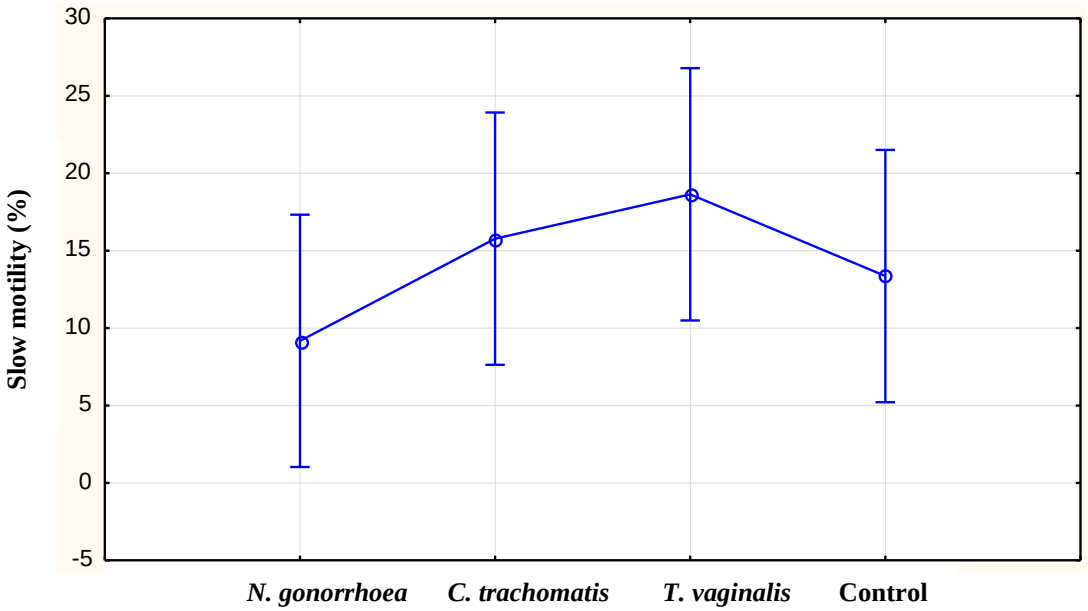
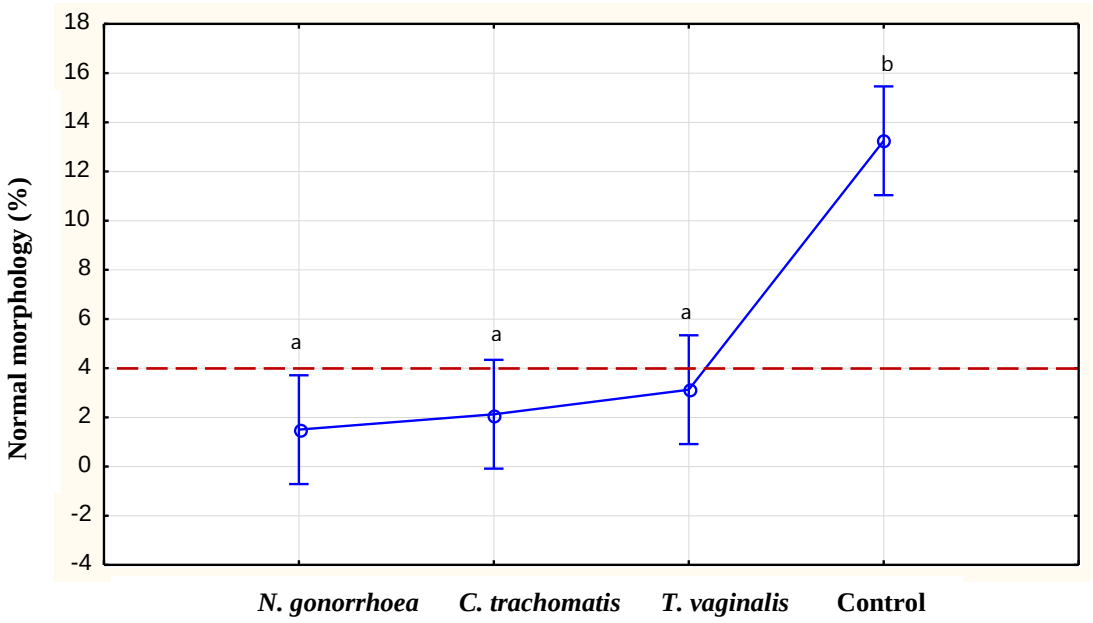


Figure 21: Slow swimming spermatozoa (%) of the four sample groups

4.2.7 Morphology

The characterisation of spermatozoa morphology graded according to the WHO criteria is shown in Figure 22. The sample collection of semen identified as *N. gonorrhoea* positive had the lowest percentage of spermatozoa with normal morphology (1.50 ± 0.38 %), The STI positive sample groups' *C. trachomatis* (2.13 ± 0.52 %) and *T. vaginalis* (3.13 ± 0.67 %) had a normal morphology that falls below the WHO lower reference limit of 4% (WHO, 2010). The control group (13.25 ± 1.95 %) had a mean normal morphology that was statistically significantly ($p<0.05$) higher than the three STI positive groups ($p=0.0021$).

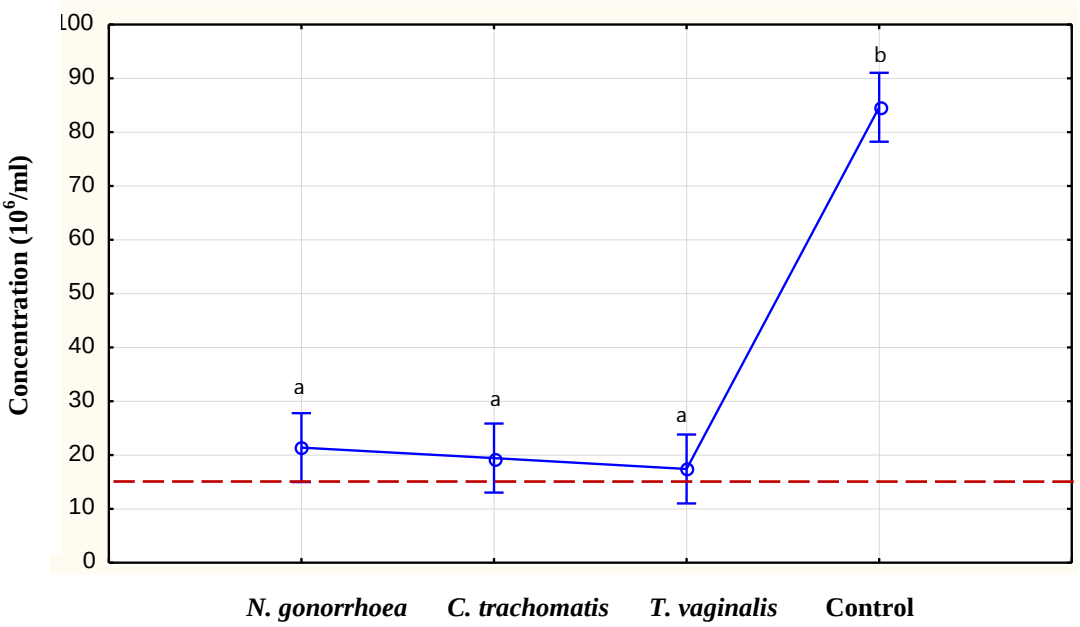


Means with different letters denote significance ($p<0.05$)

Figure 22: Normal morphology (%) of the four sample groups

4.2.8 Concentration (10^6/ml)

The spermatozoa concentration per millilitre of semen is represented in Figure 23, which shows the highly statistically significant difference between the control group versus the three STI positive sample populations. *T. vaginalis* (17.43 ± 3.45 x10^6/ml) showed the lowest mean concentration of spermatozoa, followed by *C. trachomatis* (19.45 ± 2.49 x10^6/ml) and *N. gonorrhoea* (21.39 ± 3.39 x10^6/ml). However, all four sample groups had a concentration of spermatozoa per millilitre above the WHO reference limit of 15 x10^6/ml (WHO, 2010). Statistically significant difference ($p<0.05$) was found between the spermatozoa concentration of the control group (84.64 ± 3.22 x10^6/ml) versus the three STI positive groups ($p=0.0014$).

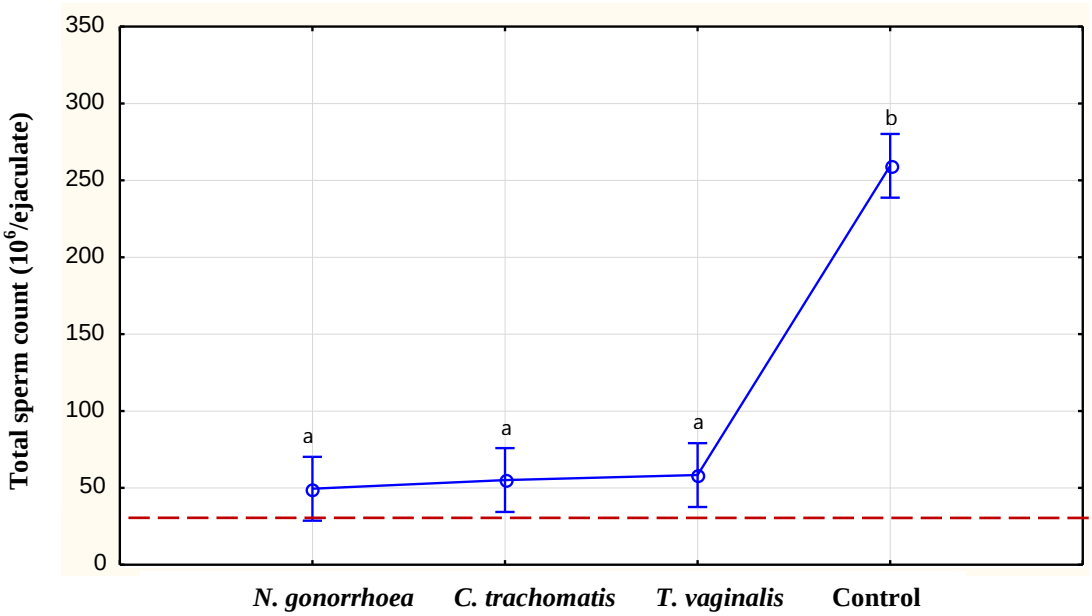


Means with different letters denote significance ($p<0.05$)

Figure 23: Concentration (10^6/ml) of the four sample groups

Total sperm count (10^6/ejaculate)

The sample group positive for *N. gonorrhoea* (49.44 ± 14.39 x10^6/ejaculate) had the lowest mean total sperm count amongst the sample groups as shown in Figure 24, followed by *C. trachomatis* (55.11 ± 8.82 x10^6/ejaculate) and *T. vaginalis* (58.35 ± 8.86 x10^6/ejaculate). The control group was significantly ($p<0.05$) higher than all three STI positive groups (258.99 ± 0.34 x10^6/ejaculate) ($p=0.0027$). All four sample groups had a total sperm count above the WHO- reference value of 39 x10^6/ejaculate (WHO, 2010).

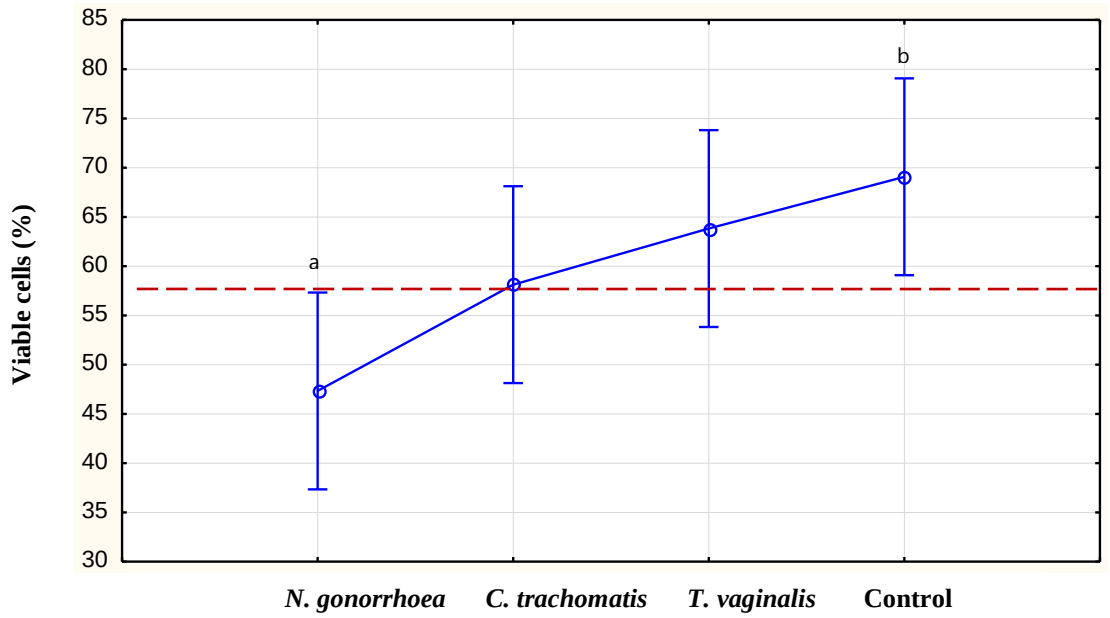


Means with different letters denote significance ($p<0.05$)

Figure 24: Total sperm count (10^6/ejaculate) of the four sample groups

4.2.9 Viability

Figure 25 represents the results of the dye exclusion technique to assess spermatozoa viability. The percent of viable spermatozoa was decreased in the STI positive sample groups in comparison to the control group, with samples positive for *N. gonorrhoea* to significantly($p<0.05$) have the lowest percentage of viable sperm (47.34 ± 5.91 %) falling below the WHO reference value of 58% (WHO, 2010), in comparison to the control (69.01 ± 4.88 %) ($p=0.041$). Despite the percentage of viable cells being lower in the samples positive for *C. trachomatis* (58.13 ± 5.72 %) and *T. vaginalis* (63.83 ± 5.49 %), there was no significant difference ($p=0.06$) when compared to the control group.

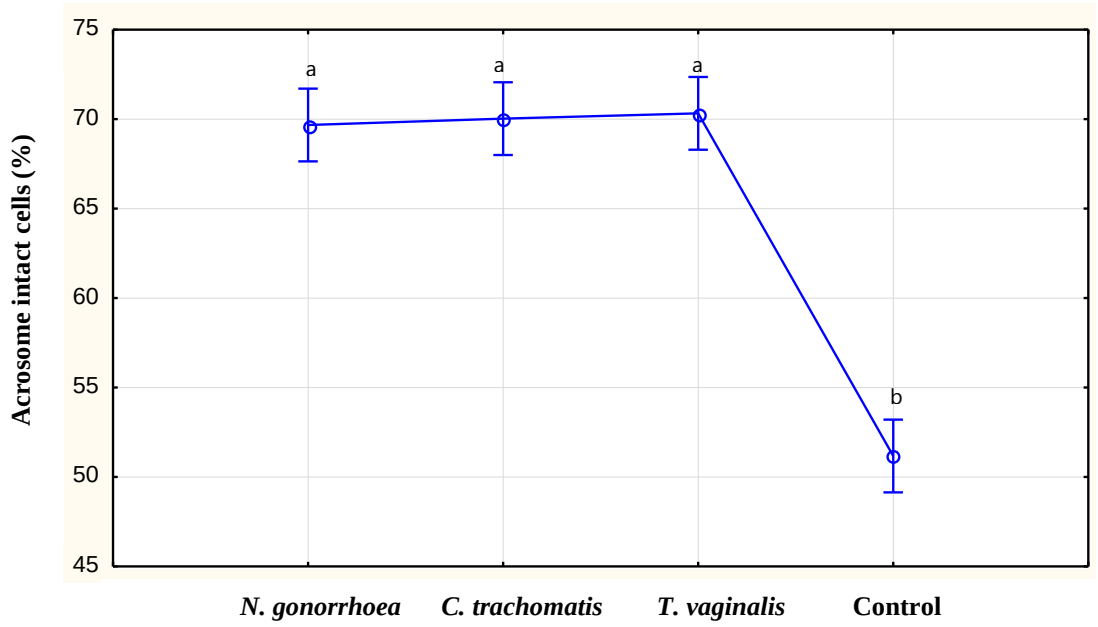


Means with different letters denote significance ($p<0.05$)

Figure 25: Viable Cells (%) of the four sample groups

4.2.10 Acrosome status

Figure 26 shows the percentage of acrosome intact spermatozoa as detected by green fluorescence microscopy, using FITC-PSA. The results show that the percentage of acrosome intact spermatozoa was increased in the samples identified as positive for the STIs tested in this study, in comparison to the control group. Samples positive for the *T. vaginalis* bacterium had the highest percentage of acrosome intactness (70.32 ± 0.75 %), followed by *C. trachomatis* (70.03 ± 0.79 %) and *N. gonorrhoea* (69.67 ± 1.07 %). When compared to the control group (51.18 ± 1.27 %), the STI samples had statistically significantly ($p<0.05$) more acrosome intact unstimulated cells ($p=0.00015$).



Means with different letters denote significance ($p<0.05$)

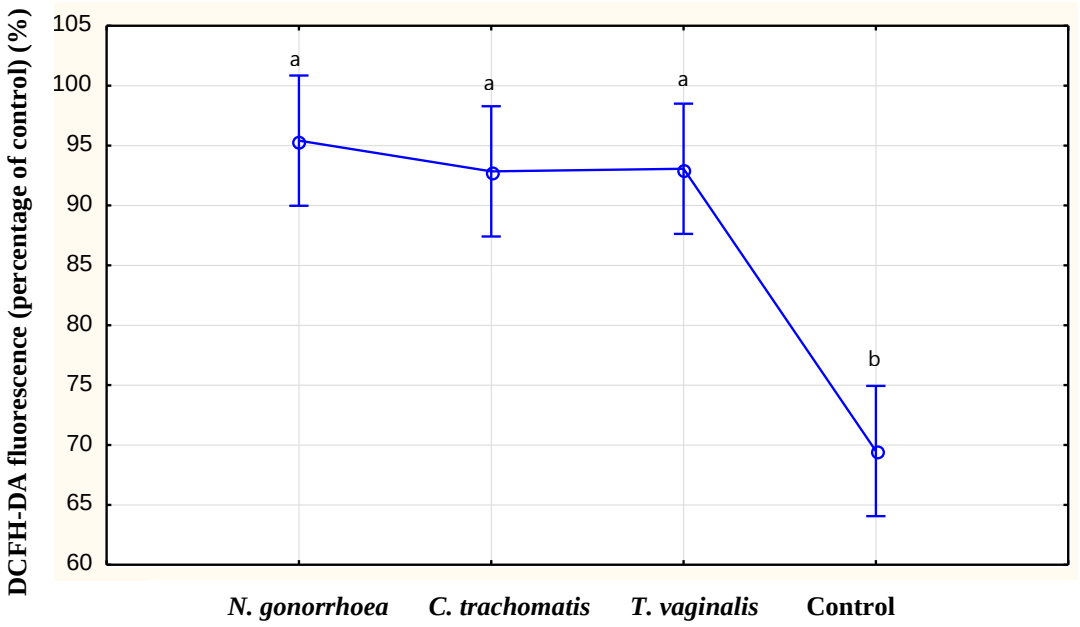
Figure 26: Acrosome intact cells (%) of the four sample groups

Objective 3:

Investigate the impact of infection on the generation of ROS and the possible impact on the spermatozoa's DNA.

4.3.1 ROS

The levels of ROS among the four groups is represented in Figure 27. These results were obtained by using DCFH-DA, a cell permeable probe detecting intracellular ROS. Statisitically significantly ($p<0.05$) increased mean percentages of DCFH-DA fluorescence were observed in the STI positive populations, with the *N. gonorrhoea* positive samples having the highest degree of detected ROS (95.41 ± 1.64 %). All three of the STI positive groups (*N. gonorrhoea*, 95.41 ± 1.64 %; *T. vaginalis*, 93.06 ± 4.12 %; *C. trachomatis*, 92.80 ± 4.44 %). were statistically higher in ROS than the control group (69.50 ± 13.64 %) ($p=0.0038$).

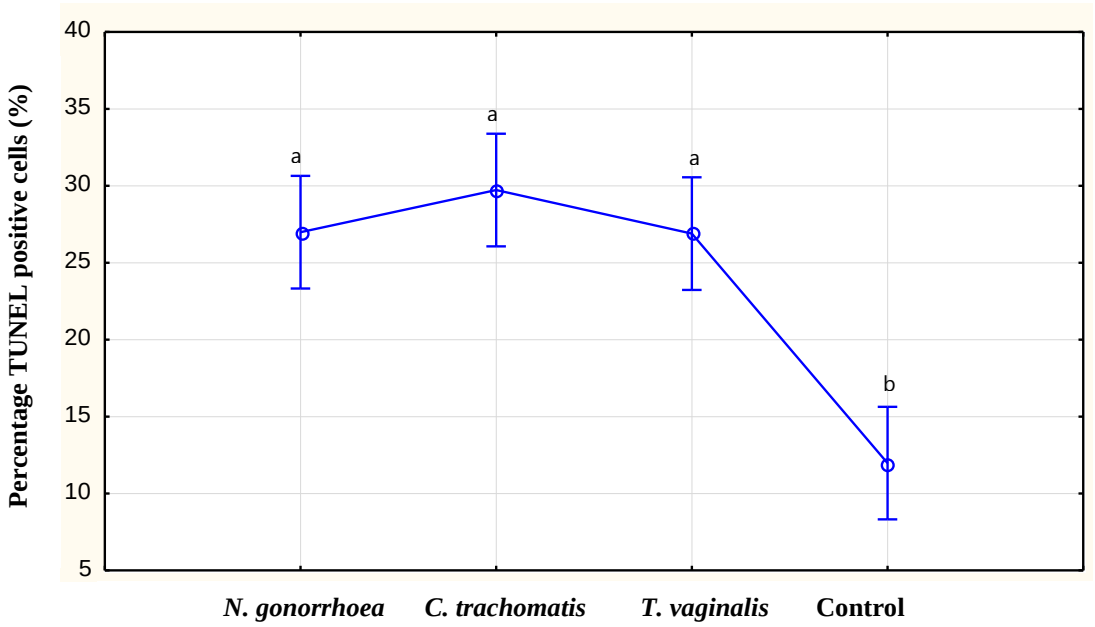


Means with different letters denote significance ($p<0.05$)

Figure 27: DCFH-DA fluorescence (percentage of control) of the four sample groups

4.3.2 DNA fragmentation

The fluorescence, which indicates the extent of DNA fragmentation, as detected by the labeling of the OH^- group, is represented in Figure 28. The group of *C. trachomatis* positive ejaculates (29.73 ± 1.99 %), displayed the highest percentage of emitted fluorescence followed by *N. gonorrhoea* (26.99 ± 1.91 %) and *T. vaginalis* (26.90 ± 2.13 %). Statistical significance ($p<0.05$) was observed between the control (11.98 ± 0.76 %) group and the three STI positive sample groups ($p=0.0029$).



Means with different letters denote significance ($p<0.05$)

Figure 28: Percentage of TUNEL positive cells of the four sample groups

4.3.4 Correlation Analysis: Objective 2

Correlation analysis between the parameters assessed in objective two is summarised in Tables 5-8 (Appendix B). From the four groups, the parameters, which displayed statistical significance in the correlations, will be represented in Figures 32-34.

Among the samples positive for *C. trachomatis*, a statistically significantly ($p<0.05$) negative correlation ($r= -0.768$; $p=0.0042$) was found between an increase in semen viscosity and corresponding decrease in the percentage of spermatozoa with normal morphology. As shown in Figure 29, the viscosity measurements of this sample group were found to range from 6.9 - 11.5 cP.

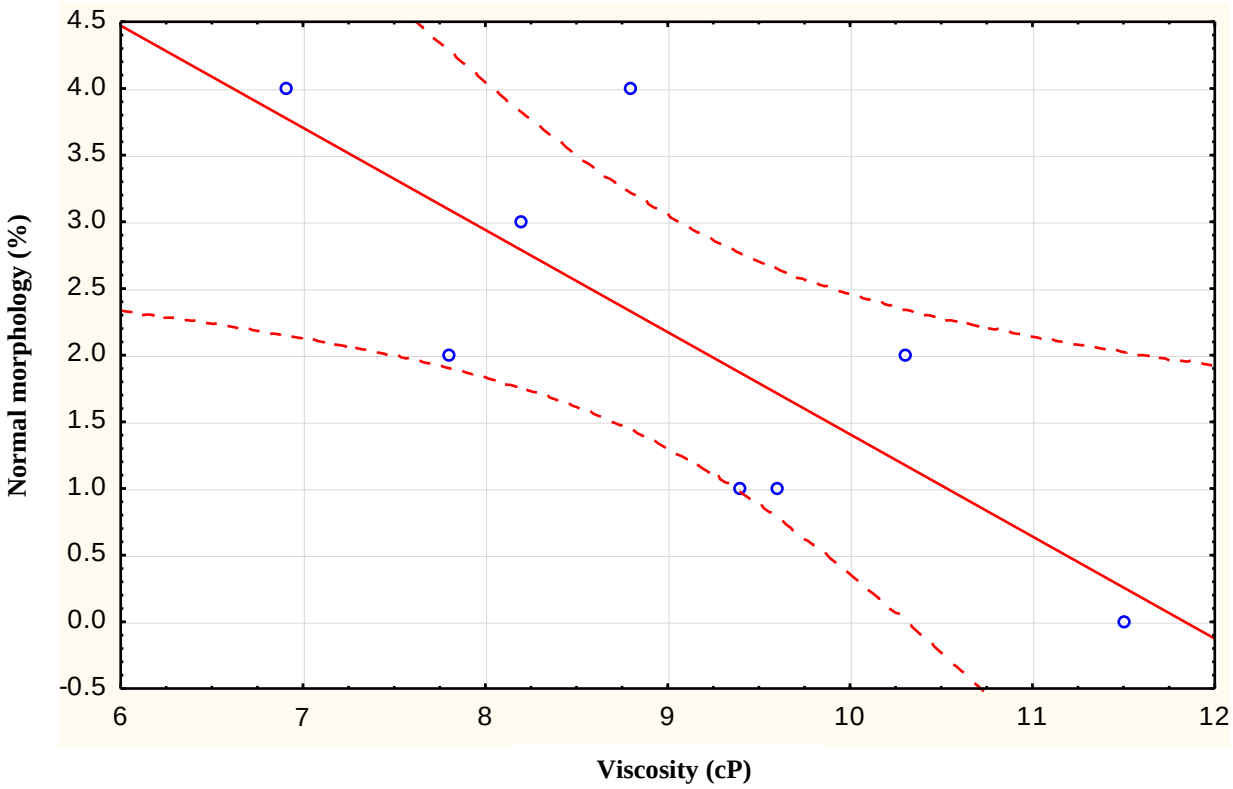


Figure 29: Correlation analysis between semen viscosity (cP) and normal spermatozoa morphology (%)

In the sample group positive for *C. trachomatis*, Figure 30 shows the statistically significant ($p<0.05$) negative correlation ($r= -0.653$; $p=0.0044$) between DCFH-DA fluorescence (percentage of control) and the percentage of total motile spermatozoa. The marker of ROS ranged from 39.46 % to 80.68 %, whilst total motile spermatozoa ranged from 24.7 % to 53.8 %.

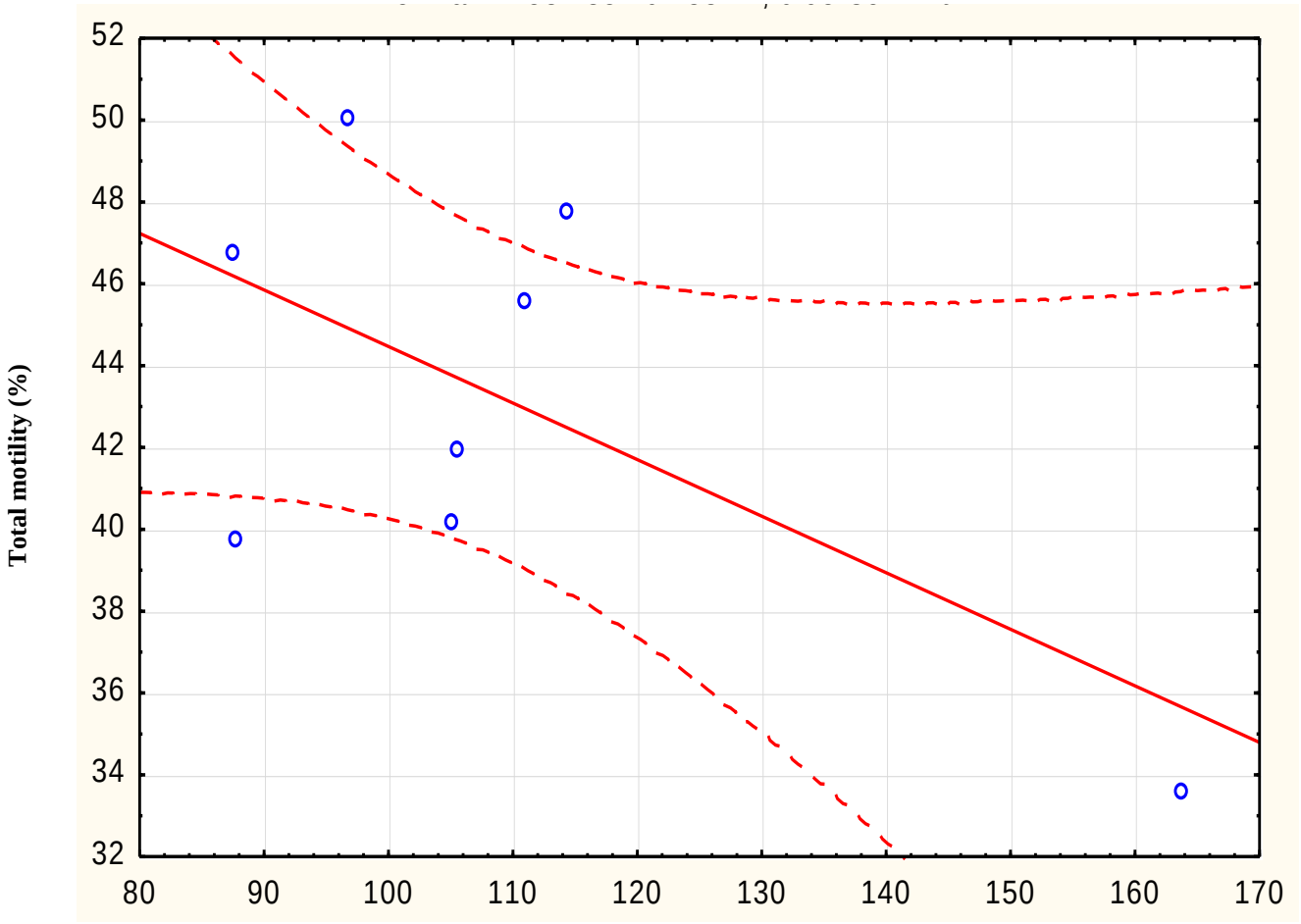


Figure 30: Correlation analysis between DCFH-DA fluorescence (percentage of control) and total motility in the sample group positive for *C. trachomatis*

4.3.5 Correlation analysis: Objective 3

Correlation analysis of the results of ROS and spermatozoa DNA fragmentation showed statisitical significance ($p<0.05$) in the sample group positive for *N. gonorrhoea.* Figure 31 shows the expected outcome of a significant positive correlation ($r= 0.494$; $p=0.0037$). The percentage of TUNEL positive cells ranged from 19-35 %, whereas the DCFH-DA fluorescence ranged from 65-117 %.

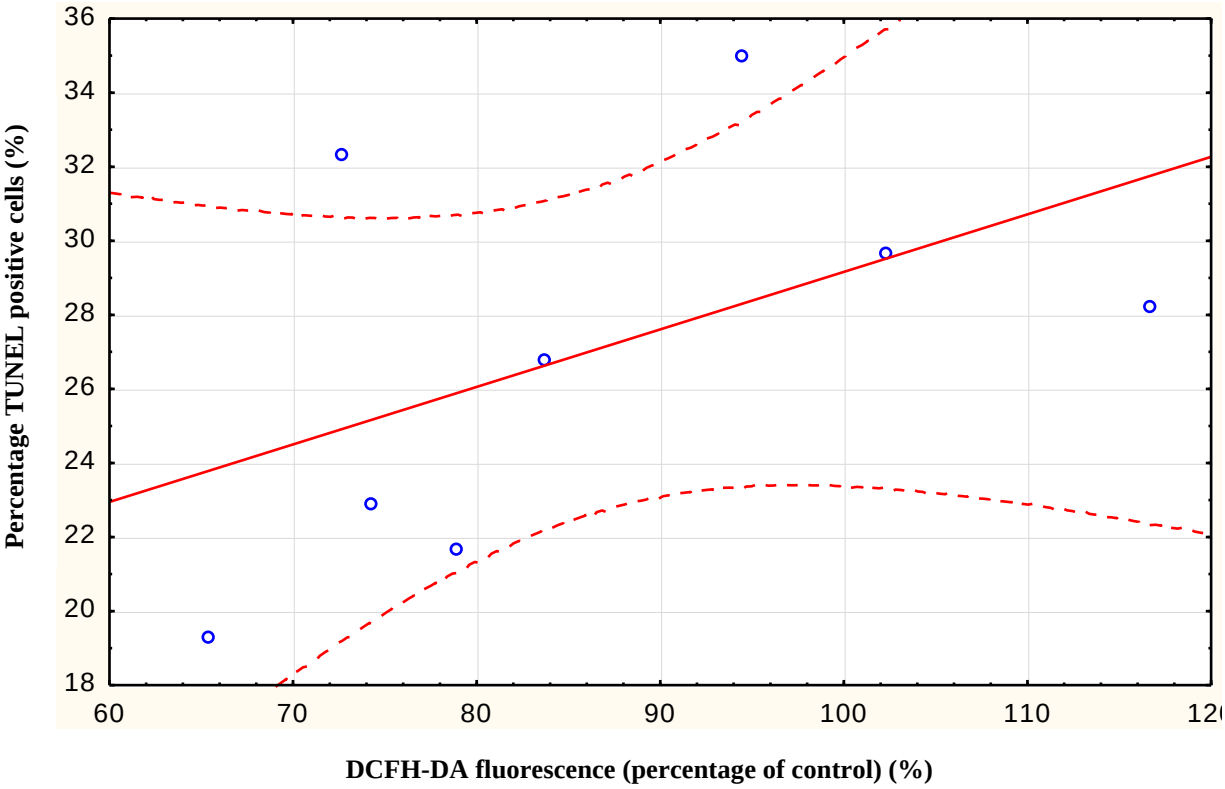


Figure 31: Correlation analysis between DCFH-DA fluorescence (%) and TUNEL positive cells (%)

Objective 4:

Investigate the effect of N. gonorrhoea, C. trachomatis and T. vaginalis on the secretory function of the prostate and seminal vesicles in comparison to the control, determined through the quantification of seminal plasma citric acid and fructose concentrations.

4.4.1 Citric acid

The mean citric acid levels per ejaculate of the four groups quantified in this study is shown in Figure 32. The sample group positive for *N. gonorrhoea* had the lowest mean concentration of citric acid (9.60 ± 0.78 mg/ejaculate). The three STI positive sample groups had a mean above the WHO reference value of 9.36 mg or more per ejaculate (WHO, 2010), with the following values: *C. trachomatis* (11.39 ± 0.58 mg/ejaculate) and *T. vaginalis* (12.04 ± 0.81 mg/ejaculate). Interestingly, the control group's citric acid concentration (7.66 ± 0.47 mg/ejaculate) fell below the reference value. No statistical significance was observed among the four groups (p=0.055).

Table 6: Summary of the citric acid and fructose concentrations (mean ± SEM)

	N. gonorrhoea **(n=25)**	***C. trachomatis*** **(n=19)**	***T. vaginalis*** **(n=11)**	**Control (n=65)**
Citric Acid (mg/ejaculate)	9.60 ± 0.78	11.39 ± 0.58	12.04 ± 0.81	7.66 ± 0.47
Fructose (mg/ejaculate)	1.79 ± 0.26[a]	2.58 ± 0.40	2.91 ± 0.31	3.41 ± 0.16[b]

Different letters in superscript following means in the same row indicate statistical significance (p<0.05)

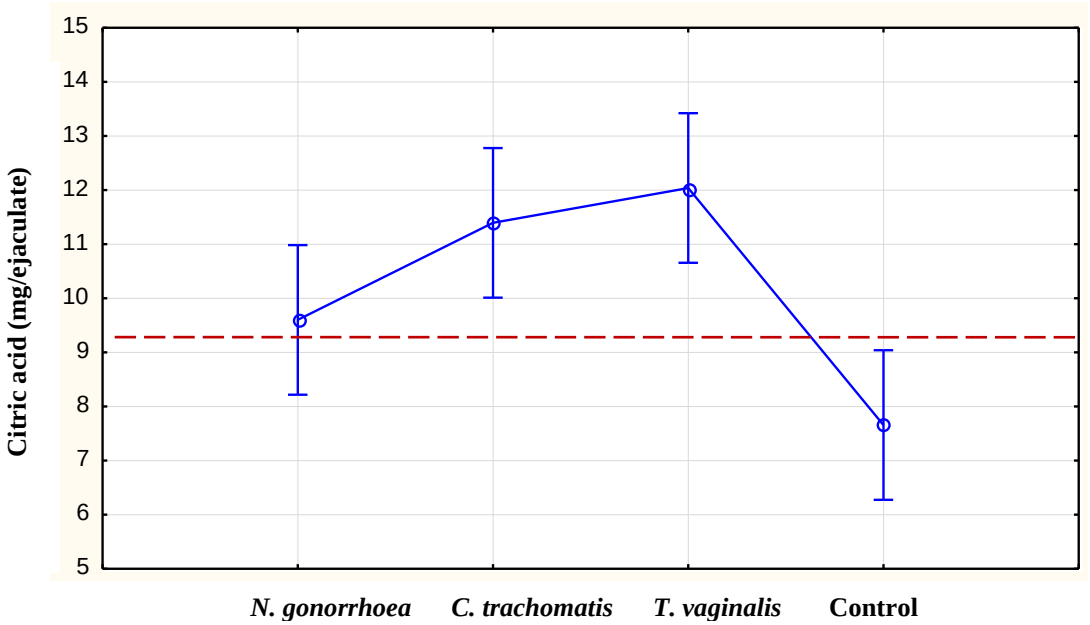
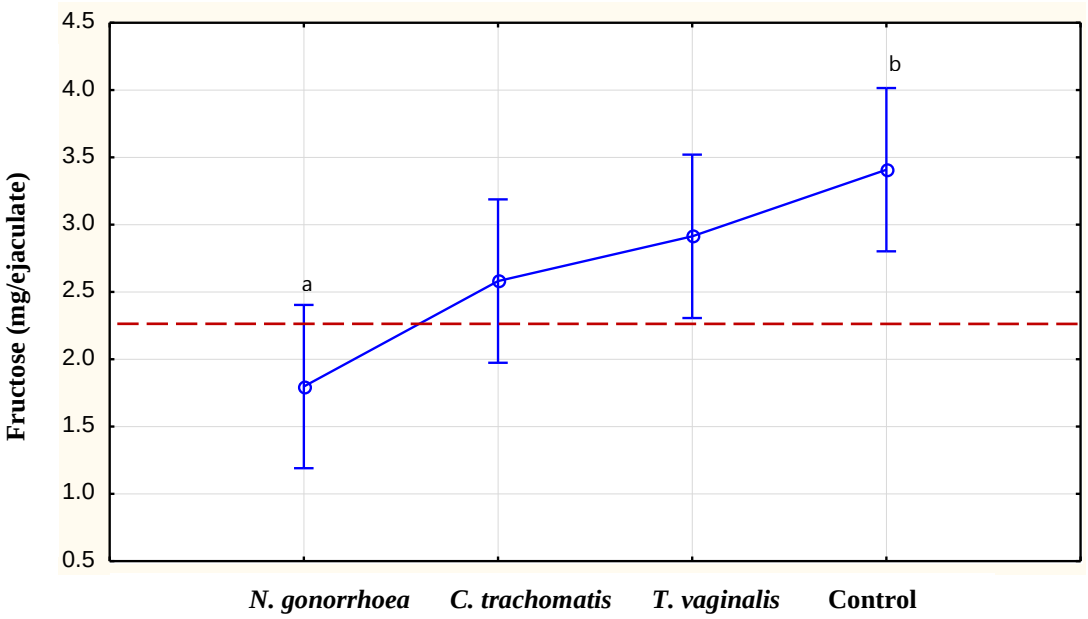


Figure 32: Citric acid (mg/ejaculate) of the four sample groups

4.4.2 Fructose

Figure 33 illustrates the mean concentrations of fructose per ejaculate. The samples positive for *N. gonorrhoea* (1.79 ± 0.26 mg/ejaculate) were the only semen samples in this study that fell below the WHO normal reference value of 2.34 mg or more per ejaculate (WHO, 2010). *C. trachomatis* (2.58 ± 0.40 mg/ejaculate) and *T. vaginalis* (2.91 ± 0.31 mg/ejaculate) were not statistically significant, however, the concentration of fructose in the *N. gonorrhoea* sample group was statistically significantly ($p<0.05$) lower than the control group (3.41 ± 0.16 mg/ejaculate) ($p=0.049$).



Means with different letters denote significance ($p<0.05$)

Figure 33: Fructose (mg/ejaculate) of the four sample groups

4.5 Objective 5:

Determine the differentially expressed proteins in the spermatozoa isolated from samples positive for N. gonorrhoea, C. trachomatis and T. vaginalis.

All the samples from the four groups included in the study were satisfactorily digested as judged by the total ion chromatograms. For the global proteomic assessment, 791 proteins were identified, with two peptides or more and a false-discovery rate threshold of 1%. From this total, 76% of the proteins were reproducible amongst the four groups and were omitted to obtain the expressed proteins unique to each sample group. As a result, 166 DEPs were then isolated and are represented in Table 19 (Appendix B). Of the 166 DEPs, the molecular mass (kDa) ranged from 4.7 - 789.4 and the isoelectric point (pI) ranged from 5.5 – 8.1. The Gene Ontology database (www.geneontology.org) was used to search for the subcellular location of the total proteins identified in the study and is represented in Figure 37. The predominant site of the total number of 791 proteins within the spermatozoa was found to be localised within the cytoplasm (315), followed by 184 proteins identified as being expressed within the mitochondria. 153 proteins, where found in intracellularily, including proteins found within the nucleus, whilst 98 proteins were localised to the extracellular matrix. 33 identified proteins were specified to the membrane, whilst eight proteins were unclassified according to their cellular site within the spermatozoa.

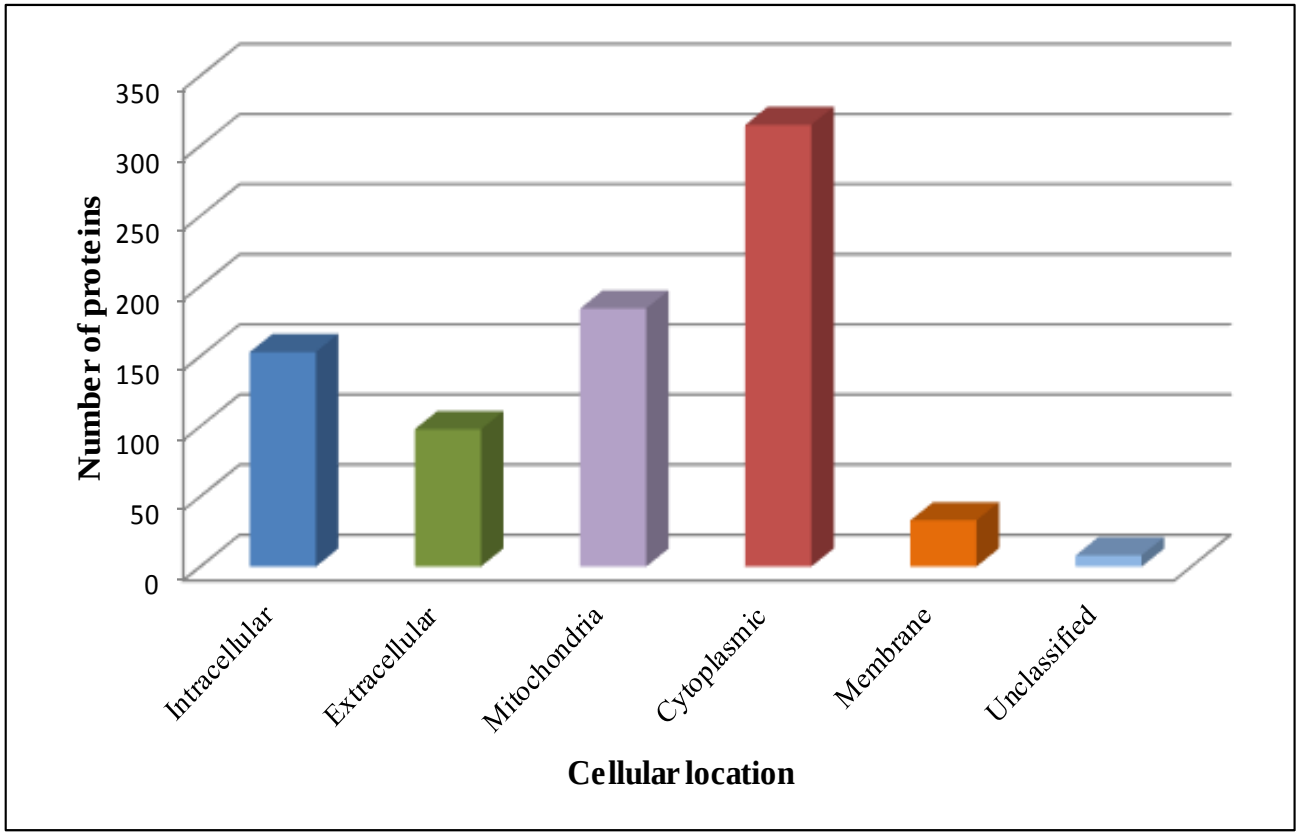


Figure 34: A summary of the cellular distribution of the DEPs

The evaluation of the proteins that were up- and down-regulated between the STI positive groups in comparison to the controls, found a total of 109 down- and 69 up-regulated proteins. 49 DEPs were identified from the *N. gonorrhoea* sample population, with 35 down- and 14 up-regulated *(Table 11).* The samples positive for *C. trachomatis* were found to be the most abundant in DEPs, with 69 down- and 23 up-regulated *(Table 12).* The third population group positive for the bacterium *T. vaginalis*, showed 11 down- and 32 up-regulated proteins *(Table 13).*

Table 7: Summary of the down-regulated DEPs from samples positive for *N. Gonorrhoea*

Accession number	Gene name	Description	Sequence coverage (%)	Molecular mass (kDa)	pI	Fold change	p-value
C9IZE4	**PSMD6**	**26S proteasome non-ATPase regulatory subunit 6**	14.71	51.9	6.74	0.44	1.000
P24752	**ACAT1**	**Acetyl-CoA acetyltransferase, mitochondrial**	52.93	45.2	8.85	0.58	0.530
P23526	**AHCY**	**Adenosylhomocysteinase**	25.93	47.7	6.34	0.14	0.583
B7ZB63	**ARF3**	**ADP-ribosylation factor 3**	49.31	16.1	7.34	0.41	0.555
Q96IU4	**ABHD14B**	**Alpha/beta hydrolase domain-containing protein 14B**	37.14	22.3	6.4	0.38	0.012
P18859	**ATP5J**	**ATP synthase-coupling factor 6, mitochondrial**	65.74	12.6	9.52	0.28	0.004
O75952	**CABYR**	**Calcium-binding tyrosine phosphorylation**	41.18	52.7	4.55	0.35	0.195
Q13939	**CCIN**	**Calicin**	26.7	66.5	8.18	0.42	0.362
Q14093	**CYLC2**	**Cylicin-2**	10.34	39.1	9.74	0.06	0.583
P27487	**DPP4**	**Dipeptidyl peptidase 4**	22.45	88.2	6.04	0.45	0.010
P04844	**RPN2**	**Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2**	24.09	69.2	5.69	0.67	0.560
J3QTJ6	**FSIP2**	**Fibrous sheath-interacting protein 2**	12.49	789.4	6.68	0.19	0.548
P15104	**GLUL**	**Glutamine synthetase**	35.39	42	6.89	0.11	0.365
Q5VVW2	**GARNL3**	**GTPase-activating Rap/Ran-GAP domain-like protein 3**	13.72	112.8	7.65	0.39	0.038
O00410	**IPO5**	**Importin-5**	14.04	123.5	4.94	0.3	0.530

P50213	**IDH3A**	**Isocitrate dehydrogenase (NAD) subunit alpha**	29.23	39.6	6.92	0.27	1.000
P04035-2	**HMGCR**	**Isoform 2 of 3-hydroxy-3-methylglutaryl-coenzyme A**	15.45	92	6.96	0.24	0.167
Q96RQ9-2	**IL4I1**	**Isoform 2 of L-amino-acid oxidase**	18.68	65.3	8.51	0.55	0.325
P02751-7	**FN1**	**Isoform 7 of Fibronectin**	23.55	268.7	5.53	0.43	0.205
J3KQU0	**NUCB2**	**Nefastin-1**	14.1	46.6	5.22	0.49	0.048
Q9H361	**PABPC3**	**Polyadenylate-binding protein 3**	26.15	70	9.67	0.33	0.434
F8W0W8	**PPP1CC**	**Serine/threonine-protein phosphatase**	22.29	38.2	6.83	0.39	0.137
P54709	**ATP1B3**	**Sodium/potassium-transporting ATPase subunit beta-3**	28.32	31.5	8.35	0.29	0.247
A1A5C7	**SLC22A23**	**Solute carrier family 22 member 23**	18.22	73.7	7.74	0.26	0.754
P38646	**HSPA9**	**Stress-70 protein, mitochondrial**	41.24	73.6	6.16	0.27	0.070
Q9UJT2	**TSKS**	**Testis-specific serine kinase substrate**	24.83	65	5.97	0.42	0.567
O94832	**MYO1D**	**Unconventional myosin-Id**	14.51	116.1	9.39	0.24	0.398
D6RBV2	**LMAN2**	**Vesicular integral-membrane protein VIP36**	35.08	36.5	6.65	0.38	0.119

**>1.5 down-regulated; <0.77 down-regulated (Jung et al., 2009; Karp et al., 2010; Breitwieser et al., 2011)*

Table 8: Summary of the up-regulated DEPs from samples positive for *N. Gonorrhoea*

Accession number	Gene name	Description	Sequence coverage (%)	Molecular mass (kDa)	pI	Fold change	p-value
B7ZB63	**ARF3**	**ADP-ribosylation factor 3**	49.31	16.1	7.34	2.28	0.583
P18859	**ATP5J**	**ATP synthase-coupling factor 6, mitochondrial**	65.74	12.6	9.52	2.16	0.167
Q9UII2	**ATPIF1**	**ATPase inhibitor, mitochondrial**	32.08	12.2	9.35	2.11	0.003`
P20160	**AZU1**	**Azurocidin**	60.56	26.9	9.5	2.93	0.457
Q13939	**CCIN**	**Calicin**	26.7	66.5	8.18	2.45	-0.588
P08238	**HSP90AB1**	**Heat shock protein HSP 90-beta**	47.38	83.2	5.03	3.18	0.043
Q9H361	**PABPC3**	**Polyadenylate-binding protein 3**	26.15	70	9.67	6.65	0.269
P12273	**PIP**	**Prolactin-inducible protein**	80.82	16.6	8.05	4.25	0.167
F8W0W8	**PPP1CC**	**Serine/threonine-protein phosphatase**	22.29	38.2	6.83	2.04	0.575
P54709	**ATP1B3**	**Sodium/potassium-transporting ATPase subunit beta**	28.32	31.5	8.35	2.56	0.566
P00441	**SOD1**	**Superoxide dismutase (Cu-Zn)**	34.42	13.38	15.9	2.99	0.813
Q9UJT2	**TSKS**	**Testis-specific serine kinase substrate**	24.83	65	5.97	4.12	0.250
D6RBV2	**LMAN2**	**Vesicular integral-membrane protein VIP36**	35.08	36.5	6.65	3.3	0.141

**>1.5 down-regulate; <0.77 down-regulated (Jung et al., 2009; Karp et al., 2010; Breitwieser et al., 2011)*

Table 9: Summary of the down-regulated DEPs from samples positive for *C. Trachomatis*

Accession number	Gene name	Description	Sequence coverage (%)	Molecular mass (kDa)	pI	Fold change	p-value
B8ZZL8	**HSPE1**	**10 kDa heat shock protein, mitochondrial**	54.46	51.93	10.7	0.15	0.555
B7Z6B8	**DECR1**	**2,4-dienoyl-CoA reductase, mitochondrial**	51.53	146.9	35	0.35	0.325
P15880	**RPS2**	**40S ribosomal protein S2**	47.44	44.16	31.3	0.35	0.457
F5H7U0	**PGD**	**6-phosphogluconate dehydrogenase, decarboxylating**	18	35.33	50.8	0.48	1.000
P24752	**ACAT1**	**Acetyl-CoA acetyltransferase, mitochondrial**	52.93	162.23	45.2	0.58	0.130
P00568	**AK1**	**Adenylate kinase isoenzyme 1**	54.12	63.17	21.6	0.49	0.504
P27824	**CANX**	**Calnexin**	14.86	66.77	67.5	0.28	0.360
P47985	**UQCRFS1**	**Cytochrome b-c1 complex subunit Rieske, mitochondrial**	25.18	28.18	29.6	0.39	0.023
P20674	**COX5A**	**Cytochrome c oxidase subunit 5A, mitochondrial**	66	95.34	16.8	0.25	0.482
P12074	**COX6A1**	**Cytochrome c oxidase subunit 6A1, mitochondrial**	28.44	22.43	12.1	0.15	0.094
D6RGV5	**COX7A2**	**Cytochrome c oxidase subunit 7A2, mitochondrial (Fragment)**	56.31	20.27	11.5	0.25	0.283
E9PEX6	**DLD**	**Dihydrolipoyl dehydrogenase, mitochondrial**	42.39	88.22	51.8	0.31	0.365
P10515	**DLAT**	**Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial**	38.95	147.07	69	0.15	0.199
E5RHW4	**ERLIN2**	**Erlin-2 (Fragment)**	25.74	31.92	37.7	0.45	0.108

P06396	**GSN**	**Gelsolin**	15.98	27.51	85.6	0.27	0.012
Q9UBQ7	**GRHPR**	**Glyoxylate reductase/hydroxypyruvate reductase**	22.26	12.08	35.6	0.28	0.205
E7ES43	**HSPA4L**	**Heat shock 70 kDa protein**	21.72	72.46	97.6	0.48	0.588
P54652	**HSPA2**	**Heat shock-related 70 kDa protein 2**	73.55	1245.16	70	0.16	0.130
E7ENR4	**HK1**	**Hexokinase-1**	32.67	124.03	106.2	0.4	1.000
O43837	**IDH3B**	**Isocitrate dehydrogenase (NAD) subunit beta, mitochondrial**	32.21	13.85	42.2	0.33	0.056
P45880-1	**VDAC2**	**Isoform 1 of Voltage-dependent anion-selective channel protein 2**	39.16	239.25	33.4	0.24	0.280
Q9NRX4-2	**PHPT1**	**Isoform 2 of 14 kDa phosphohistidine phosphatase**	16.94	6.67	13.7	0.13	0.236
P62195-2	**PSMC5**	**Isoform 2 of 26S protease regulatory subunit 8**	23.37	44.49	44.8	0.21	0.365
Q9Y6Q9-2	**NCOA3**	**Isoform 2 of Nuclear receptor coactivator 3**	15.33	33.37	153.7	0.27	0.266
P11177-2	**PDHBODPB**	**Isoform 2 of Pyruvate dehydrogenase E1 component subunit beta, mitochondrial**	49.27	123.74	37.2	0.24	0.536
P40227-2	**CCT6A**	**Isoform 2 of T-complex protein 1 subunit zeta**	16.05	45.85	53.3	0.4	0.643
Q92526-2	**CCT6B**	**Isoform 2 of T-complex protein 1 subunit zeta-2**	23.09	41.36	53.1	0.17	0.717
Q86XR7-2	**TICAM2**	**Isoform 2 of TIR domain-containing adapter molecule 2**	13.37	20.38	46.1	0.42	0.872
Q96JC1-2	**VPS39**	**Isoform 2 of Vam6/Vps39-like protein**	12.34	23.63	100.7	0.18	0.004
Q9BS86-2	**ZPBP**	**Isoform 2 of Zona pellucida-binding protein 1**	22	42.22	40	0.43	0.048
O75569-3	**PRKRA**	**Isoform 3 of Interferon-inducible double-stranded RNA-dependent protein kinase activator A**	15.97	9.42	31.6	0.31	0.043
Q13596-3	**SNX1**	**Isoform 3 of Sorting nexin-1**	19.57	27.86	63	0.29	0.504
Q9H0B3-4	**KIAA1683**	**Isoform 4 of Uncharacterized protein KIAA1683**	34.46	161.78	147.2	0.41	0.056

O15296-3	**ALOX15B**	**Isoform C of Arachidonate 15-lipoxygenase B**	23.18	29.07	69.1	0.44	1.000
Q7L266	**ASRGL1**	**Isoaspartyl peptidase/L-asparaginase**	40.58	81.66	32	0.21	0.075
Q9HC84	**MUC5B**	**Mucin-5B**	14.11	49.62	596	0.36	0.062
Q9C0I1	**MTMR12**	**Myotubularin-related protein 12**	10.04	34.09	86.1	0.15	0.452
P00387	**CYB5R3**	**NADH-cytochrome b5 reductase 3**	38.21	19.69	34.2	0.45	0.244
O75113	**N4BP1**	**NEDD4-binding protein 1**	15.74	34.63	100.3	0.21	0.257
P80188	**LCN2**	**Neutrophil gelatinase-associated lipocalin**	16.67	11.51	22.6	0.35	0.069
Q7Z3B4	**NUP54**	**Nucleoporin p54**	17.36	32.21	55.4	0.13	0.258
Q9BVA1	**ODF2**	**Outer dense fiber protein 2**	58.49	995.69	80.8	0.48	0.202
Q13451	**FKBP5**	**Peptidyl-prolyl cis-trans isomerase**	15.75	15.75	51.2	0.34	0.195
P30044	**PRDX5**	**Peroxiredoxin-5, mitochondrial**	54.21	93.06	22.1	0.35	0.434
P12273	**PIP**	**Prolactin-inducible protein**	80.82	16.6	8.05	0.47	0.269
P04554	**PRM2**	**Protamine-2**	35.29	266.6	13	0.15	0.360
Q8IYX7	**FAM154A**	**Protein FAM154A**	10.97	25.52	54.6	0.22	0.280
Q6J272	**FAM166A**	**Protein FAM166A**	33.12	82.42	36.1	0.12	0.075
Q92597	**NDRG1**	**Protein NDRG1**	25.38	76.46	42.8	0.24	1.000
Q4VC12	**MSS51**	**Putative protein MSS51 homolog, mitochondrial**	22.17	23.54	51.3	0.13	0.196
P14618	**PKM**	**Pyruvate kinase PKM**	50.85	412.01	57.9	0.22	0.188
Q9Y265	**RUVBL1**	**RuvB-like 1**	34.43	79.72	50.2	0.39	0.446
P10768	**ESD**	**S-formylglutathione hydrolase**	18.79	28.61	31.4	0.27	0.375

Q8NCR6	**SMRP1**	**Spermatid-specific manchette-related protein 1**	33.21	79.52	30.1	0.35	0.678
B7Z7K7	**SPATA32**	**Spermatogenesis-associated protein 32**	21.49	19.25	40.3	0.162	0.014
G3XAD8	**STIP1**	**Stress-induced-phosphoprotein 1**	28.98	26.67	68	0.44	0.016
P57105	**SYNJ2BP**	**Synaptojanin-2-binding protein**	40.69	22.16	15.9	0.18	0.362
Q9NVR7	**TBCCD1**	**TBCC domain-containing protein 1**	10.95	6.41	63.5	0.22	0.137
P78371	**CCT2**	**T-complex protein 1 subunit beta**	32.9	91.37	57.5	0.44	0.167
Q8NI27	**THOC2**	**THO complex subunit 2**	13.18	47.3	182.7	0.14	0.023
B8ZZU8	**TCEB2**	**Transcription elongation factor B (SIII), polypeptide**	25.66	11.12	12.5	0.45	0.236
Q16594	**TAF9**	**Transcription initiation factor TFIID subunit 9**	22.73	15.1	29	0.18	0.062
P60174	**TPI1**	**Triosephosphate isomerase**	79.37	339.72	30.8	0.26	0.196
P22314	**UBA1**	**Ubiquitin-like modifier-activating enzyme 1**	28.54	129.43	117.8	0.4	1.000
Q8WW14	**C10orf82**	**Uncharacterized protein C10orf82**	11.74	23.37	25.9	0.47	0.098
Q86SX3	**C14orf80**	**Uncharacterized protein C14orf80**	22.63	32.37	54.2	0.55	0.117
Q6NSI4	**CXorf57**	**Uncharacterized protein CXorf57**	15.2	32.37	97.5	0.22	0.243
D6RBV2	**LMAN2**	**Vesicular integral-membrane protein VIP36**	35.08	57.83	36.5	0.49	0.631
O75083	**WDR1**	**WD repeat-containing protein 1**	26.9	80.14	66.2	0.31	0.014

**>1.5 down-regulated; <0.77 down-regulated (Jung et al., 2009; Karp et al., 2010; Breitwieser et al., 2011)*

Table 10: Summary of the up-regulated DEPs from samples positive for *C. Trachomatis*

Accession number	Gene name	Description	Sequence coverage (%)	Molecular mass (kDa)	pI	Fold change	p-value
Q01518	**CAP1**	**Adenylyl cyclase-associated protein 1**	30.11	26.14	51.9	2.05	0.038
C9J690	**AOC1**	**Amine oxidase**	26.75	39.47	87.2	2.45	0.119
P15907	**ST6GAL1**	**Beta-galactoside alpha-2,6-sialyltransferase 1**	37.93	12.74	46.6	2.04	0.449
G8JLH6	**CD9**	**CD9 antigen (Fragment)**	27.19	69.1	25.4	2.16	0.108
Q02487	**DSC2**	**Desmocollin-2**	25.97	43.21	99.9	8.33	0.872
Q8NFZ0	**FBX018**	**F-box only protein 18**	22.05	67.38	117.6	5.96	0.202
Q12841	**FSTL1**	**Follistatin-related protein 1**	21.1	30.41	35	4.42	0.016
Q96PY5	**FMNL2**	**Formin-like protein 2**	23.2	79.07	123.2	6.14	0.389
D6RD60	**HINT1**	**Histidine triad nucleotide-binding protein 1**	26.21	5.99	11.5	5.04	0.364
P16403	**HIST1H1C**	**Histone H1.2**	37.56	66.91	21.4	5.6	0.053
P01880	**IGHD**	**Ig delta chain C region**	33.33	31.41	42.2	6.65	0.440
E7ET7	**ING3**	**Inhibitor of growth protein 3**	44.17	27.83	44.8	2.29	0.488
P58499-2	**FAM3B**	**Isoform A of Protein FAM3B**	24.45	13.21	30.4	2.92	0.494
Q9NR34	**MAN1C1**	**Mannosyl-oligosaccharide 1,2-alpha-mannosidase IC**	18.41	16.5	70.9	4.17	1.000
P01033	**TIMP1**	**Metalloproteinase inhibitor 1**	54.59	79.84	23.2	4.37	0.365

Q6N021	**TET2**	**Methylcytosine dioxygenase**	28.22	98.95	223.7	2.14	0.398
B7Z7A9	**PGK1**	**Phosphoglycerate kinase**	60.67	69.87	41.4	3.01	0.141
P07737	**PFN1**	**Profilin-1**	45.71	18.96	15	3.5	0.199
Q4KMP7	**TBC1D10B**	**TBC1 domain family member 10B**	22.65	28.61	87.1	4.96	0.717
P29401	**TKT**	**Transketolase**	34.51	38.31	67.8	2.3	0.258
O94854	**KIAA0754**	**Uncharacterized protein KIAA0754**	21.22	30.75	135.1	4.25	0.014
P36543	**ATP6V1E1**	**V-type proton ATPase subunit E 1**	23.01	13.61	26.1	2.11	0.014
B4DMI1	**ZNF786**	**Zinc finger protein 786**	13.02	24.26	85.2	2.16	0.012

**>1.5 down-regulated; <0.77 down-regulated (Jung et al., 2009; Karp et al., 2010; Breitwieser et al., 2011)*

Table 11: Summary of the down-regulated DEPs from samples positive for *T. Vaginalis*

Accession number	Gene name	Description	Sequence Coverage (%)	Molecular mass (kDa)	pI	Fold change	p-value
O75969	**AKAP3**	**A-kinase anchor protein 3**	57.21	94.7	6.18	0.49	0.548
P06733	**ENO1**	**Alpha-enolase**	35.02	47.1	7.39	0.44	0.567
Q9Y371	**SH3GLB1**	**Endophilin-B1**	15.34	40.8	6.04	0.31	0.250
P63241	**EIF5A**	**Eukaryotic translation initiation factor 5A-1**	32.47	16.8	5.24	0.45	0.365
P04406	**GAPDH**	**Glyceraldehyde-3-phosphate dehydrogenase**	62.69	36	8.46	0.48	0.643
Q8WW32	**HMGB4**	**High mobility group protein B4**	25.27	22.5	10.2	0.46	0.069
Q9Y5Z4-2	**HEBP2**	**Isoform 2 of Heme-binding protein 2**	34.24	20.8	4.78	0.15	0.678
P30086	**PEBP1**	**Phosphatidylethanolamine-binding protein 1**	37.97	21	7.53	0.32	0.631
Q6UW49	**SPESP1**	**Sperm equatorial segment protein 1**	29.43	38.9	5.73	0.34	0.003
Q9BUD6	**SPON2**	**Spondin-2**	29	35.8	5.52	0.39	0.466
P15374	**UCHL3**	**Ubiquitin carboxyl-terminal hydrolase isozyme L3**	14.35	26.2	4.92	0.25	0.344

**>1.5 down-regulated; <0.77 down-regulated (Jung et al., 2009; Karp et al., 2010; Breitwieser et al., 2011)*

Table 12: Summary of the up-regulated DEPs from samples positive for *T. Vaginalis*

Accession number	Gene name	Description	Sequence coverage (%)	Molecular mass (kDa)	pI	Fold change	p-value
H0Y512	**APMAP**	**Adipocyte plasma membrane-associated protein (Fragment)**	36.67	45.4	5.66	2.15	0.082
P12429	**ANXA3**	**Annexin A3**	48.3	83.96	36.4	4.37	0.817
P08118	**MSMB**	**Beta-microseminoprotein**	48.25	12.9	5.5	2.03	0.339
O75390	**CS**	**Citrate synthase, mitochondrial**	28.54	51.7	8.32	2.78	0.059
P39656	**DDOST**	**Dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48 kDa subunit**	21.93	50.8	6.55	12.68	0.313
P04843	**RPN1**	**Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1**	25.37	68.5	6.38	3.73	0.027
Q96BH3	**ELSPBP1**	**Epididymal sperm-binding protein 1**	21.97	26.1	6.62	2.67	0.036
Q16610	**ECM1**	**Extracellular matrix protein 1**	17.41	60.6	6.71	2.01	0.056
Q8N335	**GPD1L**	**Glycerol-3-phosphate dehydrogenase 1-like protein**	35.61	38.4	7.02	4.76	1.000
E7ENR4	**HK1**	**Hexokinase-1**	32.67	106.2	7.06	6.01	0.093
P45880-1	**VDAC2**	**Isoform 1 of Voltage-dependent anion-selective channel protein 2**	39.16	33.4	7.59	3.55	0.364
Q5T9G4	**ARMC12**	**Isoform 3 of Armadillo repeat-containing protein 12**	38.18	37.4	7.88	2.79	0.199
O75369-2	**FLNB**	**Isoform 2 of Filamin-B**	19.63	275.5	5.78	2.33	0.320
Q1ZYL8-2	**IZUMO4**	**Isoform 2 of Izumo sperm-egg fusion protein 4**	38.79	24.5	6.92	2.53	0.295

Q86XR7-2	**TICAM2**	**Isoform 2 of TIR domain-containing adapter molecule 2**	13.37	46.1	5.12	3.26	0.109
P51159-2	**RAB27A**	**Isoform Short of Ras-related protein Rab-27A**	49.77	24	5.69	7.59	0.486
Q7Z4W1	**DCXR**	**L-xylulose reductase**	62.3	25.9	8.1	3.48	0.349
P11310	**ACADM**	**Medium-chain specific acyl-CoA dehydrogenase**	42.04	55.3	85	2.31	0.055
Q9Y6C9	**MTCH2**	**Mitochondrial carrier homolog 2**	24.42	33.3	7.97	2.5	0.119
P24158	**PRTN3**	**Myeloblastin**	20.7	27.8	8.35	12.75	0.421
Q14697	**GANAB**	**Neutral alpha-glucosidase AB**	16.1	106.8	6.14	8.07	0.805
P05154	**SERPINA5**	**Plasma serine protease inhibitor**	44.09	45.6	9.26	2.43	0.664
P13796	**LCP1**	**Plastin-2**	19.62	70.2	5.43	2.67	0.093
P28066	**PSMA5**	**Proteasome subunit alpha type-5**	41.08	26.4	4.79	2.06	1.000
P07237	**P4HB**	**Protein disulfide-isomerase**	34.06	57.1	4.87	6.28	0.781
P49221	**TGM4**	**Protein-glutamine gamma-glutamyltransferase 4**	57.46	77.1	6.76	3.01	0.403
F5GZV2	**RIBC2**	**RIB43A-like with coiled-coils protein 2**	14.59	44.9	9.58	7.3	0.497
F8W0W8	**PPP1C**	**Serine/threonine-protein phosphatase**	22.29	38.2	6.83	2.14	0.420
P55809	**OXCT1**	**Succinyl-CoA:3-ketoacid coenzyme A transferase 1,**	31.15	56.1	7.46	2.18	0.167
Q99536	**VAT1**	**Synaptic vesicle membrane protein VAT-1 homolog**	28.75	41.9	6.29	12.08	0.860
D6RBV2	**VIP36**	**Vesicular integral-membrane protein VIP36**	35.08	36.5	6.65	4.98	0.023
Q9Y277	**VDAC3**	**Voltage-dependent anion-selective channel protein 3**	49.82	30.6	8.66	3.61	0.102

**>1.5 down-regulated; <0.77 down-regulated (Jung et al., 2009; Karp et al., 2010; Breitwieser et al., 2011)*

Analysis of the summarised DEPs using the search tool Reactome (http://www.reactome.org) for pathway analysis, showed 152 of the proteins to be categorised to various cellular events, with a remaining 14 DEPs not being allocated to a specific pathway. Figure 35 represents the functional distribution of the proteins from the three STI sample groups (*N. gonorrhoea, C. trachomatis* and *T. vaginalis*), determined by the database. Proteins involved with metabolism predominated at 40%, followed by the second most abundant group, which constitutes the proteins playing a role in gene expression (8%), as well as immune response (8%). Nine DEPs were categorised to the heat shock response (6%), with a similar number of proteins found to be involved in signal transduction. Five proteins (3%) play a role in the transmembrane transport of small molecules, whilst three proteins (2%) were involved in programmed cell death.

As previously mentioned, results showed that majority of the identified DEPs were associated with metabolism (40%). From this specific group, several metabolic roles were isolated and are represented in Figure 36, which includes the metabolism of proteins, which dominates with 29%. The metabolism of lipids and lipoproteins accounted for 20% of the metabolic proteins. 15% were categorised to the metabolism of carbohydrates; specifically those involved in hexose transport, and the pentose phosphate pathway. 13 were grouped to the citric acid (TCA) cycle and respiratory electron transport, whilst 12% of the proteins were categorised to the metabolism of glucose and 9% were categorised to amino acid metabolism. Proteins involved in nucleotide metabliosm accounted for 2% of the metabolic proteins.

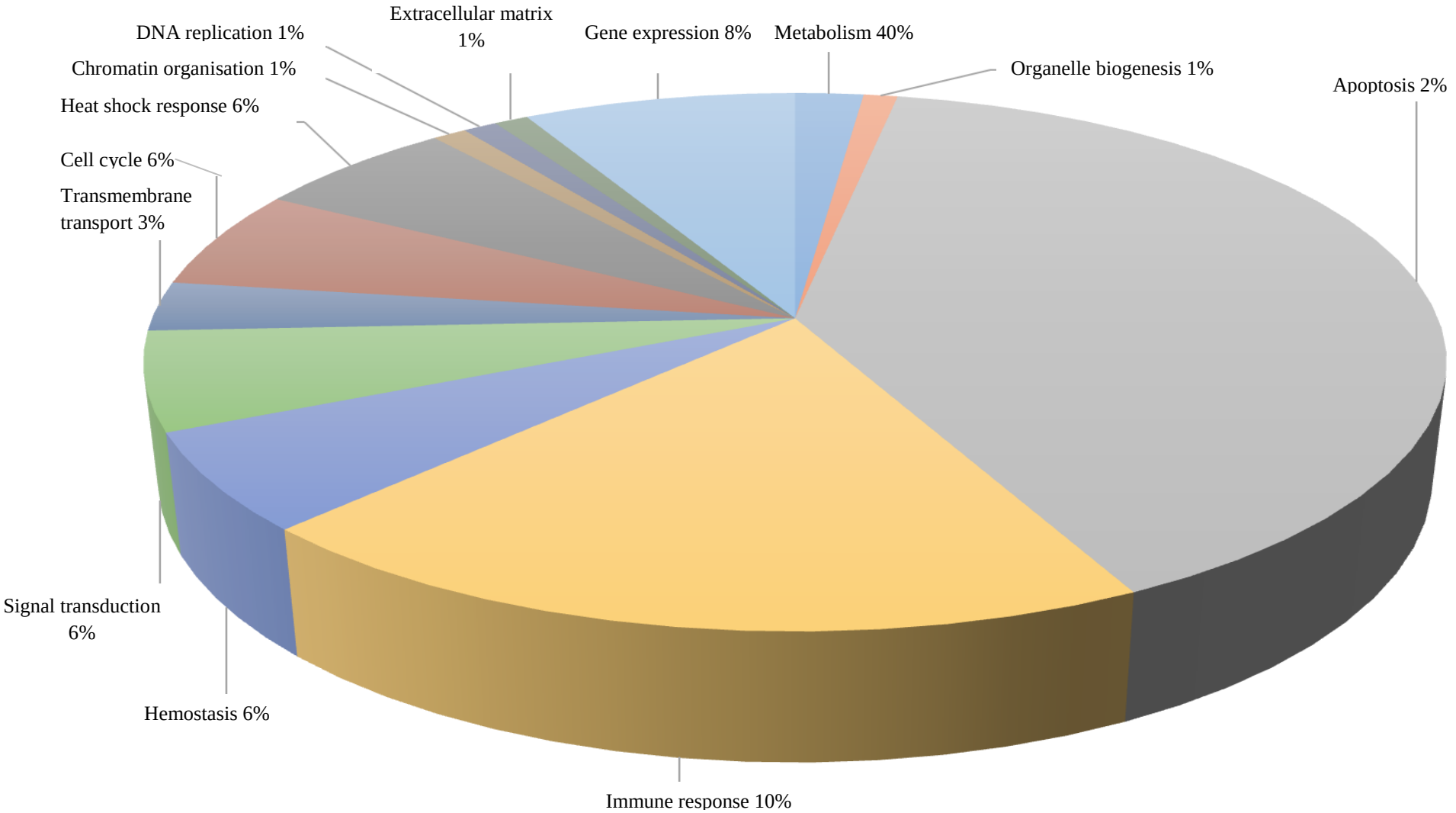


Figure 35: Schematic representation of the distribution of the DEPs as determined by Reactome according to molecular function

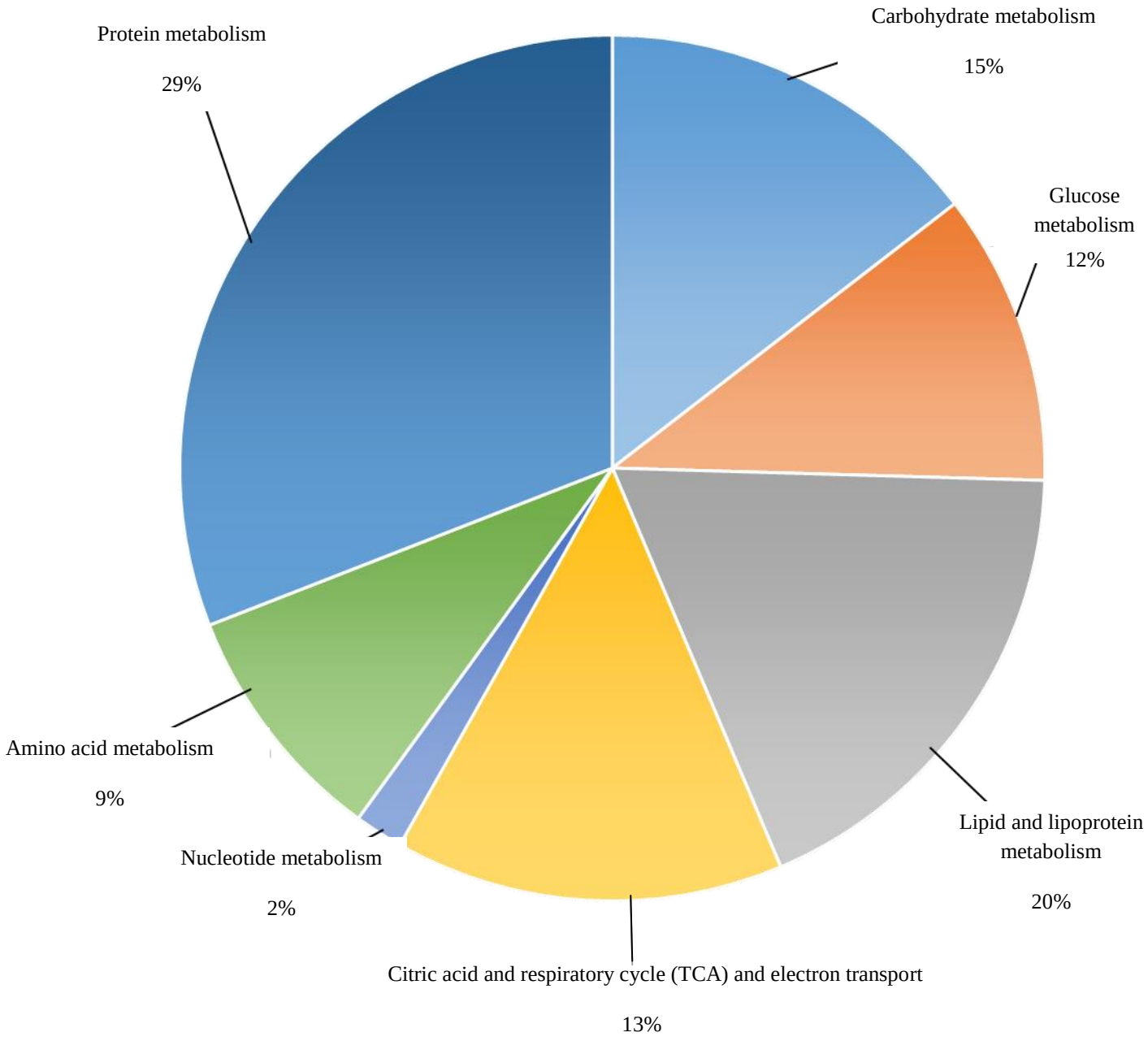


Figure 36: Pie chart representing the distribution of the metabolic proteins involved in specific metabolic pathways

Chapter 5: Discussion

5.1 Objective 1:

Determine the prevalence of N. gonorrhoea, C. trachomatis and T. vaginalis isolated from semen samples.

5.1.1 *Neisseria gonorrhoea*

A study undertaken in 2011 revealed that infection with the *N. gonorrhoea* bacterium represented 88 million of the estimated 448 million new cases of curable STIs globally (WHO, 2011). The African continent had the highest prevalence of *N. gonorrhoea* infections (WHO, 2005) and it was demonstrated over a three year period (2005-2008), the infection rate of *N. gonorrhoea* increased by 21%, from 37.7 million cases to 106.1 million cases (WHO, 2008). The results of this study showed that from the total number of 120 ejaculates that were tested for the possible presence of *N. gonorrhoea*, 15.8% (n=19) of the subjects tested positive for the presence of the bacterium. This finding is in contrast to a previous study conducted by the Centre for Epidemiological Research at the South African Medical Research Council (Mathews *et al*., 1998). This particular retrospective study found that over a twelve-month period, *N. gonorrhoea* was the most reported infection amongst the 171 male subjects attending STI clinics in the Cape Town area of the Western Cape. A factor to consider in this study is that the men included were attending the RBU for a routine semen analysis for fertility concerns. Therefore, for future studies into the incidence rate of this particular STI, a consideration is that studies concentrate on clinics or units within TBH, which address these conditions specifically.

5.1.2 *Chlamydia trachomatis*

A publication by the WHO (WHO, 2005) which focused on the incidence rate of the STIs included in this study, as well as syphilis, reported that depsite the infection rate of *C. trachomatis* being considerably higher per 1000 females, Africa still had an incidence rate of 23 men per 1000 positive for the bacterium. When considering infections in the upper MGT, numerous studies have suggested that infection with the *C. trachomatis* is the main causative factor behind non-gonococcal urethritis and epididymitis (Berger *et al*., 1978; Eley *et al*., 1992; Furuya *et al*., 2004; Motrich *et al*., 2006). The effect of *C. trachomatis* on the spermatozoa parameters has led to the conlusion that it may compromise the male's fertility status by acting directly on spermatozoa (Eley *et al*., 2005) whereby seminal IgA's against *C. trachomatis* correlate with the peroxidation of lipids present in the sperm membrane (Segnini *et al*., 2003). Past research focused on the infection has demonstrated the damaging effect that suffering from *C. trachomatis* may have on male reproductive potential. A study in Tunisia in 2001 (Gdoura *et al*. 2001), found that among 92 men experiencing fertility challenges, 36% tested positive for the bacterium.

Amongst the 120 subjects included in the present study, 9.2% (n=11) were tested positive for *C. trachomatis*. With the study's findings of compromised spermatozoa parameters of compromised total and progressive motility, as well as poor morphology amongst spermatozoa isolated from ejaculates positive for *C. trachomatis*, it can be stated that the men suffering from the condition may have compromised fertility outcomes through the inflammatory response.

5.1.3 *Trichomonas vaginalis*

T. vaginalis has been globally recognised as the most prevalent non-viral STI when compared to *C. trachomatis* and *N. gonorrhoea*, with an estimated 248 million new cases observed per annum (WHO, 2005). Within the context of Africa, the bacterium has been calculated to have an incidence rate amongst men of 311 per 1000, which is in stark contrast to the incidence rate observed in Europe (52 per 1000) and the Americas (118 per 1000) (WHO, 2005). The results of this study are in accordance with this, as amongst the total number of 120 ejaculates included in this study, *T. vaginalis* was the most common pathogen detected with 20.8% (n=25) of the 120 samples presenting with the bacterium following the inoculation of the semen onto selective chocolate agar. With a global rate of approximately 180 million woman possibly infected (Swygard *et al*., 2004), within the context of sub-Saharan Africa, the estimated rate of women infected is 32 million (WHO, 2010).

In a study conducted in 1990 amongst Malawian men attending Dermatology and STI clinics (Hobbs *et al*. 1990), it was found that amongst the 293 men tested, 51 (17.4%), were positive for *T. vaginalis*. In 2008, the WHO reported that the bacterium is responsible for substantially more occurrences of STIs amongst sexually active individuals than *C. trachomatis* and *N. gonorrhoea* combined (WHO, 2008). An important line of research into indicating the possible detrimental impact of *T. vaginalis*, have been studies investigating the correlations between this bacterium and the occurrence of prostate cancer (Dennis and Dawson, 2002; Sutcliffe *et al*., 2006). In comparison to *N. gonorrhoea* and other bacteria behind STIs, such as *T. pallidum* (the bacterium causing syphilis), *T. vaginalis* presents with fewer symptoms. This may extend the period that the bacteria is present in the MGT and can result in an inflammatory response as a consequence and subsequent infection of the prostatic epithelium (Gardner *et al*., 1986).

5.1.4 Future considerations

The seminal plasma of subfertile men is a recognised site for the presence of microorganisms (Cottell *et al*., 2000); however, the infiltration and activation of leukocytes in response to colonisation of the MGT can result in negative impact on the male carrier's fertility status (Comhaire *et al*., 1999). With the research that has focused on the incidence of seminal microflora, the implementation of a routine semen culture of samples undergoing a spermiogram for fertility assessment has been encouraged (Korrovits *et al*., 2006). It has been suggested that prior to undergoing ART, patients who have presented with ejaculates that are identified positive for bacteria, routinely receive antibiotic treatment to treat infection (Fourie *et al*. 2011). Based on this study's findings regarding the occurrence rate of STIs amongst the men seeking fertility assessment at TBH, future laboratory based applications could be included to combat the occurrence of particular micoorganisms in semen samples. A study conducted at Steve Biko Academic Hospital, Pretoria, South Africa, demonstrated the efficacy of utilising a polypropylene centrifuge insert during semen sample washing. The results showed that employing the particular insert, whilst aspirating the sperm pellet formed during double-density gradient centrifugation, resulted in 96% more bacteria being removed from the semen sample in comparison to the conventional approach (Fourie *et al*., 2011). The successful elimination of bacteria species which is economically accommodating could be valuable in a South African Government Hospital offering fertility assistance, such as TBH.

5.2 Objective 2:

Examine the effects of N. gonorrhoea, C. trachomatis and T. vaginalis, in comparison to the control group, on the standard semen parameters which includes; pH, volume, leukocyte count, viscosity and polymorphonunclear elastase concentration, and the spermatozoa parameters including; motility, concentration, morphology, viability and acrosome reaction.

The analysis of a semen sample is generally considered to be the primary laboratory assessment method to identify the fertility status of the male partner (Rodríguez-Martínez H, 2007). There are a variety of contributory factors that may be responsible for compromised sperm parameters, such as: abnormal spermatogenesis, environmental and lifestyle factors, as well as post-testicular damage in the epididymis (WHO, 2010). Possible deviations of spermatozoa parameters from the standardised WHO values, such as the concentration, viability, morphology and acrosome intactness, can help to identify a possible underlying pathological condition. Results from the analyses of the parameters included in this study have demonstrated the negative effect of the presence of an STI on the spermatozoa's functional capacity. This is to be elobaroted further in the discussion below.

5.2.1 pH

Secretions from the accessory sex glands interact to dictate the pH level of semen. Semen is multi-glandular in origin with the acidic prostatic and alkaline seminal vesicle secretions combining to produce an alkaline fluid with a high pH ranging from approximately 7.2 to 8.0. This neutralising effect is important as sperm function more advantageously in an alkaline environment and require a buffer from the hostile environment of the female reproductive tract, which can present with cervical mucus and vaginal secretions which are acidic fluids (WHO, 1992).

Results of this study found no significant difference in the pH values between the three STI postive groups and the control. The pH values differed only slighty, with the most alkaline being the control group with a mean pH of 7.60 ± 0.04, whereas the samples positive for the bacterium *N. gonorrhoea* was slightly higher at 7.76 ± 0.09 *(Figure 15; Table 2).*

As the pH of the semen is a reflection of the ASGs secretions, abnormal pH levels in a semen analysis can signify glandular dysfunction (Weidner *et al*., 1999). The assesment of the levels of citric acid and fructose in the seminal plasma, can be indicative of the functioning of the prostate and seminal vesicles respectively. The quantification of the citric acid (mg/ejaculate) found the samples positive for *N. gonorrhoea, C. trachomatis* and *T. vaginalis* to fall above the WHO reference level of 9.36 mg/ejaculate, therefore, indicating normal prostate function. *N. gonorrhoea* was the only sample group that fell below the reference value of 2.34 mg/ejaculate of fructose, a marker of seminal vesicle function, with a mean of 1.79 ± 0.26 mg/ejaculate. It can be stated that although infection was detected in the ejaculates postive for *C. trachomatis* and *T. vaginalis,* ASG functioning of the prostate and therefore, the pH was not a parameter that was compromised.

5.2.2 Volume

Upon assesment of the volume of the ejaculate, it was found that none of the four groups had a mean volume below the WHO reference value of 1.5 ml. This is in accordance with the findings of a study by Bezold *et al*., which examined the relationship between STIs and semen variables (Bezold *et al*., 2007). Results of this study demonstrated that the *N. gonorrhoea* positive population had the lowest semen volume of 2.49 ± 0.64 ml *(Figure 13; Table 2)* however, there was no statistically significant difference in the mean volume between *N. gonorrhoea, C. trachomatis*, *T. vaginalis* and the control group. One factor contributing to this finding could be the results of objective 4, which focused on the secretory function of the ASGs, in particular the seminal vesicles and the prostate. The latter is a a 4-lobe gland which contributes between 15-30 % to the ejaculates volume (Nieschlag and Behre, 2000),

with a WHO-defined reference value of 9.36 mg of citric acid per ejaculate. The prostate is the ASG that is primarily affected by infection of the MGT (Wolff *et al*., 1991). However in this study, the spectrophotometric quantification of the levels of mean citric acid per ejaculate amongst the sample groups positive for *N. gonorrhoea, C. trachomatis* and *T. vaginalis* all fell above the WHO reference value, thus indicating that the secretory function was not compromised. Although infection and inflammation may affect the volume of the ejaculate, this was not observed in this study and no significant differences were observed.

5.2.3 Leukocyte count and viscosity

The immunological response to the presence of pathogens in the MGT can result in high concentrations of leukocytes (Sharma *et al*., 2001). With the elimination of possible urethritis or a bladder infection, leukocytospermia has been suggested to be a diagnostic approach in identifying an infection in the genital tract (Weidner *et al*., 1999; Kokab *et al*., 2010). The principle behind the histochemical test used in this study is based on the granules in leukocytes containing peroxidase, which together with the H_20_2, form H_20 and free 0_2 ions. These 0_2 ions oxidise the benzidine, staining the cells brown, which allows for the differentiation between peroxidase positive round cells from peroxidase negative round cells. As expected in an immune defense system response, the three STI positive groups *(N. gonorrhoea, C. trachomatis* and *T. vaginalis)* had concentrations of leukocytes with more than 10^6 WBCs per milliliter of semen (WHO, 2010).The concentration of the WBCs in these leukocytospermic samples were significantly higher than the control group (0.34 ± 0.09 $x10^6$/ml) *(Figure 14; Table 2)*. The samples positive for the *T.vaginalis* bacterium presented with the highest mean concentration of peroxidase positive cells (1.38 ± 0.17 $x10^6$/ml) which was significantly increased in comparison to the control. Likewise, this population group also displayed the most viscous semen when quantified in centipoise (10.96 ± 0.99 cP) *(Figure 18; Table 2).*

5.2.4 PMN Elastase

Infection of the genito-urinary glands and the resulting attraction and infiltration of leukocytes to the site can result in the release of the protease, termed elastase. This occurs through cellular degranulation (Kessopoulou *et al*., 1992; Eggert-Kruse *et al*., 2009). The ELISA approach used in the study for the detection of activated PMN leukocytes is recognised as a practical and effective means of screening for leukocytospermia in semen samples (Ricci *et al*., 2000). The quantitative detection of extracellular PMN-elastase can be used as a marker of a clinically quiescent infection (Jochum *et al*., 1986) as it serves as an indicator of up-regulated cell-mediated immunity and is proven to be present in higher concentrations in samples from fertility compromised men (Zorn *et al*., 2003; Moretti *et al*., 2009).

The assessment of the enzymatic activity of PMN elastase showed the STI positive sample groups to have a mean ± SEM that ranged from 538.25 ± 27.50 to 603.25 ± 20.00 ng/ml, in comparison to the control group 217.63 ± 16.34 ng/ml *(Figure 16; Table 2)*. The significantly higher levels of the excreted proteinase in the STI positive sample groups correlates to the expected outcome, as the presence of PMN granulocytes is elevated in response to the inflammatory reaction to the presence of infectious microorganisms. The concentration at which the particular proteolytic enzyme is considered pathological has been contested and various threshold levels have been promoted. Studies such as those by Zorn *et al*. (2003) and Henkel *et al*. (2007), adopted a cut off of 250 ng/ml, which has been widely accepted as the level indicative of inflammation via the elevated PMN elastase concentration (Jochum *et al*., 1986). A study in 2012 (Flint *et al*., unpublished data), which likewise adopted the cut-off value of 280 ng/ml, showed that 65% of the patients presenting with seminal concentrations of PMN elastase exceeded the cut-off value of 280 ng/ml. In the present study, 76% of the subjects had a PMN elastase concentration greater than 280 ng/ml, however, all the samples in the control group fell below this reference value.

5.2.5 Motility

Spermatozoa motility is a crucial variable in the assessment of the spermatozoa parameters and is vital in predicting subfertility as it is indicative of a decrease in the functional competence of spermatozoa (Gunalp *et al*., 2001). In comparison to the manual evalution on a wet mount slide, the implementation of CASA offers increased objective assessment of motility, as well as allowing for a greater degree of accurracy (Maree and van der Horst, 2013).

CASA results showed that the total motility of all three STI positive groups (*N. gonorrhoea*: 37.10 ± 3.18 %; *C. trachomatis*: 37.76 ± 4.23 %; *T. vaginalis*: 47.60 ± 2.15 %) were statistically lower in comparison to the control group (82.14 ± 1.06 %) ($p<0.05$) *(Figure 17; Table 3).* Sample positive for *N. gonorrhoea* (37.10 ± 3.18 %) and *C. trachomatis* (37.76 ± 4.23 %) fell below the WHO reference value for total motility of 40% (WHO, 2010).

A similar trend was observed in the assessment of progressively motile spermatozoa (*N. gonorrhoea*: 13.40 ± 1.93 %; *C. trachomatis*: 13.63 ± 2.34 %; *T. vaginalis*: 5.28 ± 1.97 %) when compared to the control group (51.79 ± 2.51 %). Progressive motility amongst the STI samples all fall below the WHO lower reference value of 32% (WHO, 2010). Progressive motility has been recognised as a crucial component of spermatozoa's ability to migrate and penetrate the cervical mucus, thereby facilitating oocyte fusion (Mortimer S, 1997). The observed decrease in this motility parameter is an indicator of the compromised functional capacity of the spermatozoa from the STI positive sample populations. The SCA® results showed the sample group positive for *N. gonorrhoea* to have the lowest percentage total motile (37.10 ± 3.18 %), as well as progressively motile spermatozoa (13.40 ± 1.93 %), which falls below the WHO reference value of 40% (WHO, 2010). Incubation of semen samples with *C. trachomatis* has shown to result in reduced motility parameters (Hosseinzadeh *et al*., 2001). A similar finding was observed in this study, with the CASA results from the *C. trachomatis* positive samples

showing a significantly decreased percentage of total motile spermatozoa (37.76 ± 4.23 %), in comparison to the control group (82.14 ± 1.06 %) *(Figure 17; Table3).*

As of present, SCA® has no standarised quantitative cut-off reference values to determine the various swimming speeds of spermatozoa, therefore this study adopted arbitary set values. CASA results showed the three STI sample groups (*N. gonorrhoea:* 6.36 ± 3.18 %; *C. trachomatis*: 13.96 ± 7.08 %; *T. vaginalis*: 15.89 ± 2.97 %) to have statistically lower percentages of rapid swimming spermatozoa in comparison to the control group (42.41 ± 6.09 %) *(Figure 22; Table 3).* Similarily, a lower percentage of medium swimming spermatozoa was observed (*N. gonorrhoea*; 12.29 ± 2.21 %; *C. trachomatis*: 11.13 ± 7.42 %; *T. vaginalis*; 20.33 ± 1.93 %) in comparison to the control group (29.49 ± 4.17 %) *(Figure 20; Table 3).* No significance was observed in the slow swimming spermatozoa *(Figure 21; Table 3).*

The SCA® motility parameters showed a significant decrease ($p<0.05$) in the STI positive groups when compared to the control in the following velocity parameters; curvilinear (VCL), straight line (VSL) and average path (VAP). No significant difference was observed in the linearity-, straightness-, and oscillation-index's *(Table 3).* The VCL of the sample groups positive for *N. gonorrhoea* (20.59 ± 5.11 µm/s) and *C. trachomatis* (22.85 ± 14.92 µm/s) was significantly lower than the control group (42.39 ± 27.76 µm/s) ($p<0.05$). A similar trend was observed in the VAP recordings: *N. gonorrhoea* (10.50 ± 2.62 µm/s); *C. trachomatis* (12.18 ± 1.87 µm/s); *T.vaginalis* (18.86 ± 1.82 µm/s) and the control group (28.58 ± 3.66 µm/s) ($p<0.05$). The *N. gonorrhoea* sample group had a statistically lower VSL parameter (9.23 ± 3.77 µm/s) in comparison to the control group (19.11 ± 1.95 µm/s). Spermatozoa motion parameters measured by CASA are recognised as one of the several significant predictors of successful oocyte fertilisation (Lu *et al*., 2014). The study's findings of compromised

VAP amongst all three STI groups in comparison to the control, is an indicator of the compromising effect of infection on spermatozoa motility.

Statistical analysis showed a significant negative correlation ($r= -0.653$; $p<0.05$) between elevated DCFH-DA fluorescence and the percentage of total motile spermatozoa *(Figure 30)* in the sample group positive for *C. trachomatis*. Under controlled physiological conditions, the generation of a low level of reactive oxidants are essential for maintaining normal physiological processes such as sperm-oocyte fusion, capacitation and hyperactivation (De Lamirande and Gagnon, 1995). In order to maintain cellular stability, this small amount of ROS needs to be continuously inactivated (Cocuzza *et al*., 2007). However, in contrast to a beneficial role these oxidising agents can play in various cellular events, it must be strongly considered that all cellular components, including nucleic acids, lipids and proteins are potentially OS targets as a result of supra-physiological concentrations of ROS (Saleh *et al*., 2002). Due to the fact free radicals predominantly attack the closest stable molecule, which subsequently turns that specific particle into a free radical; ROS can be involved in a cascade of reactions which can damage a wide variety of biomolecules (Sanocka and Kurpisz, 2004). The action which occurs on a molecular level by the interaction of ROS is the removal of hydrogen molecules (Fariello *et al*., 2009), which results in a loss of motility (Athayde *et al*., 2007), as observed in the sample group positive for *C. trachomatis.*

5.2.6 Concentration

Anaylsing concentration of the spermatozoa in a routine semen analysis is the oldest parameter to be reported (Andrade-Rocha *et al*., 2005). In this study, the three STI positive sample population groups (*N. gonorrhoea*, *C. trachomatis* and *T. vaginalis*) had a total number of spermatozoa that exceeded the WHO lower reference limit of $15x10^6$ per millilitre (WHO, 2010). The statistical analysis found a statistically lower spermatozoa concentration per millilitre in the infected groups' ejaculates when compared to the spermatozoa in the control group ($p<0.05$). Results of the mean spermatozoa

concentration ranged from: 17.43 ± 3.45 x10^6/ml (*T. vaginalis*); 19.45 ± 2.49 x10^6/ml (*C. trachomatis*) to 21.39 ± 3.39 x10^6/ml (*N. gonorrhoea*) *(Figure 23)*, whereas the mean concentration for the control group was 84.64 ± 3.07 x10^6/ml. Therefore, although a lowered sperm concentration was measured in the STI positive populations in comparison to the control, the results were all above the WHO lower reference limits and can be considered a parameter to not be affected by infection.

The calculation of the total sperm count showed a similar trend, whereby the STI positive groups had a significantly lower total sperm count (*N. gonorrhoea:* 53.26 ± 2.17 x10^6/ejaculate; *C. trachomatis:* 56.41 ± 1.27 x10^6/ejaculate*; T. vaginalis:* 63.79 ± 1.79 x10^6/ejaculate) in comparison to the control group (258.99 ± 0.34 x10^6/ejaculate) ($p<0.05$) *(Figure 24).*

Despite the correlations showing no statistical significance, high levels of ROS is a known causative factor for decreased spermatozoa concentrations (Pasqualotto *et al*., 2000). The aforementioned state of leukocytospermia, as well as elevated PMN elastase concentrations can result in a state of OS and subsequent compromised concentrations of spermatozoa. However, varying factors, such as the subject's period of abstinence can result in a decreased sperm count. In must be considered that the subjects included in the study may not have always adhered to minimum two days of abstinence. Several components can effect the concentration of spermatozoa in the ejaculate such as the volume of epididymal sperm reserve, can influence the concentration (WHO, 2010). All of these confounding elements can result in deviation from the standard reference values and can subsequently alter their indicative value.

5.2.7 Morphology

The morphological assessment of a subject's spermatozoa is regarded as an essential parameter for establishing the fertility status of the male partner (Franken *et al*., 1999). During the maturation of spermatozoa, the spermatogenic process can result in imperfections and anomalies which can be seen in a routine semen analysis. A previous study conducted at the RBU, which included the evaluation of semen samples over a 39 year period (1969-2008), found a considerable decrease in the the mean morphology values of the patient's undergoing a spermiogram (Menkveld *et al*., 2010).

Results of this study found the three STI positive sample groups (*N. gonorrhoea, C. trachomatis* and *T. vaginalis*) to have a normal morphology that falls below the WHO reference limit of 4% (WHO, 2010). Based on this study's findings, it can be postulated that spermatogenic process in the testis is affected by infection. However, despite elevated concentrations of leukocytes resulting in a state of OS, it must be considered that several factors can influence the spermatogenesis process such as chemical and environmental factors which could result in anomalies in the morphology of spermatozoa (Auger J, 2001).

The observed elevated leukocyte counts and increased PMN elastase concentrations in the sample groups positive for *N. gonorrhoea, C. trachomatis* and *T. vaginalis* can be a cause for the poor SCA® morphology results. Statisitical analysis showed the sample group positive for *N. gonorrhoea* to have lowest percentage of normal morphology (1.50 ± 0.38 %), followed by *C. trachomatis* (2.13 ± 0.52 %) and *T. vaginalis* (3.13 ± 0.67 %) *(Figure 19; Table 4).* In comparison to the percentage of morphologically normal spermatozoa in the control group (13.25 ± 1.95 %), all three of the STI positive sample groups were significantly lower ($p<0.05$).

A significant negative correlation (r= -0.768; p<0.05) *(Figure 29)* was found between the increased semen viscosity and the percentage of normal morphological spermatozoa in the sample group positive for *C. trachomatis*. A possible reason for this observation could be the relationship between Increased leukocytes and a state of hyperviscosity (Flint *et al*., 2013). The excess concentration of leukocytes observed in hyperviscous semen is a known causative factor for high levels of ROS, which can cause morphological abnormalitites (Aitken and Buckingham, 1994). The CASA morphology results of the *C. trachomatis* sample group showed a significant decrease in normal morphology (3.13 ± 0.67 %) in comparison to the control group (13.25 ± 1.95 %) (p<0.05) *(Figure 22; Table 4)*. The spermatozoa membrane consists of a fatty acid pattern of membrane phospholipids as well as plasmologens, an ether glycerophospholipid (Lenzi *et al*., 1996), which allows for membrane fluidity. However, this membrane lipid composition can render spermatozoa highly suspceptible to ROS-induced damage to the membrane, which can cause peroxidative damage and a subsuequent increased permeability (Alkan *et al*. 1997), thereby causing morphological abnormalities as observed in the study.

5.2.8 Viability

Vibality is affected by numerous variables such as abnormal spermatogenesis and vesicular and prostatic fluids. The principle of the test is based on the exclusion of dye by the sperm membrane. In living cells, the membrane remains intact and therefore excludes the dye from penetrating. However, in dead sperm the membrane's integrity has been compromised and hence cellular staining by the dye occurs (Andrade-Rocha F, 2005).

According to the WHO criteria for semen analysis (WHO, 2010), the lower reference limit of 58% viability was not met by the samples positive for *N. gonorrhoea* (47.34 ± 5.91 %). In comparison to the control group (69.08 ± 4.88 %), the samples positive for *N. gonorrhoea* had a significantly lower percentage of viable cells (p<0.05) *(Figure 22)*. The samples positive for the presence of *C.*

trachomatis had a viability score only slightly higher than the WHO criteria, with a mean percentage of viable sperm at 58.13 ± 5.72 %, whilst the viability of the sample group positive for *T. vaginalis* was 63.83 ± 5.49 %.

This decreased number of viable sperm in the three STI postive population groups is in accordance with past research which has suggested that damage to the spermatozoa membrane and penetration of a dye indicates spermatozoa cell death and is a parameter considered to be indicative of infection of the MGT (Andrade-Rocha *et al*., 2005). A reason for the observed decreased percentage of viable cells could be attributed to the state of leukocytospermia detected in the STI positive samples. As mentioned previously, the spermtozoa's plasma membrane is highly sensitive to excess ROS. In addition, despite the cytoplasm of a mature spermatozoa containing a low concentration of antioxIdants such as SOD and catalase, the volume thereof is still small, which can result in free radical induced damage and compromised viability.

5.2.9 Acrosome reaction

The acrosome reaction, an exocytotic occurrence of the fusion of the spermatozoa outer plasma membrane with the oocyte, is initially stimulated by the glycoproteins situated in the zona pellucida (Deppe *et al*., 2008). Following the fusion and release of hydrolytic enzymes, a rise in cytosolic Ca^{2+} occurs, resulting in the opening of the membrane's Ca^{2+} ion channels. The outcome of this is acrosomal exocytosis and ultimately the penetration of the zona pellucida (Vasudevan *et al*., 2010). An intact acrosome is a vital parameter to facilitate sufficient binding to the zona pellucida (Du Plessis *et al*., 2002).

To allow for the assessment of acrosome integrity, PSA which is a carbohydrate-binding protein from the pea plant, can be used to distinguish between spermatozoa with intact acrosome membranes from those with compromised acrosomes. The lectin PSA binds to the two compounds; galactose and mannose, which are present in the acrosomal matrix (Cross *et al*., 1986). The results of this study which utilised FITC-PSA found an increased percentage of unstimulated spermatozoa with intact acrosomes in the three STI positive groups *(N. gonorrhoea* 69.67 ± 1.07 %; *C. trachomatis* 70.03 ± 0.79 %; *T. vaginalis* 70.33 ± 0.75 %), in comparison to the control group (51.18 ± 1.27 %) *(Figure 23).* The results of the acrosomal status of the spermatozoa in this study does not follow past literature. Low concentrations of ROS are crucial to spermatozoa physiological functioning, which includes the vital step of the acrosome binding to the zona pellucida (Desai *et al*., 2009). It has been established that supra-physiological concentrations of ROS can negatively impact the acrosome (Sanocka and Kurpisz, 2004; Chen *et al*., 2013) whereby a pro-oxidant load under the condition of leukocytospermia, can overwhelm the antioxidant capacity and result in damage to the acrosomal structure (Cocuzza *et al*., 2007). However, this study is in contrast to the negative impact of excess ROS concentrations on the acrosomal integrity, which can hinder the spermatozoa's functional capacity for successful oocyte fertilisation.

5.3 Objective 3:

Investigate the impact of infection on the generation of ROS and the possible impact on the spermatozoa's DNA.

The immunological triggering of leukocytes can result in a 100-fold elevation in the cellular concentration of ROS (Blake *et al*., 1987) and the high concentration of PMN leukocytes that were observed in this study can release oxygen radicals, which include 0_2^- and H_20_2, compounds with an established toxicity against spermatozoa, the latter which can penetrate the plasma membrane (Aitken

et al., 1994; Lewis *et al*., 1995; Vicari *et al*., 1999; Sharma *et al*., 2001; Alvarez *et al*., 2002; Makker *et al*., 2009). Antioxidants can decrease the concentration of OS through the molecular mechanism of breaking oxidative chains. However, the excessive generation of free radicals in response to pathogens and the resultant leukocyte infiltration can cause potential DNA damage in the sperm genome, which acts in a cascade-like manner (Sharma *et al*., 2001; Esfandiari *et al*., 2003). In view of the fact that only subjects with greater than 1 $x10^6$ WBCs/ml on the wet preperation were inluded as study cases, the changes observed were indeed from true infecitons and not only changes observed in the presence of micoorganisms in the ejaculate.

The results of this study showed the increased concentrations of ROS in the STI positive samples (*N. gonorrhoea*, 95.41 ± 1.64 %*; C. trachomatis*; 92.80 ± 4.44 %; *T. vaginalis*, 93.06 ± 4.12 %), which were significantly higher in comparison to the control group (69.50 ± 13.64 %) ($p<0.05$) (*Figure 27).* Exccess ROS can result in DNA effects such as: base modification, strand breaks as well as chromatin cross-linkage (Cocuzza *et al*., 2007). Results showed a relation ($r= 0.494$; $p<0.05$) *(Figure 33)* between the percentage of DCFH-DA fluoresence and TUNEL-positive spermatozoa in the *N. gonorrhoea* positive sample group.

This correlation may demonstrate the possible effect of elevated concentrations of ROS on spermatozoa DNA, with the flow cytometry analysis also showing *N. gonorrhoea* samples to have the highest degree of DCFH-DA fluoresence. A characteristic of this cellular damage is the activation of nucleases, which leads to the degradation of nuclear DNA into fragments, approaching a length of approximately 200 base pairs (Arends *et al*., 1990). The kit utilised in this study provides 5'-bromo-2'-deoxyuridine 5'-triphosphate (BrdUTP), which serves as the labelling analog of both single and double stranded DNA break sites. The addition of an anti-BrdU antibody allowed for the detection of damage under fluorescence, which was visualised using fluorescent microscopy.

The relationship between elevated ROS and the detection of the labelled exposed 3-OH^- groups of the spermatozoa with fragmented DNA shows the extensive impact that infection can cause on the fertilising capability of spermatozoa. The TUNEL assay is recognised for its main advantages of direct objective, high repeatability, as well as proven correlations with semen parameters (Sharma *et al*., 2013). However, a lack of standardised threshold values remains one of the disadvantages of utilising this particular approach when assessing DNA fragmentation (Sharma *et al*., 2010). In review of literature, the cut-off values for DNA damage range from between 10-40% (Duran *et al*., 2002; Benchaib *et al*., 2003; Henkel *et al*., 2003).

In the present study, a threshold value of 19.25% was adopted, based on a study by Sharma *et al*. (2010), which aimed at standardising a reference value for DNA damage between fertile and infertile men, using the TUNEL approach and observed 100% specificity. When assessing the level of DNA damage with the TUNEL assay approach, it has been shown that DNA fragmentation that exceeds 12 % compromises the fertility outcome in ART (Duran *et al*., 2002). The mean percentage of DNA damage in the control group was 11.98%, which is similar to the findings of Sergerie *et al*. (Sergerie *et al*., 2005), which observed a mean of 13.1%, amongst the samples taken from subjects with proven fertility. However, a statistically significant higher percentage of TUNEL positive cells were observed in the STI postive groups (*N. gonorrhoea*, 26.99 ± 1.91 %; *C. trachomatis;* 29.73 ± 1.99 %*; T. vaginalis*, 26.90 ± 2.13 %*)*, which were significantly higher in comparison to the control group (11.98 ± 0.76 %) ($p<0.05$) *(Figure 28).* In order to facilitate successful fertlisation, the maintenance of condensed DNA within spermatozoa is crucial (Rybar *et al*., 2012). As previoulsy discuseed, spermatozoa are particularly susceptible to cellular damage in a state of OS, due to the high concentrations of PUFAs found in the membranes, in particular docosahexaenoic, which on exposure to elevated levels of OS can result in the oxidation of the lipid's double bonds (Sanoka *et al*., 2004a; Du Plessis *et al*., 2010).

In addition to this, the sperm is a cell with a limited supply of antioxidants, exacerbating the effect of elevated ROS in males experiencing fertility challenges (Saleh *et al*., 2002a). Researchers have suggested that the assessment of the degree of DNA fragmentation to be included in the traditional sperm parameters, as an enhanced assessment tool when considering the fertility status of the male patient (Sharma *et al*., 2010). The results of this study have demonstrated the correlation between increased concentrations of ROS and subsequent DNA damage to spermatozoa.

5.4 Objective 4:

Investigate the effect of N. gonorrhoea, C. trachomatis and T. vaginalis on the secretory function of the prostate and seminal vesicles in comparison to the control, determined through the quantification of seminal plasma citric acid and fructose concentrations.

Seminal fluid is a complex assortment of various substances, which ultimately are responsible for optimising spermatozoa functioning. The ASGs provide a variety of secretions vital to the overall composition of semen. The seminal vesicles are an important part of the male reproductive system as they are the main and final contributors towards the seminal plasma, together supplying up to 85% of the total volume of semen (Heath and Young 2000)**.** Infection of the ASGs may be a causative factor behind inflammatory conditions such as prostato-vesiculitis and epididymitis (Comhaire *et al*., 1999; WHO, 2010). Colonisation of the MGT and the subsequent inflammation of the prostate and seminal vesicles can be a result of pathogens entering via the urethra or blood stream (Robbins and Kumar, 1987).

The assessment of levels of citric acid and fructose can serve as biomarkers of a possible pathophysiological condition of the prostate and of the seminal vesicles (Lewis S, 2007). Per ejaculate, 9.36 mg (52 µmol) of citric acid and 2.34 mg (13 µmol) of fructose are the reference

concentrations of the enzymes classified according to the WHO (WHO, 2010) and it has been suggested that deviations from these parameters may indicate glandular dysfunction (Ahlgren *et al*., 1995; Keck *et al*., 1998; Andrade-Rocha F, 2003). Statistical analysis showed no significant difference in the citric acid (mg/ejaculate) concentrations amongst the four groups: *N. gonorrhoea* (9.60 ± 0.78); *C. trachomatis* (11.39 ± 0.58); *T. vaginalis* (12.04 ± 0.81) and control (7.66 ± 0.47) *(Figure 32) (Table 6).* Therefore, it can be concluded that the presence of *N. gonorrhoea, C. trachomatis* and *T. vaginalis* has no impact on the secretory activity of the prostate gland.

The concentrations of fructose (mg/ejaculate) in the STI positive sample groups were as follows: *N. gonorrhoea*, 1.79 ± 0.26*; C. trachomatis*, 2.58 ± 0.40*; T. vaginalis*, 2.91 ± 0.31 *(Figure 36) (Table 6).* The only population group which fell below the WHO reference value (WHO, 1992) of 2.34 mg/ejaculate were the samples positive for *N. gonorrhoea* (1.79 ± 0.26 mg/ejaculate), which was significantly lower than the control group's mean fructose concentration (3.41 ± 0.16 mg/ejaculate) ($p<0.05$). As previously mentioned, CASA analysis showed the sample group positive for *N. gonorrhoea* to have the lowest percentage total motile (37.10 ± 3.18 %) *(Figure 18),* as well as progressively motile spermatozoa (13.40 ± 1.93 %) *(Figure 19).*

A possible explanantion for this decreased motility, could be the low concentration of the monosaccharide in this sample group. Fructose is a major source of glycoltic energy crucial for maintaining adequate spermatozoa motility (WHO, 1992). The infiltration of bacteria and pathogens can result in post-inflammatory atrophy of the seminal vesicles epithelium, which can cause a decrease in the concentration of fructose in the ejaculate (Mikhailichenko and Esipov, 2005; Eggert-Kruse *et al*., 2009). In addition, it has been suggested that leukocytospermia may affect the functioning of the seminal vesicles (Bezold *et al*., 2007). With compromised glandular functioning of the seminal vesicles amongst the ejaculates positive for *N. gonorrhoea,* as well as the samples being

leukocytospermic (1.20 ± 0.01 x10^6 WBCs/ml) *(Figure 14)*, it can be stated that this particular STI can result in reduced motility, impacting the chances of successful fertility outcomes.

5.5 Objective 5:

Determine the differentially expressed proteins in the spermatozoa isolated from samples positive for N. gonorrhoea, C. trachomatis and T. vaginalis.

Advancements in the field of proteomics have allowed for the crucial assessment of DEPs, which offers new perspectives into biological pathways on the molecular scope in both the normal physiological, as well as infected state. A crucial advancement in the field of proteomics was the introduction of reagents, which facilitates multiple samples to be differentially labelled and allows several samples to be run concurrently (Graham *et al*., 2004). The proteomic approach used in this study adopted a mass analyser, which operates with a high resolution and mass accuracy. This resulted in the quantification of a substantial number of proteins that provided further insight into protein variations and their interacting pathways within the sperm cell.

Past research utilising 1-D gel electrophoresis has facilitated the construct of the spermatozoa‘s proteins and their corresponding functions in a normal, as well as abnormal physiological condition (Mengual *et al*., 2003, Fung *et al*., 2004). With the introduction of new technological advancements utilising high-throughput MS, it is now possible to identify and study a considerably greater number of proteins than what has been achieved previously (Dasilva *et al*., 2012). With the considerable total number of 186 DEPs identified in the STI positive sample groups, the discussion will focus on specific proteins according to their role in male fertility. The most abundant up- and down-regulated DEPs as assessed by UniProt are represented in Tables 17 and 18. The findings of this study shall be

discussed according to the following: metabolism; structural related proteins; OS; stress related proteins; immune response and DNA condensation.

Table 13: Summary of the most abundant down-regulated DEPs as assessed by UniProt

Gene number	Description	Sum coverage (%)	Number of unique peptides	Mascot score	MW (kDa)	pI	Sample group	Fold change	Description
ODF2	**Outer dense fibre protein 2**	34.38	99	996	80.8	8.66	*C. trachomatis*	0.48	Ensure adequate movement of the sperm's flagellum (Brohmann *et al*., 1997)
AKAP4	**A-kinase anchor protein 4**	71.36	86	968	93.4	7.06	*N. gonorrhoea*	0.28	Localised to the fibrous sheath; involved in achieving motility (Vivayaraghavan *et al*., 1999; Tanii *et al*., 2007)
AKAP3	**A-kinase anchor protein 3**	21.34	83	846	94.7	6.18	*T. vaginalis*	0.49	Found within the flagellum, plays a role in motility (Luconi *et al*., 2004)
PKM	**Pyruvate kinase**	22.79	52	412	57.9	7.84	*T. vaginalis*	0.22	Energy metabolism, involved in glycolysis (Medrano *et al*., 2006)
PIP	**Prolactin-inducible protein**	34.93	38	398	16.6	8.05	*N. gonorrhoea*	0.47	Localised to the post-acrosomal region (Bergamo *et al*., 1997), postulated as playing an immune-modulatory role (Gaubin *et al*., 1999)

TPI1	**Triosephosphate isomerase**	41.26	40	339	30.8	5.92	*C. trachomatis*	0.21	Involved in the glycolytic pathway (Ijiri *et al*., 2013)
PRM2	**Protamine-2**	35.29	8	266	13	11.9	*C. trachomatis*	0.15	Maintains DNA integrity (De Mateo *et al*., 2007)
CABYR	**Calcium-binding tyrosine phosphorylation-regulated protein**	19.47	28	254	52.7	4.55	*N. gonorrhoea*	0.35	Role in the Ca^{2+} signalling pathway (Ho and Suarez, 2003)
GAPDH	**Glyceraldehyde-3-phosphate dehydrogenase**	32.84	36	218	36	8.46	*T. vaginalis*	0.48	Glycolytic enzyme found in the spermatozoa's flagellum (Margaryan *et al*., 2015)
FSIP2	**Fibrous sheath-interacting protein 2**	44.00	112	200	789.4	6.68	*N. gonorrhoea*	0.19	Role in maintaining flagellum competence and sperm motility (Miki *et al*., 2002)
HSPA9	**Stress-70 protein**	19.00	44	169	73.6	6.16	*N. gonorrhoea*	0.27	Mitochondrial protein (Bae *et al*., 2013)
ACAT1	**Acetyl-CoA acetyltransferase, mitochondrial**	30.00	30	162	45.2	8.85	*N. gonorrhoea*	0.58	Involved in ketone body metabolism (Schwer *et al*., 2006)
KIAA1683	**Isoform 4 of Uncharacterized protein**	13.24	58	161	147.2	10.17	*N. gonorrhoea*	0.41	Uncharacterised protein
DLAT	**Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial**	18.55	38	147	69	7.84	*C. trachomatis*	0.15	Part of the pyruvate dehydrogenase complex, role in providing energy (Dahl *et al*., 1990)

DECR1	**2,4-dienoyl-CoA reductase, mitochondrial**	22.09	29	146	35	8.9	*C. trachomatis*	0.35	Facilitates β-oxidation, role in fatty acid metabolism (Alphey *et al*., 2005)
UBA1	**Ubiquitin-like modifier-activating enzyme 1**	41.00	41	129	117.8	5.76	*C. trachomatis*	0.41	Role in the enzymatic cascade responsible for protein ubiquitination (Yi *et al*., 2011)
HK1	**Hexokinase-1**	10.19	44	124	106.2	7.06	*C. trachomatis*	0.40	Involved in the glycolytic pathway (Nakamura *et al*., 2008)
PDHB	**Isoform 2 of Pyruvate dehydrogenase E1 component subunit beta, mitochondrial**	21.99	24	123	37.2	5.9	*C. trachomatis*	0.24	Attributed to the metabolic pathways, role in motility (Piomboni *et al*., 2011)
GLUL	**Glutamine synthetase**	15.82	22	95	42	6.89	*N. gonorrhoea*	0.11	Amino acid metabolism (Martínez-Heredia *et al*., 2006)
COX5A	**Cytochrome c oxidase subunit 5A, mitochondrial**	56.00	14	95	16.8	6.79	*C. trachomatis*	0.25	Peptide involved in mitochondrial electron transport (Hüttemann *et al*., 2003)
SPESP1	**Sperm equatorial segment protein 1**	11.71	13	93	38.9	5.73	*T. vaginalis*	0.34	Role in the acrosome reaction (Wolkowicz *et al*., 2003)
PRDX5	**Peroxiredoxin-5, mitochondrial**	18.22	17	93	22.1	8.7	*C. trachomatis*	0.35	Cellular response to ROS (Xu *et al*., 2012)

CCT2	**T-complex protein 1 subunit beta**	13.83	23	91	57.5	6.46	*C. trachomatis*	0.44	Involved in protein turnover (Martínez-Heredia *et al*., 2006)
DLD	**Dihydrolipoyl dehydrogenase, mitochondrial**	24.00	24	88	51.8	7.96	*C. trachomatis*	0.31	Mitochondrial fatty acid β-oxidation (Kurtz *et al*., 1990)
ANXA3	**Annexin A3**	19.00	19	83	36.4	5.92	*T. vaginalis*	0.37	Role in protein transport (Utleg *et al*., 2003)
PEBP1	**Phosphatidylethanolamine-binding protein 1**	10.00	10	83	21	7.53	*T. vaginalis*	0.32	Postulated as having several roles in the male reproductive system (Frayne *et al*., 1997)
FAM166A	**Protein FAM166A**	13.00	13	82	36.1	7.81	*C. trachomatis*	0.22	Involved in spermatogenesis (Wang *et al*., 2014)
ASRGL1	**Isoaspartyl peptidase/L-asparaginase**	18.18	16	81	32	6.24	*C. trachomatis*	0.21	Localised to the sperm tail, converts the amino acid L-asparagine into L-aspartate (Hashemitabar *et al*., 2015)
RUVBL1	**RuvB-like 1**	21.00	21	79	50.2	6.42	*C. trachomatis*	0.39	Role in transcription, ATPase and DNA helicase actions (Martínez-Heredia *et al*., 2006)

SMRP1	**Spermatid-specific manchette-related protein 1**	8.78	10	79	30.1	8.4	*T. vaginalis*	0.35	May be involved in spermatogenesis (Matsuoka *et al*., 2008)
NDRG1	**Protein NDRG1**	11.93	16	76	42.8	5.82	*C. trachomatis*	0.24	Stress-responsive protein (Kachhap *et al*., 2007)

Table 14: Summary of the most abundant up-regulated DEPs as assessed by UniProt

Gene number	Description	Sum coverage (%)	Number of unique peptides	Mascot score	MW (kDa)	pI	Sample group	Fold change	Description
VDAC3	**Voltage-dependent anion-selective channel protein 3**	49.82	10	179	30.6	8.66	*T. vaginalis*	3.18	Postulated in modulating the flagellum's integrity (Hinsch *et al*., 2003)
MSMB	**Beta-microseminoprotein**	48.25	4	131	12.9	5.5	*T. vaginalis*	2.03	Prostatic secretory protein with a binding site on sperm surface (Whitaker *et al*., 2010)
HSP90AB1	**Heat shock protein HSP 90-beta**	47.38	12	124	83.2	5.03	*N. gonorrhoea*	3.18	Crucial in protein folding (Bukau *et al*., 2006); maintain cellular homeostasis (Eggert-Kruse *et al*., 2002)
TET2	**Methylcytosine dioxygenase TET2**	28.22	1	99	223.7	7.99	*C. trachomatis*	2.14	Role in DNA methylation (Ito *et al*., 2011)
P4HB	**Protein disulfide-isomerase**	34.06	7	96	57.1	4.87	*T. vaginalis*	6.28	Suggested as playing a role in zona pellucida binding (Tokuhiro *et al*., 2012)
ACADM	**Medium-chain specific acyl-CoA dehydrogenase, mitochondrial**	42.04	5	85	46.6	8.37	*T. vaginalis*	2.31	Plays a role in fatty acid oxidation (Tolwani *et al*., 2005)
DCXR	**L-xylulose reductase**	62.30	7	85	25.9	8.1	*T. vaginalis*	3.48	Various enzymatic activities (Akintayo *et al*., 2015)
ANXA3	**Annexin A3**	48.30	5	84	36.4	5.92	*T. vaginalis*	4.37	Belongs to a family of calcium binding proteins implicated in cell differentiation (Schostak *et al*., 2009)
TIMP1	**Metalloproteinase inhibitor 1**	54.59	8	80	23.2	8.1	*C. trachomatis*	4.39	Proteolytic enzyme, plays a role in protein degradation (Buchman-Shaked *et al*., 2002)

FMNL2	**Formin-like protein 2**	23.20	1	79	123.2	7.4	*C. trachomatis*	6.14	Involved in cellular structure (Evangelista *et al*., 2003)
OXCT1	**Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial**	31.15	3	79	56.1	7.46	*T. vaginalis*	2.18	Role in energy metabolism (Siva *et al*., 2010)
SERPINI5	**Plasma serine protease inhibitor**	44.09	6	78	45.6	9.26	*T. vaginalis*	4.76	Reproductive function not fully constructed (Murer *et al*.,2001)
FLNB	**Isoform 2 of Filamin-B**	62.00	62	102	275.5	5.78	*T. vaginalis*	2.33	Recruits leukocytes in response to inflammation (Kanters *et al*., 2008)

5.5.1 Metabolism

Glyceraldehyde-3-phosphate dehydrogenase

In order to maintain the spermatozoa's functional capacity, the cells require sufficient energy metabolism and ATP concentrations at a considerably higher level than other cellular units (Miki K, 2007). The physiological event termed capacitation, can be defined as; "the series of transformations that spermatozoa undergo during their migration through the female genital tract, in order to reach and bind to the zona pellucida, undergo the acrosome reaction, and fertilize the egg" (De Lamirande *et al*., 1997). Several crucial events ensue during this process such as protein phosphorylation, enzymatic activation and modifications of the spermatozoa membrane (Ferramosca and Zara, 2014). Accompanying capacitation is the occurrence of sperm hyperactivation, observed through the spermatozoa's flagellum, which shows an increased beat asymmetry and bends amplitude (Yanagimachi R, 1969). This hyperactivation of spermatozoa is considered imperative in the detachment from the oviductal wall and to enhance the penetration of the zona pellucida (Suarez and Ho, 2003; De Jonje C, 2005).

Several metabolic pathways, each making use of various substrates, occur throughout the progression from spermatogenesis through to hyperactivation and fertilisation. In the production of ATP, an essential energy substrate for spermatozoa, oxidative phosphorylation and glycolysis have been identified as the two predominant metabolic pathways (Farramosca and Zara, 2014). Spermatozoa are compartmentalised cells, with certain metabolic pathways that are localised to specific subcellular regions. Oxidative phosphorylation occurs only in the mitochondria of the midpiece region (Farramosca and Zara, 2014), whereas glycolysis occurs predominantly in the flagellum, whereby the enzymes involved in the reactions are localised in the fibrous sheath (Kim *et al*., 2007). One such glycolytic enzyme found in the spermatozoa's flagellum, which is approximately 0.5 µm in diameter and 50 µm in length (Ford W, 2006), is glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which consists of four, 36-kDa, identical subunits (Margaryan *et al*., 2015). The role of GAPDH in

spermatozoa motility was demonstrated in an animal study by Miki *et al*. (2004), where it was shown that GAPDH knock-out mice had significantly decreased total and progressive motility, with the measured ATP levels in the knock-out strain being only at 10% when compared to the wild-type.

In this study, proteomics revealed GAPDH to be down-regulated in the sample group positive for *T.vaginalis (Table 11).* The significantly decreased total motility (37.10 ± 3.18 %) *(Figure 17)* in this sample group can be associated with the down-regulation of GAPDH and It can be stated that the down-regulation of this protein can be a marker for compromised motility.

Triosephosphate isomerise

An additional DEP found in the present study that influences motility is triosephosphate isomerise (TPI), found to be down-regulated in the ejaculates identified as positive for the bacterium *C. trachomatis (Table 9).* This glycolytic enzyme plays a crucial role in energy metabolism (Zhao *et al*., 2007), and has been found localised to the acrosomal region of spermatozoa (Auer *et al*., 2004). TPI functions as a facilitator in the conversion between GAPDH, and dihydroxyacetone phosphate and has been identified as being down-regulated in asthenospermic samples (Siva *et al*., 2010). The down-regulation of this particular enzyme can be seen as a causative factor in the decreased motility in the spermatozoa from the samples positive for *C. trachomatis* (37.76 ± 4.23 %) *(Figure 17).* Thus, highlighting the role of TPI in maintaining energy metabolism required for maintaining motility and achieving successful oocyte fertilisation.

A-kinase anchor protein 3 and fibrous sheath-interacting protein 2

Following testicular maturation, spermatozoa are morphologically differentiated; however, mature spermatozoa only gain full motility after transit through the epididymis and upon ejaculation (Marin-

Briggiler *et al*., 2005). A signalling pathway that has been identified as one of the predominant modulators of achieving motility is the adenosine-3', 5'-cyclic monophosphate (cAMP) protein kinase A (PKA) pathway (Ho and Suarez, 2003). Proteomic analysis in this study revealed the A-kinase anchor protein 3 (AKAP3) to be-down-regulated in the sample group positive for *N. Gonorrhoea (Table 7).* This protein is essential in achieving motile spermatozoa, as it is involved in regulating the flagella's energy supply (Li *et al*., 2011). In conjunction with AKAP4, the anchor protein found in this study is one of the most abundant structural proteins and is crucial to achieving an activated flagellum (Eddy *et al*., 2003). AKAP3 plays a role in the anchoring of cAMP dependant PKA to the spermatozoa's fibrous sheath, which subsequently heightens the rate of tyrosine phosphorylation, leading to the activation of the flagellum (Eddy *et al*., 2003). Inhibition of the phosphorylation of AKAP has been highlighted as leading to compromised fertility in males (Miki *et al*., 2002). The under-expression of this particular structural protein in the *N. gonorrhoea* positive group may be seen as a causative factor for the significantly decreased motility amongst this sample cohort (37.10 ± 3.18 %) *(Figure 17).* It is suggested that the down-regulation of AKAP3 may result in the inadequate activation of the flagellum, resulting in compromised motility of the spermatozoa.

Furthermore, the sample group positive for *N. Gonorrhoea* showed a down-regulation of the fibrous sheath-interacting protein 2 (FSIP2) *(Table 7).* FSIP2 is one of several proteins related to the spermatozoa's fibrous sheath, including AKAP3 and AKAP4. Beyond the fibrous sheath's role in providing structural support to the flagella (Brown *et al*., 2003), identified proteins associated with the cytoskeletal structure have shown the sheath to have a role in optimising motility by providing a scaffold for glycolytic enzymes. The decreased motility observed in the *N. Gonorrhoea* sample group, which falls below the WHO reference-value of 40%, can be contributed to the down-regulation of FSIP2, in conjunction with the aforementioned metabolic proteins.

Calcium-binding tyrosine phosphorylation-regulated protein

In addition to the cAMP-PKA pathway, the second crucial molecular process vital for achieving adequate spermatozoa motility is the Ca^{2+} signalling pathway (Ho and Suarez, 2003). The Ca^{2+} pump, localised in the plasma membrane (Schuh *et al*., 2004), allows for the uptake of Ca^{2+}, which is essential not only in the acrosome reaction as previously discussed, but as well as maintaining hyperactivation (Krasznai *et al*., 2006). Ca^{2+}-binding tyrosine phosphorylation-regulated protein (CABYR) is a sperm and testis specific protein, localised within the fibrous sheath, which encloses the principal piece of the spermatozoa's flagellum (Eddy *et al*., 2003) and is expressed post-meiosis (Naaby-Hansen *et al*., 2002).

In this study, the samples presenting with the bacterium *N. gonorrhoea* displayed down-regulation of this particular protein *(Table 7).* In addition to this, the CASA results for this sample group demonstrated the lowest values for both total (37.10 ± 3.18 %) *(Figure 17; Table 3)* and progressive motility (13.40 ± 1.93 %) *(Figure 18; Table 3).* It can be postulated that a down-regulation of the CABYR protein can elicit compromised motility and may be used as a biomarker for this particular sperm parameter.

5.5.2 Structural related proteins

Outer dense fibre 2

As previously discussed, considerable levels of ATP are required to maintain the spermatozoa's flagellum, which consists of the inner axoneme, encapsulated by flagellar units and outer dense fibres (ODFs) (Miki *et al*., 2004). In order to maintain the elastic composition of the spermatozoa's flagellum, as well as facilitating recoil and providing protection during the ejaculatory and epididymis transit phases, compact fibres are required (Pixton *et al*., 2004). Various polypeptides compose the ODF, including ODF2 (Brohmann *et al*., 1997), which is identified as a crucial protein in this

cytoskeletal accessory structure surrounding the axoneme (Brohmann *et al*., 1997). It has been suggested that ODFs act as biomarkers in fertility-compromised men, when it was published that sperm motility is compromised by the absence of the aforementioned polypeptides (Peterson *et al*. 1999). This was substantiated further in 2004, when the sperm proteome of a patient who had experienced failed IVF fertilisation was compared against fertile donors and ODF2 was isolated as one of the proteins found to be more intensely expressed in the infertile individual's proteome (Pixton *et al*., 2004). A study by Hashemitabar *et al*. (2015), demonstrated the under-expression of ODF2 in a population group of asthenozoospermic men, versus the normozoospermic proteomic profiles of male cohorts.

The findings of the present study show the down-regulation of ODF2, from samples positive for *C. trachomatis (Table 9).* It was established that the observed substantial decrease in the total (37.76 ± 4.23 %) *(Figure 17; Table 3)* and progressive (13.63 ± 2.34 %) *(Figure 18; Table 3)* motility values for this specific sample group may be attributed to the compromised structural pattern in this group of spermatozoa's flagella, resulting in compromised motility.

5.5.3 Oxidative stress

Superoxide dismutase 1

In normal physiological functioning of the spermatozoa cell, compounds such as 0_2^- are present in the cellular environment (Sanocka and Kurpisz, 2004) and elicit a defence against possible radical induced damage to the DNA, organelles and membranes (Schoor *et al*., 2001). Research into the function of 0_2^- in spermatozoa has proven the regulatory role they play in both the initiation of the acrosome reaction, as well as hyperactivated motility (De Lamirande and Gagnon, 1993; Griveau *et al*., 1995). These compounds secondarily dismutate to H_2O_2 via superoxide dimutase (SOD) (Alvarez

et al., 1987), a metalloprotein that is a highly specific scavenging enzyme for these particular free radicals (Kobayashi *et al*., 1991). However, in a state of elevated ROS and heightened SOD activity, spermatozoa's functional capacity has been shown to be compromised (Aitken *et al*., 1996). In a publication reporting on the levels of 0_2^- between infertile men and healthy control donors, elevated levels of this particular compound was found among the infertile cohort (Said *et al*., 2004).

The limited amount of cytoplasm, compartmentalised to the midpiece of the spermatozoa, heightens the spermatozoa's compromised antioxidant capacity (Aitken *et al*., 1996). The crucial damaging impact of excessive concentrations of this compound happens in the process of lipid peroxidation (Aitken *et al*., 1993). As previously mentioned, the high concentrations of PUFAs in the spermatozoa's membrane, in particular, docosahexaenoic acid (Jones *et al*., 1979), render these cells highly susceptible to oxidation of the lipid double bonds (Sanoka *et al*., 2004a), which may negatively impact the spermatozoa's functional capacity. Significant negative correlations exist between SOD activity and numerous spermatozoa parameters including morphology, as well as decreased rapid and progressive motility.

Results of this study's proteomic analysis found SOD1 (Cu-Zn) to be up-regulated in the sample group of spermatozoa that were isolated from ejaculates positive for the bacterium *N. gonorrhoe*a *(Table 8).* Excess H_2O_2 generated by the up-regulation of SOD and the concurrent increase in the levels of OS as a result of this imbalance, may be seen as a causative factor for the observed mean total motility (37.10 ± 3.18 %) *(Figure 17; Table 3)*, below the WHO reference value of 40% (WHO, 2010). Amongst the three STI sample groups, the ejaculates positive for the bacterium *N. gonorrhoea* had the lowest CASA progressive motility (13.40 ± 1.93 %) *(Figure 18; Table 3),* as well as poor normal morphology (1.50 ± 0.38 %) *(Figure 19; Table 4).*

The significant positive correlation (r= 0.494; p<0.05) *(Figure 33)* observed in the *N. gonorrhoe*a sample group between the percentages of DCFH-DA and TUNEL positive cells demonstrates the effects of increased concentrations of ROS on spermatozoa DNA. Due to the elevated ROS in this particular sample cohort, it can be postulated that DNA damage through the detected high degree of fragmentation was due in part to the up-regulation of SOD and the resultant increased H_2O_2.

Peroxiredoxin 5

The peroxiredoxin enzyme family consists of three groups, with six isoforms, ranging from 20–31-kDa (Rhee *et al*., 2001). These acidic proteins are highly expressed in a nearly all species and are found localised in the spermatozoa to the post-acrosomal segment, as well as the midpiece (O'Flaherty and De Souza, 2011). Within the context of ROS, peroxiredoxin's fulfil a dual role as scavengers of excess free radicals, as well as facilitating ROS-dependant signalling within the cell (Gong *et al*., 2012). The substrates' enzymatic activity has shown that fertility compromised males exhibiting high levels of OS, show a decrease in peroxiredoxins (O'Flaherty and De Souza, 2011; Gong *et al*., 2012).

Amongst the samples positive for *C. trachomatis*, peroxiredoxin 5 (PRDX5) *(Table 9)*, was found to be down-regulated. This subunit provides protection against OS through the reduction of H_2O_2 (Manevich *et al*., 2005). The assessment of the levels of ROS with DCFH-DA in this study found a significantly high level of ROS in the sample group positive for *C. trachomatis* (92.80 ± 4.44 %) in comparison to the control group (69.50 ± 13.64 %) *(Figure 27; Table 3)*. The DCFH-DA cell-permeable probe is specific to intracellular H_2O_2 as a marker of OS. The identification of the down-regulation of this particular protein may be a factor for the high concentration of free radicals, illustrating the spermatozoa's functional response to oxidative injury under the cellular response to infection.

5.5.4 Stress related proteins

Heat shock proteins

Heat shock proteins (HSPs) are recognised for playing a critical role in the cellular defence mechanism of spermatozoa and serve two fundamental functions. Firstly, they are involved in protein folding, translocation across membranes and the compilation of multi-protein structures (Bukau *et al*., 2006). Secondly, HSPs are vital in maintaining cellular homeostasis and act as "molecular chaperones", allowing for the regulation of apoptosis and cell division (Eggert-Kruse *et al*., 2002). This cytoprotection is achieved through HSPs facilitating various protein interactions, whereby the proteome is guarded from possible aggregation and misfolding of proteins, as well as proteasomal degradation (Balch et al., 2008). HSPs have been shown to have a significant impact on fertility through the abnormal or absence of expression of the genes. The induction of HSPs are mediated by physiological stresses such as; increased temperature, free radicals, or infection. Such scenarios can result in HSPs facilitating a cytoprotective action by maintaining protein homeostasis in the cell (Beere H, 2004) and is termed the 'heat shock response' (Neuer *et al*., 2000). Conversely, the pre-treatment on cells with a level of stress low enough to induce HSP expression can result in cellular protection and stabilisation, a phenomenon termed 'stress tolerance' (Neuer *et al*., 2000).

Several groups' make-up the mammalian HSP family, which includes HSP27, HSP100, HSP60, HSP90 and HSP70 and are classified based on the respective molecular weight (Naaby-Hansen and Herr, 2010). Amongst the HSPs, HSP70 proteins are the most conserved mammalian proteins (Edward E, 1999). Proteomic analysis of the *N. gonorrhoea* sample group found the down-regulation of the 70-kDa heat shock protein (HSPA9) *(Table 7).* This protein is localised on a cellular level within the mitochondria and can be found in the spermatozoa's acrosome, as well as the principal piece (Neuer *et al*., 2000). The down-regulation of HSPA9 amongst this sample group could be a contributing factor to the observed poor morphology parameter. HSPs are known to be induced under

a state of cellular stress, such as infection and increased ROS. However, with a down-regulation of HSPA9, the cytoprotective effects of the HSP can be compromised, which can be one of the contributing factors for the poor morphology of the *N. gonorrhoea* sample group's spermatozoa, which had the lowest percentage of spermatozoa with normal morphology (1.50 ± 0.38 %) *(Figure 19).*

The relationship between immune reactivity and HSPs is intriguing, as these proteins are immunodominant antigens for several common infectious agents, which allows the immune system to isolate these microbes via the HSP epitope (Neuer *et al*., 2000). Upon an increase in the microbial HSP, a release of cytokines and a subsequent immune response is elicited (Tabona *et al*., 1998). Within the context of the genitourinary system, a correlation is found between the severity and duration of an infectious disease, and continuous bacterial HSP expression (La Verda *et al.*, 2000). The detrimental effect on the fertility status of an enduring inflammatory response has been shown in previous studies, whereby microbial HSPs have been associated with the induction of antisperm antibodies (ASAs) (Munoz *et al*., 1996; Witkin *et al*. 1996a,b).

In this study, proteomic analysis revealed the chaperone protein, HSP 90-kDa-beta member 1 (HSP90B1), to be up-regulated in samples positive for *N. gonorrhoea (Table 8).* It is crucial to note that this group of *N. gonorrhoea* positive ejaculates displayed the highest levels of ROS, as detected by the percentage of DCFH-DA fluorescence (95.41 ± 1.64 %) *(Figure 27).* The increased fluorescent intensity is indicative of intracellular ROS, in particular H_20_2. This finding concurs with a past study, which identified that HSP90B1 was over-expressed in spermatozoa from ejaculates with increased levels of OS (Sharma *et al*. 2013). This up-regulation of this protein can be indicative of the spermatozoa's response to a state of cellular stress (Feder and Hofmann, 1999; Katschinski D, 2004). In this study, the finding of this particular up-regulated chaperone protein in a state of genitourinary

infection revealed that colonisation of *N. gonorrhoea* can result in a stress response, which can suggest this protein can be used as a potential biomarker for OS.

5.5.5. Immune response

Prolactin-inducible protein

The prolactin-inducible protein (PIP) is a single-chain polypeptide, consisting of 146 amino acid sequences, with a molecular weight of 17-kDa (Murphy *et al*., 1987) and has been found expressed in numerous sites throughout the body, such as the nasal and tracheal glandular tissues (Haagensen *et al*., 1990). PIP is involved in various biological actions including: metastasis; immune regulation; as well as apoptosis (Kumar *et al*., 2012). In the diagnostic scenario, this specific protein is a profoundly sensitive and specific marker for breast cancer, as it highly raised levels in the fluids isolated from metastatic breast tissue and is also known as gross cystic breast cyst fluid protein-15 (GCDFP-15) (Mazoujian *et al*., 1984).

In the male reproductive system, PIP has been isolated as a secretory glycoprotein of the seminal vesicles (Akiyama and Kimura, 1990) additionally; the prostate has been isolated as a site of expression for the PIP gene (Hassan *et al*., 2009). In spermatozoa, PIP is localised to the post-acrosomal region (Bergamo *et al*., 1997) and presents in several isoforms (Chiu *et al*., 2003). It has been suggested that the PIP plays an immunomodulatory role, with past studies demonstrating the polypeptide exerts an anti-apoptotic effect on T-cells, by binding to CD4+ cells and blocking T-lymphocyte apoptosis (Gaubin *et al*., 1999). An additional immune response the protein may initiate is through the binding nature of PIP to the Fc fragment of IgG (Witkin *et al*., 1983; Hassan *et al*., 2008). Furthermore, PIP has been found to bind to ASAs (Chiu and Chamley, 2002), which can be isolated in approximately 20% of males suffering from compromised fertility (Marshburn *et al*., 1994) and their immunological activities and fertility impact on the men have been extensively studied. It

has been demonstrated that ASAs act on an oocyte's receptor for spermatozoa, thereby eliminating possible fertilisation through sperm-ovum interaction (Hjort T, 1999). In the present study, proteomic assessment revealed the down-regulation of this particular protein in the sample cohort positive for the *C. trachomatis* bacterium *(Table 9).* Amongst the four groups, the TUNEL assay's results indicative of the degree of DNA fragmentation was the highest in the *C. trachomatis* samples (29.73 ± 1.99 %) *(Figure 28; Table 2).* This observation can be considered an important indicator of the effect of infection on the male fertility status as the down-regulation of PIP may result in the loss of the immunomodulatory role of the protein, which could result in increased DNA fragmentation.

Azurocidin

Azurocidin (AZU1) is a member of the subfamily of serine proteases that are involved in the immune defence regulation against microorganisms (Jenne D, 1994). This 37-kDa glycoprotein is stored within two compartments of the PMN granulocytes, the first being the storage vesicles suspended within PMN leukocytes, and the second being the primary granules within the cytoplasm. This allows for optimal antimicrobial action by early release from by degranulation from PMN granulocytes upon activation of the immune response, as well as a prolonged effect when reaching the site of inflammation (Tapper *et al.*, 2002). Upon infection, AZU1, a strongly positively charged protein is released from PMN granulocytes and is exposed to the negatively charged surface of the endothelium, thereby facilitating the inflammatory response and recruitment of leukocytes (Pereira *et al*., 1990).

With a microbicidal action that is preferentially Gram-negative based, this antimicrobial protein was found to be up-regulated in the sample group positive for *N. gonorrhoea (Table 8).* This finding can be attributed to the high mean concentration of PMN elastase (582.38 ± 7.72 ng/ml) *(Figure 16; Table 3)* in this sample group. The expression of AZU1 and the correlating markers of inflammation in the *N. gonorrhoea* samples is an example of the role played by the immune defence to inflammatory conditions in the MGT.

Filamin-B

During the inflammatory response and corresponding cytokine stimulation, the endothelium and leukocytes greatly increase the concentration of the circulating intercellular adhesion molecule-1 (ICAM-1) (Wang *et al*., 1996). This transmembrane protein plays a role in immune surveillance and promotes the recruitment and adhesion of inflammatory cells, such as leukocytes. Filamin-B (FLNB) is a protein, which binds to the intracellular domain of ICAM-1, and the ICAM-1-filamin complex allows for the recruitment and regulation of leukocytes (Kanters *et al*., 2008).

In this study, isoform 2 of FLNB was found to be up-regulated in the sample group positive for *T. vaginalis (Table 12).* Upon histochemical assessment, the mean leukocyte count in the *T. vaginalis* (1.38 ± 0.17 x10^6/ml) *(Figure 14; Table 3)* was the highest amongst the sample groups included in this study. The observed state of leukocytospermia and increased number of peroxidase positive cells STI positive sample population could be a result of the mediated migration of these cells through the action of ICAM-1-FNB function during inflammation.

5.5.6 DNA condensation

Protamine-2

When compared to mitotic chromosomes, a mammalian spermatozoon's DNA is considered to be greater than six-fold more compact, thereby making this particular cell type the most compact in the context of eukaryotic DNA packaging (Ward and Coffey, 1991). During spermatogenesis, DNA undergoes a process of considerable remodelling through post-translational modification of the histones, which includes the following; acetylation, ubiquitination, methylation and phosphorylation (Chen *et al*., 1998; Godmann *et al*., 2007). During the process of condensation, approximately 85% of loosely arranged histones are replaced by protamines (Barone *et al*., 1994). This restructuring is crucial in ensuring a compact chromatin, which can maintain DNA integrity and offer protection from

possible fragmentation. Maintaining the functional competence of spermatozoa for fertilisation and embryonic development depends on intact sperm chromatin. Damage chromatin can have a damaging effect on reproductive outcomes if it presents with defects, such as ART failure and spontaneous abortion (Boe-Hansen *et al*., 2006; Bungum *et al*., 2007). The compact DNA-protamine structure is achieved by the intramolecular attraction between DNA, emitting a negative charge and positively charged protamines (Aoki and Carrel, 2003). A decrease in the quantity of protamines correlating with the retention of histones can render the spermatozoa more susceptible to DNA damage through factors such as OS (Aoki *et al*., 2006; Simon *et al*., 2011).

This study's findings showed Protamine-2 (PRM2) to be down-regulated in the samples positive for *C. trachomatis (Table 9).* This is a significant finding, as this particular sample cohort exhibited extensive DNA damage as detected by the highest percentage of TUNEL positive cells (29.73 ± 1.99 %) *(Figure 28; Table 3).* Based on this observation, it can be suggested that spermatozoa's compromised DNA is linked to the down-regulation in protamines, which may negatively affect the fertility outcome of the male.

5.6 Future proteomics research

Proteomic studies in the field of male reproductive biology have focused predominantly on the protein profile of spermatozoa and have provided a valuable understanding of the functional status of the cell (Du Plessis *et al*., 2011). Despite the considerable growth identifying sperm proteomes which are selective biomarkers of male infertility, there has been significantly less attention focused on comparative analysis of seminal plasma proteins (Wang *et al*., 1999; Davalieva *et al*., 2012). The seminal plasma contains a mixture of proteins (33-55 g/L) originating from the MGT (Duncan and Thompson 2007; Davalieva *et al*., 2012). Complex studies have led to the recent identification of over 2000 proteins in the seminal plasma and undoubtedly there will be further research into the proteins

that are cell- and tissue-specific to the ASGs, testis, vas deferens and epididymis (Batruch *et al*., 2011). To distinguish the origin of seminal proteins remains challenging and the approach of including men who have undergone a vasectomy is the most logical approach (Batruch *et al*., 2011). This would allow for proteins that originate from the testis and epididymis to be excluded and the seminal plasma would contain proteins from the seminal vesicles and prostate (Batruch *et al*., 2011).

5.8 Limitations of the study

The lack of reported population characteristics of the subject's included in the study is a noted limitation. Information such as individual smoking habits, number of sexual partners etc. would offer insight into the possible effects elicited on the fertility outcomes. Due to the study's focus on the molecular aspect of the sperm-specific proteome, this was not considered, however future studies should include a demographic description of the sub-cohorts. A possible limitation of the study was the lack of ELISA and PCR-based detection method in the isolation of the study's bacteria. Despite the fact that selective agar plates are considered an essential means of identifying microbial pathogens, the aforementioned diagnostic approaches can allow for a more comprehensive diagnosis. An essential limitation of the present study was the lack of validation of the proteomics data. Due to limited laboratory time available once the proteomic analysis had been completed, the validation of the proteins was not performed. Upon reflection of the results, it can be stated that future research in this field should always include this aspect of proteomic, as well as validation by Western Blots.

Chapter 6: Conclusion

The challenging aspect of addressing possible diagnostic approaches and awareness of the effects of STIs on the reproductive profile of the male partner is the economic situation in South Africa's public health sector. Despite feedback on the substantial advance in the country's approaches for the prevention of STIs and a decrease in the incident rate in African populations (Taha *et al.*, 1998a; 1998b; Creek *et al*., 2005), South Africa still remains a populace with a high incidence rate (Johnson *et al*., 2011). Amongst the three microorganisms focused on in this study, the most prevalent occurrence rate was the detection of *T. vaginalis*. The bacteria's asymptomatic manifestations increase the chances of the pathogen's transfer during sexual intercourse. The predominant group of men included in this study were the partners of couples seeking fertility assessment. Previous research has not observed the rate of occurrence of *N. gonorrhoea*, *C. trachomatis*, and *T. vaginalis*, amongst the sample cohort of patients attending TBH.

Publications into the occurrence of urethral pathogens and infection amongst South African men consistently promote the need for modification of the diagnosis and treatment tactic in the government health sector (Black *et al*., 2008; Le Roux *et a*l., 2008). A study into the efficacy of reliable and time efficient tests for bacterial strains may be able to treat the rate of STI's within the Western Cape. Access to rapid and cost-effective screening for STIs can circumvent the related complications such as compromised fertility. Based on this study's findings of the occurrence rate of STIs observed at TBH, the introduction of screening tests for the patients seeking fertility assessments can be a suggestion for future implementation.

The results of the effects of the studied STIs on the spermatozoa parameters demonstrate the negative impact of the microorganisms on the fertility outcome of the male partner. One factor to consider, is that the majority of the men (n=100) whose semen samples were included in this study, were

attending the RBU for primary, secondary or idiopathic infertility. Therefore, compromised spermatozoa parameters were an expected outcome to a certain degree. However, the observed WBCs as well as the increased concentrations of PMN elastase in the STI positive ejaculates, allows for the consideration that the inflammatory response to infection were confounding variables in the compromised fertility of the patients. In view of the fact that only subject's with >1 $x10^6$ WBC/ml were included as study cases, the changes observed were indeed from true infections and not only changes observed in the presence of these organisms in the ejaculate.

The observed negative correlation between ROS and DNA fragmentation in samples positive for *N. gonorrhoea* demonstrates the impact of a pro-oxidant overload on spermatozoa DNA integrity. The results of this study have shown the relationship between leukocyte-derived ROS and DNA fragmentation, which can significantly impair the fertility outcome. Additionally, the findings of decreased concentrations of fructose in the ejaculates positive for *N. gonorrhoea*, demonstrates the effect of an STI on the glandular functioning of the secretory activity of the seminal vesicles. This particular ASG is essential in providing sufficient levels of the monosaccharide to meet the spermatozoa's glycolytic energy requirements and a decrease in the concentration of fructose can compromise spermatozoa function.

Despite the considerable advances in proteomics, there remains a lack of research into spermatozoa proteins isolated from ejaculates that are positive for STIs. The utilisation of a high-output approach such as LC-MS/MS, as adopted in this study, allows for a considerable majority of the spermatozoa proteome to be identified. With the considerable total number of 186 DEPs identified in the STI positive sample groups, the study focused on specific proteins according to their role in male fertility. The insight the role DEPs may play in spermatozoa metabolism and the impact on the motility was discussed with the findings of the down-regulation of the following proteins: A-kinase anchor protein

3 *(N. gonorrhoea)*; triosephosphate isomerise *(C. trachomatis)*: glyceraldehyde-3-phosphate dehydrogenase *(T. vaginalis)*; fibrous sheath-interacting protein 2 *(N. gonorrhoea)* and the calcium-binding tyrosine phosphorylation-regulated protein *(N. gonorrhoea)*.

Amongst structural related proteins, the down-regulation of the outer dense fibre 2, down-regulated in samples positive for *C. trachomatis* was evaluated. OS-related proteins included the up-regulated superoxide dismutase 1 in the ejaculates positive for *N. gonorrhoea* and down-regulated peroxiredoxin 5 in the *C. trachomatis* positive group. From the proteins involved in the response to physiological stress, heat shock proteins were discussed with the down-regulation of the 70-kDa heat shock protein and the up-regulated HSP 90-kDa-beta member 1, both identified in samples positive for *N. gonorrhoea*. Among immune response proteins, the following were down-regulated: prolactin-inducible protein *(C. trachomatis)*, whilst azurocidin (*N. gonorrhoea)* and filamin-B *(T. vaginalis)* were up-regulated. Proteins involved in DNA condensation included down-regulated protamine-2 *(C. trachomatis)*.

Changes in protein expression and the corresponding impact on the spermatozoa's functional capacity were discussed. Understanding the role a DEP may play in the male fertility status can advance the role of proteomics in treating compromised fertility. The identification of proteins that are differentially expressed between spermatozoa from samples positive for STIs can provide crucial insight into their possible influence on male fertility and potential biomarkers for further research. Despite the ongoing research focusing on the sperm proteome, it can be stated with reasonable certainty, that this is the first study into the proteomic profile of spermatozoa isolated from semen samples positive for STIs and the corresponding parameters.

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Appendices:

Appendix A: Labelling solutions and buffers

DNA-labelling solution

10 µl Reaction buffer

0.75 µl TdT enzyme

8.0 µl BrdUTP

31.25 µl dH_20

Lysis buffer (made up to 20 ml with Milli-Q H_20):

1 ml TRIS (1M TRIS stock solution) (pH 7.5)

2 ml NaF (500 mM stock)

2 ml $ZnCl^-$ (200 µM stock solution)

200 µl $NaVO_4$ (10 mM stock solution)

100 µl 0.5% NP-40

2 ml 1% DOC (10% stock solution)

4 µl Aprotonin (10 µg/µl) (-20°C)

4 µl Leupeptin (10 µg/µl)

100 µl stock solution of 0.5% PMSF

iTRAQ Lysis Buffer:

100 mM TEAB (pH 7.8)

6 M Guanidine Chloride

100 mM $NaCl^-$

10 mM TCEP

2 mM EDTA

1% OGB (N-Octyl B-D Glucopyranoside)

Protease Inhibitor Cocktail

Protein spike:

60 pmol/sample of Lysozyme

Appendix B: Correlation results of objectives 2 and 3

Table 15: Correlation analysis of the parameters measured in objective 2 and 3 from the semen samples positive for *N. gonorrhoea*

	pH	**Vol (ml)**	**Leukocyte count ($x10^6$ /ml)**	**Viscosity (cP)**	**PMN elastase (ng/ml)**	**Total motility (%)**	**Progressive motility (%)**	**Normal morphology (%)**	**Concen-tration ($x10^6$ /ml)**	**Total sperm count ($x10^6$/ ejacul)**	**Viable cells (%)**	**Acrosome intact cells (%)**	**DCFH-DA fluores-cence (%)**	**TUNEL positive cells (%)**
pH	1.000	-0.530	0.583	0.555	0.012	-0.004	-0.195	-0.362	0.583	0.010	-0.560	-0.548	-0.365	0.038
Vol (ml)	-0.530	1.000	0.167	-0.325	-0.205	0.048	0.434	0.137	-0.247	0.754	0.070	0.567	0.398	-0.119
Leukocyte count ($x10^6$/ml)	0.583	0.167	1.0000`	0.457	-0.588	0.043	0.269	0.167	0.575	0.566	0.813	0.250	0.141	0.449
Viscosity (cP)	0.555	-0.325	0.457	1.000	-0.130	0.504	-0.360	0.023	0.482	-0.094	-0.283	-0.365	0.199	0.108
PMN elastase (ng/ml)	0.012	-0.205	-0.588	-0.130	1.000	-0.056	-0.280	-0.236	-0.365	-0.266	0.536	-0.643	0.717	-0.872
Total motility (%)	-0.004	0.048	0.043	0.504	-0.056	1.000	0.075	0.062	0.452	0.244	-0.257	0.069	-0.258	-0.202

Progressive motility (%)	-0.195	0.434	0.269	-0.360	-0.280	0.075	1.000	0.196	-0.188	0.446	-0.375	0.678	0.014	-0.016
Normal morphology (%)	-0.362	0.137	0.167	0.023	-0.236	0.062	0.196	1.000	0.098	0.117	-0.243	0.631	-0.014	0.389
Concentration ($x10^6$/ml)	0.583	-0.247	0.575	0.482	-0.365	0.452	-0.188	0.098	1.000	0.362	-0.797	-0.003	-0.012	0.364
Total sperm count ($x10^6$/	0.010	0.754	0.566	-0.094	-0.266	0.244	0.446	0.117	0.362	1.000	-0.517	0.466	0.101	-0.053
Viable cells (%)	-0.560	0.070	-0.813	-0.283	0.536	-0.257	-0.375	-0.243	-0.797	-0.517	1.000	-0.344	0.073	-0.440
Acrosome intact cells (%)	-0.548	0.567	0.250	-0.365	-0.643	0.069	0.678	0.631	-0.003	0.466	-0.344	1.000	0.401	0.488
DCFH-DA fluorescence (%)	-0.365	0.398	0.141	0.199	-0.717	0.258	0.014	-0.014	-0.012	0.101	0.073	0.401	1.000	0.494*
TUNEL positive cells (%)	0.038	-0.119	0.449	0.108	0.872	-0.202	-0.016	0.389	0.364	-0.053	-0.440	0.488	0.494*	1.000

**p<0.05*

Table 16: Correlation analysis of the parameters measured in objective 2 and 3 from the semen samples positive for *C. trachomatis*

	pH	Vol (ml)	Leukocyte count ($x10^6$/ml)	Viscosity (cP)	PMN elastase (ng/ml)	Total motility (%)	Progressive motility (%)	Normal morphology (%)	Concen-tration ($x10^6$ /ml)	Total sperm count ($x10^6$/ ejacul)	Viable cells (%)	Acrosome intact cells (%)	DCFH-DA fluores-cence (%)	TUNEL positive cells (%)
pH	1.000	0.110	-0.184	-0.174	0.443	-0.079	-0.044	-0.362	0.463	0.397	0.262	-0.318	-0.115	0.024
Vol (ml)	0.110	1.000	0.686	-0.566	0.444	0.315	0.185	0.359	-0.141	0.619	0.325	0.160	-0.220	0.483
Leukocyte count ($x10^6$/ml)	-0.184	0.686	1.000	0.187	-0.237	0.013	-0.437	0.209	-0.160	0.390	0.219	0.549	-0.538	0.169
Viscosity (cP)	-0.174	-0.566	-0.187	1.000	-0.309	0.144	-0.456	-0.768*	0.440	0.027	0.260	0.140	-0.462	-0.287
PMN elastase (ng/ml)	0.443	0.444	-0.237	-0.309	1.000	0.449	0.608	-0.023	-0.011	0.393	0.521	-0.128	0.005	0.346
Total motility (%)	-0.079	0.315	0.013	0.144	0.449	1.000	0.400	-0.028	0.331	0.581	0.463	-0.156	-0.653*	0.743

Progressive motility (%)	-0.044	0.185	-0.437	-0.456	0.608	0.400	1.000	0.566	-0.017	0.125	0.118	-0.218	0.604	0.216
Normal morphology (%)	-0.362	0.359	0.209	-0.768*	-0.023	-0.028	0.566	1.000	-0.395	-0.110	-0.247	0.130	0.545	0.131
Concentration ($x10^6$/ml)	0.463	-0.141	-0.160	0.440	-0.011	0.331	-0.017	-0.395	1.000	0.660	0.408	-0.191	-0.130	-0.052
Total sperm count ($x10^6$/ejac)	0.397	0.619	0.390	0.027	0.393	0.581	0.125	-0.110	0.660	1.000	0.713	0.097	-0.374	0.314
Viable cells (%)	0.262	0.325	0.219	0.260	0.521	0.463	0.118	-0.247	0.408	0.713	1.000	0.552	-0.626	-0.029
Acrosome intact cells (%)	-0.318	0.160	0.549	0.140	-0.128	-0.156	-0.218	0.130	-0.191	0.097	0.552	1.000	-0.660	-0.447
DCFH-DA fluorescence (%)	-0.115	-0.220	-0.538	-0.462	0.005	-0.653*	0.604	0.545	-0.130	-0.374	-0.626	-0.660	1.000	0.324
TUNEL positive cells (%)	0.024	0.483	0.169	-0.287	0.346	0.743	0.216	0.131	-0.052	0.314	-0.029	-0.447	0.324	1.000

$p<0.05$

Table 17: Correlation analysis of the parameters measured in objective 2 and 3 from the semen samples positive for *T. vaginalis*

	pH	Vol (ml)	Leukocyte count ($x10^6$/ml)	Viscosity (cP)	PMN elastase (ng/ml)	Total motility (%)	Progressive motility (%)	Normal morphology (%)	Concen-tration ($x10^6$ /ml)	Total sperm count ($x10^6$/ ejacul)	Viable cells (%)	Acrosome intact cells (%)	DCFH-DA fluores-cence (%)	TUNEL positive cells (%)
pH	1.000	-0.421	0.152	-0.032	0.117	0.414	-0.244	-0.227	0.576	0.306	0.090	-0.767	-0.114	0.401
Vol (ml)	-0.421	1.000	0.313	-0.545	-0.238	-0.230	-0.325	0.397	-0.438	0.125	0.033	-0.072	0.149	-0.393
Leukocyte count ($x10^6$/ml)	0.152	0.313	1.000	0.124	0.339	0.668	0.198	-0.058	0.223	0.509	-0.582	-0.056	-0.219	-0.275
Viscosity (cP)	-0.032	-0.545	-0.124	1.000	-0.278	0.167	-0.073	0.263	0.626	0.479	-0.676	0.489	-0.034	0.178
PMN elastase (ng/ml)	0.117	-0.238	0.339	-0.278	1.000	0.253	0.672	-0.503	-0.082	-0.289	0.092	0.174	0.131	-0.105
Total motility (%)	0.414	-0.230	0.668	0.167	0.253	1.000	0.326	-0.261	0.219	0.300	-0.438	-0.113	-0.271	0.390

Progressive motility (%)	-0.244	-0.325	0.198	-0.073	0.672	0.326	1.000	-0.776	-0.373	-0.561	-0.058	0.375	-0.415	0.117
Normal morphology (%)	-0.227	0.397	-0.058	0.263	-0.503	-0.261	-0.776	1.000	0.245	0.572	-0.179	0.232	0.682	-0.401
Concentration ($x10^6$/ml)	0.576	-0.438	0.223	0.626	-0.082	0.219	-0.373	0.245	1.000	0.794	-0.568	-0.128	-0.005	-0.053
Total sperm count ($x10^6$/ejac)	0.306	0.125	0.509	0.479	-0.289	0.300	-0.561	0.572	0.794	1.000	-0.730	-0.061	0.099	-0.188
Viable cells (%)	0.090	0.033	-0.582	-0.676	0.092	-0.438	-0.058	-0.179	-0.568	-0.730	1.000	-0.390	0.284	0.112
Acrosome intact cells (%)	-0.767	-0.072	-0.056	0.489	0.174	-0.113	0.375	0.232	-0.128	-0.061	-0.390	1.000	0.273	-0.298
DCFH-DA fluorescence (%)	-0.114	0.149	-0.219	-0.034	0.131	-0.271	-0.415	0.682	-0.005	0.099	0.284	0.273	1.000	-0.269
TUNEL positive cells (%)	0.024	0.483	0.169	-0.287	0.346	-0.743	0.216	0.131	-0.052	0.314	-0.029	-0.447	0.324	1.000

Table 18: Correlation analysis of the parameters measured in objective 2 and 3 from the semen samples of the control group

	pH	Vol (ml)	Leukocyte count ($x10^6$/ml)	Viscosity (cP)	PMN elastase (ng/ml)	Total motility (%)	Progressive motility (%)	Normal morphology (%)	Concen-tration ($x10^6$ /ml)	Total sperm count ($x10^6$/ ejacul)	Viable cells (%)	Acrosome intact cells (%)	DCFH-DA fluores-cence (%)	TUNEL positive cells (%)
pH	1.000	-0.040	0.440	-0.131	0.607	-0.285	-0.125	-0.057	0.082	0.109	0.210	-0.155	0.149	-0.211
Vol (ml)	-0.040	1.000	0.144	-0.043	0.269	0.196	0.518	-0.454	0.817	0.486	0.135	-0.453	0.488	-0.054
Leukocyte count ($x10^6$/ml)	0.440	0.144	1.000	0.425	0.201	-0.858*	-0.334	0.158	-0.339	-0.349	-0.246	-0.064	-0.115	-0.377
Viscosity (cP)	-0.131	-0.043	-0.425	1.000	0.147	0.341	0.173	-0.303	0.059	0.055	-0.300	-0.027	0.102	-0.530
PMN elastase (ng/ml)	0.607	0.269	0.201	0.147	1.000	-0.048	-0.313	0.244	-0.313	0.119	0.125	0.355	0.738*	-0.121
Total motility (%)	-0.285	0.196	-0.858*	0.341	-0.048	1.000	0.485	-0.365	-0.027	0.421	0.104	-0.174	0.318	0.366

Progressive motility (%)	-0.125	0.518	-0.334	0.173	-0.313	0.485	1.000	-0.938	-0.036	0.805	0.385	-0.782	0.097	0.153
Normal morphology (%)	-0.057	-0.454	0.158	-0.303	0.244	-0.365	-0.938	1.000	0.056	-0.664	-0.167	0.860	-0.023	0.102
Concentration ($x10^6$/ml)	0.082	0.817	-0.339	0.059	-0.313	-0.027	-0.036	0.056	1.000	0.093	0.364	0.199	-0.320	0.295
Total sperm count ($x10^6$/ejac)	0.109	0.486	-0.349	0.055	0.119	0.421	0.805	-0.664	0.093	1.000	0.781	-0.403	0.497	0.420
Viable cells (%)	0.210	0.135	-0.246	-0.300	0.125	0.104	0.385	-0.167	0.364	0.781	1.000	-0.062	0.296	0.565
Acrosome intact cells (%)	-0.155	-0.453	-0.064	-0.027	0.355	-0.174	-0.782	0.860	0.199	-0.403	-0.062	1.000	0.308	0.261
DCFH-DA fluorescence (%)	0.149	0.488	-0.115	0.102	0.738*	0.318	0.097	-0.023	-0.320	0.497	0.296	0.308	1.000	0.366
TUNEL positive cells (%)	-0.211	-0.054	-0.377	-0.530	-0.121	0.366	0.153	0.102	0.295	0.420	0.565	0.261	0.366	1.000

$p<0.05$

Appendix C: Summary of the DEPs

Table 19: Summary of the DEPs

Accession Number	Gene name	Description	Sequence Coverage (%)	Number of peptides	Mascot Score	Molecular Mass (kDa)	Iso-electric Point (pI)
B8ZZL8	**HSPE1**	**10 kDa heat shock protein, mitochondrial**	54.46	3	52	10.7	9.58
B7Z6B8	**DECR1**	**2,4-dienoyl-CoA reductase, mitochondrial**	51.53	9	147	35	8.9
C9IZE4	**PSMD6**	**26S proteasome non-ATPase regulatory subunit 6**	14.71	9	8	51.9	6.74
P15880	**RPS2**	**40S ribosomal protein S2**	47.44	1	44	31.3	10.24
F5H7U0	**PGD**	**6-phosphogluconate dehydrogenase, decarboxylating**	18.00	2	35	50.8	8.03
P23526	**AHCY**	**Adenosylhomocysteinase**	25.93	13	26	47.7	6.34
P00568	**AK1**	**Adenylate kinase isoenzyme 1**	54.12	5	63	21.6	8.63
Q01518	**CAP1**	**Adenylyl cyclase-associated protein 1**	30.11	15	26	51.9	8.06
H0Y512	**APMAP**	**Adipocyte plasma membrane-associated protein (Fragment)**	36.67	19	95	45.4	5.66
B7ZB63	**ARF3**	**ADP-ribosylation factor 3**	49.31	4	33	16.1	7.34
O75969	**AKAP3**	**A-kinase anchor protein 3**	57.21	39	846	94.7	6.18
P06733	**ENO1**	**Alpha-enolase**	35.02	4	71	47.1	7.39
C9J690	**AOC1**	**Amine oxidase**	26.75	26	39	87.2	7.17
P15144	**ANPEP**	**Aminopeptidase N**	28.65	34	147	109.5	5.48
P12429	**ANXA3**	**Annexin A3**	48.30	19	84	36.4	5.92
P18859	**ATP5J**	**ATP synthase-coupling factor 6, mitochondrial**	65.74	2	33	12.6	9.52

Q9UII2	**ATPIF1**	**ATPase inhibitor, mitochondrial**	32.08	3	44	12.2	9.35
P20160	**AZU1**	**Azurocidin**	60.56	4	134	26.9	9.5
B1AM15	**BANF2**	**Barrier-to-autointegration factor-like protein (Fragment)**	21.43	1	6	4.7	5.87
P15907	**ST6GAL1**	**Beta-galactoside alpha-2,6-sialyltransferase 1**	37.93	13	13	46.6	9.01
P08118	**MSMB**	**Beta-microseminoprotein**	48.25	11	131	12.9	5.5
O75952	**CABYR**	**Calcium-binding tyrosine phosphorylation-regulated protein**	41.18	2	255	52.7	4.55
Q13939	**CCIN**	**Calicin**	26.70	4	42	66.5	8.18
P27824	**CANX**	**Calnexin**	14.86	4	67	67.5	4.6
G8JLH6	**CD9**	**CD9 antigen (Fragment)**	27.19	13	69	25.4	6.52
O75390	**CS**	**Citrate synthase, mitochondrial**	28.54	17	42	51.7	8.32
Q14093	**CYLC2**	**Cylicin-2**	10.34	2	30	39.1	9.74
P47985	**UQCRFS1**	**Cytochrome b-c1 complex subunit Rieske, mitochondrial**	25.18	3	28	29.6	8.32
P20674	**COX5A**	**Cytochrome c oxidase subunit 5A, mitochondrial**	66.00	5	95	16.8	6.79
P12074	**COX6A1**	**Cytochrome c oxidase subunit 6A1, mitochondrial**	28.44	1	22	12.1	9.32
D6RGV5	**COX7A2**	**Cytochrome c oxidase subunit 7A2, mitochondrial**	56.31	1	20	11.5	9.91
Q02487	**DSC2**	**Desmocollin-2**	25.97	21	43	99.9	5.34
E9PEX6	**DLD**	**Dihydrolipoyl dehydrogenase, mitochondrial**	42.39	4	88	51.8	7.96
P10515	**DLAT**	**Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial**	38.95	7	147	69	7.84
P27487	**DPP4**	**Dipeptidyl peptidase 4**	22.45	21	58	88.2	6.04

P39656	**DDOST**	**Dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48 kDa subunit**	21.93	12	24	50.8	6.55
P04843	**RPN1**	**Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1**	25.37	20	67	68.5	6.38
P04844	**RPN2**	**Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2**	24.09	15	39	69.2	5.69
Q9Y371	**SH3GLB1**	**Endophilin-B1**	15.34	2	17	40.8	6.04
Q96BH3	**ELSPBP1**	**Epididymal sperm-binding protein 1**	21.97	8	51	26.1	6.62
E5RHW4	**ERLIN2**	**Erlin-2 (Fragment)**	25.74	1	32	37.7	5.62
P63241	**EIF5A**	**Eukaryotic translation initiation factor 5A-1**	32.47	2	40	16.8	5.24
Q16610	**ECM1**	**Extracellular matrix protein 1**	17.41	14	39	60.6	6.71
Q8NFZ0	**FBX018**	**F-box only protein 18**	22.05	21	67	117.6	8.25
Q12841	**FSTL1**	**Follistatin-related protein 1**	21.10	11	30	35	5.52
Q96PY5	**FMNL2**	**Formin-like protein 2**	23.20	24	79	123.2	7.4
P06396	**GSN**	**Gelsolin**	15.98	1	28	85.6	6.28
P15104	**GLUL**	**Glutamine synthetase**	35.39	7	96	42	6.89
P04406	**GAPDH**	**Glyceraldehyde-3-phosphate dehydrogenase**	62.69	12	218	36	8.46
Q8N335	**GPD1L**	**Glycerol-3-phosphate dehydrogenase 1-like protein**	35.61	17	47	38.4	7.02
Q9UBQ7	**GRHPR**	**Glyoxylate reductase/hydroxypyruvate reductase**	22.26	1	12	35.6	7.39
Q5VVW2	**GARNL3**	**GTPase-activating Rap/Ran-GAP domain-like protein 3**	13.72	17	25	112.8	7.65
E7ES43	**HSPA4L**	**Heat shock 70 kDa protein 4L**	21.72	3	72	97.6	5.95
P08238	**HSP90AB1**	**Heat shock protein HSP 90-beta**	47.38	58	624	83.2	5.03

P54652	**HSPA2**	**Heat shock-related 70 kDa protein 2**	73.55	22	1245	70	5.74
E7ENR4	**HK1**	**Hexokinase-1**	32.67	44	124	106.2	7.06
Q8WW32	**HMGB4**	**High mobility group protein B4**	25.27	2	19	22.5	10.2
D6RD60	**HINT1**	**Histidine triad nucleotide-binding protein 1**	26.21	2	6	11.5	6.79
P16403	**HIST1H1C**	**Histone H1.2**	37.56	16	67	21.4	10.93
P01880	**IGHD**	**Ig delta chain C region**	33.33	11	31	42.2	7.93
O00410	**IPO5**	**Importin-5**	14.04	19	41	123.5	4.94
E7ET7	**ING3**	**Inhibitor of growth protein 3**	44.17	17	28	44.8	6.84
Q7L266	**ASRGL1**	**Isoaspartyl peptidase/L-asparaginase**	40.58	4	82	32	6.24
O43837	**IDH3B**	**Isocitrate dehydrogenase (NAD) subunit beta, mitochondrial**	32.21	1	14	42.2	8.46
P50213	**IDH3A**	**Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial**	29.23	12	21	39.6	6.92
P45880-1	**VDAC2**	**Isoform 1 of Voltage-dependent anion-selective channel protein 2**	39.16	20	239	33.4	7.59
Q9NRX4-2	**PHPT1**	**Isoform 2 of 14 kDa phosphohistidine phosphatase**	16.94	1	7	13.7	7.47
P62195-2	**PSMC5**	**Isoform 2 of 26S protease regulatory subunit 8**	23.37	3	44	44.8	8.18
P04035-2	**HMGCR**	**Isoform 2 of 3-hydroxy-3-methylglutaryl-coenzyme A reductase**	15.45	16	26	92	6.96
O75369-2	**FLNB**	**Isoform 2 of Filamin-B**	19.63	4	103	275.5	5.78
Q9Y5Z4-2	**HEBP2**	**Isoform 2 of Heme-binding protein 2**	34.24	2	26	20.8	4.78
Q1ZYL8-2	**IZUMO4**	**Isoform 2 of Izumo sperm-egg fusion protein 4**	38.79	7	17	24.5	6.92
Q96RQ9-2	**IL4I1**	**Isoform 2 of L-amino-acid oxidase**	18.68	14	22	65.3	8.51

Q9Y6Q9-2	**NCOA3**	**Isoform 2 of Nuclear receptor coactivator 3**	15.33	1	33	153.7	7.37
P11177-2	**PDHBODPB**	**Isoform 2 of Pyruvate dehydrogenase E1 component subunit beta, mitochondrial**	49.27	8	124	37.2	5.9
A0MZ66-2	**KIAA1598**	**Isoform 2 of Shootin-1**	9.43	5	20	52.6	5.43
P40227-2	**CCT6A**	**Isoform 2 of T-complex protein 1 subunit zeta**	16.05	1	46	53.3	7.27
Q92526-2	**CCT6B**	**Isoform 2 of T-complex protein 1 subunit zeta-2**	23.09	1	41	53.1	7.99
Q86XR7-2	**TICAM2**	**Isoform 2 of TIR domain-containing adapter molecule 2**	13.37	6	20	46.1	5.12
Q96JC1-2	**VPS39**	**Isoform 2 of Vam6/Vps39-like protein**	12.34	1	24	100.7	7.12
Q9BS86-2	**ZPBP**	**Isoform 2 of Zona pellucida-binding protein 1**	22.00	3	42	40	9.28
Q5JQC9-2	**AKAP4**	**Isoform 2 of A-kinase anchor protein 4**	71.36	167	2968	93.4	7.06
O75569-3	**PRKRA**	**Isoform 3 of Interferon-inducible double-stranded RNA-dependent protein kinase activator A**	15.97	1	9	31.6	8.43
Q13596-3	**SNX1**	**Isoform 3 of Sorting nexin-1**	19.57	1	28	63	5.48
Q9H0B3-4	**KIAA1683**	**Isoform 4 of Uncharacterized protein KIAA1683**	34.46	9	162	147.2	10.17
P02751-7	**FN1**	**Isoform 7 of Fibronectin**	23.55	68	289	268.7	5.53
P58499-2	**FAM3B**	**Isoform A of Protein FAM3B**	24.45	8	13	30.4	9
O15296-3	**ALOX15B**	**Isoform C of Arachidonate 15-lipoxygenase B**	23.18	2	29	69.1	6.93
P51159-2	**RAB27A**	**Isoform Short of Ras-related protein Rab-27A**	49.77	16	71	24	5.69
Q7Z4W1	**DCXR**	**L-xylulose reductase**	62.30	21	85	25.9	8.1
Q9NR34	**MAN1C1**	**Mannosyl-oligosaccharide 1,2-alpha-mannosidase IC**	18.41	11	17	70.9	7.46
P11310	**ACADM**	**Medium-chain specific acyl-CoA dehydrogenase**	42.04	5	85	46.6	8.37

P01033	**TIMP1**	**Metalloproteinase inhibitor 1**	54.59	22	80	23.2	8.1
Q6N021	**TET2**	**Methylcytosine dioxygenase**	28.22	62	99	223.7	7.99
Q9Y6C9	**MTCH2**	**Mitochondrial carrier homolog 2**	24.42	9	57	33.3	7.97
Q9HC84	**MUC5B**	**Mucin-5B**	14.11	1	50	596	6.64
P24158	**PRTN3**	**Myeloblastin**	20.70	7	20	27.8	8.35
Q9C0I1	**MTMR12**	**Myotubularin-related protein 12**	10.04	1	34	86.1	6.62
P00387	**CYB5R3**	**NADH-cytochrome b5 reductase 3**	38.21	1	20	34.2	7.59
O75113	**N4BP1**	**NEDD4-binding protein 1**	15.74	1	35	100.3	5.36
J3KQU0	**NUCB2**	**Nefastin-1**	14.10	8	20	46.6	5.22
Q14697	**GANAB**	**Neutral alpha-glucosidase AB**	16.10	21	61	106.8	6.14
P80188	**LCN2**	**Neutrophil gelatinase-associated lipocalin**	16.67	1	12	22.6	8.91
Q7Z3B4	**NUP54**	**Nucleoporin p54**	17.36	1	32	55.4	7.02
Q9BVA1	**ODF2**	**Outer dense fiber protein 2**	58.49	40	996	80.8	8.66
Q13451	**FKBP5**	**Peptidyl-prolyl cis-trans isomerase**	15.75	2	16	51.2	5.9
P30044	**PRDX5**	**Peroxiredoxin-5, mitochondrial**	54.21	5	93	22.1	8.70
P30086	**PEBP1**	**Phosphatidylethanolamine-binding protein 1**	37.97	4	83	21	7.53
B7Z7A9	**PGK1**	**Phosphoglycerate kinase**	60.67	25	70	41.4	7.33
P05154	**SERPINA5**	**Plasma serine protease inhibitor**	44.09	21	78	45.6	9.26
P13796	**LCP1**	**Plastin-2**	19.62	19	52	70.2	5.43
Q9H361	**PABPC3**	**Polyadenylate-binding protein 3**	26.15	2	55	70	9.67

P07737	**PFN1**	**Profilin-1**	45.71	7	19	15	8.27
P12273	**PIP**	**Prolactin-inducible protein**	80.82	12	398	16.6	8.05
P04554	**PRM2**	**Protamine-2**	35.29	7	267	13	11.9
P28066	**PSMA5**	**Proteasome subunit alpha type-5**	41.08	12	71	26.4	4.79
P07237	**P4HB**	**Protein disulfide-isomerase**	34.06	22	96	57.1	4.87
Q8IYX7	**FAM154A**	**Protein FAM154A**	10.97	3	26	54.6	8.37
Q6J272	**FAM166A**	**Protein FAM166A**	33.12	5	82	36.1	7.81
Q92597	**NDRG1**	**Protein NDRG1**	25.38	5	76	42.8	5.82
P49221	**TGM4**	**Protein-glutamine gamma-glutamyltransferase 4**	57.46	78	411	77.1	6.76
Q4VC12	**MSS51**	**Putative protein MSS51 homolog, mitochondrial**	22.17	1	24	51.3	6.98
P14618	**PKM**	**Pyruvate kinase PKM**	50.85	2	412	57.9	7.84
F5GZV2	**RIBC2**	**RIB43A-like with coiled-coils protein 2**	14.59	10	29	44.9	9.58
Q9Y265	**RUVBL1**	**RuvB-like 1**	34.43	6	80	50.2	6.42
F8W0W8	**PPP1CC**	**Serine/threonine-protein phosphatase**	22.29	2	28	38.2	6.83
P10768	**ESD**	**S-formylglutathione hydrolase**	18.79	2	29	31.4	7.02
P54709	**ATP1B3**	**Sodium/potassium-transporting ATPase subunit beta-3**	28.32	2	16	31.5	8.35
A1A5C7	**SLC22A23**	**Solute carrier family 22 member 23**	18.22	15	30	73.7	7.74
Q6UW49	**SPESP1**	**Sperm equatorial segment protein 1**	29.43	5	93	38.9	5.73
Q8NCR6	**SMRP1**	**Spermatid-specific manchette-related protein 1**	33.21	5	80	30.1	8.4
B7Z7K7	**SPATA32**	**Spermatogenesis-associated protein 32**	21.49	2	19	40.3	4.74

Q9BUD6	**SPON2**	**Spondin-2**	29.00	3	27	35.8	5.52
G3XAD8	**STIP1**	**Stress-induced-phosphoprotein 1**	28.98	2	27	68	7.74
P55809	**OXCT1**	**Succinyl-CoA:3-ketoacid coenzyme A transferase 1**	31.15	18	79	56.1	7.46
P00441	**SOD1**	**Superoxide dismutase (Cu-Zn)**	34.42	6	13	15.9	6.13
Q99536	**VAT1**	**Synaptic vesicle membrane protein VAT-1 homolog**	28.75	11	24	41.9	6.29
P57105	**SYNJ2BP**	**Synaptojanin-2-binding protein**	40.69	2	22	15.9	6.3
Q4KMP7	**TBC1D10B**	**TBC1 domain family member 10B**	22.65	19	29	87.1	9.19
Q9NVR7	**TBCCD1**	**TBCC domain-containing protein 1**	10.95	1	6	63.5	8.69
P78371	**CCT2**	**T-complex protein 1 subunit beta**	32.90	5	91	57.5	6.46
Q9UJT2	**TSKS**	**Testis-specific serine kinase substrate**	24.83	3	57	65	5.97
Q8NI27	**THOC2**	**THO complex subunit 2**	13.18	1	47	182.7	8.44
Q8TBK7	**TPT1**	**TPT1 protein**	21.01	4	16	15.6	5.07
B8ZZU8	**TCEB2**	**Transcription elongation factor B (SIII)**	25.66	1	11	12.5	4.97
Q16594	**TAF9**	**Transcription initiation factor TFIID subunit 9**	22.73	1	15	29	8.66
P29401	**TKT**	**Transketolase**	34.51	18	38	67.8	7.66
C9J3L8	**SSR1**	**Translocon-associated protein subunit alpha**	26.42	14	21	29.6	4.30
P60174	**TPI1**	**Triosephosphate isomerase**	79.37	19	340	30.8	5.92
P15374	**UCHL3**	**Ubiquitin carboxyl-terminal hydrolase isozyme L3**	14.35	2	14	26.2	4.92
P22314	**UBA1**	**Ubiquitin-like modifier-activating enzyme 1**	28.54	10	129	117.8	5.76
H7C417	**H7C417**	**Uncharacterized protein (Fragment)**	15.38	8	11	37.4	8.19

Q8WW14	**C10orf82**	**Uncharacterized protein C10orf82**	11.74	1	23	25.9	8.47
Q86SX3	**C14orf80**	**Uncharacterized protein C14orf80**	22.63	1	32	54.2	8.05
Q6NSI4	**CXorf57**	**Uncharacterized protein CXorf57**	15.20	1	32	97.5	8.41
O94854	**KIAA0754**	**Uncharacterized protein KIAA0754**	21.22	22	31	135.1	4.21
O94832	**MYO1D**	**Unconventional myosin-Id**	14.51	21	37	116.1	9.39
Q9P0L0	**VAPA**	**Vesicle-associated membrane protein-associated protein A**	27.31	3	34	27.9	8.62
D6RBV2	**VIP36**	**Vesicular integral-membrane protein VIP36**	35.08	14	58	36.5	6.65
Q9Y277	**VDAC3**	**Voltage-dependent anion-selective channel protein 3**	49.82	27	179	30.6	8.66
P36543	**ATP6V1E1**	**V-type proton ATPase subunit E 1**	23.01	4	14	26.1	5.87
O75083	**WDR1**	**WD repeat-containing protein 1**	26.90	2	80	66.2	6.65
B4DMI1	**ZNF786**	**Zinc finger protein 786**	13.02	10	24	85.2	9.32

Appendix D: Publications (2012-2015)

Flint, M., Agarwal, A., du Plessis, SS. *Leukocytospermia and Oxidative Stress*. In: Studies on Mens Health and Fertility. (2012)

Flint, M., Lampiao, F., Agarwal, A., du Plessis, SS. *Sperm Assessment: Traditional approaches and their indicative value*. In: Practical Manual of In Vitro Fertilization: Advanced Methods and Novel Devices (2012)

Flint, M., du Plessis, SS, Menkveld, RM. *Revisiting the assessment of semen viscosity and its relationship to leukocytospermia*. Andrologia 34(3) September 2013.

Flint, M., du Plessis, SS. 2013 Trichomonas vaginalis in Sub-Saharan Africa: occurrence and diagnostic approaches for the male partner. South African Journal of Medical Biotechnology, 26 (1) June 2013.

Maartens, J., **Flint, M**., Agarwal, A., du Plessis, SS 2013. *Ionizing radiation. Male Infertility - A Complete Guide to Lifestyle and Environmental Factors*, Ed Du Plessis, SS., Sabanegh, ES Jr., Agarwal, Springer Life Sciences, October 2014.

Flint M, McAlister DA, Agarwal A, du Plessis SS. *Male accessory sex glands: Structure and function*. In: Endocrinology and Male Reproductive Biology Ed. Shio Kumar Singh, PHI Learning, Varanasi, India. September 2015