EFFECTS OF ATRIPLA® AND ALCOHOL WITH LOW PROTEIN DIET INTAKE IN THE EPIDIDYMIS OF ADULT MALE SPRAGUE DAWLEY RATS

Trust Nyirenda

Supervisor: Prof. E.F. Mbajiorgu

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DECLARATION

I Trust Nyirenda declare that the work contained in this dissertation is my original work. It is being submitted for the Master of Science in Medicine degree at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

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Trust Nyirenda

25 July 2018.

DEDICATION

To my mother, my daughter Elsa, my wife Shamiso and all the family members who were a source of strength, comfort and inspiration in the challenging times that prevailed throughout the course of this study, they always reminded me:

"... [To] glory in tribulation also, knowing that tribulation works patience, and patience character and character, hope: and hope will never fail..." (Romans 5:3-5, Bible).

For:

"...we are co-labourers together with Christ..." (1 Corinthians 3:9, Bible).

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Presentations

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1. Effects of Atripla® and ethanol co-administration on the rat caput epididymis: alterations in the immunoreactivity of androgen receptors. It was presented at the Anatomical Society of Southern Africa (ASSA) conference in Cape Town South Africa, on the 23th of April 2017.
2. Histo-morphological effects of Atripla® and ethanol co-administration on rat epididymis. It was presented at University of the Witwatersrand, 8th Cross Faculty Symposium on the 25th of October 2017.

Publication work

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1. **Nyirenda, T.**, A. Abdulkadir, L.R. Sena, J.V.C. Feliu, K.H. Erlwanger, E. F. Mbajiorgu. Effects of Atripla® and ethanol co-administration on the rat caput epididymis: A study on morphometry and androgen receptor immunoreactivity.

ABSTRACT

The efficacy and high compliance of Atripla® (efavirenz, emtricitabine and tenofovir disoproxyl fumarate) has increased demand for therapeutic use among HIV/AIDS patients, thereby necessitating investigation into drug toxicity. Therefore, the present study was designed to investigate the *in vivo* effects of Atripla® and ethanol co-administration in the epididymis of protein malnourished rats. Sixty-four adult male Sprague Dawley rats were randomised into two diet groups: normal protein diet (NP, 23%) and low protein diet (LP, 6%) groups. Each diet group was further divided into four sub-groups (with eight rats each). Under both normal and low protein diet groups, the first group served as control (NPC, LPC) and received vehicle only. The second group received Atripla® (ATp) only [NPATp, LPATp], the third group received ethanol (E) only [NPE, LPE] and the fourth group received both ATp and E (NPEATp, LPEATp). Atripla® (27.5 mg/kg body weight per rat) was administered using gelatine rectangles ($2cm^3$) once a day orally. 6% ethanol in distilled water was provided *ad libitum* to appropriate groups. After ninety days of treatment, the rats were euthanised, epididymides harvested, weighed and processed for analyses. Changes in epididymal histo-achitecture, histo-morphometric indices (epididymal tubular diameter [ETD], tubular area [A_C] and epithelial height [EH]) and principal cell nuclear androgen receptor immuno-reactivity [AR-ir] percentages were evaluated. Epididymal tissue alterations which include depletion of cauda sperm concentration, increased interstitial space and increased luminal debri were observed in all treatment groups compared to NPC (study control group). Severity in tissue alterations was greater in E only treated groups relative to ATp only treated groups within both NP and LP diet groups. Incidence and severity in tissue alterations was higher among the LP treated groups compared to NPC group and their NP treated cohorts. Severe tissue alterations and significant reductions in ETD values were recorded in the LPE and LPEATp groups compared to NPC ($p=0.01$). AR-ir percentage values were significantly low in all treatment groups compared to NPC group, but generally lower among LP treated groups. The study reveals that both Atripla® and ethanol can independently and concurrently cause epididymal functional derangements. Ethanol caused greater toxicity to the epididymis relative to Atripla®. Protein malnutrition exacerbated reproductive toxicity in the epididymis due to Atripla® and ethanol, especially when the two are used concurrently.

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Above all, I am thankful to the Lord God Almighty, for being my ever present refuge and strength. The accomplishment of this study and the compilation of this dissertation is a living miracle I will forever cherish. The Lord allowed me to go through extremely challenging and hopeless situations through the course of this study to build my character, to strengthen my resolve and faith. Hence, better resilience, faith and a strengthened character are some of the most cherished traits I now have apart from any success that can come out of this study. Thank you Lord for being: '*a shield for me, my glory and the lifter of my head' (Psalms 3:3, Bible).*

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LIST OF ABBREVIATIONS

µm: micrometres

A_C: epididymal tubular area

AIDS: Acquired immunodeficiency syndrome

AR: androgen receptor

AR-ir: androgen receptor immunoreactivity

ART: antiretroviral therapy

ATp: Atripla®

DHT: dihydrotestosterone

E: ethanol

EFV: efavirenz

EH: epididymal epithelial height

ETD: epididymal tubular diameter

FTC: emtricitabine

g: centrifugal force (1.118 x 10^5)R S2 where [R is the radius of the rotor in centimeters and S is the speed of the centrifuge].

HAART: Highly active antiretroviral therapy

HE: heamatoxylin and eosin

HIV: Human immunodeficiency virus

LPATp: low protein diet Atripla® only group

LPC: low protein diet control group

LPE: low protein diet ethanol only group

LPEATp: low protein diet, Atripla® and ethanol group

m: meters

mg: milligrams

NNRTIs: non-nucleotide reverse transcriptase inhibitors

NPATp: normal protein Atripla® only group

NPC: normal protein diet control group

NPE: normal protein diet and ethanol only group

NPEATp: normal protein diet, Atripla® and ethanol group

NtRTI: nucleotide reverse transcriptase inhibitor

PLWHAs: People living with HIV/AIDS

PM: protein malnutrition

SSA: Sub-Sahara African region

T: testosterone

TDF: tenofovir disoproxyl fumarate

VG: Van Gieson stain

CHAPTER 1: INTRODUCTION

Towards the dawn of the twenty first century, highly active antiretroviral therapy (HAART) was developed to treat HIV/AIDS (Brechtl *et al.*, 2001, Vella *et al.*, 2012, Andrade *et al.*, 2017). This ushered in a new era by which HIV/AIDS has been gradually transforming from a fretful infectious disease characterised by high mortality and morbidity, to a more treatable chronic condition (Kayode and Kayode, 2015, Baynes *et al.*, 2017). In recent years, drug companies have combined HAART drugs: efavirenz, emtricitabine and tenofovir disoproxil fumarate into a single pill drug regimen called Atripla® that is taken once daily to further increase compliance (Squibb, 2010, Horberg and Klein, 2010a, Truong *et al.*, 2015). Atripla® has registered successful use among developed countries and has also been adopted for use among developing countries including those in the Sub-Saharan Africa (SSA) region (Squibb, 2010).

However, Atripla® like many other antiretroviral drugs before it continue to be widely used among the resource limited countries of the SSA region where major hypo-fertility factors like protein malnutrition and alcohol consumption are endemic (Meeting and Organization, 2006, Singer, 2011, HIV/AIDS, 2014). It is estimated that over a quarter of the total number of people who are chronically undernourished (with deficits mostly in protein) in the world reside in SSA (FAO, 2014). It is in this same region where about 71% of all HIV infected people also live (HIV/AIDS, 2015). Meanwhile, various studies have shown that the estimated alcohol consumption per annum among SSA countries is one of the highest globally (Morojele *et al.*, 2013a, Rehm and Imtiaz, 2016). This suggests that a prevalence of Atripla® use, alcohol consumption and protein malnutrition may occur concurrently within the same population. This combination of conditions may be rampant among male individuals of active reproductive age, who are increasingly becoming interested in procreation due to improved quality of life enabled by antiretroviral therapy success (Azu *et al.*, 2014, Adilo and Wordofa, 2017). Therefore, there is a need to investigate the combined effects of Atripla®, chronic alcohol consumption and protein malnutrition on male reproductive health.

1.1 Rationale and problem statement

Atripla® is one of the widely used high compliance antiretroviral drugs that have facilitated a manageable treatment routine leading to a better-quality of life among people living with HIV/AIDS [PLWHAs] (Horberg and Klein, 2010a, Chen *et al.*, 2013). However, successes in HIV/AIDS treatment results from a lifetime use of antiretroviral drugs. This brings into question the possible toxicity that may result from chronic antiretroviral drug use especially in male reproductive health. More importantly, it necessitates the need to research on the effects of Atripla® and its concurrently use with chronic alcohol consumption (a commonly abused drug) and protein malnutrition which is especially endemic in the SSA region.

In addition, there is paucity of literature describing the combined effects of Atripla®, alcohol and protein malnutrition on male reproduction involving the epididymis tissues. Multiple drug therapy involve the presence of two or more drug in the body at the same time. This may lead to drug-drug interaction which may result in modification of the effects of the drugs. Additionally, interaction may occur between the drugs and food or its components resulting in drug-nutrition interactions (Köhler *et al.*, 2000, Kaufman *et al.*, 2002, Alomar, 2014). Such interactions may have different implications to male reproductive health since adequate nutrition is crucial in male reproductive sufficiency. Reproductive competence is reported to be related to structural integrity of reproductive organ such that alterations to tissue structure affects the function (Lanning *et al.*, 2002, De Grava Kempinas and Klinefelter, 2014). However, in most male reproductive health studies the toxicological effects of various xenobiotics are investigated on the tissues of the testis, while the epididymis is often overlooked (Cornwall, 2009, De Grava Kempinas and Klinefelter, 2014). Furthermore, sperm maturational disorders are associated with about 40% of all infertility cases reported (Cornwall, 2009) which may not be unrelated with drug toxicities and/or drug – nutrition interaction. Sperm maturation occurs in the epididymis; hence, the evaluation of potential effects of toxicants on the epididymis becomes necessary (Cornwall, 2009, Sullivan and Mieusset, 2016). Therefore, the present study is designed to explore the combined effects of Atripla®, alcohol and protein malnutrition on the epididymis of male Sprague Dawley rat model.

The use of an experimental animal model is commonly recommended for toxicity because it is not possible to carry out such studies on humans and the related ethical issues involved. Hence

the design of the present study (Lanning *et al.*, 2002, Cornwall, 2009, Azu *et al.*, 2014, Kayode and Kayode, 2015). Therefore, it is hoped that this study will provide invaluable data for clinicians and researchers as well dealing with male reproductive health issues involving toxicity of substances in the management of HIV/AIDS patients relative to alcohol and protein malnutrition.

1.2 Hypothesis

In previous studies, HAART, alcohol and protein malnutrition have all been independently linked to testicular tissue derangements which are associated with male infertility (De Souza Santos *et al.*, 2004, Azu, 2012, Albadri, 2013). It is hypothesised that the combined effects of Atripla®, alcohol and protein malnutrition would be deleterious on the histo-architecture of the epididymis resulting in functional derangements and consequent fertility challenges.

1.3 Aim and objectives

1.3.1 Aim:

- To investigate the effects Atripla®, ethanol and protein deficient diet co-administration on the epididymis of adult male Sprague Dawley rats through the evaluation of histo-morphometric, histo-pathological and Immuno-histochemical parameters.

1.3.2 Specific objectives:

1. To determine the effects of Atripla® and/or alcohol with low protein dietary intake on rat feeding parameters (feed and fluid intake) and biometric parameters (body weight changes, epididymal weight and relative epididymal weight).
2. To determine the effects of Atripla® and/or alcohol with low protein dietary intake on the histo-architecture of epididymis.
3. To determine the adverse effects of Atripla® and/or alcohol with low protein dietary intake by evaluating changes in the histo-morphometric/stereological indices of the epididymis.
4. To investigate the effects of Atripla® and/or alcohol with low protein dietary intake on the connective tissue profile (collagen staining) of the epididymis using Van Gieson stain.

5. To determine any changes in the levels of serum testosterone following exposure to Atripla® and/or alcohol with low protein dietary intake.
6. To determine any changes in the expression of androgen receptors (AR) in epididymal tissues following exposure to Atripla® and/or alcohol with low protein dietary intake.
7. To determine any association in the level of serum testosterone levels with the changes in the expression of androgen receptors on exposure to Atripla® and/ or alcohol with low protein dietary intake.

CHAPTER 2: LITERATURE REVIEW

2.1 HIV/AIDS epidemiology in Sub-Sahara Africa

Improved accessibility to antiretroviral therapy (ART) has saved millions of lives globally (Singer, 2011, HIV/AIDS, 2012, WHO, 2012, Wandera *et al.*, 2015, Iacob *et al.*, 2017). The situation is set to be even better as the new guidelines for initiating ART upon testing gets fully applied (Franco and Saag, 2013, UNAIDS, 2016). Correspondingly, mortality and morbidity rates have also decreased among SSA countries like South Africa, Nigeria, Botswana, Swaziland and Zimbabwe where HIV/AIDS is most prevalent (Sheet, 2016). This implies that life expectancy among PLWHAs is on the rise and more are expected to have a prolonged life. Nevertheless, eradication of the HIV virus remains elusive. Thus, besides improving the effectiveness of antiretroviral therapy, researchers and health professionals need to focus on the toxicity of chronic antiretroviral drug use to obviate the emergence of any unforeseen health challenges in future (Julg and Bogner, 2008).

2.2 Development of HIV/AIDS therapy

2.2.1 Antiretroviral therapy era

The human immunodeficiency virus (HIV) is a retrovirus that causes the acquired immunodeficiency syndrome [AIDS] (Broder and Gallo, 1984, Clay *et al.*, 2008, Li *et al.*, 2017b). There are two major types of the human immunodeficiency viruses; HIV-1 and HIV-2.(Squibb, 2006, Horberg and Klein, 2010b). The former was discovered first and is the most widespread type worldwide while the latter is less prevalent and less pathogenic (disease-causing), it is found principally in western Africa (Squibb, 2006). Therefore, major advancements in HIV/AIDS therapy have been based upon the structure of the virus (HIV-1). One of the earliest treatment strategies was the development of antiretroviral drugs (Palmisano and Vella, 2011, Group, 2015). The history of ART begins with the first clinical trial of zidovudine that was conducted in 1986 (Fischl *et al.*, 1987, Broder, 2010, Adediran *et al.*, 2016). Zidovudine is a nucleoside reverse transcriptase inhibitor that acts by competitively inhibiting the transcription of the HIV virus (Adediran *et al.*, 2016). Several new drugs were introduced during

the decade from 1986-1996 and a dual therapy strategy also became well established (Broder, 2010). However, antiretroviral therapy did not register much success in the treatment of HIV during these first ten years due to lack of drug efficacy and challenges in adherence amongst other factors (Bartlett, 2006, Broder, 2010).

2.2.2 HAART era

During the mid-90s (1995 to 1996) a group of antiretroviral drugs called non-nucleotide reverse transcriptase inhibitors and protease inhibitors (PI) were developed. Combined therapy of these new drugs with pre-existing nucleoside reverse transcriptase inhibitors established the triple drug therapy called highly active antiretroviral therapy (HAART) (Julg and Bogner, 2008, Usach *et al.*, 2013). HAART was established through the research efforts of various researchers notably Gulick and co-workers who illustrated the efficacy and better tolerance of a triple drug therapy (Gulick *et al.*, 1997, Bartlett, 2006, Wolff *et al.*, 2017). Therefore, implementation of HAART into the treatment of HIV/AIDS resulted in a decline in HIV prevalence, mortality and morbidity rates of up to 80% (Palella Jr *et al.*, 1998, Broder, 2010). Hence, The decade from 1996-2006 was characterised by significant gains in the treatment and management of HIV/AIDS (Bartlett, 2006). However, strict adherence to this triple drug therapy (HAART) was essential to achieve optimal therapeutic efficacy.

2.2.3 Development of HAART into a single once-daily pill: Atripla®

Despite the efficacy of HAART, higher pill burden and severe drug adverse reactions were among the major factors that contributed to challenges in treatment compliance among PLWHAs (Chesney, 2003, Horberg and Klein, 2010a). The earlier HAART regimens required several dosages during the day associated with intake of several pills at each dosage (Bartlett *et al.*, 2001). However, by the late 1990s efavirenz was developed as a once daily dose pill thus reducing the overall pill burden (Staszewski *et al.*, 1999, Horberg and Klein, 2010b). The development of tenofovir disoproxil fumarate then followed and was also approved as a single pill in the year 2001 (Dando and Wagstaff, 2004, Horberg and Klein, 2010b). Tenofovir disoproxyl fumarate was then co-formulated with emtricitabine into a single pill taken once a day, further reducing the pill burden (Horberg and Klein, 2010b, Truong *et al.*, 2015). This

combination improved compliance to treatment and it became widely used among the developed countries. In the year 2006, efavirenz, emtricitabine and tenofovir disoproxyl fumarate drugs were then formulated into a single once-daily tablet called Atripla® thus establishing the single pill once-daily dosage for HIV/AIDS treatment (Horberg and Klein, 2010b, Truong *et al.*, 2015).

2.3 Atripla®

Atripla® drug consists of 600 mg of efavirenz, 300 mg of emtricitabine and 200 mg of tenofovir disoproxil fumarate as active components (Truong *et al.*, 2015). The three drugs are constituted with other inactive substances such as colorants, croscarmellose sodium, hydroxypropyl cellulose, magnesium stearate and purified water (Squibb, 2010). The effect of Atripla® on the tissues of epididymis has not been extensively researched. However, the active component drugs of Atripla® have been reported to cause testicular tissue alterations which have been linked to fertility challenges in males (Frapsauce *et al.*, 2015).

2.3.1 Efavirenz

Efavirenz (marketed as Stutiva®) is one of the earliest non-nucleoside reverse transcriptase inhibitor to be developed and approved for use in HIV/AIDS treatment (Julg and Bogner, 2008, Frapsauce *et al.*, 2015). Efavirenz (EFV) acts by non-competitively inhibiting the replication of HIV-1and it has been widely used in combination with other antiretroviral drugs as part of the HAART triple drug therapy (Clay *et al.*, 2008). This is because efavirenz like many NNRTs is associated with relatively high compliance levels among HIV/AIDS patients (Usach *et al.*, 2013). Efavirenz is a crystalline chemical which is insoluble in water but dissolves in organic solvents (Squibb, 2006).

2.3.1.1 Efavirenz pharmacokinetics, pharmacodynamics and side effects

With regards to the pharmacokinetic profile, efavirenz is highly bound to plasma proteins with a mean free fraction of 0.57%, 0.58%, and 0.25-0.5% in rats, rhesus monkeys and human plasma respectively (Squibb, 2010). Oral bioavailability is estimated around 16% and 42%-45% in rats and rhesus monkeys respectively (Kasim *et al.*, 2004). The distribution of EFV is broad in rats

after oral dosing, it crosses the placenta and is excreted in milk (Squibb, 2010). Efavirenz is metabolised through cytochrome P450 liver enzymes (Clay *et al.*, 2008). Efavirenz half-life is estimated around 40 hours in humans compared to about 0.8 to 1.9 hours in rats, it is mainly excreted in urine and feaces in rats, monkeys and humans (Squibb, 2006).

EFV crosses both the blood-brain and blood-testis barriers and this has been associated with several of its side effects in patients that use the drug (Gaida *et al.*, 2016). In the former case, EFV has been reported to cause neuropsychiatric effects in approximately 50% of all patients that use the drug; these include insomnia, dizziness, impaired concentration and abnormal dreams (Gutierrez-Valencia *et al.*, 2009, Gaida *et al.*, 2016). Meanwhile, in the latter case EFV has been reported to cause impaired sperm motility mainly in heterosexual men (Pavili *et al.*, 2010, Frapsauce *et al.*, 2015). However, the exact mechanisms of how EFV causes sperm alterations remain elusive. In a previous study, EFV was reported to cause impaired sperm mitochondrial respiration which in turn affects sperm motility (Pavili *et al.*, 2010). While in other studies, EFV has been reported to alter the vitamin D depended maturation processes in the epididymis (Childs *et al.*, 2012, Frapsauce *et al.*, 2015).

2.3.2 Emtricitabine

Emtricitabine (marketed as Emtriva®) is a pyrimidine nucleoside analog, a nucleoside reverse transcriptase inhibitor (NRTI) (Julg and Bogner, 2008). Emtricitabine mode of action is through two mechanisms. The first involves competitive inhibition of HIV-1 replication and the second is through incorporation into HIV-1 deoxyribonucleic acid (DNA) causing the termination of the viral chain elongation (Frampton and Croom, 2006). However in the former mechanism of action, FTC has to be phosphorylated into emtricitabine-5-triphosphate which then competes with the natural substrate deoxycytidine 5'-triphosphate on HIV reverse transcriptase (Julg and Bogner, 2008). FTC is a white powder that dissolves in various aqeous solutions (Squibb, 2010).

2.3.2.1 Emtricitabine pharmacokinetics, pharmacodymics and side effects

Oral administration of FTC results in rapid absorption with highest plasma concentration recoded from 1 to 2 hours after administration (Masho *et al.*, 2007, Squibb, 2010). With regards to

distribution, FTC protein plasma binding was recorded to be approximately 4% in human while the mean bioavailability was around 93% (Saag *et al.*, 2004). FTC is metabolised by the liver but it does not inhibit the cytochrome P450 enzymes in humans and its plasma half-life is approximately 10 hours (Squibb, 2010). Most of FTC is excreted in urine (86%) following oral administration and studies in pregnant mice, rabbits and humans demonstrated that it crosses the placenta barrier (Squibb, 2006). Emtricitabine has a limited penetration through the blood-brain-barrier into the central nervous system (Squibb, 2006).

Headache, insomnia, diarrhea, nausea, vomiting and rash are amongst the most common side effects recorded among patients using FTC (Saag *et al.*, 2004, Masho *et al.*, 2007). However, chronic use of NRTs like FTC has been associated with mitochondrial toxicity (Squibb, 2010). Clinical adverse effects include polyneuropathy, cardiomyopathy, lactic acidosis, pancreatitis and lipodystrophy among others (Squibb, 2010). As reported for EFV, alterations to mitochondrial respiration have been linked to affect sperm motility and thus FTC has been linked to alterations in sperm characteristics among HIV infected patients using the drug (Masho *et al.*, 2007). Therefore, the combination of EFV and FTC in Atripla® might be a risk for the synergism of the adverse effects on sperm motility.

2.3.3 Tenofovir disoproxil fumarate

Tenofovir disoproxyl fumarate (TDF) is marketed as Viread®. It is a nucleotide analog, a nucleotide reverse transcriptase inhibitor (NtRTI). TDF mode of action involves competitive inhibition of HIV reverse transcriptase inhibitor in a similar mechanism as that of NRTIs (Suzuki *et al.*, 2017). However, TDF is mono-phosphorylated hence referred as nucleotide analog (Julg and Bogner, 2008). TDF is a white powder which is soluble in most aqueous solutions but sparingly soluble in water (Squibb, 2010).

2.3.3.1 Tenofovir disoproxyl fumurate phamacokinetics, pharmacodynamics and side effects

TDF is absorbed rapidly after oral administration and it has the highest plasma concentration from 25 minutes to one and a half hours (Squibb, 2010). The half-life in humans is about 17

hours, however, in studies involving rats a half-life of 6 hours was recorded (Clay *et al.*, 2008, Squibb, 2010). In *in-vitro* studies TDF has a plasma protein binding percentage of about 7.2% and bioavailability of up to 20% and 25% in rodents and humans respectively (Clay *et al.*, 2008). The bioavailability of TDF is dependent upon two variables: drug dose and concurrent administration with food (Squibb, 2010). Food is thought to decrease the bioavailability of TDF by increasing the drugs absorption, while bioavailability is inversely proportional to drug dosage (Hamzah *et al.*, 2015). TDF metabolism does not involve any inhibition of cytochrome enzymes, but some studies suggest induction action to the cytochrome CYP1A1 enzyme activity (Clay *et al.*, 2008). TDF is mostly excreted in urine (up to 80%) (Hamzah *et al.*, 2015).

While mitochondrial toxicity is widespread with the use of NRTIs, several studies suggest a low potential for TDF to affect mitochondrial functions (Squibb, 2010). TDF was reported not to have caused adverse effects on fertility, reproductive performance, embryo-fetal development and post-natal development (Squibb, 2010). However, the major target organs for toxicity post oral administration with TDF in animal studies were gastrointestinal tract, kidneys and bone (Hamzah *et al.*, 2015). Morphological changes in the renal tubule epithelium which include cell necrosis, tubular dilatation, pigment accumulation and interstitial nephritis were the most sensitive indicators of kidney toxicity in studies involving animal models (Suzuki *et al.*, 2017). Meanwhile, chronic administration of TDF to animals resulted in bone alterations ranging from decrease in bone mineral density, content and osteomalatia (Hamzah *et al.*, 2015). Therefore, morphological changes are often indicators of organ malfunction and toxicity pointing to how structure is related to function. While TDF was reported to not affect reproduction in previous studies, the interactive effects of TDF with other drug components in ATp on reproductive tissues needs to be clarified.

2.4 HAART success: rise in quality of life and intentions to reproduce among PLWHAs

The triple drug therapy (HAART), with better patient compliance, has proved to be effective in decreasing HIV viral loads to undetectable levels, thus reducing the incidence of HIV related opportunistic infections (Ford *et al.*, 2018). Therefore, HIV/AIDS treatment has virtually transformed from being a temporary remedy in the face of imminent death, to a survivable chronic disease for the majority of HIV/AIDS patients on treatment who are living in SSA

[Figure 2.1] (Chen *et al.*, 2013). This resultant effect is set to improve with high compliance drugs (one pill a day) such as Atripla®. However, the improvement in the quality of life among PLWHAs has been coupled with an increase in reproductive intentions (Chen *et al.*, 2001, Iliyasu *et al.*, 2009, Adilo and Wordofa, 2017). Procreation is an inherent human characteristic, hence, the desire to have children is also expected among PLWHAs (Bunnell *et al.*, 2005, Adilo and Wordofa, 2017). Indeed, several studies indicate that HIV positive men including women continue desiring to have children even after learning of their HIV status (Table 2.1). The desire to procreate among men just like among females is viewed as a sign of normalcy in most societies (Kirshenbaum *et al.*, 2004, Ashimi *et al.*, 2017). In addition, most societies in SSA remain strongly pro-natal, hence, procreation even among PLWHAs is a determinant and a denominator for establishing male status, fulfillment in life and continuity (Cooper *et al.*, 2007, Van Zyl and Visser, 2015, Ashimi *et al.*, 2017).

Table 2.1: Studies on the reproductive intentions among PLWHAs

Researchers	Estimated percentage of PLWHAs with reproductive intentions	Country
Chen *et al* (2001)	**29% overall**, in a population of 2864 (n=2864), 28% of males and 29% of females, over the age of 18 years	United States of America
Sherr and Barry (2004)	**43.8% overall** (n=32), heterosexual males, over the age of 18 years	United Kingdom
Tamene and Fantahun (2007)	**40.2% overall** (n=460), 35.2% of males and 44.7% of females, mean age of 33.3 years	Ethiopia
Mmbaga *et al* (2013)	**37.1% overall** (n=410), 39.7% of males and 35.6% females, mean age of 34.2 years	Tanzania
Wekesa and Coast (2014)	**34% overall** (n=554), mean age of 38 years	Kenya
Agbo and Rispel (2017)	**46% overall** (n=430), 63% of males and 37% females, mean age of 36.4 years	South Africa

2.5 Lifestyle challenges in Sub-Sahara Africa: prevalence of protein malnutrition and excessive alcohol use

2.5.1 Malnutrition (Protein deficiency)

Originally one of the United Nations millennium development goals was to reduce the prevalence of world hunger by half by the year 2015, however, hunger and malnutrition continue to be on the increase mainly in Africa [Figure 2.2] (Bain *et al.*, 2013, Butler, 2015). Malnutrition is broadly defined as a state in which the physical function of an individual is impaired to the point where he or she can no longer maintain adequate bodily performance process such as growth, pregnancy, lactation, physical work and resisting and recovering from disease (Liete *et al.*, 2011, Bain *et al.*, 2013). While malnutrition includes both over- and under- nutrition, undernutrition is wide spread in SSA such that one in four people is reported to be undernourished (Bain *et al.*, 2013, FAO, 2015). This has been linked to poverty, socio-political instability, illiteracy, general increase in population and corruption among other factors endemic in the region (Bain *et al.*, 2013). The HIV/AIDS scourge has also been associated with a decline in agricultural output since the turn of the millennium especially in SSA (Muzari, 2014, Mabuza and Dlamini, 2017). In addition, it has been forecasted that global climate change will impact negatively on SSA especially on food production and security (Parry *et al.*, 2007, FAO, 2016). Unfortunately, it is protein food sources that become critically low in supply in times of food deficits. A healthy balanced diet comprises of adequate amounts of protein, carbohydrates, fats and micronutrients. However, protein remains one of the most scarce and costly components of diet in most resource limited settings globally (Bain *et al.*, 2013). Moreover, higher nutritional needs are required for PLWHAs than those without (De Waal and Whiteside, 2003, Thapa *et al.*, 2015) suggesting the need to document the reproductive effects of Atripla® use in protein deficiency.

2.5.2 Alcohol consumption

Concurrent with protein malnutrition, is alcohol consumption which is a lifestyle phenomenon in SSA. It is reported that SSA region has the highest rate of heavy episodic alcohol drinking per

drinker globally (Morojele *et al.*, 2013b) [Figure 2.3]. According to WHO heavy episodic alcohol use is defined as consumption of 60 or more grams of pure alcohol (6+ standard drinks in most countries) on at least one single occasion at least monthly, which has a high potential for causing health or social harm (WHO,2014, Rehm and Imtiaz, 2016). In addition, several studies have reported the association between HIV infection, alcohol use and abuse in the SSA (Rosenberg *et al.*, 2015, Rehm *et al.*, 2017). Studies by Fisher *et al* (2007) suggested that alcohol drinkers have more than a 70% chance of being HIV positive when compared to those who do not consume alcohol. It is also estimated that approximately 33% of HIV patients receiving treatment in major referral clinics in the metropolitan regions of SSA have reported the use of alcohol (Wandera *et al.*, 2015), suggesting concomitant intake of alcohol and antiretroviral treatment.

In addition, increased alcohol consumption rates have been reported among males not only in SSA, but across the globe (Fisher *et al.*, 2007, Francis *et al.*, 2015). This is due to social customs/norms and values associated with alcohol consumption among men (Lemle and Mishkind, 1989, Wandera *et al.*, 2015). In this regard, high alcohol intake among PLWHAs especially among males in SSA may be inferred. Given that concurrently, the SSA region is struggling with a prevalence of protein deficiency mentioned previously, there is a possibility of a high incidence of PLWHAs on Atripla® treatment living a lifestyle affected by both phenomenon. It is therefore imperative to study and document the combined effects of the Atripla®, protein malnutrition and chronic alcohol intake on the male reproductive function focusing on the epididymis. The epididymis plays a critical role in sperm maturation which is key to reproductive success (Azu *et al.*, 2014, Sullivan and Mieusset, 2016).

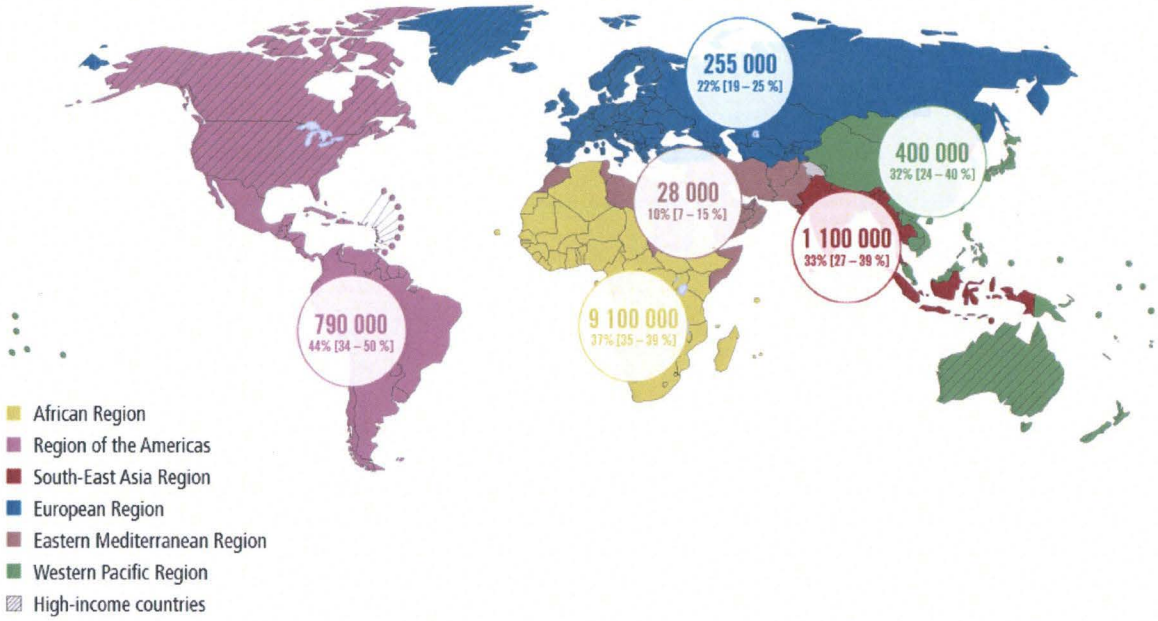


Figure 2.1: Population of PLWHAs on antiretroviral therapy by WHO regions (Bhatti *et al.*, 2016). Note the WHO African region has the highest number of PLWHAs on treatment (the percentages are for the population on treatment from the total population infected).

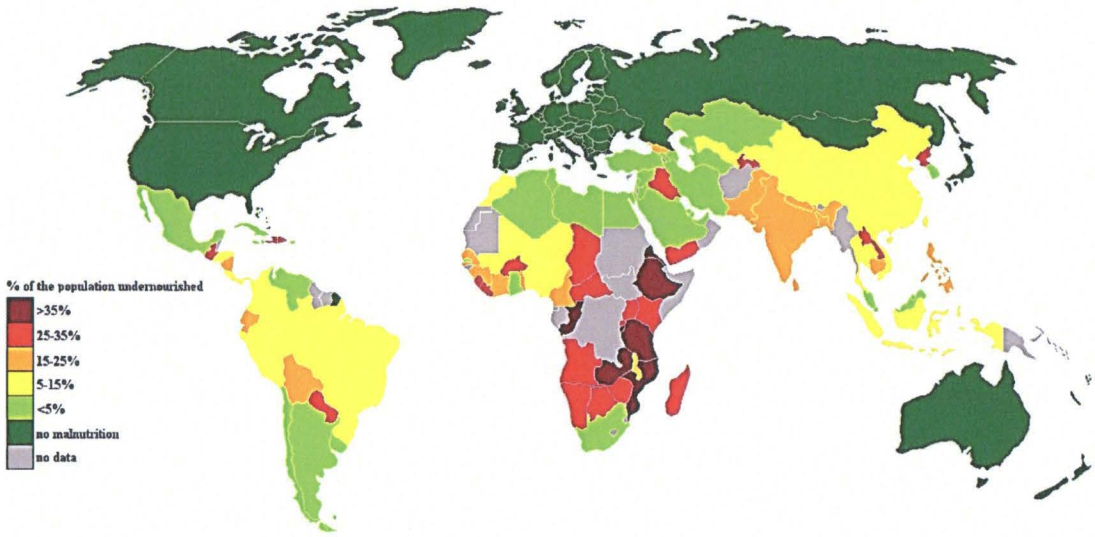


Figure 2.2: Global map for undernutrition (Education, 2012). Higher levels generally above 25% were recorded in the SSA region as shown in the colour red on the map.

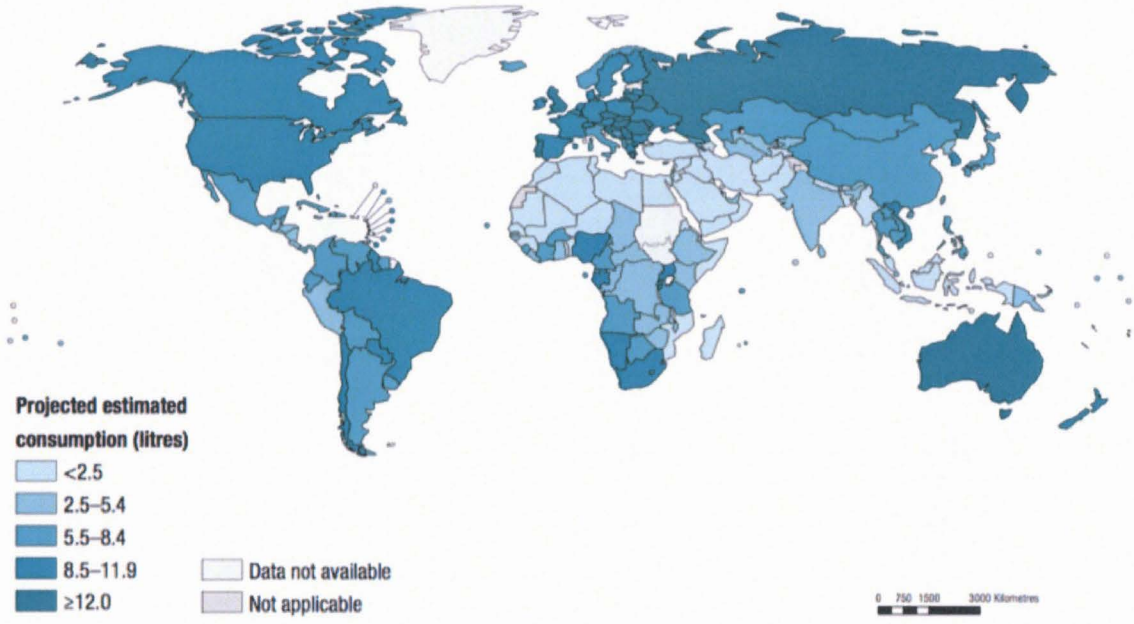


Figure 2.3: The global estimated alcohol consumption per capita in litres of pure alcohol (Organization and Unit, 2014). SSA region has one of the highest values globally.

2.6 Organ of study: Epididymis

The epididymis is a male reproductive organ that connects the efferent ducts to vas deferens. It is formed by the joining of efferent ducts which form a single highly convoluted duct that is organised into an organ (Robaire *et al.*, 2006, Sullivan and Mieusset, 2016). The epididymal duct is very long having lengths of up to 7 metres in men and approximately 3.2 metres in rats (Sullivan, 2004, Turner, 2008). The major function of the epididymis is mainly the differentiation of spermatozoa into mature, motile cells that are able to fertilise an ovum (De Grava Kempinas and Klinefelter, 2014), though, the process by which the epididymis performs this function is yet to be fully understood. However, it is believed that the epididymis induces physiological, biochemical and morphological changes to spermatozoa as it passes along the organ that prepare them to actualise their fertilisation function (Gatti *et al.*, 2004, Arrotéia *et al.*, 2012). This is because post testicular sperms are immotile and unable to fertilize an oocyte (Yanagimachi *et al.*, 1985, Flesch and Gadella, 2000, Arrotéia *et al.*, 2012).

2.6.1 Macroscopic, microscopic features and functions of the epididymis

The gross anatomical features of the epididymis consists of the proximal region (which includes the initial segment and caput), the corpus/body and the distal region or the cauda/tail [Figure 2.4] (Robaire and Henderson, 2006, Arrotéia *et al.*, 2012). In mammals, each region is further divided into intraregional compartments/segments that are separated by connective tissue septa [Figure 2.5] (Jelinsky *et al.*, 2007, Arrotéia *et al.*, 2012). These septa function as both internal support for the organ and functional boundaries between the segments (Kirchhoff, 1999, Cornwall, 2009). The intraregional segments have been reported to be of functional importance because of independent gene and protein expression in each intraregional segment (Jelinsky *et al.*, 2007).

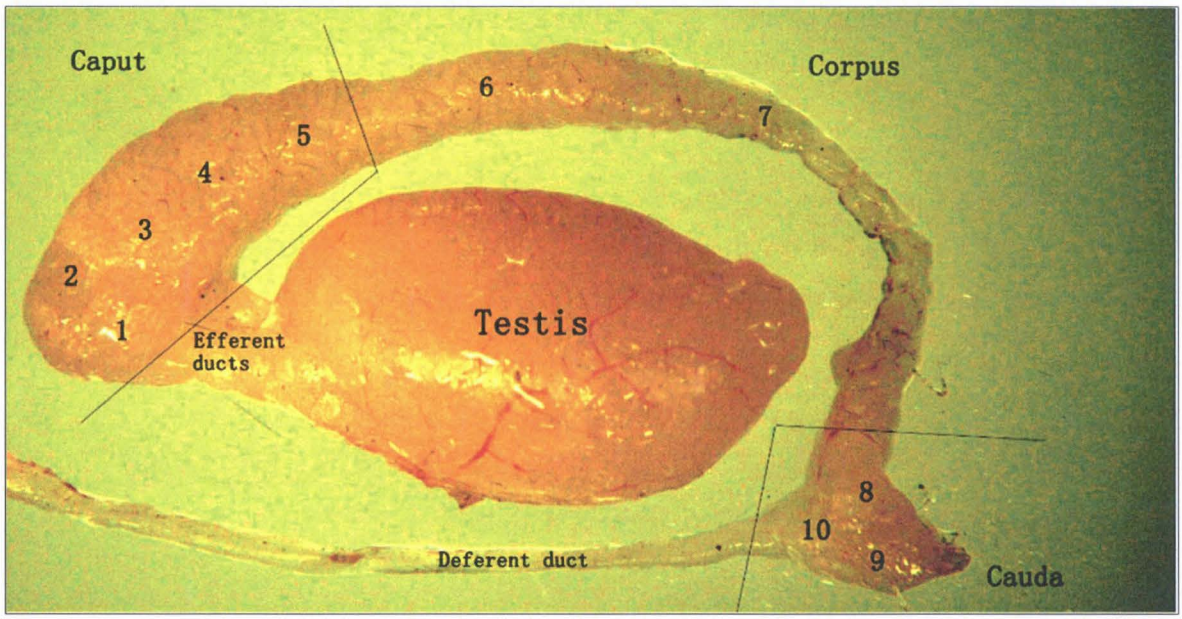


Figure 2.4: Testis and epididymis of a mouse (Li *et al.*, 2017a). The epididymis is divided into regions 1 to 10. The caput (region 1 to 5), corpus (region 6 and 7) and cauda (region 8 to 10).

Various types of cells with different functions are located in the epithelium of the epididymis. The most common are principal and basal cells and they are present in all regions of the epididymis [Figure 2.6]. Other cell types such as apical, narrow, clear and halo cells are also present in the epithelium of the epididymis and they vary within each region. The most abundant cell type in the initial segment is the principal cell and it makes up to 80% of the total epithelium cells in that region. The concentration of principal cells gradually decreases to 65% of the total cells in the epithelium of the cauda (Robaire and Hermo, 1988, Arrotéia *et al.*, 2012). The second most abundant cells type in the epididymis epithelium is called basal cell. Basal cells make up to 20% of the total epithelium population in the epididymis. The cells are located in the base of the epithelium and are triangular in shape (Robaire *et al.*, 2000, Cornwall, 2009). Apical cells constitute about 10% of the total epithelial cell population in the epididymal epithelium but they make up about 1% of the total epithelial cell population in the cauda epididymis (Adamali and Hermo, 1996, Arrotéia *et al.*, 2012).

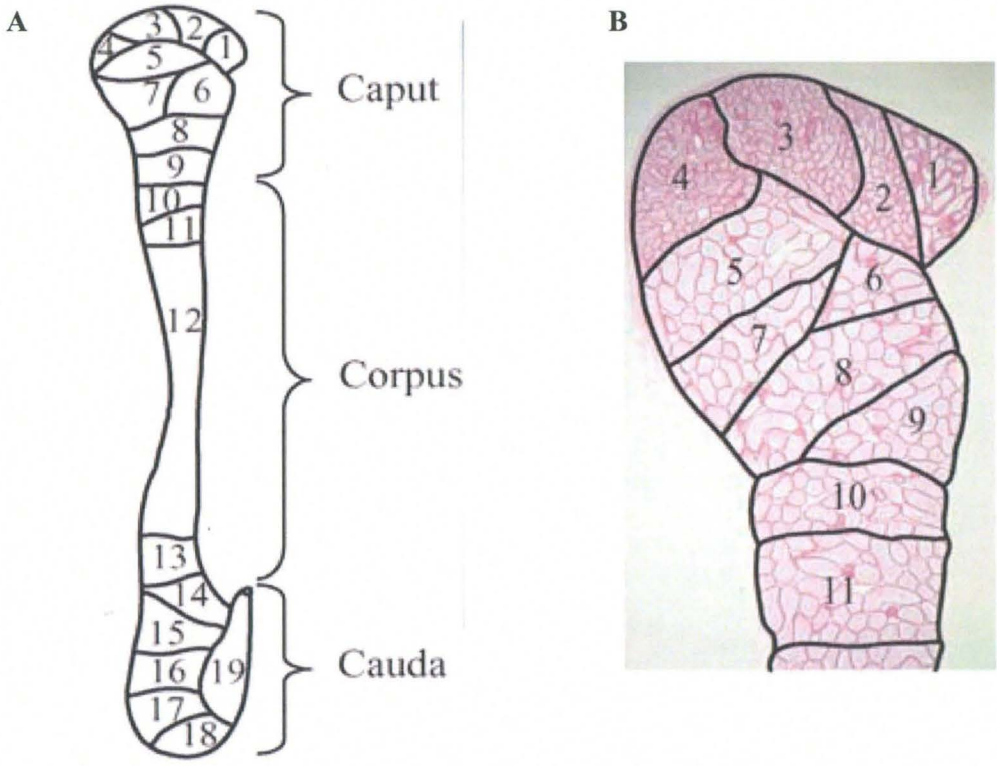


Figure 2.5: Schematic diagram that shows the segmental structure of the epididymis [longitudinal section] (Jelinsky *et al.*, 2007). **A**: intraregional segments of the epididymis (numbered) that are separated by connective tissue septa (in black). **B**: panoramic view of the intraregional segments of the proximal region of the epididymis (Initial segment has segments 1 to 4; caput has segments 5 to 11.

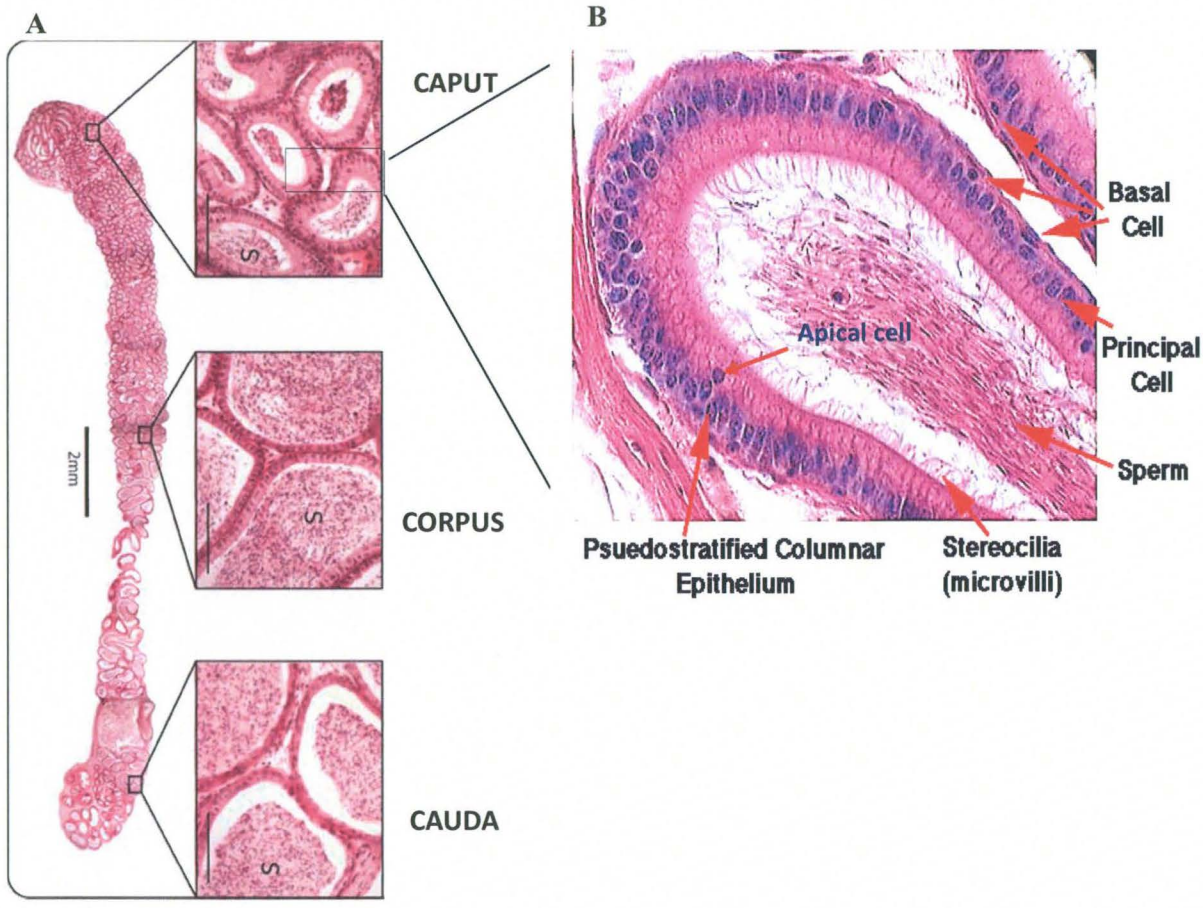


Figure 2.6: Panoramic view of epididymis histology (Borg *et al.*, 2009) [modified]. **A:** longitudinal section of the epididymis that shows the overall view of all the regions **B**: longitudinal section showing the various cell types in the epithelium of the epididymal tubule (principal cells, basal cells, apical cells and sperm cells).

In addition, epithelial height varies along the length of the epididymis, it is higher in the proximal caput and it is lower in the cauda region. On the contrary, the thickness of the peritubular smooth muscle layer and the diameter of the lumen increases from the proximal to the distal regions of the epididymis (Toshimori, 2003, Arrotéia *et al.*, 2012). Likewise with luminal sperm concentration, there is less concentration of sperm cells in the initial segment but cauda epididymis is filled with sperm cells (Cornwall, 2009, Arrotéia *et al.*, 2012). Whilst the

fertilisation capacity of sperm cells is acquired as the sperm migrate throughout the entire length of the epididymis, epididymal regions are thought to have specific functions. The initial segment consists of highly columnar principal cells that are involved in the reabsorption of water, ions and small organic molecules (De Grava Kempinas and Klinefelter, 2014). The principal cells in the caput secrete proteins which modify the membrane of sperm cells. The corpus is responsible for the modification of the lipid component of sperm plasma membrane; hence, the principal cells in that region have abundant lipids in their supranuclear region (De Grava Kempinas and Klinefelter, 2014, Bedford, 2015). The cauda epithelium has clear cells and thinner epithelium lining; the clear cells engulf excess proteins and cytoplasmic droplets from maturing sperm (De Grava Kempinas and Klinefelter, 2014). Mature spermatozoa are mainly stored in the cauda epididymis (Amann and Howards, 1980, Bedford, 2015).

The functions of the epididymal regions is further enhanced by presence of epithelial tight junctions that hold the epithelial cells together thus forming the blood-epididymis-barrier which is essential for the intricate functions of the epididymis (Robaire *et al.*, 2006). In various research studies, sperm fertilisation capacity is reported to be acquired mostly in the proximal caput up to the distal half of the corpus epididymis (Takeda *et al.*, 1990, Sullivan and Mieusset, 2016). Furthermore, regulation of testicular fluid pH and reabsorption of testicular fluid is also associated with the proximal caput epididymis (Cosentino and Cockett, 1986, Sullivan and Mieusset, 2016). Hence, in most toxicological studies, researchers evaluate the effect of xenobiotics in the proximal region of the epididymis (Takeda *et al.*, 1990, Gür *et al.*, 2011, Menad *et al.*, 2014).

2.6.2 Hormonal regulation of epididymal functions

The development and function of the epididymis is highly dependent on a group of hormones called androgens. There are two types of androgen hormones: testosterone (T) and its derivative dihydro-testosterone (Robaire *et al.*, 2006, Ramaswamy and Weinbauer, 2014). Testosterone is the principal androgen and it is involved in the differentiation of the epididymis, vas deferens and the seminal vesicles from the Wolffian duct system during embryonic development (Arrotéia *et al.*, 2012). DHT is the most effective metabolite of testosterone and it is formed by the reduction

of T into DHT through 5 α -reductase enzyme (Jin and Penning, 2001, Davey and Grossmann, 2016). In the mature epididymis, T and DHT are key in the maintenance of the structure, secretory and absorptive functions of epididymal epithelium (Trybek *et al.*, 2005).

2.6.2.1 Androgen receptors

Testosterone and dihydro-testosterone actions are exerted through the intracellular receptor known as the androgen receptor [AR] (Gür *et al.*, 2011, Boukenaoui-Ferrouk *et al.*, 2017). The localisation of AR in the epididymis as well as in other male sexual organs has been reported in several species including rats, monkeys and in humans (Trybek *et al.*, 2005, Rinaldi *et al.*, 2013, El-Meseeh *et al.*, 2016). The binding of androgens to AR regulates specific effects in target tissues through the transcription of specific proteins (De Souza Santos *et al.*, 2004, Davey and Grossmann, 2016). In the epididymis, androgen receptor immunoreactivity (AR-ir) was reported to be highest in the proximal parts of the epididymis decreasing to the cauda region (Takeda *et al.*, 1990, Zhou *et al.*, 2002, Gür *et al.*, 2011). Furthermore, AR-ir is more prominent in nuclei of principal cell than any other type of cell in the epididymis (Sar *et al.*, 1990, Gür *et al.*, 2011). The high distribution of androgen receptors in the caput epididymis especially among principal epithelial cells might be linked to its role in the production and secretion of epididymal proteins for the modification of the maturing sperm cells (Arrotéia *et al.*, 2012).

2.6.3 Investigating toxicity in the epididymis

Toxicological effects of xenobiotics on the epididymis can be mediated via two categories: androgen deprivation (indirect toxicity) and direct chemical toxicity (De Grava Kempinas and Klinefelter, 2014). Since the epididymis is functionally highly depended on androgens, the most prominent histological derangements have been due to androgen deprivation. Significant androgen deprivation is associated with a decrease in epididymal tubule diameters, epithelial height and induction of apoptosis in most regions of the epididymis (De Grava Kempinas and Klinefelter, 2014). Various conditions such as castration, testosterone/estrogen implants and xenobiotics capable of reducing androgen synthesis have been associated with these deleterious effects on the epididymis and consequently infertility (Hamzeh and Robaire, 2009, Marcoccia *et al.*, 2017). This reveals how structural integrity is related to function in the epididymis. Meanwhile, several chemicals have been shown to cause tissue derangements in the epididymis

directly (Creasy *et al.*, 2012, Marcoccia *et al.*, 2017). Direct toxicity to the epididymal epithelium may be associated with the deterioration, necrosis and exfoliation of principal cells (Creasy *et al.*, 2012). In some instances, toxins that act directly on the epididymis cause an increase rather than a decrease in epithelium height (De Grava Kempinas and Klinefelter, 2014).

2.6.3.1 Epididymal tissue alterations due to androgen deprivation

The effect of various xenobiotics has been associated with depletion in the concentration of sperm stored in the cauda epididymis with little or no reduction in sperm production (Fernandez *et al.*, 2008, De Grava Kempinas and Klinefelter, 2014). This implies that toxicity is mainly in the epididymis and the transit of sperm is being accelerated by exposure to the chemicals. The action has been linked to the effect on androgen deprivation, however, recent studies have demonstrated the direct action of several compounds on the epididymis can cause accelerated transit time (De Grava Kempinas and Klinefelter, 2014). When there is accelerated transit time, sperm cells take less time to be transported through the entire duct of the epididymis and this compromises sperm quality (Klinefelter *et al.*, 1997, Lanning *et al.*, 2002, De Grava Kempinas and Klinefelter, 2014).

Androgen deprivation has also been associated with presence of cellular debris in the lumen of the epididymis (Creasy *et al.*, 2012, De Grava Kempinas and Klinefelter, 2014). Debris is usually generated from exfoliation of principal cells from the epithelium and from degenerating sperm. In androgen deprivation, numerous round spermatids will be observed in the lumen (De Grava Kempinas and Klinefelter, 2014). The presence of exfoliated material may be a sign of testicular toxicity and the material is transported through the lumen into the epididymis signaling epithelial injury to the organ (Lanning *et al.*, 2002, Romualdo *et al.*, 2002). However, luminal debris can also arise from chemical that act directly on the epididymis or testis.

Cribriform change is a hyperplastic histo-architectural alteration in the tissues of the epididymis characterised by folded epithelium that forms pseudoglandular structures (Creasy *et al.*, 2012, De Grava Kempinas and Klinefelter, 2014). Cribriform change has been associated with gonal toxicity in rats but the real causes remain elusive (De Grava Kempinas and Klinefelter, 2014).

However, it has been remotely connected to androgen deprivation secondary to testicular toxicity (De Grava Kempinas and Klinefelter, 2014). The infolding of epithelium may be associated with lack of sperm, testicular fluid and epididymal atrophy. Cribriform change was observed in 42% of cases in 167 human samples following the surgical removal of one or both testes due to various medical conditions (Shah *et al.*, 1998).

2.6.3.2 Epididymal alterations due to direct toxicity

While increases in interstitial space between epididymal tubules can be an indication of tubular atrophy caused by androgen deprivation; increased interstitial space can also be an indication of interstitial oedema (Lanning *et al.*, 2002). Movement of luminal sperm in the epididymis is dependent upon a balance in the amount of fluid produced by sertoli cells in the testis and that absorbed in the efferent ductules and initial segment (De Grava Kempinas and Klinefelter, 2014). Alterations in the fluid dynamics especially due to epithelial damage by xenobiotics may cause impairment in fluid reabsorption by epithelium cells of the efferent ducts and the initial segment resulting in interstitial oedema and/or tubular dilatation (Lanning *et al.*, 2002).

The action of some substances has also been reported to provoke an inflammatory response in the epididymis which is commonly observed in the interstitium of tissue (Creasy *et al.*, 2012, De Grava Kempinas and Klinefelter, 2014). The population of tissue inflammatory infiltrates usually comprises of neutrophils, lymphocytes, fibroblasts and sperm cells (De Grava Kempinas and Klinefelter, 2014). While the exact cause of tissue inflammatory infiltrates remains elusive, it is believed that they arise from the rapture of an epididymal tubule due to increased luminal pressure (De Grava Kempinas and Klinefelter, 2014). The break of luminal contents into the interstitium is thought to be followed by an inflammatory response that forms a nodule of inflammatory cells encapsulating the leaked contents (Gregory and Cyr, 2014).

Clear cells are abundant in the cauda epididymis, they are more prominent there because they contain a lot of lysosomes which are key to their role in engulfing cytoplasmic droplets from maturing sperms (Creasy *et al.*, 2012). In some cases, clear cells disappearance is a sign of toxicity to the epididymis (Klinefelter *et al.*, 1990). However, in other instances, toxicity in the

cauda epididymis is revealed by an abundance of clear cells dominating the epithelial cell of the epididymal tubules a phenomenon called clear cell hyperplasia (De Grava Kempinas and Klinefelter, 2014). Clear cell hyperplasia was reported in the tissues of the epididymis after treatment with chemical compound called gossypol (De Andrade *et al.*, 2006). Clear cell hyperplasia is often observed in association with the luminal debri in the corpus and cauda regions of the epididymis due to the need to engulf the exfoliated epithelial cells in the lumen (Creasy *et al.*, 2012). Therefore in this study, combined effect of ATp, E and PM was evaluated under direct and indirect toxicity on the epididymis.

2.7 Effects of HAART on male reproductive hormones, testicular and epididymal tissues

The effect of HAART on male reproductive health continues to be an elusive subject among researchers. Some studies have reported depressed levels of testosterone, follicle stimulating hormone and luteinising hormone with antiretroviral use among PLWHAs (Wagner *et al.*, 1995, Rochira *et al.*, 2011, Gomes *et al.*, 2016). These disturbances in hormonal levels have been thought to cause disruptions in the hypothalamo-pituitary-gonadal (HPG) axis that translate into reproductive dysfunction, but the actual mechanism by which it happens remains unclear (Rochira *et al.*, 2011, Gomes *et al.*, 2016). In contrast, antiretroviral drug use was also reported to cause increased levels of testosterone in HIV patients and the hormonal disruptions in the HPG axis were not associated with sexual dysfunction (Collazos *et al.*, 2002).While these authors did not give reasons backing up their findings, differences in methodological techniques may be contributory factors. Clearly, further studies is needed for clarity of effect in this regard.

Meanwhile, a recent study on the histo-architecture of the testis revealed that HAART causes testicular tissue derangements involving decreases in tubular and epithelial areas, necrosis and a decline in the spermatogenesis index (Azu *et al.*, 2014). In addition, HAART has been linked to disruptions in the structural integrity of the testicular epithelium including the blood-testis-barrier through disruptions of the cell junctions and depletion of mitochondria in sertoli cells including the spermatozoa (Azu, 2012). These disruptions in testicular tissue associated with antiretroviral use have been thought to result in reproductive incompetence (Azu *et al.*, 2014). Whilst most studies indicate altered reproduction capacity with HAART use, other studies maintain that it does not cause alterations to sperm parameters such sperm morphology and count (Krieger *et al.*,

1991, Oyeyipo *et al.*, 2018). Instead, HAART was even associated with improved semen quality (Robbins *et al.*, 2001, Azu *et al.*, 2014). The discordance in these reports may also be due to different combinations of antiretroviral drugs used. Hence, the present study explores the effect of the combination in Atripla® (EFV, FTC, TDF), combined with alcohol and protein deficient diet, on the levels of testosterone and histo-architecture of the epididymis.

2.8 Effects of malnutrition on male reproductive hormones, testicular and epididymal tissues

Nutrition associated toxicity has become a topic of current concern (Bain *et al.*, 2013, Jadán-Piedra *et al.*, 2017, Guo and Katta, 2017). Undernutrition is reported to be associated with imbalances in the production of gonadotropin hormones (Clarke and Henry, 1999). Deficiencies in protein and/or other micronutrients can also result in growth and maturational retardation that may alter the function of reproductive organs (Mcdonald *et al.*, 1992). In several studies involving maternal protein malnutrition in rats, there was a decrease in the levels of luteinising hormone, follicle-stimulating hormone and testosterone accompanied by decreased weight of reproductive organs of the pups (Ramos *et al.*, 2010, Rinaldi *et al.*, 2013, Khorram *et al.*, 2015). Meanwhile in some studies, maternal protein malnutrition in pups (of rats) was reported to cause tissue structural changes by altering epithelial cell configuration and androgen receptor expression which affects the function of the epididymis, testis and prostate in pups (Vicente *et al.*, 2004, Rinaldi *et al.*, 2013).

However in a study involving adult rats, protein malnutrition did not cause changes in rat feed intake, biometric parameters (body and organ weights) and testosterone levels but only caused a decrease in AR protein levels in the testis (De Souza Santos *et al.*, 2004, Teixeira *et al.*, 2007). In another study, protein malnutrition was reported to potentiate the hypo-fertility and toxic effects of pirimiphos-methyl (pesticide), cadmium (found in industrial pollutants) and sulfasalazine (rheumatoid arthritis drug) in the testis and epididymis tissues of adult rats when administered concurrently (Hassanein and Hamed, 2008). Thus, protein malnutrition becomes an important factor that influences male reproductive capacity and it may interact adversely with Atripla® and alcohol. The documentation of the combined effects of these factors is thus of clinical importance

in the management and treatment of male reproductive health issues especially in this era of HIV/AIDS.

2.9 Effects of alcohol on male reproductive hormones, testicular and epididymal tissues

Various studies have implicated alcohol abuse as a precursor for male infertility (Albadri, 2013, Oremosu and Akang, 2015, Erol *et al.*, 2017). It has been reported that approximately 42% of men with infertility problems consume alcohol (Chia *et al.*, 2000, Jensen *et al.*, 2014). However, the exact mechanism by which alcohol causes infertility in the males is not clearly understood. In some studies, alcohol consumption has been reported to cause infertility solely through the disruption of regulatory feedback mechanism that result from decreased testosterone levels that negatively impact the hypothalamo-pituitary-gonadal (HPG) axis (Maneesh *et al.*, 2006, Dosumu *et al.*, 2012). However, recent studies assert that alcohol consumption causes disruptions in male reproductive health through a combination of pathways that involves decreased male reproductive hormones (testosterone, follicle stimulating hormone and leutinising hormone), testicular tissue derangements and direct toxicity on the hypothalamus (Albadri, 2013, Oremosu and Akang, 2015).

2.10 Interactive effects of alcohol and/or nutrition on drug pharmacology

Multiple drug therapy involves the presence of two or more drug in the body at the same time. This may lead to drug-drug interaction which may result in modification of the effects of the drugs. Additionally, interactions may occur between the drugs and food or its components resulting in drug-nutrition interactions (Köhler *et al.*, 2000, Kaufman *et al.*, 2002, Alomar, 2014). Such interactions may have different implications to male reproductive health since adequate nutrition is crucial in male reproductive sufficiency.

2.10.1 Interactive effects of alcohol and HAART (or other xenobiotics)

Alcohol has been associated with several harmful effects on general health. More importantly, alcohol affects the body's ability to metabolise xenobiotics including antiretroviral drugs (Chan and Anderson, 2014). The metabolism of alcohol is via the liver enzymes cytochrome P450 (CYP2E1) and alcohol dehydrogenase (ADH) and these are the same enzymes that also

metabolise xenobiotics. Hence, pharmacological interactions between drugs and alcohol are likely to ensue. However, alcohol is metabolised through various pathways by these liver enzymes. ADH is more actively involved in the biotransformation of alcohol with casual light alcohol consumption, while, CYP2E1 is extensively involved in the metabolism of alcohol during chronic heavy drinking (Aubert *et al.*, 2011, Cederbaum, 2012). Therefore, the effects of alcohol and xenobiotics interactions may be different in acute or chronic alcohol consumption.

Alcohol has also been shown to affect the degradation of drugs by competitively inhibiting their metabolism by liver enzymes resulting in increased bioavalability of the drug, thus, increased adverse effects (Hoyumpa Jr and Schenker, 1982, Fagerberg *et al.*, 2015). In contrast, chronic alcohol consumption may activate drug-metabolizing enzymes which increase the activity of microsomal ethanol oxidase system (MEOS) thereby enhancing drug elimination and consequently reducing adverse effects (Lieber, 1992, Cederbaum, 2012). In some instances, induction of liver enzymes by chronic alcohol consumption may also transform some drugs into harmful metabolites that can cause impairment of the liver function (Cederbaum, 2012).

Additionally, interaction of alcohol with antiretroviral drugs metabolised by the same cytochrome enzyme systems may result in differences in alcohol pharmacodynamics and pharmacokinetics. For instance, ritonavir inhibits the activity of cytochrome enzyme CYP 450 3A4 the same enzymes responsible for alcohol metabolism (Kumar *et al.*, 1996, Kumar *et al.*, 2015) . Therefore, ritonavir might potentiate the effects of alcohol when these drugs are co-administered (Li and Chan, 1999, Kumar *et al.*, 2015). On the contrary, efavirenz is a potent activator of cytochrome CYP 3A4 enzymes and it was reported to cause a decrease in blood alcohol levels when co-administered with alcohol (Mccance-Katz *et al.*, 2013).

2.10.2 Nutrition – drug interactions

The nutritional status of the individual is important in the body's metabolic and physiological processes that support and maintain life. Research shows that nutrition is a major determinant of drug action and hence drug metabolism (Reen *et al.*, 1999, Péter *et al.*, 2017). A multitude of mechanisms are related to food effects including solubilisation of drugs by food, integration of drugs with food components and modulation of drug-metabolising enzymes (Reen *et al.*, 1999,

Harris *et al.*, 2003, Bushra *et al.*, 2011). Furthermore, bio-transforming enzyme systems such as the mixed-function oxidase system (MFOS) depends on many dietary nutrients and can be decreased by deficiencies in protein, essential amino acids, ascorbic acid, tocopherol, and retinol (Bibi, 2008, Bartelink *et al.*, 2015). Thus, drug metabolism may be altered in states of nutritional deficiency or nutritional manipulation. Importantly, deficiencies in protein leads to alteration and changes in body composition, and consequently affects drug disposition by altering protein-binding and volume of distribution (Haken, 2000, Bartelink *et al.*, 2015).

2.10.3 Alcohol and nutrition

The body requires sufficient nutrients from diet to cater for the energy requirements and to sustain the bodily structure and function (Raiten, 2011, Bartelink *et al.*, 2015). However, chronic alcohol consumption is often associated with reduced intake of food which results in limited supply of essential nutrients thus affecting both energy supply and structure maintenance (Ouwens *et al.*, 2003). In addition, alcohol interferes with food metabolism by altering the digestion, storage, utilisation, and excretion of nutrients (Lieber, 1988, Lieber, 2003). In food digestion, alcohol is reported to affect the production of pancreatic enzymes that breakdown nutrients into usable components (Korsten, 1989, Yeomans, 2004). Meanwhile, alcohol is reported to interfere with nutrient absorption by causing damage to the epithelial lining of the stomach and intestines (Feinman, 1989, Ouwens *et al.*, 2003). In some instances after nutrients are digested and absorbed, alcohol can impair their use by altering their transport, storage, and excretion (Thomson and Pratt, 1992, Ouwens *et al.*, 2003). Thus alcohol intake in conditions of protein malnutrition may further deteriorate the body homeostasis which putatively may lead to more drug toxicity or otherwise.

2.10.4 Drug-drug interaction

The consumption of drugs is often associated with the incidence of adverse drug reactions. Adverse drug reactions are undesirable effects that may be experienced with the use of a drug as part of the drug pharmacological action or as a unique occurrence (Edwards and Aronson, 2000,

Liu *et al.*, 2017). ADRs often result from drug interactions which are classified as either pharmacodynamic or pharmacokinetic in nature. Pharmacodynamic interactions include those that result in additive or antagonistic effects (Brunton *et al.*, 2005, Rodrigues and Oliveira, 2016). Pharmacokinetic interactions result in alterations in the body's ability to metabolise, distribute, absorb and excrete drugs (Alomar, 2014, Heuberger, 2012).

In pharmacodynamic interactions, an additive effect occurs when the combined effect of two or more drugs, given concomitantly, is greater than the expected action for one of the drugs (Hansten and Horn, 2006). The result of addition of the common effects produced by the drugs may be less than (infra-addition), equal to (simple-addition), or more than (supra-addition) that produced by simple addition of their individual effects (Kierland, 1972, Hansten and Horn, 2006). Summation of effects thus occurs when the effects of two or more drugs are equal to the sum of the individual effects that are produced when drugs are administered alone. Interaction of drugs can also result in inhibition of one drug by another. This may include antagonism, infra-addition or any type of drug interaction that occurs when a substance given either previously, concurrently or subsequently prevents a drug from exerting its action and producing its full effects in a patient (Griffin and D'arcy, 1997, Hansten and Horn, 2006). The mechanisms involved may include enzyme induction, if the metabolites are more active than the original drug (Kierland, 1972, Hansten and Horn, 2006). In some situations interactive effects of drugs results in potentiation of effects. This is a situation which involves the enhancement of the effect of a drug by another substance or drug (Griffin and D'arcy, 1997, Horberg and Klein, 2010b, Alomar, 2014).

Finally apart from ADRs, some drug interactions may have desirable and intended results. These occur when a combination of medications improves therapy, lower toxicity and/or enhances potency with diminished side effects (Alomar, 2014). Occasionally, patients are given several drugs because they require intensive multiple drug therapy to correct a very serious condition as in the case of HIV/AIDS treatment. Therefore, the present study also explores the interactive effects of Atripla® constituent drugs (efavirenz, emtricitabine and tenofovir disoproxy fumarate) together with protein deficient diet and alcohol intake on male reproductive health.

2.11 Summary

Chapter 1 and 2 comprises of the introduction and background (literature review) to the study respectively. The chapters review the independent effects of HAART, E and PM on the male reproductive function in aspects of reproductive hormone regulation and on reproductive tissues. Literature review also highlights the different mechanisms by which alcohol and nutrition affects the pharmacology of xenobiotics. Review of such mechanisms also gives insight on the combined effects of ATp, E and PM on the tissues of the epididymis.

CHAPTER 3: MATERIALS AND METHODS

3.1 Ethical and legal considerations

All experimental procedures were performed according to the standard protocol for animal research experimentation which was approved by the Animal Ethics Screening Committee (AESC) of the University of Witwatersrand, Johannesburg, South Africa. The clearance certificate number: 2015/08/33/C (Appendix I).

3.2 Drugs/chemicals

Atripla® (efavirenz 600 mg, emtricitabine 300 mg, tenofovir disoproxyl fumarate 200 mg) [Gilead Inc, Bistol and Mayers] was purchased from Dischem Pharmacy (Johannesburg, South Africa). Absolute ethanol (100%) [Merck, South Africa] was obtained from the School of Anatomical Sciences laboratory stocks and diluted with distilled water to 6% ethanol for use in the experiment (Appendix II).

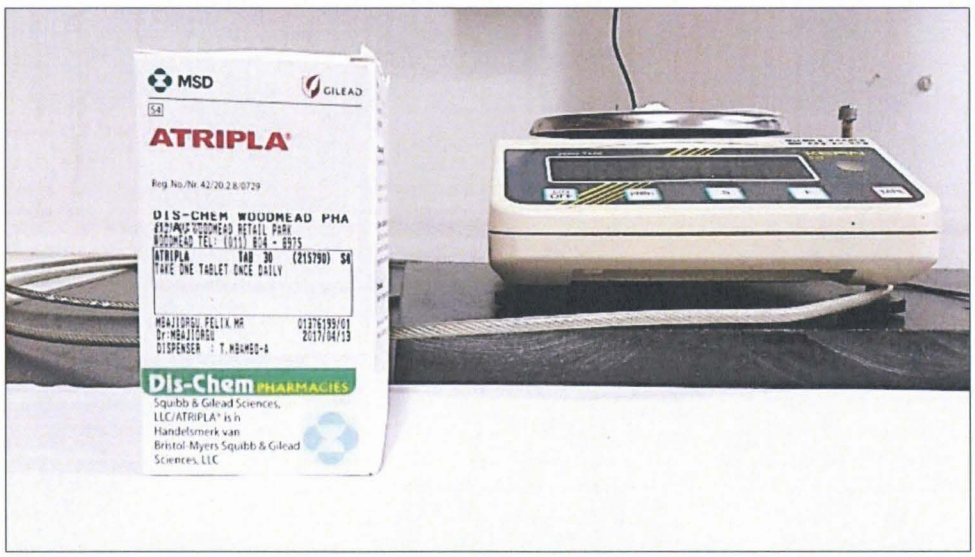


Figure 3.1: Box of Atripla® tablets and an electronic balance (Kern and Sohn Gmbh, Germany).

3.3 Experimental treatment diets

Normal protein diet was procured from Labchef Research Nutrition® (Northwest, South Africa). Low protein diet was then formulated and reconstituted from this normal protein diet according to animal diet standards under the guidance of an animal nutritionist (Reeves *et al.*, 1993, De Souza Santos *et al.*, 2004, Mbajiorgu *et al.*, 2008, Rinaldi *et al.*, 2013). This was done by mixing ground normal protein diet pellets with cassava (tuberous roots) powder using a ratio of 1:6 respectively as described in Appendix III. Commercially prepared powdered cassava (tuberous roots) was procured from a local vegetable market (Johannesburg, South Africa). Cassava (tuberous roots powder) was used in the formulation of low protein diet because it is widely used in the formulation of animal feeds (Tewe and Egbunike, 1992, Balagopalan, 2018). It also has a low crude protein of approximately 2.1% and an energy value between 11.7 to 12.6 MJ/kg (Bhuiyan and Iji, 2015). Such an ingredient was essential because a diet with a significantly low protein concentration with comparable energy and micronutrient levels to normal protein diet was required for the study. Moreover, the protein concentration was required to be at most 7% or less to properly reflect the impact of protein deficiency on the rats (Vawdaw and Mandlwana, 1990). Consequently, the final mixture of grounded normal diet powder and cassava powder (mixed at a ratio of 1:6 respectively) had 5.7% protein content and an energy value of 15.97 MJ/kg.

The energy value of the low protein diet (15.97 MJ/kg) was at a comparable level to that of the normal protein diet (16.58 MJ/kg) to allow for the study of protein deficiency (Table 3.1). Hence, protein concentration was 23% in the normal diet and 5.7% (which is approximately 6%) in the low protein diet. Protein content and other components of the normal and low protein diets were tested at the Agricultural Research Center (ARC), Irene analytic services testing lab (South African National Accreditation System [SANAS] approved testing laboratory). The diets were also tested at the University of the Free State, Department of Animal and Wildlife testing laboratory because the energy value for low protein diet could not be acquired due to a technical fault at the ARC testing lab. Hence, the protein and energy values used for this study were an average of the values from the two testing centers (except the energy value for low protein diet) [Appendix IV]. The proximate composition levels of the two diets are summarised in Table 3.1.

Table 3.1: Proximate composition levels of the normal and low protein diets

Components (%)	[a]Normal protein diet 23% of protein	[b]Low protein diet 6% of protein
Dry Matter	91.11	92.78
Moisture	8.89	7.22
Ash	6.99	3.35
Fat (ether extraction)	3.34	3.25
Fiber (crude)	2.60	3.74
***Protein (N x6.25)**	22.75	5.73
Calcium	1.32	2.95
Phosphorous	0.62	0.15
Total energy (MJ/kg)	16.58	15.97[£]

*For the conversion of nitrogen content to protein content the factor 6.25 was used.
[a]Standard rodent laboratory diet (Labchef Research Nutrition®, South Africa).
[b]Low protein diet was prepared at Central Animal services, Faculty of Health Sciences, University of Witwatersrand, South Africa by mixing the standard rodent laboratory diet with commercially prepared cassava tuberous roots powder at a ratio of 1:6 respectively.
[£] Low protein diet energy value from the University of the Free State testing laboratory

3.4 Experimental animals

Sixty-four adult male sexually mature Sprague Dawley (SD) rats between 11 and 12 weeks old (420 g- 450 g) were used in the study. The rats were acquired from the Central Animal Services at the University of Witwatersrand. The rats were housed individually in perspex cages with wood shavings as bedding. The bedding was changed twice a week to prevent animal infection and maintain hygiene. A 12–hour light/12-hour dark regimen (lights on at 07:00) was maintained throughout the study. Ambient temperatures of 24°C ± 2°C were also maintained with adequate ventilation provided.

3.5 Experimental design and procedure

The rats (within their individual cages) were the randomly assigned to two major groups based on their dietary protein level: normal protein diet (NP, 23% protein concentration) and low protein diet (LP, 6% protein concentration) groups. Each diet group of rats was further sub-divided into four sub-groups (with eight rats each) giving a total of 8 groups. Under both normal and low protein diet groups, one group served as control and received no treatment (except treatment vehicles), the second group received Atripla® (ATp) only, the third group received

ethanol (E) only and the fourth group received both Atripla® and ethanol (EATp) respectively in either of the dietary protein groups for 90 days (Lanning *et al.*, 2002). The rats were acclimatised to this treatment (protein deficient diet, ethanol and gelatine) for 7 days (one week) before the actual 90 days of treatment commenced. The treatment groups were thus identified as controls on normal or low protein diet designated as NPC and LPC groups respectively. Rats treated with Atripla® only whilst on normal or low protein diet were designated as NPATp and LPATp groups respectively. Rats on ethanol treatment only whilst on normal or low protein diet were designated as NPE and LPE groups respectively. Finally, rats treated with Atripla® and ethanol whilst on normal or low protein diets were designated as NPEATp and LPEATp groups respectively. The flow chart of the experimental design is shown in Figure 3.2.

Atripla® (27.5 mg/kg body weight per rat) was administered using flavoured gelatine rectangles ($2cm^3$) (Kamerman *et al.*, 2004) once a day orally for 90 days to NPATp, LPATp, NPEATp and LPEATp groups. This dose was adjusted for animal weight to obtain the corresponding therapeutic doses for the rat model from the human therapeutic dose (Deeks and Perry, 2010). The formula for calculating the dosage and protocol for constituting the drug into the gelatine rectangles is described in Appendix V. Meanwhile, 6% ethanol in distilled water was provided *ad libitum* to NPE, LPE, NPEATp and LPEATp (Mbajiorgu *et al.*, 2008, Abdulkadir *et al.*, 2018). Distilled water, being the vehicle for E, was given to the control and ATp only treatment groups (NPC, NPATp; LPC, LPATp). Plain flavoured gelatine rectangles ($2cm^3$), the vehicle for ATp, was given to the control groups and those receiving E only (NPC, LPC, NPE, LPE). The rats were weighed once weekly and the weights recorded. After 90 days of treatment the rats were euthanised with an overdose of sodium pentobarbitone (Euthanaze®, South Africa); 200 mg/kg body weight intra peritoneal injection. Organ specimen (right epididymides) and blood samples were then collected for analysis.

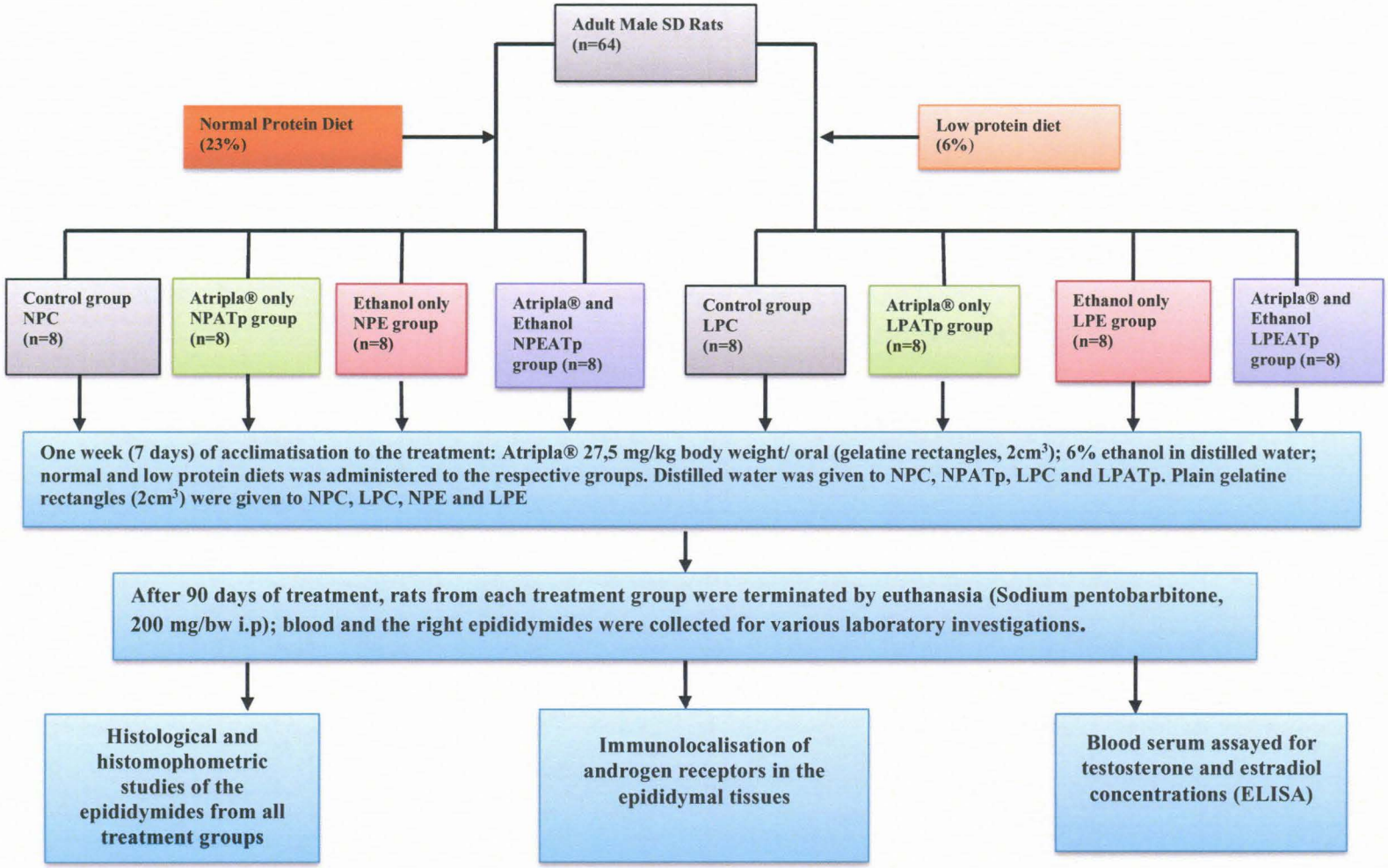


Figure 3.2: Experimental design flow diagram.

3.6 Measurement of feeding parameters: feed and fluid consumption

The amount of feed consumed by each rat in the treatment groups was recorded to evaluate the feeding patterns and the estimated total feed consumed for the treatment duration as descried by (De Souza Santos *et al.*, 2004, Mbajiorgu *et al.*, 2008, Abdulkadir *et al.*, 2018). Feed allocated to each rat was weighed by a digital analytic balance (Snowrex, Germany) at the beginning of each week and the estimated amount of feed consumed after a week was calculated by subtracting the end of week feed weight from the initial allocated feed weight. Likewise, the amount of fluid (6% ethanol or distilled water) consumed per rat was calculated by subtracting the end of week fluid value from the initial allocated fluid value. Ethanol and distilled water were both measured using a measuring cylinder (Duran, Germany) and it was administered using graduated drinking bottles.

3.7 Hormonal assay preparation of specimen and procedure

Blood samples were collected by cardiac puncture whilst the rats were anaesthetised before being euthanised (Neychev and Mitev, 2005, Abdulkadir *et al.*, 2018). The samples were collected into blood collecting tubes containing silica particles as clot activators (Vacutainer®, United Kingdom) to facilitate the collection of blood serum for hormone analysis. The clotted blood samples were then centrifuged at 4000*g* for 10 minutes using a bench centrifuge (Hettich Rotofix, Germany). The sera was then collected as the supernant in 1500 µl Eppendoff tubes (Whitehead Scientific, South Africa) and stored at -80°C before hormonal analyses. Assay of testosterone was performed using the enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocols (Appendix VI). The testosterone 96 well ELISA kit was manufactured by Demeditec (Germany).

3.8 Measurement of rat biometric parameters

3.8.1 Body weight changes

The rats in all treatment groups were weighed using a digital analytical balance (Snowrex, Germany) at the beginning of the experiment and subsequently weighed once a week up to the end of the treatment duration to evaluate the weight changes (Ramos *et al.*, 2010).

3.8.2 Organ harvesting, sampling and measurement of weights

After the rats were euthanised, rat testes were surgically removed through a lower abdominal incision and were trimmed of fat (Oremosu and Akang, 2015). The left and right epididymides were identified and dissected from the testes in all animals. The epididymides were then weighed using an analytic balance (Radwag, Poland). Various other vital organs such as the testes, liver and kidneys were also dissected out and weighed. The right epididymides from all treatment groups were then fixed in 10% buffered formalin solution for 48 hours before being processed for various tissue analysis procedures (Oremosu and Akang, 2015). The left epididymides were reserved for semen parameter tests and biochemical analysis (sperm cartinine, acetyl carnitine and glyceryphosphoryl choline). However, these procedures could not be performed due to limited time allocated to the study, lack of appropriate equipment and budgetary constraints.

3.8.3 Evaluation of relative organ weight for epididymis and other vital organs

The relative organ weights for the epididymides, testes, kidneys and liver were evaluated (De Souza Santos *et al.*, 2004, Azu *et al.*, 2014). This was done using the formula:

$$\text{Relative organ weight} = \frac{\text{organ weight}}{\text{Terminal body weight}} \times 100$$

3.9 Histological and histo-morphometric procedures:

3.9.1 Haematoxylin and eosin staining (HE)

After harvesting, weighing and fixing the right epididymides in 10% formalin solution for 48 hours, the samples were dehydrated in graded concentrations of alcohols, cleared in xylene and infiltrated with molten paraffin wax using an automated tissue processor (Shandon Citadel 1000, Germany). The epididymides were then embedded longitudinally in wax (tissue blocks), two pieces of tissue were embedded from each epididymis giving two tissue blocks. The first block encompassed the initial segment, caput and proximal corpus regions of the epididymis. The second block encompassed the distal corpus and the cauda regions of the epididymis. Tissue processing and embedding was done as described in Appendix VII.

Longitudinal sections were then cut using a rotary microtome (Leica, Germany) at 5μm thickness and mounted on gelatine coated glass slides (the process of coating slides in gelatine is described in Appendix VIII). The mounted sections were left to dry overnight at room temperature and then placed in an incubator at 36°C for 12 hours to promote tissue adherence on the slides. After drying the slides were stained with Mayer's haematoxylin and eosin (Appendix IX) and cover-slipped for histo-architectural analysis. Qualitative analysis was done in all regions of the epididymis which involved evaluating changes such as prevalence of cell types, lesions and signs of inflammation amongst other abnormalities (Lanning *et al.*, 2002).

3.9.2 Histo-morphometric investigations

Haematoxylin and eosin stained longitudinal sections of the right epididymis were also used for histo-morphometric analysis (Awobajo *et al.*, 2010, Olukole and Obayemi, 2010, Azu *et al.*, 2014). Qualitative analysis of the histo-achitecture of the epididymis and quantitative analysis of morphometric indices was performed in every first (longitudinal) serial section. In quantitative analysis, analytical evaluations of the histological measurements were performed using an Axioscope microscope (Carl Zeiss Microscopy, Gmbh, Jena, Germany) fitted with a camera (Axiocam HRc). Images were captured on a computer screen using X5, X10 and X40 objective lens and saved in jpeg format. The following measurements were taken from the initial segment

and proximal caput regions of the epididymis using Carl Zeiss (Axio-vision SE64 REL 4.9.1) image analyser software: epididymal tubular diameter (ETD), epididymal epithelial height (EH) and cross-sectional area of the epididymal tubules (A_C) (Figure 3.3). The initial segment and the proximal caput were the regions of interest in quantitative analysis in this study as most processes to do with the actualisation of sperm motility and sperm maturation occur mostly in these regions (Takeda *et al.*, 1990, Menad *et al.*, 2014). Moreover, the initial segment and proximal caput have epididymal tubular profiles that are round or almost round which can allow histo-morphometry evaluation.

Therefore, the epididymal tubular diameter with profiles that are round or nearly round were measured on each section on the minor (D_1) and major axes (D_2) and the mean diameter was obtained as an average of the two values (the tubules were only considered if D_1 divided by D_2 was ≥ 0.85 which approximated 1, a perfect circle) [Figure 3.3]. This was done to exclude longitudinal profiles which might exhibit different amounts of damage along their length and/or show irregular shrinkage as previously reported (Gundersen *et al.*, 1999, Olukole and Obayemi, 2010). Measurements for EH and A_C were also taken from the same epididymal tubule where ETD values were measured. The mean value for EH was obtained as an average of the two measurements obtained from each epididymal tubule (EH_1 and EH_2). EH_1 was measured from the lowest point in the epididymal epithelium and EH_2 was measured from the highest point in the epididymal epithelium. Cross-sectional area (A_C) of the epididymal tubule was measured by selecting the area option on the Carl Zeiss software and encircling the epididymal tubule using a wand/pointer. Histo-morphometric measurements were measured from 4 intraregional segments in the initial segments (segments 1 to 4) and 4 intraregional segments in the proximal caput (segments 5 to 8) [Figure 3.4]. 2 epididymal tubules were randomly chosen and analysed per each intraregional segment. Therefore, a total of 16 tubules were analysed per each epididymal section from all the 8 intraregional segments. 8 epididymides were analysed per treatment group, thus, a total of 128 tubules were analysed per group.

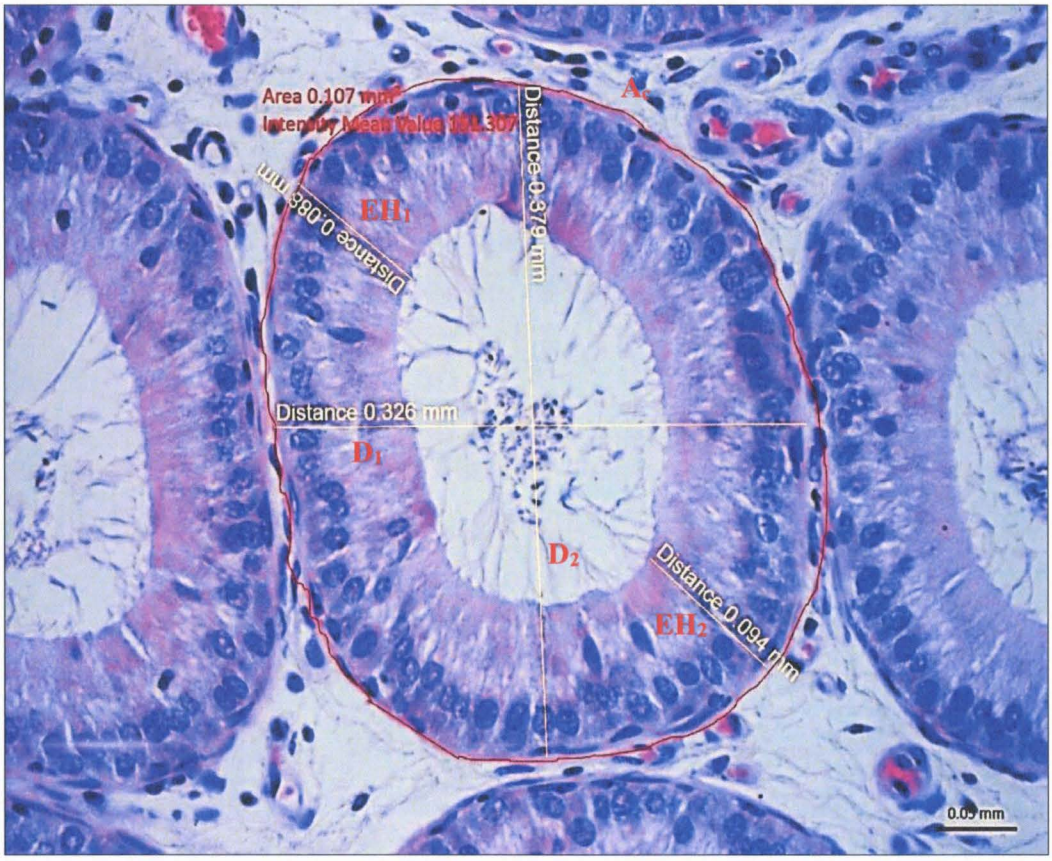


Figure 3.3: Photomicrograph showing histo-morphometric parameters that were measured on each epididymis in the initial segment and proximal caput regions (HE, longitudinal section of control tissue [NPC], X40 magnifications, scale bar=0.05mm]. D_1 and D_2 denote the minor and major axes of the diameter. EH_1 and EH_2 denote the two epididymal epithelial height measurements. The circumference line (drawn in red) was plotted by a wand/pointer on the Carl Zeiss Software and it represents the cross sectional area of the epididymal tubule (A_C) [Image was taken from epididymis of NPC group in the study].

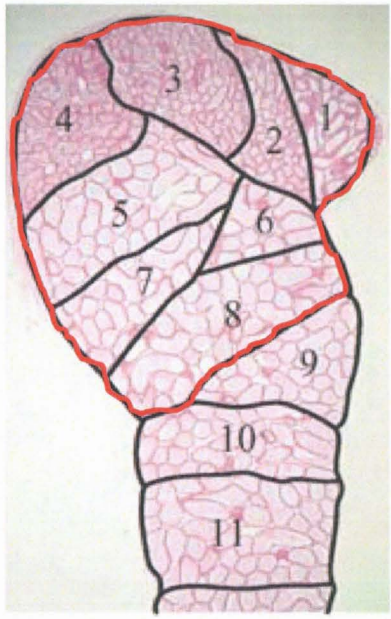


Figure 3.4: Intraregional segments of the proximal region of the epididymis [modified] (Jelinsky *et al.*, 2007). Epididymal tubules (for histo-morphometry/AR immune-staining) and fields (collagen fibre analysis) used for quantitative analysis in the study were randomly selected from intraregional segments of the initial segments (segments 1 to 4) and the proximal caput (segments 5 to 8) [region marked in red].

3.10 Van Gieson stain

Epididymal sections were stained using the Van Gieson staining technique as described in Appendix X to allow for qualitative and quantitative analysis of connective tissue profiles. Briefly, the sections (mounted on gelatine coated slides) were deparaffinised and rehydrated in distilled water. The slides were then stained with celestine blue, haematoxylin and finally with Curtis stain. The slides were then blotted and briefly dehydrated in graded alcohols before being cover slipped and viewed under Carl Zeiss Axioscope microscope fitted with a camera (Axiocam HRc).

3.10.1 Connective tissue analysis

Connective tissue profiles were qualitatively and quantitatively analysed in every second serial section. Qualitative analysis of connective tissue staining was performed in all regions of the epididymis. Quantitative analysis of the connective tissue profiles in sections stained with VG stain was performed using image J® software on images captured by the Carl Zeiss microscope fitted with a camera (Axiocam HRc). Images were captured on to the screen using the X5 and X10 objective lens then saved in jpeg format and analysed quantitatively. The regions of interest for quantitative analysis were the initial segment and proximal caput based on the rationale described in the histo-morphometric analysis section. Connective tissue quantification was determined from an image taken randomly from a field measuring 1790.97 μm X 1341.94 μm in intraregional segments of the initial segment and proximal caput. Four intraregional segments in the initial segment (segments 1 to 4) and 4 intraregional segments in the proximal caput (segments 5 to 8) were analysed [Figure 3.4]. Sixteen fields were analysed per each epididymal section (2 fields per each intraregional segment) from all the eight intraregional segments. The area of the field was determined by converting the field area units from pixels to micrometres (μm) using a scale bar of 0.775 pixels=1μm (Rasband, 2008). The connective tissue area fraction was then determined by superimposing a grid (area per point=2967.1 μm^2) onto the image at X5 magnification and using the cell counter function to count the number of points hitting the red stained collagen fibres in the image.

$$\text{Collagen fibre area fraction (\%) per field:} = \frac{\text{(Number of points hitting the grid X 2967.1 } \mu m^2\text{) X 100}}{\text{Area of a single field: 1790.97 } \mu m \text{ X 1341.94 } \mu m}$$

3.11 Immuno-histochemical staining of androgen receptors

Longitudinal sections (5 μm) of the epididymis (that encompass the initial segment, caput, proximal corpus segments) were mounted on saline coated slides and processed for AR immunostaining [Appendix XI] (Gür *et al.*, 2011). Briefly, antigen retrieved sections were

incubated with 5% non-immune goat serum for 10 minutes at room temperature to block nonspecific binding. The sections were then incubated at 4°C for 16 to 20 hours in a humidified chamber with (Rabbit polyclonal) Androgen receptor primary antibody (Ab74272, Abcam, United Kingdom) diluted at 1:200 in phosphate buffered saline (PBS). The sections were also treated with biotinylated goat anti-rabbit secondary antibody (Vector laboratories, United States of America) at a dilution of 1:1000. Finally, the sections were incubated with avidin-biotin complex (Vector Laboratories, United States of America) followed by diaminobenzidine tetrachloride (DAB) working solution. Counterstaining was performed with Mayer's haematoxylin. Tissue sections were subsequently dehydrated in a series of graded alcohols, cleared in xylene and finally cover-slipped with entellan mounting solution (Merck, Germany) [the rest of the chemicals used are detailed in Appendix XII). To confirm the specificity of the anti-androgen receptor antibody, testicular tissue from an untreated rat was used as the positive control. The negative control included omission of the primary antibody and substituting it with PBS in the immunostaining of epididymal tissues.

3.11.1 Androgen receptor immuno-reactivity analysis

Androgen receptor immunoreactivity (AR-ir) in the sections of the epididymis was qualitatively and quantitatively analysed. Qualitative and quantitative analyses for AR-ir were done in every third serial section. Images from the stained sections were captured using the Carl Zeiss Axioscope microscope fitted with camera (Axiocam HRc) using the X10 and X40 objective lens. Images were saved in jpeg format and analysed. Qualitative analysis involved the evaluation of the distribution and intensity of AR-ir in all the regions of the epididymis. Immuno-staining in all regions was evaluated two times and scored according to intensity. The area of interest for AR-ir quantitative analysis was the initial segment and proximal caput based on the rationale described in the histo-morphometric section. In addition, AR-ir was found to be mostly pronounced in the principal cell nuclei located in the initial segment and proximal caput in this study as it was reported in several studies (Sar *et al.*, 1990, Gür *et al.*, 2011, Menad *et al.*, 2014). Therefore, it was appropriate to evaluate AR-ir in these regions.

In quantitative analysis, AR-ir was also considered on the principal cell nuclei in epididymal tubules with profiles that are round or nearly round. The tubules were randomly selected from four intraregional segments in the initial segment (segments 1 to 4) and four intraregional segments in the proximal caput (segments 5 to 8) [Figure 3.4]. Two epididymal tubules were randomly chosen and analysed per each intraregional segment. Therefore, a total of sixteen tubules were analysed per each epididymis from all the eight intraregional segments. Eight epididymides were analysed per treatment group, thus, a total of 128 tubules were analysed per group. Quantitative analysis of AR-ir was performed on images of the tubules captured at X40 magnification. The images were analysed using the Image J® software using the cell counter option. All principal cell nuclei in a single epididymal tubule were first counted (using a multi-point wand/pointer) as Type 1 cells. Type 2 cells were the immuno-reactive nuclei. Therefore, AR-ir percentage value was calculated:

$$\text{Principal cell nuclear AR-ir percentage} = \frac{\text{Number of Type 2 nuclei}}{\text{Number of Type 1 nuclei}} \times 100$$

3.12 Data analysis

Statistical analysis was performed using IBM SPSS version 24, data was managed and graphs constructed using GraphPad Prism version 5.02 and Microsoft Excel (2013). Data was tested for normality using the Shapiro-Wilk test and normally distributed data was analysed using one-way ANOVA followed by Turkey's post hoc multiple tests for statistical comparisons among the groups. Non parametric data was analysed using Kruskal-Wallis test followed by Dunn's multiple comparison tests. All data was expressed as mean ± standard deviation (SD). $P<0.05$ was considered as statistically significant.

In qualitative and quantitative analysis of the study parameters, data for control group (NPC) was used as the baseline in all comparative analyses [NPC compared to NPATp, NPE, NPEATp, LPC, LPATp, LPE and LPEATp]. Comparisons were also made among NP treated as well as LP treated groups and for NP treated groups with LP treated cohorts (NPC compared to LPC; NPATp compared to LPATp; NPE compared to LPE and so on).

3.12 Summary

Chapter 3 is a review of the methodology for the study. The chapter outlines the study experimental design, materials, methods, treatment periods and analysis protocols that were used for the study. This includes the formulation and provision of the nutrition diets (low and normal protein) and treatment drugs (Atripla® and ethanol) to the various treatment groups. Analysis of blood samples, tissue samples and the treatment data was also highlighted.

CHAPTER 4: RESULTS

4.1 General

There were no incidences of mortality, signs of changed behaviour and severe deformities in the general appearance of the rats in all the treatment groups after the 90 days treatment period. However, all rats in LP treated groups had thin fur, pale tails and were generally lean compared to rats from groups that received normal protein diet. Feeding and intake of the respective fluids was regular with varying differences among the treatment groups.

4.2 Effect of treatment on feeding parameters

4.2.1 Feed consumption

The mean feed intake values among the treatment groups after the 90 day period are shown in Figure 4.1. In general, higher values for mean feed intake were recorded among the NP treated groups compared to the LP treated groups. The mean feed intake value for the NPATp group was slightly lower than the control group (NPC) but higher compared to NPE ($p<0.05$) and NPEATp ($p>0.05$) among the NP treated groups. There was a significant reduction in feed consumption in LPC, LPE and LPEATp groups compared to NPC group ($p<0.05$), while the reduction in feed consumption was not significant in the LPATp group relative to the control (NPC). There was no significant differences between the NP treated groups and their LP treated cohorts, but LPC was significantly reduced ($p<0.05$) relative to NPC.

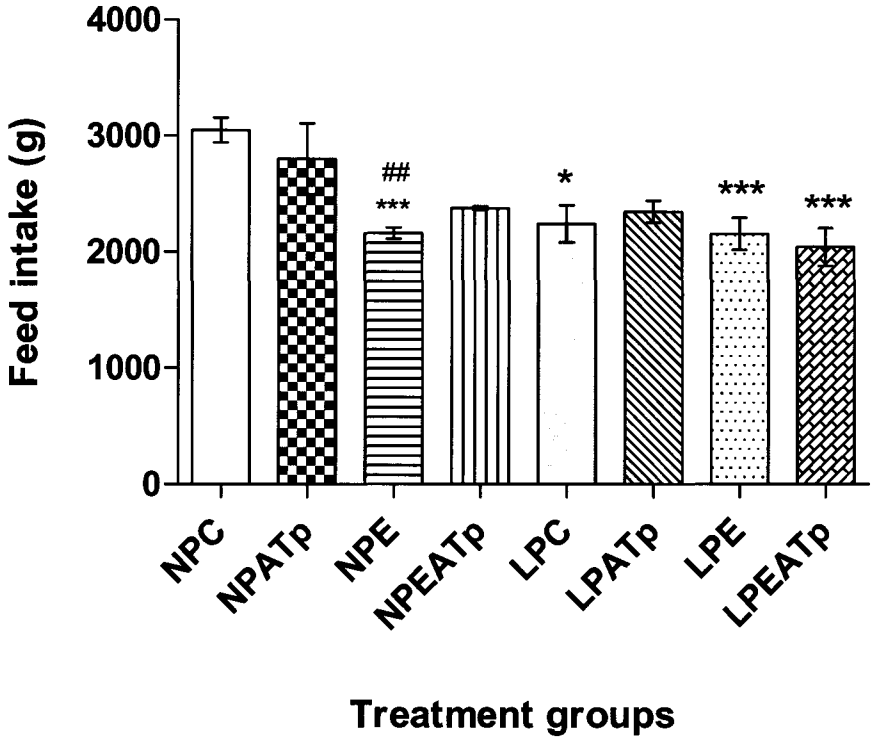


Figure 4.1: The mean feed intake values among the treatment groups after the 90 days treatment period. Data is represented as mean ± SD (n=8). *, ** and *** denotes a significant difference at $p<0.05$, $p<0.01$, $p<0.005$ respectively compared to the control (NPC). ## denote significant differences at $p<0.01$ compared to the NPATp group. Differences between diet cohorts were non-significant ($p>0.05$).

4.2.2 Fluid consumption

Figure 4.2 shows the mean fluid intake values recorded for the 90 day treatment period. In general, higher fluid intake values were recorded among NP treated groups compared to LP treated groups. There were no significant differences in mean fluid intake values among the NP as well as LP treated groups ($p>0.05$). The mean fluid intake value for NPATp was slightly lower than that of the NPC group but higher compared to NPE and NPEATp groups. There was a significant reduction in fluid intake in all the LP treated groups (LPC, LPATp, LPE, LPEATp)

compared to NPC group. There was a significant reduction in fluid intake values of LP treated groups relative to their respective NP treated cohorts ($p<0.05$).

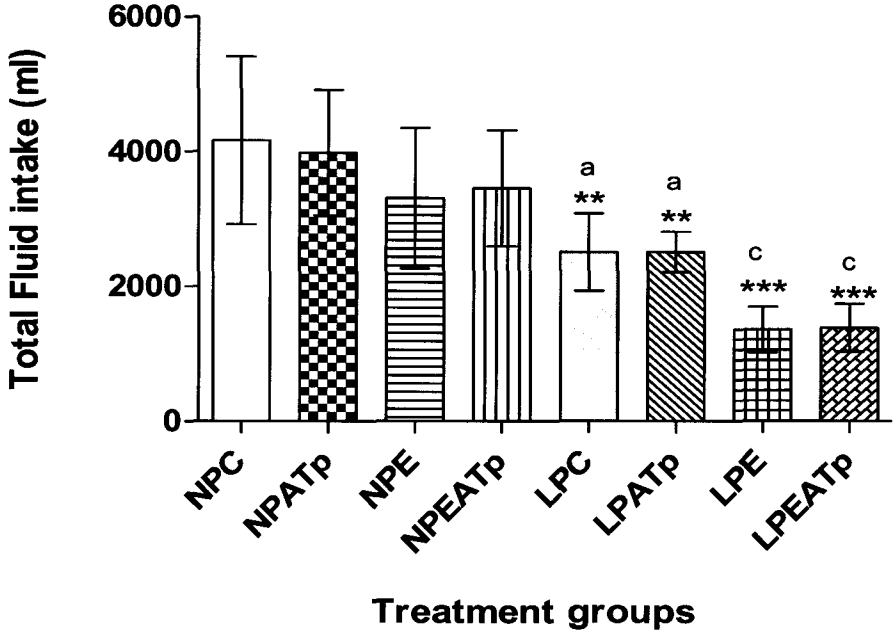


Figure 4.2: Fluid intake values after the 90 days treatment period. Data is represented as mean ± SD (n=8). *, ** and *** denotes a significant difference at $p<0.05$, $p<0.01$, $p<0.005$ respectively compared to the control (NPC). a and c denotes a significant difference between NP and LP treated cohorts at $p<0.05$ and $p<0,005$ respectively.

4.3 Effect of treatment on serum testosterone levels

Figure 4.3 is a standard curve graph that was calibrated by plotting the mean absorbance values obtained from the six standards of the testosterone ELISA kit against their respective concentration values using the 4 Parameter Logistics and Microsoft excel computer software. Samples were tested undiluted from all treatment groups and absorbance values fell within the range of the curve. Testosterone concentration in serum samples from all the treatment groups were then determined directly from the equation in the calibrated graph.

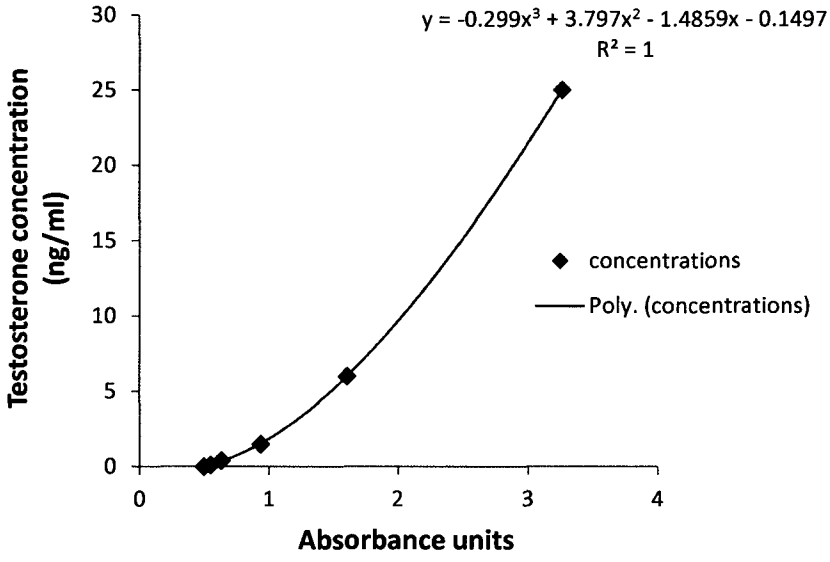


Figure 4.3: Calibration plot for testosterone absorbance values for the standards to obtain the concentration curve.

Figure 4.4 is a graphical presentation of mean serum testosterone concentration levels of the treatment groups. The mean serum testosterone concentration values for all treatment groups fell within the normal range for male rats [0.66 and 5.4 nano-grams per milliliter (ng/ml)] (Demeditec, Germany). However, there were no significant differences in the mean testosterone concentration values among all the treatment groups. Higher mean serum testosterone concentration values were generally recorded among the LP treated groups compared to the NP treated groups, except between NPATp and LPATp. Figure 4.4 also shows large SD error bars especially among the LP treated groups which indicate huge variability of values from the means.

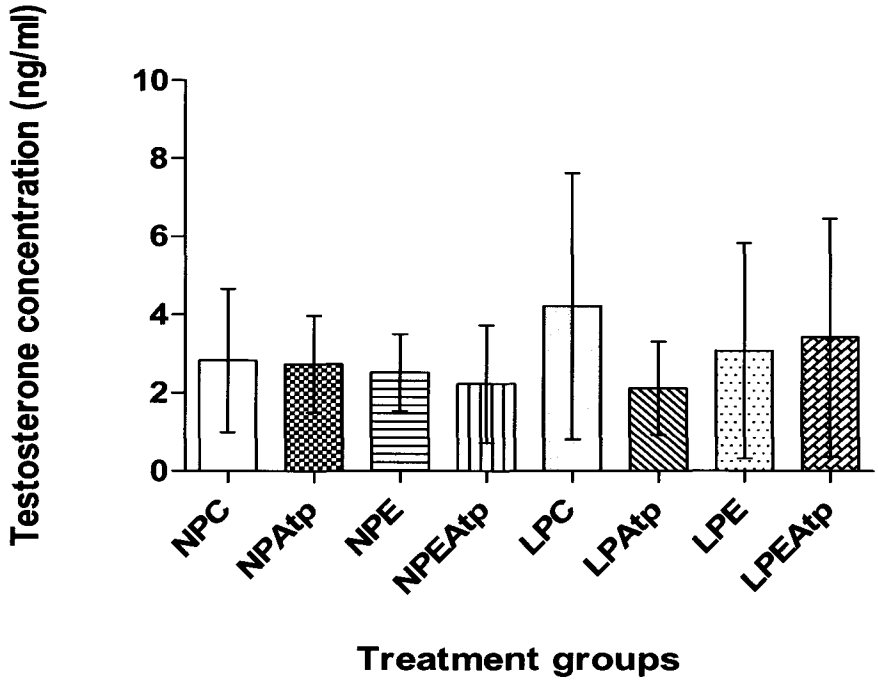


Figure 4.4: Serum testosterone concentrations levels from the treatment groups. Data is represented as mean ± SD (n=8).

4.4 Effects of treatment on rat biometric values

4.4.1 Body weight gain

Figure 4.5 shows mean body weight gain values for all the treatment groups at the end of the 90 days treatment period. In general, higher mean body weight gain values were recorded among the NP treated groups compared to the LP treated groups. There were no significant differences in mean body weight gain values among the NP as well as LP treated groups ($p>0.05$). The mean body weight gain value for the NPATp group was slightly lower than the NPC group but higher compared to both NPE and NPEATp groups values ($p>0.05$). There was a significant reduction in the weight gain values among all the LP treated groups (LPC, LPATp, LPE, LPEATp) compared to the control (NPC) [$p<0.005$]. LPATp group had the highest weight gain value while LPEATp had the lowest value among the LP treated groups. The large SD error bars for standard

deviation in the Figure 4.5 indicate a large spread of values around the means and treatment was characterised by weight loss among the LP treated groups. There was a significant reduction in weight gain values of NP treated groups relative to their respective LP treated cohorts ($p<0.05$).

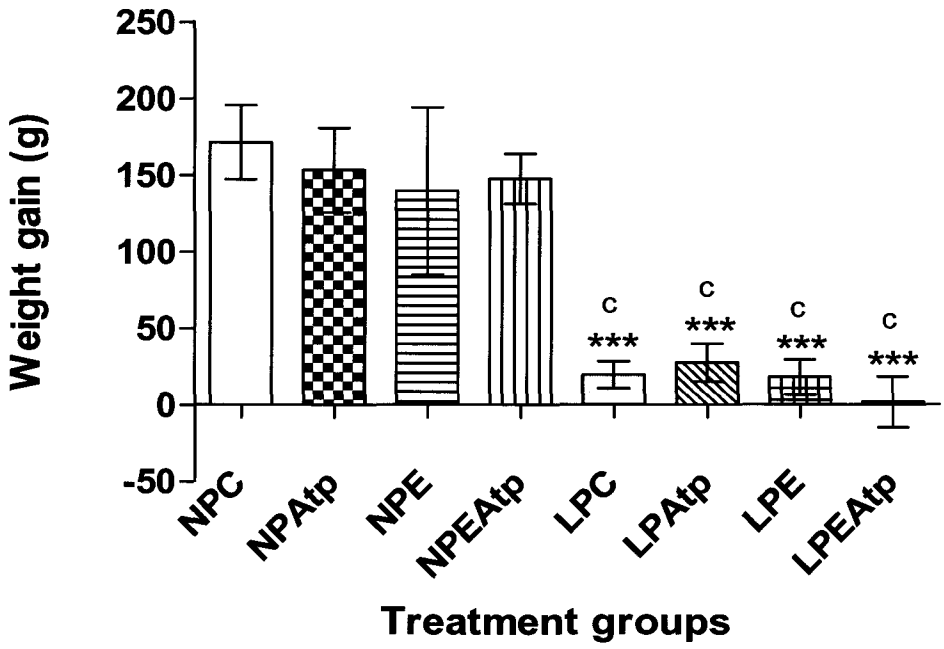


Figure 4.5: Weight gain values among the rats from the treatment groups. Data is represented as mean ± SD (n=8), *** denotes a significant difference at $p<0.005$ compared to the control (NPC). c denotes a significant difference between NP and LP treated cohorts at $p<0,005$ respectively.

4.4.2 Organ weights

The mean organ weights (epididymides, testes, liver and kidneys) for all the treatment groups after the 90 days treatment period are summarised in Table 4.1. There were no significant differences in mean weight of the epididymides among all the treatment groups ($p>0.05$). However, significantly lower mean weight values for the liver were recorded in the LPC and LPE groups compared to the control (NPC) [Table 4.1].

Table 4.1: Effects of treatment of organ weights

Organ	Experimental Group								
	NPC	**NPATp**	**NPE**	**NPEATp**	**LPC**	**LPATp**	**LPE**	**LPEATp**	**P value**
Right and left epididymides	1.67±0.22	1.64±0.34	1.59±0.31	1.62±0.20	1.77±0.18	2.06±0.38	2.04±0.30	1.72±0.19	0.275
Right and left testes	3.70±0.33	3.83±0.18	3.48±0.57	3.71±0.30	3.93±0.24	3.84±0.66	3.64±0.48	4.07±0.19	0.06
Right and left kidneys	2.71±0.88	2.79±1.00	2.79±1.14	2.78±1.06	2.47±0.24	2.34±0.38	2.12±1.15	2.97±0.55	0.650
Liver	20.42±2.68	19.97±1.66	19.64±3.09	18±7.48	15.59±1.30[c]	16.95±1.60	16.04±1.48[c]	17.13±2.13	0.00

The data is presented as mean ± SD. [a] ,[b], [c] indicates significant difference at $p<0.05$, $p<0.01$ and $p<0.005$ compared to the control (NPC).

4.4.3 Relative organ weights

Figure 4.6 shows the mean relative organ weight percentages. In general, higher relative organ weight values were recorded for the LP treated groups compared to NP treated groups. There were no significant differences in relative organ weight values among NP as well as LP treated groups ($p>0.05$). The relative organ weight percentage values for epididymides, liver, testes values were significantly higher in the LPEATp group compared to the control (NPC) ($p<0.005$) [Figure 4.6].

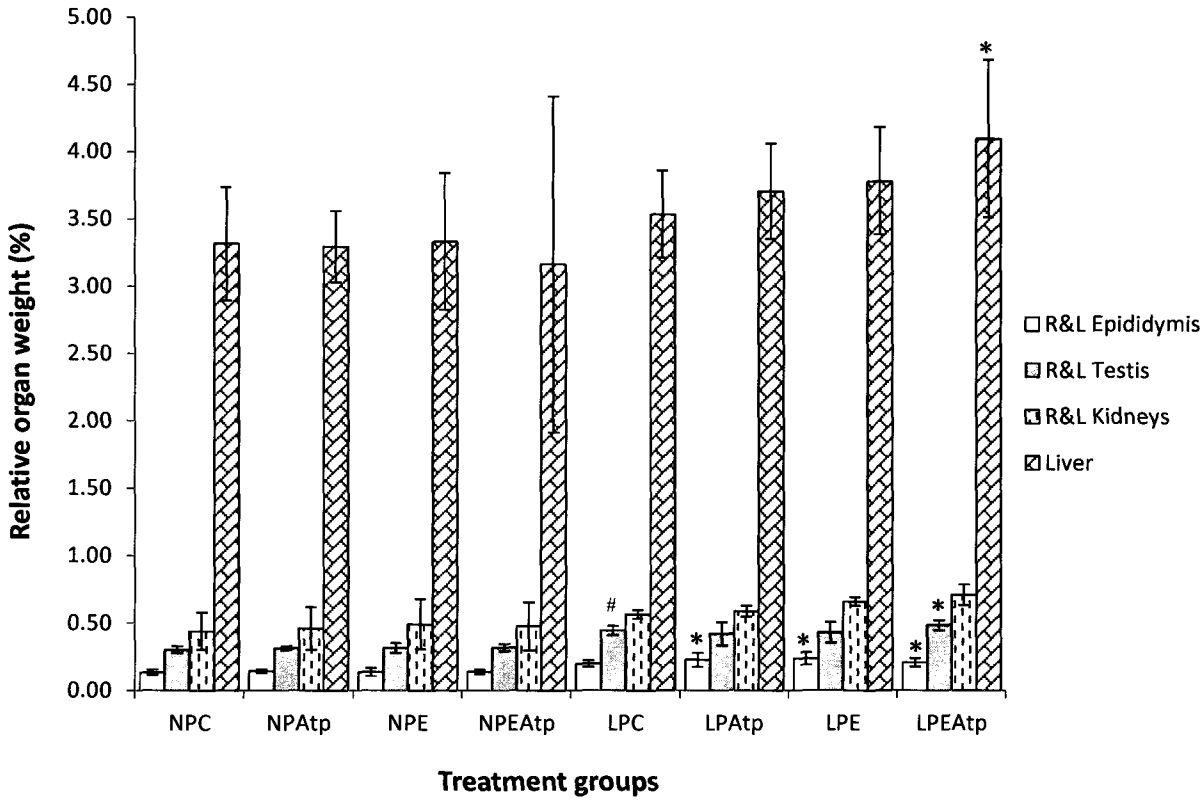


Figure 4.6: Relative organ weight values from the treatment groups. Data is represented as mean ± SD (n=8). # and * denotes a significant difference at $p<0.01$ and $p<0.005$ compared to the control (NPC) respectively.

4.5 Relationship between feeding parameters and weight gain

Table 4.2 shows the relationship of feed intake values among the treatment groups with their respective fluid intake and weight gain values. A Pearson bivariate correlation analysis showed a positive correlation between feed intake and weight gain values ($p<0.05$). There was a strong positive correlation between fluid intake and weight gain values ($p<0.05$) and there was also a positive correlation between feed intake and fluid intake values ($p<0.05$).

Table 4.2: Relationship of feed intake, fluid intake and weight gain among the treatment groups

		Feed intake	Fluid intake	Weight gain
Feed intake	Pearson Correlation	1	.627**	.633**
	Significance (2-tailed)		.000	.000
Fluid intake	Pearson Correlation	.627**	1	.825**
	Significance (2-tailed)	.000		.000
Weight gain	Pearson Correlation	.633**	.825**	1
	Significance (2-tailed)	.000	.000	

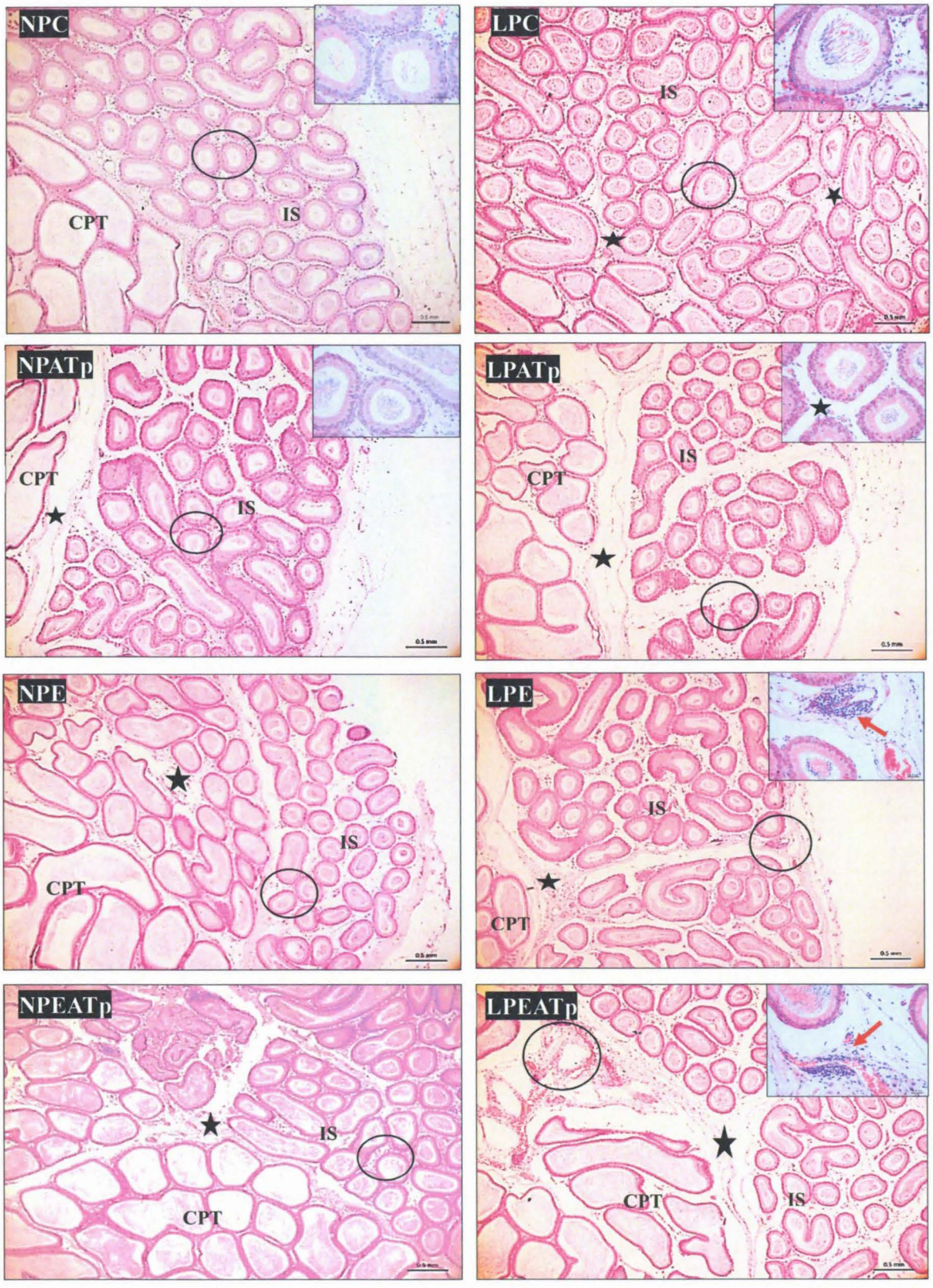
**. Correlation is significant at the 0.01 level (2-tailed).

4.6 Histological findings

The control group (NPC) epididymides had normal histology in all regions (initial segment, caput, corpus and cauda) [Figure 4.7 and 4.8]. The connective tissue septa dividing the regions and segments of the epididymis were well defined and the regions conspicuous. The epithelium of the epididymis was characterised by pseudostratified columnar epithelium. The differences in the population of principal, basal, apical cells and clear cells could be identified in all regions of the epididymis. The smooth muscle cells could also be identified surrounding the epididymal tubules especially in the initial segment. The thickness/height of the epithelium decreased from the initial segment to the cauda regions while the concentration of sperm cells within the lumen of the epididymal tubule increased from the regions of the initial segment to the cauda.

The treatment variables used in this study (Atripla®, ethanol and protein deficient diet) had wide ranging effects on the histology of epididymis as summarised in Table 4.3 and 4.4. Severe and high incidences of histopathological alterations were observed in tissues from the LP treated groups compared to the NP treated groups. Interstitial spaces between the epididymal tubules were more pronounced in tissues of the epididymides from the LP treated groups compared to NP treated groups (Figure 4.7). The tubules of the initial segments of NPC group were also more compactly arranged compared to NPATp, NPE and NPEATp. High concentration of the luminal debri clumped together (red arrow) with round spermatids and decreased epithelial height were observed in the initial segment region of the tissues of the LPE and LPEATp group compared to NPC group and to the rest of the treatment groups (Figure 4.8). Figure 4.9 shows increased exfoliated material (luminal debri) in the proximal corpus of tissues from LPEATp group compared to the control group (NPC). Luminal debri in the proximal corpus was associated with low luminal sperm concentration/empty epididymal tubules. In addition, there was a general increase in the number of clear cells from the distal caput to the cauda regions of sections from LP treated groups (especially in the LPEATp) compared to the control (NPC). Clear cell hyperplasia was observed in the distal caput, corpus and the cauda regions of several epididymal sections from the LPEATp group (Figure 4.10 and 4.11). Tissue inflammatory infiltrates were also a common observation between the epididymal tubules in the initial segment and caput regions in tissues of several sections from the LPEATp group (Figure 4.7 and 4.10). Cribriform change was observed in one of sections from the LPEATp group (Figure 4.10).

Figure 4.7: Photomicrographs of the epididymis showing the effect of ATp and E under both NP and LP dietary groups [X5 magnification scale bar=0.5 mm; insert images (X40) scale bar=0.05mm] {longitudinal sections}. The black circles in all images denote the section viewed under higher magnification insert. **NPC:** control group tissue, initial segment (IS) has round or almost round tubules having thicker epithelium linings. Proximal caput region (CPT) is characterised by irregular shaped epididymal tubules that have thinner epithelial linings and high concentrations of luminal sperm cells. Notice the compact arrangement of the epididymal tubules in the IS of NPC group compared to increased interstitial spaces (black star) in tissues from all treatment groups (**NPATp, NPE, NPEATp, LPC, LPATp, LPE** and **LPEATp**). Interstitial space was more pronounced among LP treated groups compared to NP treated cohorts. LPEATp group had the greatest increase in interstitial space in epididymal tissues. Inflammatory infiltrates (red arrow) were observed epididymal tissues in LPE and LPEATp groups.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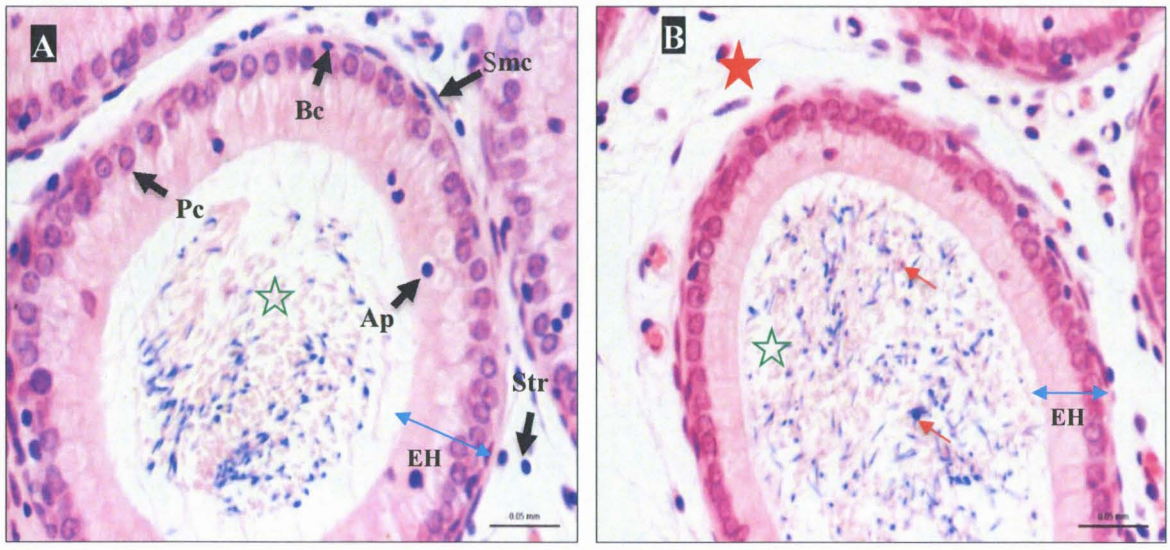


Figure 4.8: Histological observations in the tissues of the epididymis (HE, X63, magnifications, scale bar=0.05mm). **A:** Initial segment from the control group (NPC). Black arrows in the image show the nuclei of various types of cells in the epithelium of the epididymis, principal cell (Pc), basal cell (Bc), apical cell (Ap), stroma cell (Str) and smooth muscle cell (Smc). Note the concentration of luminal contents with more elongated spermatids in image A (green star) .**B**: Histology of the initial segment from LPEATp group, note the high concentration of the luminal debri (red arrow) with round spermatids clumped together and increased interstitial space (red star). Note also the decreased epithelial height (EH) with shortened stereocilia in the tubule from image B compared to A (blue double arrows).

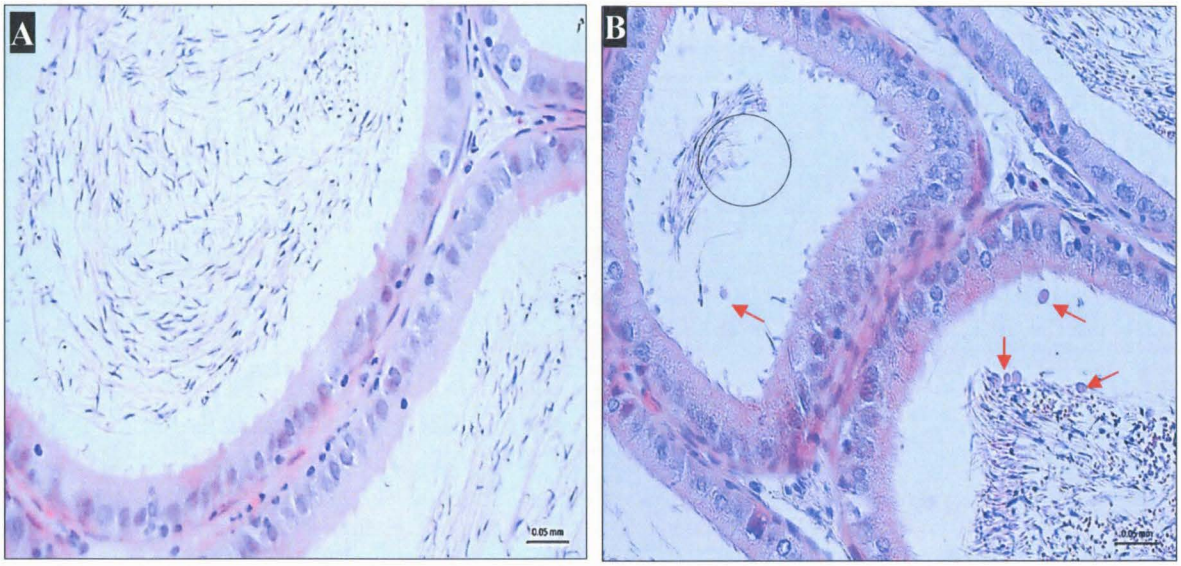


Figure 4.9: Photomicrographs showing the effect of treatment on the integrity of the epithelium in the proximal corpus (X40 magnification scale bar=0.05mm [50µm]). **A:** epididymal tissue from the control group (NPC) with a high concentration of sperm cells and no exfoliated materials. **B**: abundance of exfoliated material (red arrows, encircled) and low concentration of luminal sperm cells in the epididymal tubules of tissues from the LPEATp group.

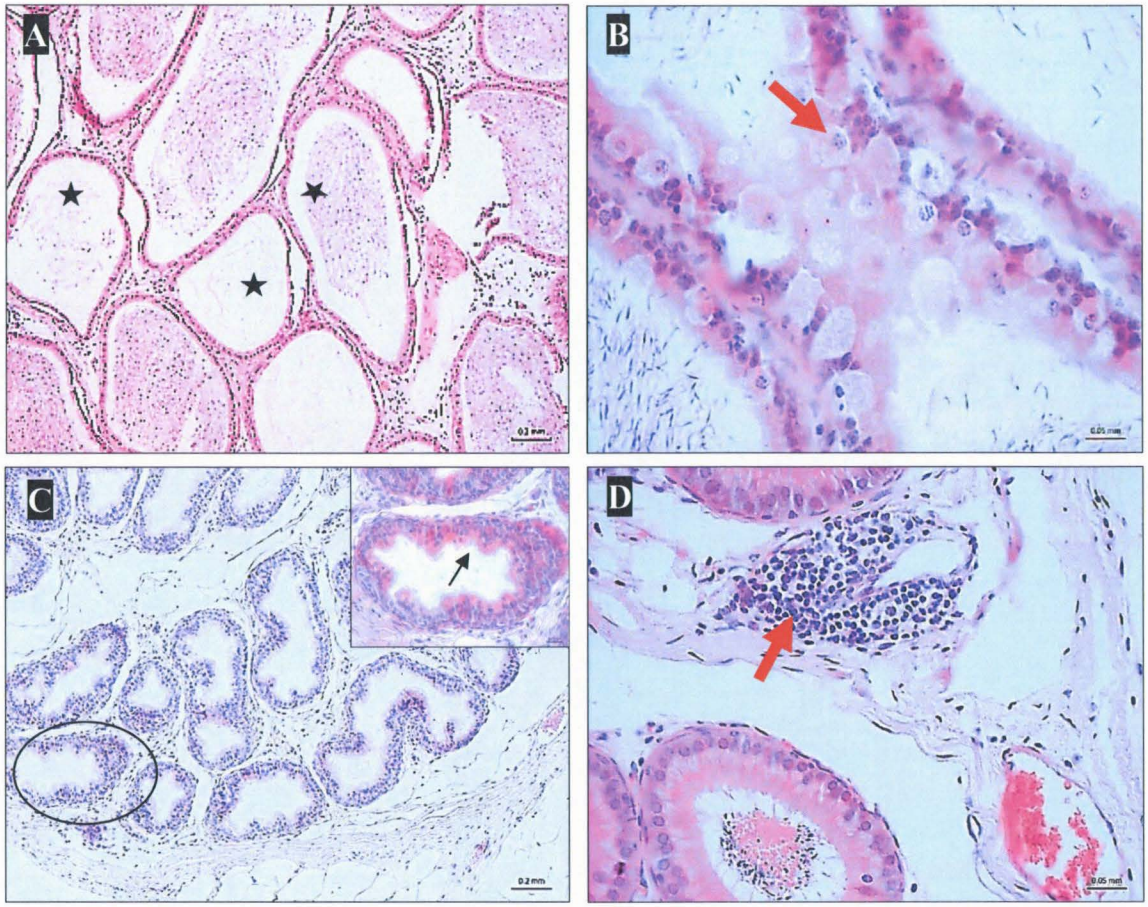


Figure 4.10: Photomicrographs showing the histo-pathological alterations in the LPEATp group (X10, X40, X 63 magnifications [insert], scale bar=0.2 mm, 0.05 mm and 0.05 mm respectively) **A:** shows cauda epididymis with empty epididymal tubules (black star) and decreased concentration of luminal sperm cells **B:** shows clear cell hyperplasia in the proximal corpus of the epididymis. **C:** shows cribriform change (shown by black arrow, insert X63), the black circle denotes the section viewed insert under higher magnification. **D:** shows inflammatory infiltrates (red arrow) observed in the initial segment of the epididymis.

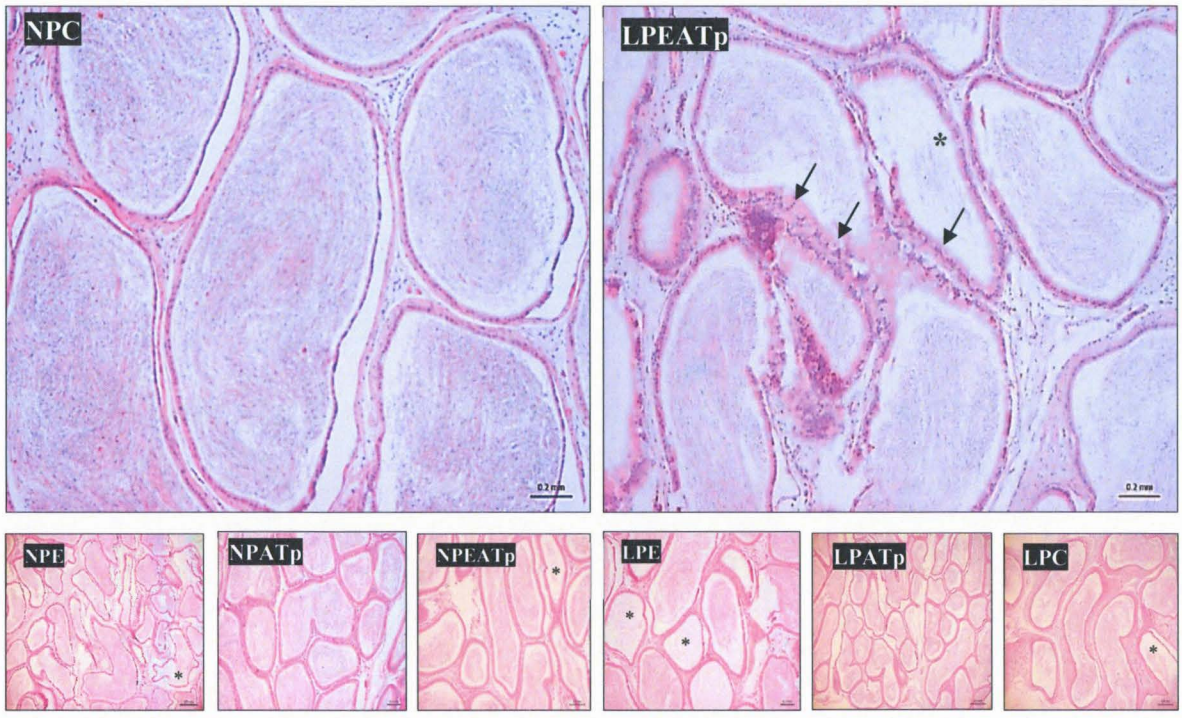


Figure 4.11: Photomicrographs showing the effects of treatment on the cauda epididymis (HE, X10 magnification scale bar=0.2 mm). Note the high concentration of luminal sperm cells in the epididymal tubules from the **NPC** group compared to the sparsely populated tubules (asterisk) in the **LPEATp** group coupled with clear cell hyperplasia in the epithelium (black arrows). Fewer luminal sperm cells and empty epididymal tubules were observed in the tissues of **NPE**, **NPEATp**, **LPATp**, **LPE** and **LPC** group (asterisks).

4.7 Histo-morphometric/stereological parameters analysis

Histo-morphometric parameters which include ETD, A_C and EH were measured in the initial segment and proximal caput of the epididymis on haematoxylin and eosin stained sections in all the treatment groups (Figure 4.12). In general, higher values for mean ETD and A_C values were recorded among the NP treated groups compared to LP treated groups. The mean ETD, A_C and EH values recorded for the NPATp group were non-significantly lower than the values of the control group (NPC) but higher compared to NPE and NPEATp values. Among the NP treated groups, NPE recorded the lowest values for ETD, A_C and EH and the reduction in ETD and EH values was significant compared to the control (NPC) [$p<0.05$]. There were no significant differences in the mean ETD, A_C and EH values among the LP treated groups. However, significant reduction in the mean ETD values was recorded in LPE and LPEATp compared to the control (NPC) [$p<0.05$].

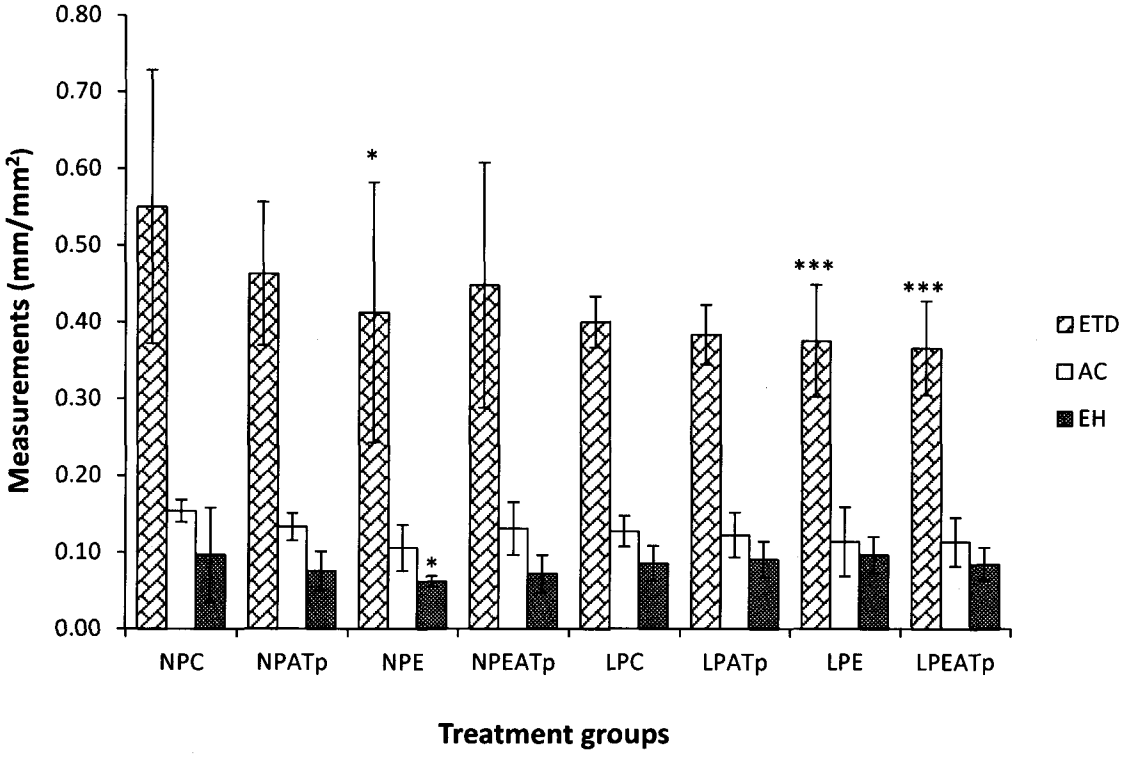
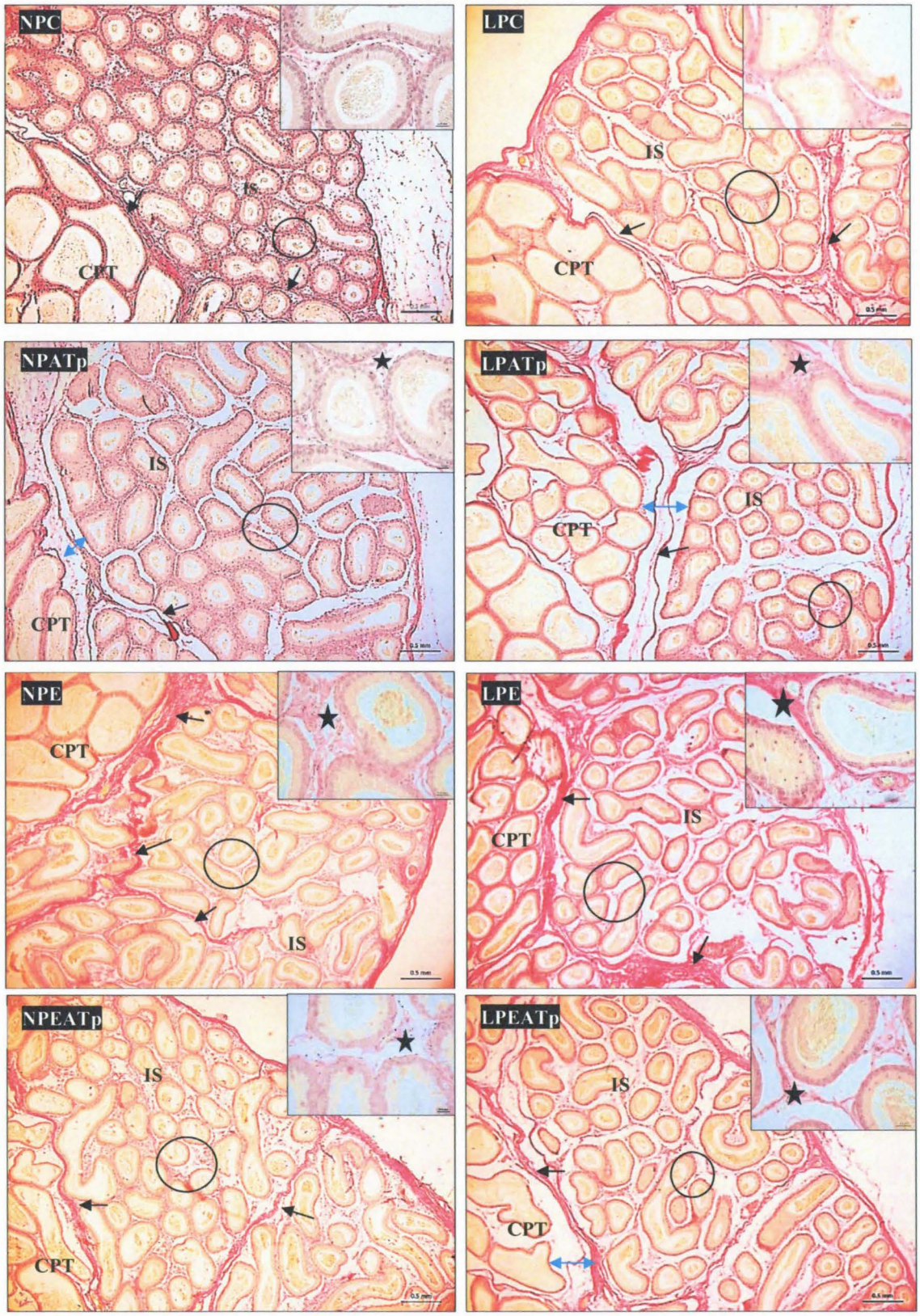


Figure 4.12: Histo-morphometric values from the treatment groups. Data is represented as mean ± SD (n=8). * and *** denotes a significant difference at $p<0.05$ and ($p<0.005$) respectively compared to the control group (NPC).

4.8 Connective tissue histology

Qualitative analysis of VG stained sections showed more connective tissue septa separating the intraregional segments of the initial segment and caput regions of the epididymides from the LP treated groups compared to the NP treated groups (Figure 4.13). Figure 4.14 shows the differences in the quantitative analysis of the mean collagen tissue area fraction. There were no significant differences in the collagen area fraction values among all the treatment groups. However, higher values for collagen tissue area fraction were generally recorded after treatment with either ATp and/or E in both NP and LP treated groups.

Figure 4.13: Photomicrographs showing the effects of ATp, E and PM in the initial segment (IS) and proximal caput regions (CPT) of the epididymis (VG, X5 and X40 insert magnification scale bar=0.5 mm and 0.05 mm respectively) [longitudinal sections].The black circles denote the section viewed insert at X40 magnification. Notice the general increase in staining of collagen fibres (red stained) in all treatment groups compared to the **NPC**. Staining of collagen fibres was intense and more expressed among **NPE**, **NPEATp**, and **LPE** and **LPEATp** groups. In addition, staining of collagen fibres and interstitial space (blue double arrows and black stars) was greater among LP treated groups compared to their NP treated cohorts,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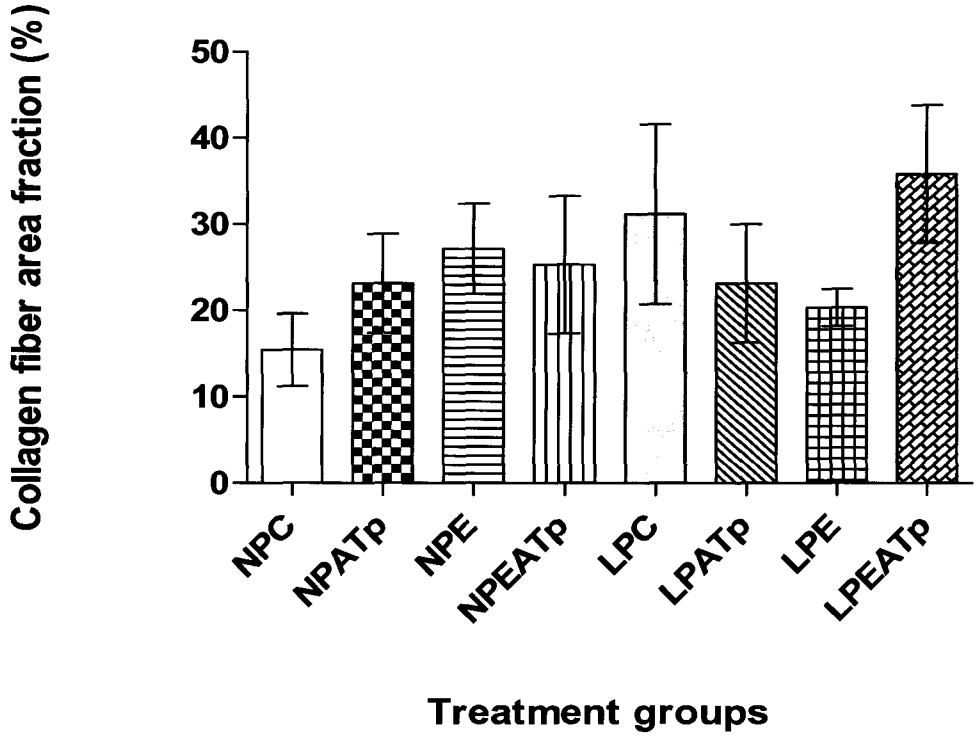


Figure 4.14: Collagen fiber area fraction values in the initial segment and proximal caput of tissues from the treatment groups. Data is represented as mean ± SD (n=8). No significant differences were recorded among the treatment groups.

Table 4.3: Summary of the histo-achitectural derangements caused by ATp, E and PM on epididymal tissues

Treatment Groups	Initial Segment (IS) and Caput epididymis	Proximal Corpus of epididymis	Epididymis Cauda
NPC	Normal histology, normal sperm cells in the lumen of the epididymal tubule, normal cell types		
NPATp	Increased spaces between epididymal tubules	Low sperm concentration Luminal debri	Low sperm concentration
NPE	Increased luminal debri in the IS Increased spaces between epididymal tubules Increased connective tissue staining	Increase clear cell concentration Inflammatory infiltrates Luminal debri Low sperm concentration	Low sperm concentration
NPEATp	Increased luminal debri in the IS Increased spaces between epididymal tubules Increased connective tissue staining	Low sperm concentration Luminal debri	Low sperm concentration
LPC	Increased spaces between epididymal tubules Increased connective tissue staining	General increase in clear cell concentration Luminal debri	Low sperm concentration
LPATp	Increased spaces between epididymal tubules Increased connective tissue septa staining	General increase in clear cell concentration	Low sperm concentration
LPE	Increased luminal debri in the IS Increased spaces between epididymal tubules Connective tissue staining epididymal tissue inflammatory infiltrates	Clear cell hyperplasia	Low sperm concentration Increase in luminal exfoliated material/debri Clear cell hyperplasia
LPEATp	Increased luminal debri in the IS Increased spaces between epididymal tubules Increased connective tissue staining epididymal tissue inflammatory infiltrates Cribriform change	Clear cell hyperplasia Increased in luminal debri Inflammatory infiltrates Cribriform change	Low sperm concentration Clear cell hyperplasia Increased in luminal debri Cribriform change

Table 4.4: Effect of treatment on the incidence of histopathological alterations in treatment groups

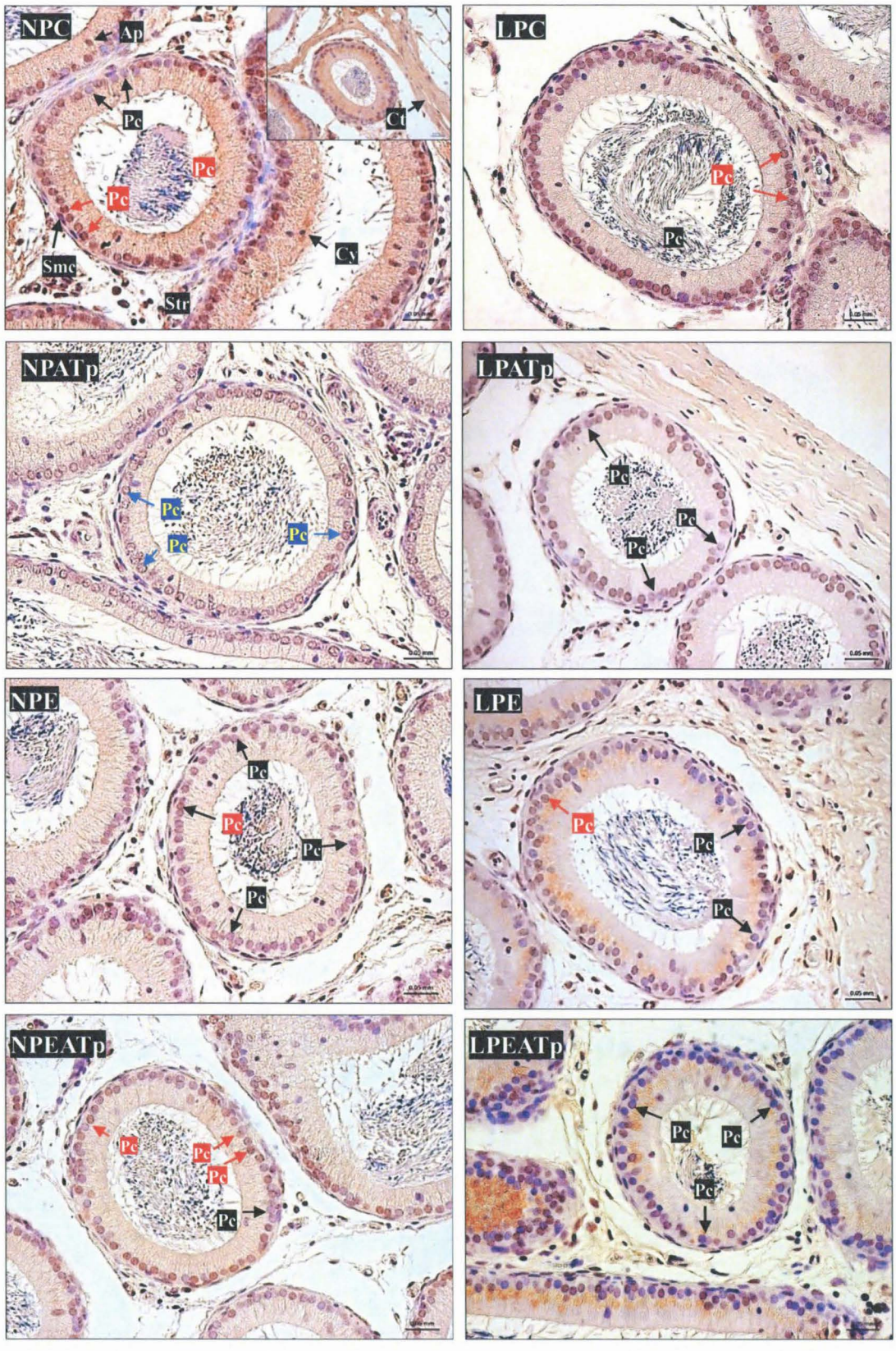
Parameter	Experimental Group							
	NPC	**NPATp**	**NPE**	**NPEATp**	**LPC**	**LPATp**	**LPE**	**LPEATp**
luminal debri (IS) [%]	0	0	25 (2/8)	25 (2/8)	0	0	37.5 (3/8)	50 (4/8)
Luminal debri [in proximal corpus, cauda] (%)	0	25 (2/8)	37.5 (3/8)	37.5 (3/8)	25 (2/8)	25(2/8)	37.5 (3/8)	50 (4/8)
Inflammatory infiltrates (%)	0	0	25 (2/8)	0	0	0	25 (2/8)	37.5 (3/8)
Clear cell hyperplasia (%)	0	0	0	0	0	0	37.5 (3/8)	37.5 (3/8)
Cribriform change (%)	0	0	0	0	0	0	0	12.5 (1/8)
Low sperm concentration (due to accelerated transit time/ low sperm concentration) (%)	0	25 (2/8)	62.5 (5/8)	37.5 (3/8)	37.5 (3/8)	25 (2/8)	50 (4/8)	75 (6/8)
Increased interstitial spaces between epididymal tubules (%)	0	25 (2/8)	50 (4/8)	37.5 (3/8)	50 (4/8)	50 (4/8)	50 (4/8)	75 (6/8)

IS represents the initial segment region; CPT represents the proximal caput region of the epididymis. Data is represented as a percentage of incidents per group (n=8).

4.9 Immuno-histochemical analysis

Androgen receptor immunoreactivity (AR-ir) was observed in all regions of the epididymides from all the treatment groups (Figure 4.15). Androgen receptors were expressed in the nuclei of all the cells of the epithelium (apical, principal and basal cells), tubular smooth muscle cells and stromal cells as well as in the cytoplasm of the epithelial cells, connective tissue and luminal sperm cells in varying degrees of intensity among the treatment groups. Strong AR-ir staining was generally observed in the proximal regions (initial segment and caput) compared to the corpus and cauda regions of the epididymal sections in all treatment groups. Therefore, AR-ir in the nuclei of principal cells from the initial segment and proximal caput region was quantitatively analysed for comparison among all treatment groups. Negative controls did not show any positive AR-immunostaining (blue nuclei) while the positive controls (untreated rat testicular tissue) were immuno-reactive (brown staining) [Figure 4.16].

Qualitative analysis scoring (semi-quantitative analysis) of AR-ir in the tissues of the epididymis is summarised in Table 4.5. The most intense AR-ir staining was observed in the nuclei of the principal cells, cytoplasm, stromal cells and connective tissue of the epididymides from the control (NPC) group compared to all the other treatment groups. Variable staining intensities were observed in the nuclei of epithelial cells and luminal sperm cells in all treatment groups but generally more expression was observed in the NPC group. Figure 4.17 show the mean principal cell nuclear AR-ir percentage values of all the treatment groups. Treatment resulted in significantly lower mean principal cell nuclear AR-ir percentage values in all treatment groups under both normal and low protein diet compared to NPC ($p<0.005$). There were no significant differences in the mean principal cell nuclear AR-ir percentage values among the NP as well as LP treated groups ($p>0.05$).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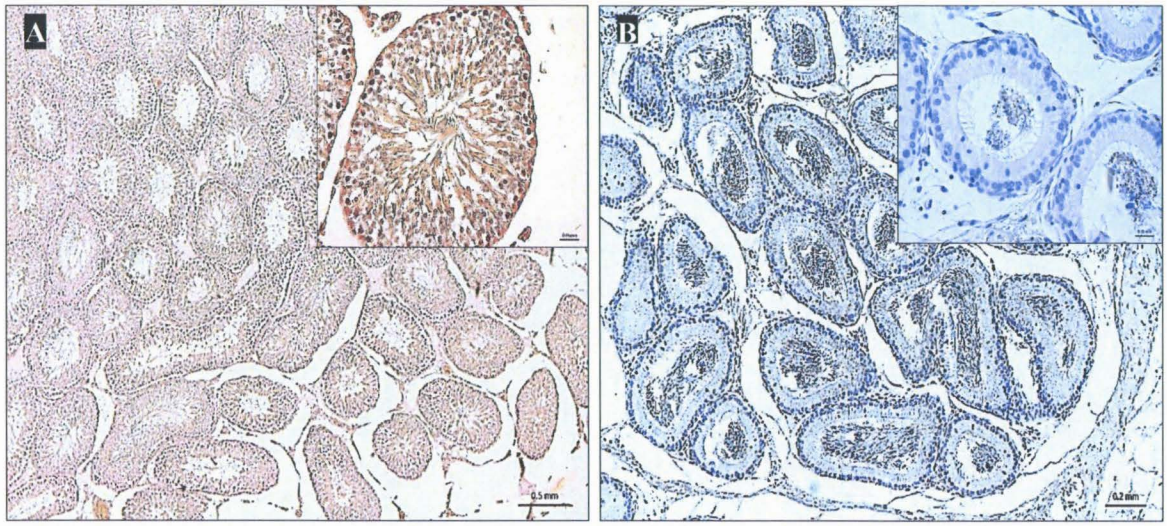

Figure 4.16: AR-ir in control photomicrographs (X5, X10 and X40 [insert] magnification, scale bar=0.5 mm, 0.2 mm and 0.05 mm respectively) [longitudinal sections]. **A**: AR-ir in a section of the testis from an untreated rat, it was used as positive control. Notice strong staining in all epithelial cells including the luminal sperm cells. **B**: no staining in a section of rat epididymis from an untreated rat, it was used as a negative control in the immunostaining of androgen receptors.

Table 4.5: Effect of treatment on the immuno-localisation of AR in the rat epididymis (scoring of qualitative analysis/semi-quantitative analysis)

AR-ir localization		**NPC**	**NPATp**	**NPE**	**NPEATp**	**LPC**	**LPATp**	**LPE**	**LPEATp**
Principal cells	N	+++	+G	++	+++	+++	+++	++	+
	C	+++	+	+	+	+	+	+	+
Basal cells	N	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Apical cells	N	+/-	-	-	-	+/-	-	-	-
Smooth muscle cells	N	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Lumen	Spz	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Stromal cells		+++	++	+	+++	++	++	+	-
Connective tissue		+++	+	+	+	+	+	+	+

The degree of AR immunostaining was designated by +++=strongly positive, ++= moderate, += weakly positive, -= negative, +/-= variable; N-nucleus; C=cytoplasm; Spz= sperm cells; G=granular staining

Table 4.6: Semi-quantitative analysis of AR-ir in the epididymis of rats from the control and LPEATp groups in this study compared to that of other species in literature.

AR-ir localisation		**This study**		**Rat**			**Human**		**Mouse**	**Goat**	**Fat Sand Rat**
		Control (NPC) **[2018]**	LPEATp **(2018)**	Takeda *et al*, 1990	Yamashati, 2004	Gur *et al* 2011	Ruizeveld de Winter *et al*., 1991	Ungefroren *et al*., 1997	Zhou *et al*., 2002	Goyal *et al*., 1997a	Menad *et al*, 2014
Principal cells	N	+++	+	++/+	++	++/-	++	++	++	++	+++
	C	+++	+	NE	NE	NE	NE	NE	NE	NE	+++G
Basal cells	N	+/-	+/-	++/+	++	+	-	++	++	+	++/-
Apical cells	N	+/-	-	++/+	++	+	NE	NE	++	+	++/-
Smooth muscle cells	N	+/-	+/-	+/-	+	+/-	++/-	++/-	+/-	+	++
Lumen	Spz	+/-	+/-	NE	NE	NE	NE	NE	NE	NE	+
Stromal cells		+++	+	NE	NE	NE	NE	NE	NE	NE	NE
Connective tissue		+++	+	NE	NE	NE	NE	NE	NE	NE	NE

The degree of AR immunostaining was designated by as +++=strongly positive, ++= moderate, += weakly positive, -= negative, +/-=variable; N-nucleus; C=cytoplasm; Spz= sperm cells; G=granular staining NE=not examine

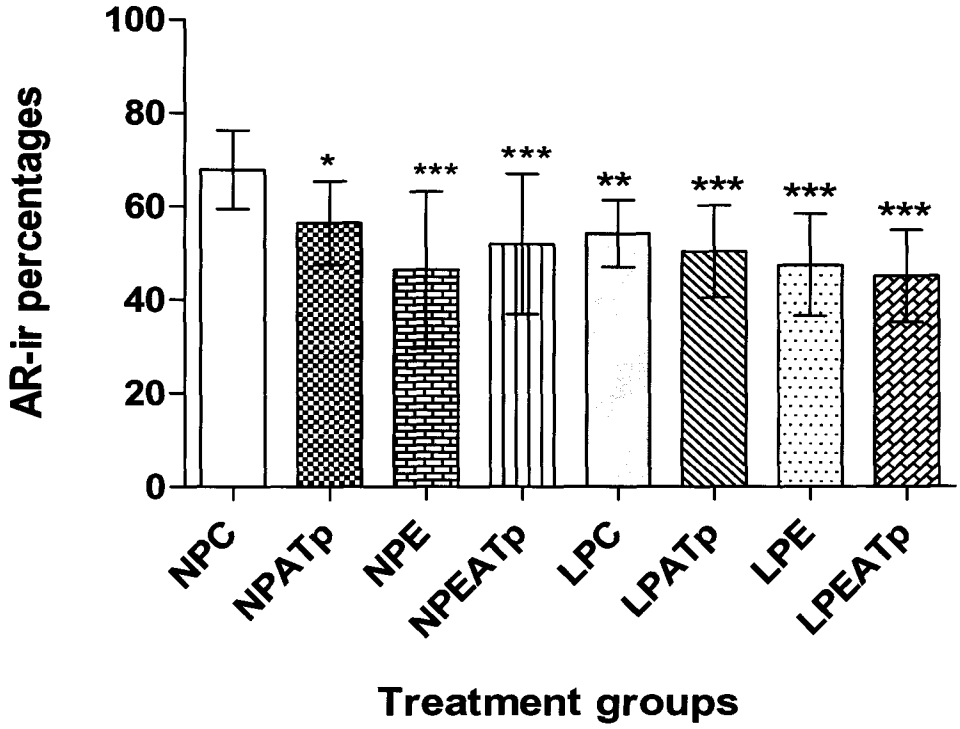

Figure 4.17: Principal cell nuclear AR-ir percentage values for the treatment groups. Data is represented as mean ± SD (n=8), *, ** and *** denotes a significant difference at $p < 0.05$, $p < 0.01$ and $p < 0.005$ respectively compared to the control (NPC).

4.10 Relationship of serum testosterone levels with principal cell nuclear AR-ir percentage index and epididymal tubular diameter (ETD) values

Table 4.6 shows the relationship of the serum testosterone levels in all treatment groups with their respective principal cell nuclear AR-ir percentage and ETD values. A Pearson bivariate correlation analysis showed no relationship between the serum testosterone levels and principal cell nuclear AR-ir index ($p>0.05$). There was a negative correlation between serum testosterone levels and ETD values but it was not significantly correlated ($p>0.05$). There is a strong positive correlation between ETD values and principal cells AR-ir percentage index ($p<0.05$).

Table 4.7: Pearson correlation analysis of serum testosterone levels with principal cell nuclear AR-ir percentage index and ETD values for the treatment groups

		Testosterone	ETD	AR-ir
Testosterone	Pearson Correlation	1	-.207	.004
	Significance (2-tailed)		.623	.993
ETD	Pearson Correlation	-.207	1	.913**
	Significance (2-tailed)	.623		.002
AR-ir	Pearson Correlation	.004	.913**	1
	Significance (2-tailed)	.993	.002	

**. Correlation is significant at the 0.01 level (2-tailed).

4.10. Summary

Chapter 4 is a comprehensive review of the results and outcomes of the study. The chapter outlines the effect of treatment on rat feeding parameters, biometric parameters, histo-architecture, histo-morphometric indices and AR-ir in the tissues of the epididymis. Treatment analysis and outcomes are presented in tables and graphs.

CHAPTER 5: DISCUSSION

5.1 General discussion

The broad aim of the present study was to probe the long-term effects of Atripla® use on male reproductive health (using the epididymis) in light of chronic alcohol use and protein malnutrition. This study thus gives a unique contextual perspective of antiretroviral adjuvant therapy and potential outcomes in chronic alcohol use and protein malnutrition (major socio-economic factors) bedeviling most of the resource limited countries of SSA region.

5.1.1 The effects of treatment on physical appearance and general well being

Protein malnutrition has been reported to cause muscle wasting, hair loss, broken nails and intense agitation in adults rats (Leite *et al.*, 2011). These findings are similar to some of the physical features observed in rats from groups treated with low protein diet in this study (though the effects were mild). The similarity in some of the major physical features observed in the two studies confirms the presence of protein malnutrition and adverse effects thereof in the present study. The findings also demonstrate the major role played by protein in the general outlook and wellbeing of adult rats. In humans, protein deficiency in diet is also associated with deterioration in physical appearances especially among children in conditions such as Kwashiorkor and Marasmus (Müller and Krawinkel, 2005, Kandala *et al.*, 2011). Additionally, protein malnutrition was the prominent factor affecting physical appearance given that there were no noticeable differences in physical appearance with ethanol and ATp treatment.

5.2 Effects of treatment on feeding parameters

Antiretroviral drugs (including Atripla®) have oftentimes been associated with nausea and vomiting (Johnson *et al.*, 2005, Squibb, 2010) which might affect food consumption. Therefore, this may have contributed to the non-significant reduction in feed/fluid intake in NPATp and LPATp relative to NPC and LPC groups respectively. The non-significant reduction in feed/fluid intake (suggestive of mild effect) may explain the high compliance level among HIV/AIDS patients reported for the drug (Horberg and Klein, 2010b, Truong *et al.*, 2015). Meanwhile, the

significant reduction in feed intake in NPE group relative to NPC among NP treated groups may be consistent with the role of ethanol in depressing appetite (Westerterp-Plantenga and Verwegen, 1999, Caton *et al.*, 2004) resulting in low intake of feed in the group. Alcohol has been reported to alter various hormonal processes that are involved in the regulation of appetite and this has been implicated in the reduction of food intake (Kokavec, 2008). The marked reductions in feed/fluid intake among the LP treated groups indicate that intake of low protein diet reduces feed/fluid consumption in adult rats. In contrast, treatment with low protein diet was reported to not alter feed consumption, body or organ weights in adult rats (De Souza Santos *et al.*, 2004). These differences in the findings may be accounted for by the different protein levels (6% and 8% respectively) and the treatment protocols in both studies. Additionally, it has been reported that deitary protien levels above 7% would have no effect on these parameters (Vawdaw and Mandlwana, 1990).

5.3 Effect of treatment on rat biometric parameters

Results from the present study suggest that treatment with either ATp and/or E does not significantly affect rat body weight gain among NP treated groups and weight gain was positively related to feed and fluid intake. However, the significant decline in feed intake value in NPE group compared to NPC accompanied by a non-significant difference in body weight gain values between NPE and NPC suggests that weight gain may not be as a result of feed intake in NPE group. Alcohol consumption has often been associated with deposition of fat in the body which can cause increases in body weight (Caton *et al.*, 2004, Traversy and Chaput, 2015, Bayerl *et al.*, 2018). Therefore, this could be the reason why reduction in feed intake was not associated with weight reduction in the group. Meanwhile, the low weights for liver, testes and epididymides recorded among LP treated groups could also be linked to the significantly lower weight gain recorded in the respective groups. Therefore, this finding reveals critically important role of the dietary protein component in stimulating growth and development. In addition, the significantly low weight gain observed in LPEATp treated rats mirrors the combinatorial severe effects of ATp and alcohol treatments in LP-dietary intake and underscores the significance of normal protein intake in HIV/AIDS treatment and management.

Relative organ weight has been demonstrated to be an economic and effective measure of identifying the target organ in toxicological studies (Asagba *et al.*, 2007, Azu *et al.*, 2014). In

reproductive studies, toxicity may be associated with hypertrophy, swelling or atrophy of reproductive organs (Afolayan and Yakubu, 2009, Azu *et al.*, 2014). According to the results in the present study, treatment with ATp only does not affect the relative epididymis/testis weight of rats when a normal protein dietary treatment is maintained. In contrast, previous HAART combinations (lamivudine, stavudine and nevirapine) were reported to cause significantly higher values for relative testicular weight, suggesting toxic insult to the organ (Azu *et al.*, 2014). The different results may be attributed to the different combination/formulation of antiretroviral drugs in ATp (EFV, FTC and TDF) which may have a mild effect to gonadal weight compared to HAART combination in their study.

Meanwhile, the increases in relative organ weights for epididymides, testes, liver and kidneys in all LP treated groups compared to NPC indicates a possibility of swelling or hypertrophy in the organs. Concurrent intake of ATp and E by protein malnourished rats resulted in significant enlargement of epididymides, testes and liver which is an indication of impaired function of the respective organs and suggests toxicity due to drug-drug/drug-nutrition interaction. Antiretroviral drugs particularly emtricitabine and efavirenz (components of ATp) have both been reported to cause steatosis which result in hepatomegaly (Cooper, 2000). Meanwhile, chronic alcohol consumption is also reported to cause steatosis (Caton *et al.*, 2004, Bayerl *et al.*, 2018). Therefore, protein malnutrition might have augmented the toxic effects of ATp and E resulting in summation of their effects in the liver.

5.4 Effects of treatment on serum testosterone levels

In previous studies, HAART and ethanol have independently been reported to cause decreases in testosterone levels as well as other male reproductive hormones which has a potential of disrupting the function of the epididymis and possibly leading to infertility problems (Rochira *et al.*, 2011, Albadri, 2013, Oremosu and Akang, 2015). The lack of significant differences in the levels of serum testosterone in all treatment groups (NP and LP treated groups) compared to control (NPC) suggests that the overall treatment may not have appreciable adverse effects on serum testosterone concentrations. However, the high serum testosterone concentration recorded among most LP treated groups is consistent with reports from previous studies involving protein malnutrition in adult rats and in pups weaned of protein malnourished mothers (rats) (De Souza Santos *et al.*, 2004, Ramos *et al.*, 2010).

5.5 Effects of treatment on the histo-architecture of the epididymis

The epididymis is primarily involved in conferring fertilisation capacity on sperm cells. Therefore, chemicals that disrupt the structure of the epithelium and interstitium of the epididymis will also alter the composition of luminal fluid, sperm morphology and function (De Grava Kempinas and Klinefelter, 2014). This is the first study to evaluate the combined effects of ATp, E and PM on the epididymis. Increased histo-achitectural alterations observed in NPATp and NPE groups (Table 4.3) compared to tissues from the control group (NPC) indicates that ATp and E can independently cause epididymal functional derangements under normal protein status (De Grava Kempinas and Klinefelter, 2014). However, the depletion of cauda sperm concentration and inflammation infiltrates in epididymal tissues from NPE group relative to NPATp group suggests greater toxicity by E compared to ATp.

The increases in interstitial space (initial segment), exfoliated material (proximal corpus and cauda) and oligospemia (cauda) observed in epididymal tissues were more pronounced in LP than NP treated rat groups which distinctly emphasises the role of protein deficiency in drug toxicity. Furthermore, the severity of these histopathological alterations observed in LP treated groups (LPATp, LPE, LPEATp) compared to NPC rats, and to their NP cohorts (NPATp, NPE, NPEATp) suggests that protein deficiency may have augmented the toxicity of these chemical probably through drug-nutrition interaction (Krishnaswamy, 1989, Oshikoya and Senbanjo, 2009). Impairment in the function of liver enzymes like cytochrome P450 involved in the metabolism of both ATp and E (Bibi, 2008, Bartelink *et al.*, 2015) may have resulted in increased bioavailability of these drugs and therefore increased toxicity to epididymal tissue. The increased collagen component of epididymis tissue of the treated rat groups also suggests fibrosis due to treatments which is indexed by increased connective tissue profiles (Lanning *et al.*, 2002, Rinaldi *et al.*, 2013) and may negatively interfere with normal function of the epididymis.

5.6 Effects of treatment on histo-morphometric/stereology indices of the epididymis

Morphological and stereological assessments have enabled accurate interpretation of reproductive tissue toxicity (Awobajo *et al.*, 2010, Azu, 2012, Azu *et al.*, 2014). Toxicity in the epididymis is reported to manifest in altered morphometric parameters (De Grava Kempinas and

Klinefelter, 2014). Therefore, the reduction in ETD (significant) and A_C values in the LPEATp group relative to NPC is highly suggestive of reproductive toxicity in the epididymis after combined treatment with ATp, E and low protein diet. Results also confirm greater toxicity with E relative to ATp as reduction in both ETD (significant) and EH values were recorded in NPE and LPE groups compared to NPATp/LPATp. The reductions in ETD and A_C values recorded could also imply that there might be a possibility of atrophy/shrinkage of epididymal tubules in the tissues of the epididymis and this can compromise the function of the organ (De Grava Kempinas and Klinefelter, 2014). Therefore, epididymal tubular atrophy could have been one of the reasons why larger interstitial spaces were observed between epididymal tubules in all treatment groups compared to NPC. Interstitial space was even more pronounced in tissues of the LP treated groups. Correspondingly, significant reductions in ETD were recorded in LPE and LPEATp groups.

The lack of association between changes in serum testosterone levels and ETD in both NP and LP treated groups may not be related to testosterone deprivation, but could be a direct toxic effect of the treatments (Lanning *et al.*, 2002, De Grava Kempinas and Klinefelter, 2014). This result is contrary to earlier reports that associated depressed androgen levels to decreased tubular diameters and epithelial height in the epididymis (Creasy *et al.*, 2012, De Grava Kempinas and Klinefelter, 2014). This lack of association between serum testosterone levels and ETD as well as EH in our study may not be unrelated to non-significant reduction recorded in these parameter as against significant reductions earlier on reported (Creasy *et al.*, 2012, De Grava Kempinas and Klinefelter, 2014). Furthermore, the increased variability in the testosterone levels observed in this study suggests disturbances in the androgen homeostasis (probably through toxicity on Leydig cells) which may be related to the tissue alteration and damages (such as increased luminal debris (round spermatids), severe depletion of cauda sperm concentration, decreases in ETD and cribriform change) observed in our study.

5.7 Effects of treatment on androgen receptor immunoreactivity in epididymal tissues

According to our knowledge, this is the first study to investigate the effects of ATp, E and PM on the immunoreactivity of androgen receptor in tissues of the epididymis. Hence, this study offers a unique perspective in the evaluation of these variables to the functionality of the epididymis. Table 4.6 shows differences in the immunolocalisation of AR in the tissues of the epididymis

from different species in published literature compared with that observed in this study. Strong and wide spread AR-ir was observed in all regions of the epididymis compared to other studies. AR-ir was also observed in the interstitial stroma cells and connective tissue in all regions of the epididymis compared to immunostaining reported in other studies. The immuno-localisation of AR in the interstitial cells in this study implies that androgen control might not be confined to cells of the epithelium only. Therefore, disruptions in the AR-ir of the interstitial cells may also have a bearing on the functions of the cells in the stroma.

The weak immunostaining and significant reduction in the mean principal cell nuclei AR-ir percentage values in tissues from all the treatment groups compared to NPC group indicate that treatment with either ATp and/or E with PM diminishes the expression of AR in the tissues of the epididymis. Weaker staining and lower mean principal cell nuclear AR-ir percentage values were observed especially among the LP treated groups compared to NP treated groups which is consistent with previous reports where low levels of AR proteins were recorded in adult male rats fed with low protein diet and in pups weaned from mothers subjected to protein malnutrition (de Souza Santos *et al.*, 2004, Vicente *et al.*, 2004, Rinaldi *et al.*, 2013). The explanation of the above findings is beyond the scope of the present study, but could possibly be due to low levels of amino acids in protein deficient diets which directly or indirectly deplete AR proteins leading to diminished immuno-expression or its downregulation.

Epididymal function is largely controlled by androgens via androgen receptors (Menad *et al.*, 2014, Boukenaoui-Ferrouk *et al.*, 2017). Hence, the downregulation of AR by ATp, E and low protein diet is evidence of functional interference. Furthermore, direct inhibitory action by a hypo-fertility compound called hydroxyflutamide on AR in reproductive tissues has been reported to cause a decline in AR-ir (Klinefelter and Suarez, 1997). Therefore, direct inhibitory action by ATp could be the reason why immunostaining was granular in the nuclei of the principal cells in the sections from NPATp group. Similar, granular AR immunostaning was also reported in the cytoplasm of the epididymis of the fat sand rat during breeding (Menad *et al.*, 2014).

The non-significant increase in mean principal cell nuclear AR-ir percentage value of NPEATp group relative to NPE group was quite unusual since both ATp and E independently caused a significant decline in AR-ir. A summation of the effects of the two drugs was expected to cause marginal decline in AR-ir percentage values as was observed in LPEATp group. This slight increase in values of NPEATp group relative to NPE among the NP treated groups was also observed in feeding parameters (feed and fluid intake), rat biometric parameters, morphometric indices, and histopathological alterations and all these may suggest ameliorative interaction between ATp and E when a normal protein diet is maintained leading to infra-addition of the adverse effects. This could be due to the fact that alcohol and ATp are both metabolised in the liver via cytochrome p450 (Chan and Anderson, 2014, De Grava Kempinas and Klinefelter, 2014). The activation of cytochrome p450 enzymes in the liver by efavirenz (a component of ATp) resulted in increased metabolism of alcohol (Deeks and Perry, 2010) and consequently low ethanol bioavailability leading to less effect on tissues of the epididymis. The above may be supported by the report of Mccance-Katz *et al* (2013) in which lower blood alcohol levels and intoxication were recorded among patients who were treated with both efavirenz and alcohol compared to those who received equal amounts of alcohol only. However, the ameliorative action of ATp on the effects of E was not observed with LP treatment. This is possibly due to depletion or impairment of P450 enzymes due to protein deficiency (Bibi, 2008, Bartelink *et al.*, 2015) leading to the marked tissue derangements observed in LP-treated rat groups and the probable reproductive incompetence.

5.5 Summary

Chapter 5 highlights all the discussion points of the outcomes from the study experiment. The derangements caused by Atripla®, ethanol and low protein diet co-administration are consistent with impaired function of the epididymis in literature. The study also revealed severe toxicity to the epididymis with ethanol relative to Atripla®.

CHAPTER 6: CONCLUSIONS

The present study revealed that concurrent treatment with Atripla®, ethanol and protein deficient diet causes alterations in rat biometric parameters, epididymal histo-architecture and androgen receptor immunoreactivity. Disruptions in these aspects affect the functioning of the epididymis and are associated with fertility challenges. In addition, this is one of the few studies to clearly demonstrate, compare and contrast how Atripla®, ethanol and protein malnutrition independently and concurrently affect the tissues of the epididymis, biometric parameters, feeding patterns and general wellbeing of adult rats. Therefore, apart from exposing the combined toxic effects of these variables, the study also revealed greater toxicity with ethanol relative to Atripla®. However, the ameliorative interaction between Atripla® and ethanol did not abrogate or significantly reduce the toxicities in NP dietary condition. Additionally, the importance of protein component in diet is hugely demonstrated in assessment of drug efficacy and toxicity in this study, thereby revealing the potentiating effects of protein deficiency in diet on the toxicity of Atripla® and ethanol to reproductive efficiency/capacity among PLWHAs. Though these findings hopefully will assist clinicians in their treatment and management of HIV/AIDs and further improve the quality of life among people living with HIV/AIDS in the SSA region and beyond, further studies are required to clearly delineate the implications of the observed adverse effects.

6.1 Research limitations

Challenges were experienced in the formulation and procurement of diets for the study especially the low protein diet. This had a huge bearing on the timelines of the study resulting in delays in completion of the study. Eventually, the formulation of a 6% protein concentration diet was successful. However, formulated samples had to be tested at two separate laboratories because we could not have the energy value for the low protein diet from the first testing laboratory (ARC Irene testing laboratory) due to a technical fault. This could be the possible reason why energy levels were not exactly the same for the normal and low protein diets since handling and protocols in the two laboratories differ. However, energy values and other components in the normal and low protein diets were comparable with no marginal differences. Normal and low protein diets with comparable energy values have also been used previously in similar study

models with success (Mbajiorgu *et al.*, 2008). The other objectives for the study were to determine the effect of treatment on the level of serum oestrogen/estradiol and semen parameter characteristics. Due to limited time allocated to the study, lack of appropriate equipment and budgetary constraints, semen characteristics and serum 17β estradiol could not be analysed.

6.2 Recommendations

It is recommended that further investigations be performed to give more information on the combined effects of Atripla®, ethanol and low protein diet on the male reproductive system. Electron microcopy is recommended to evaluate the effects of the study variables on the ultrastructure of the epididymis to understand the cellular effects and corroborate with the histological effects already observed in this study. In addition, evaluation of epididymal tissue biochemical parameters like sperm cartinine, acetyl carnitine and glyceryphosphoryl choline which are essential in sperm maturation is also advised. We suggest the evaluation of apoptosis in the tissue of the epididymis using TUNEL assay, this was an objective in our study but it could not be investigated due to funding constraints. Further hormonal assays which include the measurement of dihydrotestosterone (DHT) and oestrogen/estradiol levels is advised. It is also recommended that other reproductive organs like the testis, prostate and seminal vesicles be evaluated to give an overall picture on the effect of study variables on the male reproductive system. All these investigations will provide more information about the effect of the study variables on male reproductive health.

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APPENDICES

Appendix I-Ethics clearance certificate

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2015/08/33/C

APPLICANT: Mr T Nyirenda

SCHOOL: Schoo of Anatomical Sciences
DEPARTMENT:
LOCATION:

PROJECT TITLE: Effects of Atripla® and/or alcohol with protein malnutrition on the testis, epididymis and prostate glands of adult male Sprague Dawley rats

Number and Species

80 male Sprague Dawley rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 2015/08/25 This approval remains valid until 2017/09/24

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below.

None

Signed: _____ (Chairperson AESC) Date: 29 Sept 2015

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 29 Sept 2015

cc: Supervisor: Professor E F Mbajiorgu
Director: CAS

Works 2000/Iain/0015/AESCCert.wps

Appendix II: Preparation of 6% ethanol solution:

For 1000 ml (1liter) of 6% ethanol in distilled water, 60 ml of absolute ethanol (Merck, South Africa) was added to 940 ml of distilled water. 5000 ml was prepared every week for the rats, that is, 300ml of absolute ethanol in 4,700 ml of distilled water.

Appendix III: Preparation of the low protein diet

Low protein was constituted from a standard laboratory diet (Labchef Research Nutrition®, South Africa) which has normal protein levels (23%). The proximate composition of the normal diet is described in table 3.1. The ingredients of the diet are as follows:

a) Maize
b) Wheat
c) Soya beans
d) Soya bean protein concentrate
e) Fish meal
f) Maize protein concentrate
g) Molasses
h) Sucrose
i) Calcium carbonate
j) Sodium chloride
k) Calcium phosphate, approved accidulants
l) Approved antioxidants
m) Approved vitamins and minerals

Cassava was chosen for the constitution of low protein diet because it has low crude protein and high energy content. It is also widely used in the formulation of stock feeds (Bhuiyan and Iji, 2015).

Protocol for Preparing low protein diet

Normal protein diet is prepared as 12 mm pellets in 20 kg bags. Preparation of the low protein diet involved mixing the normal protein diet with commercially prepared cassava powder (tuberous roots). The low protein diet was prepared as follows:

1. Normal protein diet pellets were ground using a Corona® (Nigeria) hand grinder to 1 mm grain size.
2. Cassava was purchased commercially prepared as follows: Cassava tuberous roots were washed and outer skin peeled. Peeled cassava was then air dried and ground using a machine grinder to 0.5 to 1 mm grain size.
3. Normal protein diet ground pellets and cassava powder were mixed at a ratio of 1:6 respectively. Preparation of low protein diet sufficient for 5 days involved adding 500 g of ground normal diet pellets to 3000g of cassava powder whilst stirring to allow for thorough mixing. The mixture was stirred for 5 minutes until it was completely homogenous.
4. 1200 ml of distilled water was then added to the low protein powdered mixture whilst stirring until it was completely homogenous.
5. The homogenous mixture was then moulded into handmade pellets using ice tray casts to have pellet sizes of approximately 12 mm comparable to the normal protein diet pellets.
6. The handmade pellets of the low protein diet were then allowed to air dry in an oven (Labcon, Germany) overnight (10 to 12 hours).
7. After 10 to 12 hours the low protein pellets were completely dried and ready to be used for treatment.

Appendix IV-Normal and low protein diet test results

ARC • LNR

ARC-Irene Analytical Services
LNR-Irene Analitiese Dienste

sanas

Private Bag/Privaatsak X2, Irene, 0062 Tel: (012) 672 9294 Fax: (086) 607 7102

Enquiries: Penny Barnes
Tel: 012-672 9292/94

04/11/2016

The Manager
Univ of Witwatersrand (Anatomical Sciences)
School of Anatomical Sciences
Private bag X3
Wits
2050

Tel No: **(011)** 7171570
Fax No:

Attention: Mr T Nyirenda

TEST REPORT

Date received: 18/10/2016
Date accepted: 18/10/2016
Date completed: 04/11/2016
Test report no: 2016-F-355

RESULTS OF ANIMAL FEED (UNSPECIFIED)

Please take note that:

1. Test results relate only to the samples tested.
2. This report may not be reproduced without the written consent of the Quality Manager.
3. The samples received were thoroughly mixed before analysis.
4. Chromatogrammes, if applicable, are available on request.
5. Opinions and interpretations expressed herein are outside the scope of SANAS accreditation.

Yours sincerely

LE Smit
Research Team Manager: Qualitative and Quantitative Analysis

ARC-IRENE ANALYTICAL SERVICES
SERVICES

Physical Address: ARC-API, Old Olifantsfontein Rd, Irene

Page 1 of 2

Appendix IV- Normal protein diet test

Analysis	Dry matter	Moisture	Ash	Fat (ether extraction)	Fibre (crude)	* Protein (N x 6.25)	Calcium	Phosphorous	Gross energy
Method Number	ASM 013	ASM 013	ASM 048	ASM 044	ASM 059	ASM 078	ASM 042	ASM 045	ASM 053
Unit	%	%	%	%	%	%	%	%	MJ/kg
Sample Number									
1 : Concentrate feed	91.11	8.89	6.99	3.34	2.60	21.19	1.32	0.62	16.40

Sample	Sample type	Date analysis commenced
1	Animal feed (unspecified)	29/04/2016

For the conversion of nitrogen content to protein content the factor 6.25 was used.

Appendix IV- Low protein diet tests

TEST REPORT 2016-F-355

This laboratory holds SANAS accreditation for analyses with an ASM number.
Results are expressed on a wet basis, therefore as samples were received.

Analysis	Method Number	Unit	Sample Number 1 : Cassava : Casfeed 5:1	Sample Number 2 : Cassava : Casfeed 6:1
Dry matter	ASM 013	%	92.94	92.78
Moisture	ASM 013	%	7.06	7.22
Ash	ASM 048	%	3.39	3.35
*Protein (N x 6.25)	ASM 078	%	6.42	5.57
Fat (ether extraction)	ASM 044	%	6.49	3.25
Fibre (crude)	ASM 059	%	3.70	3.74
Calcium	ASM 042	%	3.41	2.95
Phosphorous	ASM 045	%	0.16	0.15

Sample	Sample type	Date analysis commenced
1-2	Animal feed (unspecified)	19/10/2016

* For the conversion of nitrogen content to protein content the factor 6.25 was used.

Results transcript shows two samples tested at the ARC laboratory, the first had a 1:5 ratio mixture of standard laboratory diet and cassava powder, it gave a crude percentage of 6.42%. The second sample was 1:6 ratio mixture of the standard laboratory diet and cassava powder and it gave a crude percentage value of 5.57% which was rounded to 6%. The second mixture was used for the study

Appendix IV: Feed test results from the University of the Free State

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA
UFS·UV
NATURAL AND AGRICULTURAL SCIENCES
NATUUR- EN LANDBOUWETENSKAPPE
Department of Animal, Wildlife and Grassland Sciences

Nyirenda, Trust

MSc Medicine Student
School of Anatomical Sciences, Faculty of Health Sciences,
University of the Witwatersrand

RESULTS

VAT No :

30-10-2017

The following samples were analysed :

Client	DM	OM	CP	GE	NDF	ADF	ADL	Fat
Sample	g / kg sample	g / kg DM	g / kg DM	MJ / kg DM	g / kg DM	g / kg DM	g / kg DM	g / kg DM
Cas Feed 1:6	934.35	949.18	58.65	15.97	152.30	51.38		7.35
Cas Feed	928.47	909.88	243.13	16.76	146.40	63.86		23.83

Please note that the information are not for commercial or advertising purposes.

Please contact us for any further enquiries.

Kind regards

* DM = droë materiaal / dry matter; OM = organiese materiaal / organic matter; RP/CP = ruproteïen / crude protein; BE/GE = bruto energie / gross energy; NBV/NDF = neutraalbestande vesel / neutral-detergent fibre; SBV/ADF = suurbestande vesel / acid-detergent fibre; SBL/ADL = suurbestande lignien / acid-detergent lignin; Minerale / Minerals (Ca,Mg,Na,K,P,pH)

205 Nelson Mandela Drive/Rylaan, Park West/Parkwes, Bloemfontein 9301, South Africa/Suid-Afrika
P.O. Box/Posbus 339, Bloemfontein 9300, South Africa/Suid-Afrika,
Tel : 051-4019712, E-Pos/E-mail : vandermerwejam@ufs.ac.za

Appendix IV: Feed test results from the University of the Free State

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
NATURAL AND AGRICULTURAL SCIENCES
NATUUR- EN LANDBOUWETENSKAPPE

Department of Animal, Wildlife and Grassland Sciences

Nyirenda, Trust

MSc Medicine Student
School of Anatomical Sciences, Faculty of Health Sciences,
University of the Witwatersrand

RESULTS

VAT No :

30-10-2017

The following samples were analysed :

Client Sample	Ca g / kg DM	Mg g / kg DM	Na g / kg DM	K g / kg DM	P g / kg DM	pH g / kg DM
Cas Feed 1:6	**3.65**				**1.91**	
Cas Feed	**21.41**				**7.95**	

Please note that the information are not for commercial or advertising purposes.

Please contact us for any further enquiries.

Kind regards

* DM = droë materiaal / dry matter; OM = organiese materiaal / organic matter; RP/CP = ruproteïen / crude protein; BE/GE = bruto energie / gross energy; NBV/NDF = neutraalbestande vesel / neutral-detergent fibre; SBV/ADF = suurbestande vesel / acid-detergent fibre; SBL/ADL = suurbestande lignien / acid-detergent lignin; Minerale / Minerals (Ca,Mg,Na,K,P,pH)

205 Nelson Mandela Drive/Rylaan, Park West/Parkwes, Bloemfontein 9301, South Africa/Suid-Afrika
P.O. Box/Posbus 339, Bloemfontein 9300, South Africa/Suid-Afrika,
Tel : 051-4019712, E-Pos/E-mail : vandermerwejam@ufs.ac.za

Appendix V: Dose calculation for Atripla®

1. The average weight of each tablet was 1600mg (n=100 tablets).
2. Atripla® consists of 600 mg of efavirenz , 200 mg emtricitabine and 300 mg tenofovir disiproxil fumurate.

Therefore: 1 tablet weighing 1600 mg has 1100mg of active ingredient

If 1600mg of tablet has 1100 mg of active ingredients

Hence 1mg of tablet = 1100 mg/1600mg tablet

1mg of tablet = **0,6875 mg of active ingredients**

3. If a 40kg person is prescribed to take **one tablet** of the Atripla® drug with a total of 1100mg of the active ingredients (Squibb, 2010).

Therefore:

40 kg body weight: 1100 mg

1 kg body weight: 1100 mg/40 kg

1 kg body weight = 27.5 mg

Hence the dose is **27.5 mg/kg of body weight**

This can also be translated as a total of **0.0275 mg of active ingredients/g of body weight**

4. The average weight of adult male rats between 11 and 12 weeks was 442.25 g. The dose was adjusted for animal weight to obtain the corresponding therapeutic doses for the rat model from the human therapeutic dose (Squibb, 2006, Clay *et al.*, 2008).

Therefore each rat received 442.25 g X 0.0275 mg/ g= **12.16 mg of Atripla® active ingredients of per day**

5. If 1mg of tablet has 0.6875 mg of Active ingredients

Hence 13.75 mg/0.6875mg per mg of tablet = 17.69 which was rounded to **18 mg of tablet**

Therefore the each rat received **18 mg of tablet per day** which consists of a total of **12.16 mg** of Atripla® active ingredients.

Protocol for adding Atripla® drug into gelatin rectangles (Kamerman *et al.*, 2004)

The ingredients to make gelatine rectangles as a medium for introducing treatment are:

a) Unflavoured gelatine (Pick and Pay supermarket, South Africa)

b) Brown Sugar (Pick and Pay supermarket, South Africa)

c) Beefy Bovril (Pick and Pay supermarket, South Africa)

d) Two laboratory beakers (100 ml)

e) Two spatulas

f) Electronic balance

g) Ice tray to mould the rectangles in

h) Two magnetic stirs

Adding Atripla® into the gelatine rectangles

1. Crush one tablet of Atripla® (which weighs 1600 mg) into powder
2. Since 100 ml makes 50 (2 ml gelatine rectangles) then one gelatine rectangle should contain 18 mg of Atripla® (from the calculations earlier)

50 gelatine cubes X 18 mg Atripla® powder= **900 mg**

Hence for 50 gelatine rectangles use 900 mg of Atripla® powder

3. Add 17 g of sugar and 8 g of gelatine into the beaker.
4. Then add 1000mg of Atripla® powder into the beaker.
5. Add 2.5 g of Beefy Bovril.
6. Add 100 ml of hot water.
7. Place mixture on a magnetic stir and mix for 3 to 5 minutes until it is thoroughly mixed
8. Using a 10 ml syringe draw 2 ml of mixture into the ice trays to mould.
9. Place tray with mixture into the fridge for 30 minutes to an hour at 2 to 4°C.
10. Thereafter, the cubes will be ready to be given for treatment.

To make **plain gelatine rectangles** (to be given as a placebo to all groups not receiving Atripla®; NPC, NPE, LPC, LPE) follow the same procedure omitting to add Atripla®.

Appendix VI-Testosterone ELISA protocol

6 ASSAY PROCEDURE

6.1 GENERAL REMARKS

- All reagents and specimens must be allowed to come to room temperature before use. All reagents must be mixed without foaming.
- Once the test has been started, all steps should be completed without interruption.
- Use new disposal plastic pipette tips for each standard, control or sample in order to avoid cross contamination.
- Absorbance is a function of the incubation time and temperature. Before starting the assay, it is recommended that all reagents are ready, caps removed, all needed wells secured in holder, etc. This will ensure equal elapsed time for each pipetting step without interruption.
- As a general rule the enzymatic reaction is linearly proportional to time and temperature.

6.2 ASSAY PROCEDURE

Each run must include a standard curve.

1. Prepare a sufficient number of microplate wells to accommodate calibrators and samples in duplicates.
2. Dispense **10 µl** of each **Calibrator and Sample** with new disposable tips into appropriate wells.
3. Dispense **100 µl** of **Incubation Buffer** into each well.
4. Add **50 µl Enzyme Conjugate** into each well.
5. Incubate for **60 minutes** at room temperature on a Microplate mixer.
   Important Note:
   Optimal reaction in this assay is markedly dependent on shaking of the microplate!
6. Discard the content of the wells and rinse the wells **4 times** with diluted **Wash Solution** (300 µl per well). Remove as much Wash Solution as possible by beating the microplate on absorbent paper.
7. Add **200 µl** of **Substrate Solution** to each well.
8. Incubate without shaking for **30 minutes** in the dark.
9. Stop the reaction by adding **50 µl** of **Stop Solution** to each well.
10. Determine the absorbance of each well at 450 nm. It is recommended to read the wells within 15 minutes.

6.3 CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of calibrators, controls and patient samples.
2. Using semi logarithmic graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration from the calibration curve.
4. Automated method: Computer programs using cubic spline, 4 PL (4 Parameter Logistics) or Logit-Log are recommended.
5. The concentration of the samples can be determined directly from this calibrator curve. Samples with concentrations higher than that of the highest calibrator have to be further diluted. For the calculation of the concentrations, this dilution factor has to be taken into account.

Conversion to SI units:
Testosterone (pg/ml) x 3.47 = pmol/l

Appendix VII-Histology tissue processing protocol

Tissues of the epididymis were processed using an automatic processor (Shandon Citadel 1000, Germany) in the following steps:

A. Dehydration

1. Immerse in 70% alcohol for 1 hour.
2. Immerse in 95% alcohol for 2 hours.
3. Immerse in 95% alcohol for 2 and half hours.
4. Immerse in 100% ethanol for 1 hour.
5. Immerse in 100% ethanol for 2 hours
6. Immerse in 100% ethanol for 1 hour.

B. Clearing

1. Immerse in chloroform for 1 hour.
2. Immerse in chloroform for 2 hours.
3. Immerse in wax for 2 hours (X2).

C. Embedding

The embedding process was done using an embedding center (Tissue-Tek®, Germany). The center has a wax heating chamber and wax dispensing nozzle, cryo-plate for cooling the blocks and warming chambers for the tissue blocks.

1. Take all cassettes with tissues from the tissue processor and place them in a pre-warmed chamber on the embedding centre.
2. Put wax in heating chamber and heat until molten (temperatures below 60°C).
3. Once the wax is molten, fill the metal mould with wax using the wax dispensing nozzle.
4. Using forceps take out tissue specimen from the cassette and gently orientate it such that the intended cutting edge faces the floor of the metal mould.
5. Briefly cool specimen in mould on the cold plate and then fit the plastic cassette accordingly.
6. Quickly dispense more molten wax on the top of the plastic cassette then transfer the mould onto the cooling plate for approximately 15 minutes
7. Thereafter, the wax block can be easily separated from the metal mould while it remains attached to the plastic cassette.

Appendix VIII: Gelatine coated Slides

Chrome alum gelatin solution

The following reagents are required to prepare chrome alum solution for coating slides:

1. Gelatine 1.5 g
2. Chromium potassium sulfate 0.25 g
3. Distilled water 500 ml

The procedure:

1. Heat water to 60°C and completely dissolve the gelatine then stir in the chromium potassium sulfate.
2. Dip racks of clean slides in the warm gelatine solution (at 40°C to 50°C) and then drain the slides onto paper towel.
3. Allow the slides to dry in the incubator overnight (10 to 12 hours) at 42°C.
4. Store in a dust free container at room temperature

Appendix IX: Haematoxylin and Eosin stain staining protocol

A. Hydration

1. Dewax mounted slides in 2 changes of xylene, 5 minutes each.
2. Gently dip 10 times in 2 changes of 100% alcohol.
3. Gently dip 10 times 95% alcohol.
4. Gently dip 10 times in 70% alcohol.
5. Wash in running tap water for 5 minutes

B. Nuclear staining

1. Stain mounted slides with Meyer's haematoxylin for 5 minutes.
2. Wash in running tap water for 5 minutes.
3. Dip 3 times in 1% acid alcohol consecutively to remove haematoxylin from cytoplasm
4. Wash in running tap water for 5 minutes to remove acid alcohol.

C. Cytoplasm staining

1. Counterstain in eosin solution for 2 minutes.
2. Wash in running tap water for 1 minute.

D. Dehydration and mounting

1. Gently dip 10 times in 70% alcohol.
2. Gently dip 10 times in 95% alcohol.
3. Gently dip 10 times in 100% alcohol.
4. Clear mounted slides in 2 changes of xylene, 5 minutes each.
5. Coverslip section in section with entellen solution.

Staining results:

- Nuclei: Blue-black
- Muscle fibres: Deep pinky red
- Cytoplasm : Varying shades of pink

Appendix X-Van Gieson staining

Preparation of solutions:

A. Celestine blue

The following reagents are required to prepare Celestine blue

1. 100 ml of 5% ammonium ferric sulphate (iron alum).
2. 0.5 g of Celestine blue.

Add the Celestine blue to the ammonium ferric sulphate and boil for 3 minutes. Filter when cool and store in a refrigerator.

B. 1% Ponceau

1. 1 g of Ponceau S (C1 27195).
2. 100 ml of distilled water.

C. Curtis stain

Prepare Curtis stain by adding:

1. 90 ml of saturated aqueous picric acid
2. 10 ml of glacial acetic acid

Van Gieson staining protocol:

1. Bring sections to distilled water.
2. Stain nuclei with Celestine blue for 5 minutes.
3. Rinse in distilled water for 5 minutes.
4. Stain in haematoxylin for 5 minutes.
5. Wash well in running water for 5 minutes.
6. Flood with Curtis stain for 5 minutes.
7. Blot slides on filter paper.

Dehydrate rapidly in alcohols

8. Dip 2 times in 70% alcohol.
9. Dip 2 times in 95% alcohol
10. Dip 2 times in 100% alcohol.
11. Clear mounted slides in 2 changes of xylene, 5 minutes each.
12. Mount in section with entellen solution

Staining results:

- Nuclei Blue
- Collagen Bright Red
- Cytoplasm muscle, fibrin and red blood cells: Yellow

Appendix XI-Immunohistochemistry protocol

A. Silane coating of slides for immunohistochemistry

Reagents for 2% 3-aminopropyl triethoxysilane (APES) include:

1. 200 ml of acetone.
2. 4 ml of xylene (3-aminopropyl triethoxysilane).
3. Xylene (3-aminopropyl triethoxysilane).

Coating Protocol

1. Immerse slides in 2% APES for 30 minutes.
2. Dip 10 times in 2 changes of acetone.
3. Dip 10 times in 2 changes of distilled water.
4. Put slides in incubator at 37°C overnight to allow them to dry.
5. Store in a clean container at room temperature.

B. Immunohistochemistry protocol for paraffin sections

Antigen retrieval solutions

Sections are microwaved in 10mM Sodium citrate buffer pH 6 and the solution is prepared by mixing:

1. Tri-sodium citrate (di-hydrate) 2.9 g
2. Distilled water 1000 ml
3. Add 500 µl of Tween 20 solution to the mixture.

Solution was calibrated to pH 6 using 1M Hydrochloric acid and tested using Meter Teledor (United States of America) pH meter and 500 µl of tween 20 was then added.

Buffer wash solutions

Sections will be washed with 1M Phosphate buffer pH: 7.4 and the solution is prepared by mixing:

1. Sodium Chloride 16 g
2. Di-Sodium hydrogen phosphate 3.5 g
3. Potassium chloride 0.4 g
4. Potassium di-hydrogen phosphate 0.4 g
5. Distilled water 2000 ml

Solution was calibrated to pH 7.4 using 1M Hydrochloric acid and tested using Meter Teledor (United States of America) pH meter.

Diaminobenzidine tetrachloride (DAB) working solution

DAB (**solution a**) was prepared in a bijou bottle using:

1. Diaminobenzidine tetrachloride 1 mg
2. Tris-Hydrochloric acid solution 2 ml

In a separate tube mix solution **b)** by adding:

29 µl of cold distilled water to 1 µl of hydrogen peroxide then add 20 µl of this solution to the DAB solution **a)**

The procedure for immunohistochemistry is conducted over 2 days:

Day 1 Immunohistochemistry procedure:

Rehydration

1. Dewax mounted slides in 2 changes of xylene, 5 minutes each.
2. Gently dip 10 times in 2 changes of 100% alcohol.
3. Gently dip 10 times in 95% alcohol
4. Gently 10 times in 70% alcohol.
5. Wash in running tap water for 5 minutes.
6. Antigen retrieval-microwave sections in Citrate buffer pH at 720 watts. Allow sections to cool at room temperature for 20 minutes.
7. Rinse slides in PBS for 5 minutes.
8. Block endogenous peroxidase with 3% hydrogen peroxide (H_2O_2) in methanol for 15 minutes.

- 3% Hydrogen peroxide in methanol was prepared by adding 6 ml of hydrogen peroxide to 194 ml of methanol to make 200 ml.

9. Wash in PBS pH 7.4 for 5 minutes in 3 consecutive washes (X3)
10. Incubate with 5% normal goat serum for 1 hour.

- 5% Normal goat serum (NGS) is prepared during the preceding third PBS wash. 100 µl is required to block a section on a single slide and it is prepared by adding 5 µl of NGS to 95 µl of PBS to make 100 µl of 5 % NGS.

11. Tap serum off slides. DO NOT WASH.

12. Incubate sections with (Rabbit polyclonal) Androgen receptor primary antibody (Ab74272, Abcam, United Kingdom) at 4°C for overnight (16-20 hours) in a moist chamber.

- AR primary antibody is prepared in the last 15 minutes of normal goat serum blocking step. 100 µl is required for every section on one slide and it is prepared by diluting the AR antibody at a ratio of 1:200. Therefore, 2 µl is added to 198 µl of PBS giving 200 µl sufficient for 2 slides.

Day 2 Immunohistochemistry procedures

13. Allow sections to reach room temperature.
14. Wash in PBS pH 7.4 for 5 minutes (X3).
15. Incubate with biotinylated secondary antibody (Vector Laboratories, United States of America) for 30minutes.
    - Prepare biotinylated secondary antibody in the preceding third PBS wash. 100 µl is required for every section on one slide and it is prepared by diluting the secondary antibody at a ratio of 1:1000. Therefore, 1 µl of secondary antibody is added to 999 µl of PBS giving 1000 µl sufficient for 10 slides.

15. Wash in PBS pH 7.4 for 5 minutes (X3).

16. Incubate with Avidin Biotin Complex for 30 minutes.

- Prepare the ABC complex in the last 10 minutes before the first wash of PBS. 100 µl is required for every section on one slide and it is prepared by adding 20 µl of solution A to 20 µl of solution B in 1000 µl of PBS. This is sufficient for 10 slides having 100 µl each.

17. Wash in PBS pH 7.4 for 5 minutes (X3)

18. Incubate with DAB working solution 5 minutes (Add 100 µl on each slide)

19. Rinse in running tap water for 5 minutes.

20. Counter stain in Haematoxylin for 1 minute.

21. Wash in running tap water for 5 minutes.

21. Dehydrate:

    a. Gently dip 10 times in 70% alcohol.
    b. Gently dip 10 times in 95% alcohol.
    c. Gently dip 10 times 100% alcohol.
    d. Clear mounted slides in 2 changes of xylene, 5 minutes each.
    e. Coverslip section in section with entellan solution.

Appendix XII: List of ingredients, reagents and chemicals

1. Atripla®, Dischem Pharmacies, South Africa.
2. Labchef Nutrition, Northwest, South Africa.
3. (Rabbit polyclonal) Androgen receptor primary antibody [Ab74272], Abcam, United Kingdom.
4. Biotinylated goat anti-rabbit secondary antibody (Vector laboratories, United States of America).
5. Avidin Biotin complex (ABC kit), (Vector laboratories, United States of America).
6. Brown Sugar (Pick and Pay supermarket, South Africa).
7. Bovril (Pick and Pay supermarket, South Africa).
8. Gelatine (Pick and Pay supermarket, South Africa).

Merck, Johannesburg, South Africa

9. Absolute Alcohol, Merck, Johannesburg, South Africa.
10. Entellan working solution, Merck, Johannesburg, South Africa.

Associated Chemical Enterprises, Johannesburg, South Africa

11. Formaldehyde.
12. Di-sodium hydrogen phosphate.
13. Tri-Sodium citrate (dehydrate).
14. Sodium chloride.
15. Potassium chloride.
16. Potassium dihydrogen phosphate.
17. Acetone.
18. Xylene.
19. Chloroform.

Sigma Aldrich, Johannesburg, South Africa

20. Diaminobenzidine tetrachloride (DAB).
21. 2% 3-aminopropyltriethoxysilane.
22. Picric acid.
23. Glacial acetate.

Appendix XIII: Statistical Analysis

One way analysis of variance	**p value**
1. Androgen receptor immunoreactivity	0.00
2. Total fluid intake	0.00
3. Body weight gain	0.00
Kruskal-Wallis test (non-parametric)	
1. Feed intake	0.00
2. Terminal body weight	0.00
3. Initial weight body weight	0.37
4. Organ weights	
a. Right epididymis	0.14
b. Right and left epididymis	0.26
c. Right and Left Testis	0.06
d. Right and left Kidneys	0.65
e. Livers	0.00
5. Relative organ weights	
a. Right epididymides	0.00
b. Right and left epididymides	0.00
c. Right and left epididymis	0.00
d. Right and Left Testis	0.09
e. Right and Kidneys	0.02
6. Connective tissue area fraction	0.67
7. Testosterone hormone concentrations	0.89
8. Morphometry indices	
a. ETD	0.00
b. A_C	0.78
c. EH	0.04

Appendix XIV: Equipment and software packages

1. Automated tissue processor (Shandom, Citadel 10000)
2. Plastic cassettes (Merk, South Africa)
3. Water bath (Leica, Germany)
4. Digital Balance:
    a. (Kern and Sohn Gmbh, Germany), serial: 8423167
    b. (Snowrex digital scale, Germany)
    c. (Radwag, Poland)
5. Laboratory glass ware (beakers, measuring cylinders, flasks): Duran, Germany
6. Bench centrifuge (Hettich Rotofix, Germany)
7. Microtome (Leica, Germany), serial: 07390329
8. Axioscope microscope (Carl Zeiss Microscopy, Gmbh, Jena, Germany)
9. Embedding station (Miles Scientific, Germany), serial: 31602-554
10. Microscope tissue slides (Lasec, South Africa)
11. Laboratory fume hood (ESCO, Germany), serial: 2011-60070

Software packages

1. 4 Parameter Logistics (My Assays, Germany)
2. Carl Zeiss (Axio-vision SE64 REL 4.9.1) image analyser software
3. Fiji, Image J® software
4. GraphPad Prism® version 5.02
5. IBM SPSS version 24
6. Microsoft Excel 2010 (Microsoft Corporation)

IN-PATIENT MORTALITY ASSOCIATED WITH PNEUMOCOCCAL MENINGITIS IN ADULTS IN SOWETO

Omphemetse Moedi

This Thesis is submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Medicine.

Johannesburg 2018

Corresponding Authors:

1. Dr. Omphemetse Moedi

Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa. P O Box 503172, Gaborone, Botswana. Email: omoedi2000@yahoo.com

2. Professor. Alan Karstaedt

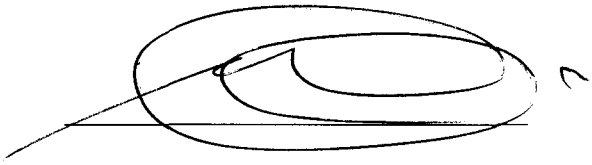
Division of Infectious Diseases, Department of Medicine, Chris Hani Baragwanath Academic Hospital, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa.

Abstract word count: 250

Total word count: 3352

DECLARATION

I Omphemetse Moedi declare that this research report is my own work. It is being submitted for the degree of Master of Medicine (in the submissible format with the protocol and literature review) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



8th day of NOVEMBER . 2018

PUBLICATIONS ARISING FROM THIS RESEARCH PROJECT

This research is also being prepared for submission to the Southern African Journal of Infectious Diseases.

ABSTRACT

Background

Bacterial meningitis caused by Streptococcal pneumonia has a high mortality especially in Africa (e.g. in Malawi, Burkina-Faso), where the reported rates are 41-54% [1]. Few studies on prognostic factors in high HIV prevalence areas have been done.

Objectives

To describe the baseline characteristics, the mortality rate, and prognostic markers for pneumococcal meningitis in adults in Soweto.

Methods

This was a retrospective study which involved secondary analysis of a database with additional selected laboratory data for blood and cerebrospinal fluid (CSF).

We studied patients aged ≥14 years with pneumococcal meningitis confirmed by CSF culture admitted to Chris Hani Baragwanath hospital between August 2013 to July 2017. We examined prognostic factors for death by logistic regression.

Results

Among 141 patients, 77% had identifiable risk factors, and 73% were HIV-infected (87% of those tested) with a median CD4-count of 180 cells/μL. The median CSF neutrophil count was 84 cells/μL. Twenty six-percent had penicillin-resistant pneumococcus, and 66% had non-PCV13 serotypes. The mortality rate was 45.4%. On univariate analysis, there was a significant association between mortality and Glasgow coma scale (GCS) (p=<0.001), antimicrobial therapy (p= 0.028), presence of risk factors (p=0.030), haemoglobin (p= 0.014), platelet count (p= 0.010), and C-reactive protein (p= 0.015). On multivariate analysis, only a high GCS was significant (OR 0.309, CI 0.121-0.781)

Conclusion

Adult pneumococcal meningitis mortality rate in South Africa is comparable to other African countries. GCS can be used as a prognostic marker as it has shown a statistically significant association with mortality in a multivariate model.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Professor Alan Karstaedt for working tirelessly, his patience and guidance to make this research a success. To my beloved wife for her unconditional support and assistance at these testing times. To my daughter, I love you both.

Lastly, I would to acknowledge GERMS-SA (The Group for Enteric, Respiratory, and Meningeal Disease Surveillance in South Africa) of the National Institute for Communicable Diseases for allowing me to analyse their data dictionary and using it as part of my research, specifically Dr. Vanessa Quan of GERMS-SA, Professor Anne Von Gottberg and Linda de Gouveia of the Center for Respiratory Disease and Meningitis. I thank you for your assistance.

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LIST OF TABLES

NOMENCLATURE

ABM:	Acute bacterial meningitis
ART:	Anti-retroviral therapy
CHBAH:	Chris Hani Baragwanath Academic Hospital
CSF:	Cerebrospinal fluid
GERMS- SA:	Group for Enteric, Respiratory, and Meningeal Disease Surveillance in South Africa
HIV:	Human immunodeficiency virus
HB:	Haemoglobin
PM:	Pneumococcal meningitis
VL:	Viral load

BACKGROUND

Acute bacterial meningitis (ABM) is a suppurative infection of the central nervous system (CNS) that affects the meninges and subarachnoid space. *Streptococcus pneumoniae* is the commonest cause of ABM in adults in Malawi and the USA [1, 2].

Risk factors in adults for invasive pneumococcal disease include human immunodeficiency virus (HIV), splenectomy, diabetes mellitus, alcoholism, malignancy, traumatic CSF (cerebrospinal fluid) leak, cochlear implant, chronic kidney and liver disease, multiple myeloma, and use of immunosuppressive drugs [3-8]. Independent predictors for mortality include a low Glasgow coma scale (GCS) on admission, occurrence of seizures, a CSF leukocyte count <1000 cells/mm^3, and a high CSF protein concentration [1, 8-10].

The mortality rate from pneumococcal meningitis (PM) varies between 20 and 37 % in high-income countries and is 54% in Malawi[1].

Since the introduction of conjugate pneumococcal vaccines into childhood immunisation programmes, there has been a decline in invasive pneumococcal disease in vaccinated children as well as in unvaccinated adults through a herd effect. In South Africa, from 2005-2012, there was a reduction of 89% in the incidence of invasive pneumococcal disease caused by vaccine serotypes and an 82% reduction in those caused by penicillin non-susceptible serotypes in children less than 2 years of age. Invasive pneumococcal disease decreased by more than 50% in unvaccinated adults aged 25-44 years and by more than 30% in unvaccinated children, highlighting the herd effect [11]. South Africa introduced the 7-valent conjugate vaccine (PCV7) in 2009 and the 13-valent conjugate vaccine (PCV13) in 2011 [12].

For meningitis caused by penicillin-resistant pneumococci, penicillin levels in CSF are insufficient [13]. Penicillin resistance in pneumococci often coincides with a decreased susceptibility to other antimicrobial agents, and multidrug-resistant bacteria have been reported to result in treatment failures for patients with pneumococcal meningitis [13]. Therefore, empirical therapy for pneumococcal meningitis generally consists of an expanded-spectrum cephalosporin (cefotaxime or ceftriaxone), as well as vancomycin if there is ceftriaxone resistance in the area, until *in vitro* susceptibility is known[8]. A South African study found that factors associated with ceftriaxone non-susceptibility included β-lactam use in preceding 24hrs and PCV13 vaccine serotypes [14]. They also found that being HIV-infected and infection with ceftriaxone non-susceptible serotypes was associated with increased mortality [14].

Since 2002, three large trials have been performed to evaluate the role of adjunctive dexamethasone therapy for adults with community-acquired bacterial meningitis[5, 15, 16]. A European trial showed a clear reduction in mortality for all suspected bacterial meningitis patients, while trials in Malawi and Vietnam did not. The Vietnam trial, however, did show a decreased rate of mortality for patients with confirmed bacterial meningitis. Currently, adjunctive dexamethasone is advised for patients with suspected bacterial meningitis in high-income countries [17].

Meningitis is reported as one of the leading causes of death in HIV-infected patients in Sub-Saharan Africa [18]. Despite advances and roll out of anti-retroviral therapy in South Africa, the burden of invasive pneumococcal disease had not decreased among the HIV-infected in the first 6 years of ART (antiretroviral therapy) [19]. In Malawi, the increased prevalence of HIV-infection has been associated with a 4-fold increase in medical admissions annually and higher fatality rates of meningitis [20].

The aim of the study was to describe the baseline characteristics, the mortality rates, and prognostic markers for pneumococcal meningitis in the adult population admitted to Chris Hani Baragwanath Academic hospital (CHBAH).

MATERIALS AND METHODS

Study site

The study was conducted at Chris Hani Baragwanath Academic Hospital (CHBAH), which is the third largest hospital in the world. It has a bed occupancy of more than 2 800 beds. It is a public sector academic hospital which serves the population of Soweto in Johannesburg, South Africa. Soweto is a township area with a population of more than 2 million.

Study design

This was a retrospective study of patients aged ≥14 years with pneumococcal meningitis, confirmed by CSF culture, admitted to CHBAH between August 2013 and July 2017. With secondary analysis of a database from GERMS-SA, we inputted additional selected laboratory data (cerebrospinal fluid (CSF) and blood tests) from the National Health Laboratory Service (NHLS) Trakcare online website.

The Group for Enteric, Respiratory, and Meningeal Disease Surveillance in South Africa (GERMS-SA) is a nationwide network of clinical microbiology laboratories (both in the public and private sector) which participate in laboratory-based surveillance programme for pathogens of public importance and is based at the National Institute for Communicable Diseases (NICD). GERMS-SA has enhanced surveillance sites at 25 public sector hospitals (CHBAH is one of them) in all 9 provinces of South Africa. These sites collect prospective demographic and clinical data. The Respiratory and Meningeal Pathogens Reference Unit (RMPRU), collaborates with GERMS-SA, and functions as a national and African reference centre for communicable respiratory diseases and meningitis.

Patients were excluded if there was a bloody tap, if there was co-infection in the CSF with another bacterium, fungus or virus, and if there was CSF evidence of meningitis but the pneumococcus was cultured only in blood and not in CSF.

Study population and data collection

We included demographics (age, gender), outcome (death or discharge and time to death), predisposing diseases, concurrent tuberculosis, CD4 count, viral load (VL) and a history of anti-retroviral therapy (ART) for HIV-infected patients, Glasgow coma scale, CSF and blood results, and serotype, and antibiotic sensitivities of the pneumococcus.

Additional laboratory data comprised CSF cell count, protein and glucose; and baseline blood tests including full blood count, albumin, alanine transaminase (ALT), C-reactive protein (CRP), sodium (Na) and creatinine.

Cerebrospinal fluid microbiology

S. pneumoniae was identified by standard laboratory methods. Isolates were sent to the NICD for serotyping by Quellung reaction using serotype-specific antisera (Statens Serum Institut, Copenhagen, Denmark). Antimicrobial susceptibility testing for penicillin and ceftriaxone was based on the broth microdilution method using commercially prepared Sensititre-SASP2 panels (Trek Diagnostics Inc., Cleveland, OH). Isolates were non-susceptible to penicillin if the MIC was $\geq$0.12 mg/L and ceftriaxone sensitive with MIC <0.5 mg/l according to Clinical and Laboratory Standards Institute (CLSI) breakpoints. PCV13 serotypes were 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F.

End points (outcome analysis)

The outcome measure was in-patient mortality in patients with pneumococcal meningitis or survival to discharge. Baseline patient characteristics and laboratory parameters were evaluated as prognostic indicators for in-patient mortality.

Ethics approval

The study was approved by the Committee for Research on Human Subjects, University of the Witwatersrand (permission no. M170964). Because this was a retrospective study with secondary analysis of an existing database, individual patient consent was not required.

Statistical analysis

STATA/IC® version 14.2 software (Stata Corporation, College Station, TX 77845, USA) was used for data analysis. Clinical and laboratory parameters were evaluated as possible prognostic indicators for in-patient mortality in pneumococcal meningitis.

Binary variables were compared by Pearson chi square test or by Fisher's exact test. Continuous variables were compared by the Student T test or unequal variances t-test with Welch's approximation when they were normally distributed. Where continuous variables were not normally distributed, the Mann Whitney's U test was applied. Medians and inter-quartile range (IQR) were also reported for certain parameters and for comparison of the two populations Wilcoxon rank-sum test was used. All statistical tests were 2 sided and a p-value of $\leq$0.05 was considered significant.

Multivariate logistic regression was built using a forward step selection with Pe (p-value to enter into model) of 0.2. The final model was run with statistically significant variables and those with p-values <0.2. The strength of association was reported in odds ratio, with 95% confidence intervals (CI).

RESULTS

Baseline characteristics

The sample was made up of 141 patients with pneumococcal meningitis, of whom 64 patients (45.4%) died, at a median of 4 days after admission (Table 1). The median stay for the patients who survived was 15 days. The patients were generally young with a median age of 40 years ranging from 14-68. There was a slight female preponderance. There were 109 (77%) patients with predisposing diseases. There were 103 (73%) HIV-infected patients (89% of those tested), while 13 (9%) were HIV non-infected and 25 (18%) had unknown serostatus. For the 68 (66%) patients with a known CD4 count, 36 (53%) were ≤ 200 cells/mm^3. Eight patients were known to be on anti-tuberculosis therapy. Another 8 patients had another predisposing illness, 6 of whom were HIV-infected. Two patients each had malignancy, cerebrovascular accident and diabetes mellitus and 1 each had pancreatitis and head injury. A decreased level of consciousness was present in 70 (72%) of 97 patients with data. Information on treatment of the meningitis was available for only 110 (78%) patients. All treated patients were initiated on empiric third generation cephalosporins. Among the 8 patients who did not receive antibiotics, one patient survived.

The median neutrophil count in CSF was 84 cells/µl. CSF neutrophils of >100 cells/µl was found in 61 patients; 32 of them survived whilst 29 died. A total of 32 patients had CSF neutrophils <20 cells/µl: 22 of them survived whilst 10 demised. 23 patients had CSF neutrophils of >1000 cells/µl: 13 of them survived whilst 10 died.

The commonest pneumococcal serotypes/serogroups of the 101 identified were 8 (13 patients), 23 (11), 12 (10), 15 (8), 1 and 6 (7 each), and 10 (6). Non-vaccine PCV-13 serotypes accounted for 66% of cases. Penicillin resistance was found in 24 (26%) of 93 patients. None were ceftriaxone resistant. Childhood serotypes (6, 14, 19 and 23) were found in 14 resistant patients, as well as serotypes 15A, 35B, 3, 9V, and 15B.

The HIV-infected patients are compared to the HIV non-infected in Table 2. Comparing the HIV non-infected to the HIV infected, there was a clear male predominance, the GCS was significantly higher ($p<0.001$), the median CSF neutrophil count was 211.5/ µl compared to 90.5/µl ($p=0.116$), penicillin resistance was less common (13% vs. 28%), and the haemoglobin level was significantly higher ($p=0.014$).

Prognostic factors for mortality on univariate analysis

In a univariate analysis 7 parameters had a significant association with mortality: GCS (and mental status), antibiotic use in this admission, presence of risk factors, duration of hospital stay, haemoglobin, platelets and CRP levels. In a multivariate analysis, only GCS turned out to have a significant association with mortality (p, 0.013). A multivariate model is depicted in table 3: mental status was omitted because it is similar to GCS, both CD4 and viral load were rejected by the model because there were few participants for the parameter. Age was forced into the model because it is clinically relevant.

Tables 1 shows the differences between the survival group and the cohort of patients who died. Mental status is important as 25 of the 27 patients who were alert on presentation made a complete recovery and this is significant (p, <0.001). The median GCS of the survival

group was higher 14 versus 13 in those who died (p, <0.001). The presence of risk factors (including retroviral status) was associated with mortality (p, 0.030).

Antibiotic use was protective of death with an OR of 0.9 and this was significant with a p-value of 0.028 (7 of 8 patients not instituted on antibiotic demised). All the patients were started on a third-generation cephalosporin.

Patients who recovered had lower haemoglobin levels (p-value 0.014), higher platelets (p-value 0.010) and higher CRP (p, 0.015)

Predisposing risk factors for pneumococcal meningitis (PM) other than HIV infection was found in 8 patients. Four of them died. A small number of patients were noted to have either smoked (15) or used alcohol (8), neither was a significant predictor of mortality.

HIV parameters and therapy of our cohort patients

In the HIV-positive group, the median CD4 count of the patients who survived was slightly higher 210 cells/μl and they had lower viral load levels 2 574 copies/ml. The 5 patients with a CD4 count more than 500 cells/μl survived (p, 0.393). Being on ART was not significant (p, 0.464). Only 32 patients were on ART. Twenty patients known to be on ART had a median CD4 count of 105 cells/μl (IQR 81-277) compared to 224 cells/μl (IQR 104-321) in 35 patients not on ART, p value 0.151. Similarly, 15 patients known to be on ART had recorded viral loads with a median 333 copies/ml (IQR 0-87 000) compared to 21 patients not on ART with a median VL 17 300 (IQR 1210-140 000), p value 0.117. Only 5 of the patients known to be on ART had virological control.

DISCUSSION

The high mortality of 45.4% in this cohort of adults with pneumococcal meningitis is in keeping with that in other studies from sub-Saharan Africa. In Malawi the mortality was 53.4% while it was 69.8% in Senegal[18]. This compares to 11% in Spain and 31% in the Netherlands[8]. Possible reasons for this higher mortality include delayed presentation and management, and possible suboptimal resuscitation and intensive care support[1]. Most common causes of death among patients with pneumococcal meningitis are stroke, status epilepticus, brain herniation and cardiorespiratory failure [13].

Baseline characteristics

This was a generally young population with a median age of 40 years, which is slightly older than that in Malawi (31 years), it is similar to Turkey (45), but younger than the 58 year mean age in the Netherlands. The slight female preponderance was similar to that in Malawi and the Netherlands, while in Turkey, 70% were male, which is equivalent to that in HIV non-infected patients in this cohort. This cohort had a high rate of predisposing disease. Although predisposing diseases were 70% in the Netherlands, they included clinical features like otitis media, sinusitis and pneumonia, and only 22% of patients were immunosuppressed. HIV infection was the predominant underlying disease in this cohort as it was in Malawi, where 87% of patients were HIV seropositive. HIV-infected patients are at an increased risk of developing PM, with relative risk of 20-150 has been reported[21, 22]. The exact mechanism is not known.

The median GCS of our patients was 14, higher than that found in Malawi (12) and Turkey (29.7% of their patients had GCS of 13-15)[23]. The median CSF white cell count of our cohort was 92 cells/μL, significantly lower than that found in Malawi (707 cells/μL) and Netherlands (2530 cell/μL). The median CSF neutrophil count was markedly lower in HIV-infected patients than in non-HIV infected patients, although this was not statistically significant (Table 2). This low CSF cell count may reflect the underlying severe immunocompromised states of our HIV-infected patients. Serotypes 8, 23 and 12 were common in our cohort of patients, whilst 44% had serotype 1 in Malawi, and serotypes 3 and 14 were commonest in Netherlands. There is no dominant serotype in this era of childhood vaccination. Non-PCV 13 serotypes dominated.

Penicillin resistance pneumococcus was found in 26% of our patients (more in HIV-infected than the non-infected) compared to 1% in Netherlands. There was no data for Malawi.

Prognostic factors for mortality:

<u>On univariate analysis</u>

Mental status and or Glasgow coma scale were found to have a strong association with mortality with both variables having a p-value of <0.001. A high proportion of patients who were alert made a complete recovery (93%) as opposed to patients who were stupor/comatose whom the majority succumbed to death (67%). This is in keeping with previous studies in which a high GCS of 13-15 had recovery percentage of up to 86% in Turkey[23].

Omission of antimicrobial therapy had a significant impact on mortality with a p-value of 0.028. It is likely that some did not get antibiotics as they died before receiving it. Most patients were initiated on empiric third generation cephalosporins, and in only a few patients (6 HIV-infected and 2 with unknown HIV status) there was no initiation of therapy, and these patients fared worse than their counterparts as all, but one died. No reason was given in the database for the fact that a patient survived who did not receive antibiotics.

In our study, the only serum laboratory markers that had significant prediction for mortality were haemoglobin (HB), platelets and CRP. Patients who survived had lower HB levels (11.1g/dl) as opposed to those who died (12.3g/dl). Previous studies have found the opposite association between HB and mortality in acute bacterial meningitis[1, 24]. This may reflect fluid hydration state and that the patients from our cohort whom died were generally ill and dehydrated and perhaps had a spuriously high HB level. This is in keeping as patients who demised had higher urea and creatinine levels reflecting a state of dehydration biochemically. Platelets were also an important predictor of mortality as patients who recovered had higher levels of platelets (227 x10^9/L). This may reflect that patients who demised were more acutely ill with sepsis and had disseminated intravascular coagulopathy with low platelets as part of the disease spectrum. Previous studies have shown that laboratory markers which had a significant prediction for mortality were CSF white cell count[24]. ESR was previously related to mortality[8], but in our cohort we could not validate it because of few patients (3) had a recorded ESR. However, CRP was shown to be a significant prredictor of mortality. We could not validate any CSF parameters such as high protein and low neutrophils (low CSF leukocyte <1000 cells/ml in Netherlands) associated with unfavourable outcome as predictors of mortality as previously shown by some studies[8].

<u>On multivariate analysis</u>:

Only GCS was determined to be a significant predictor of mortality. A high GCS was protective of death (OR 0.309, CI 0.121-0.781, coefficient -0.74). Total hospital stay, although having a significant p-value in the multivariate model, is a surrogate marker for survival as all patients would have completed at least 10 days treatment, while the median time to death was 4 days.

HIV parameters and therapy of our cohort patients

In our cohort of patients, a significant number, 103 (73%), had HIV-infection. HIV infection didn't confer a significant risk for mortality. Similarly, HIV serostatus was not a prognostic indicator in Malawi [13].

For HIV-infected patients, a recent CD4 count seemed to be protective of death. This could reflect that the patient was already known to the health system and that the patient possibly had medical attention and therapy instituted to mitigate the immunocompromised state, or that the patient died before a CD4 count could be agreed to and performed. The CD4 count or viral load were not prognostic indicators, although the patients with high CD4 counts >500/µl survived. The fact that only 36 patients were known to be on antiretroviral therapy reflects problems with testing for HIV infection before a serious opportunistic infection or initiation of ART in those tested.

LIMITATIONS AND CONCLUSION

Limitation

This study was based on surveillance data developed for epidemiologic description and not specifically for clinical outcome. Data for most fields was not complete, such as for CD4 count and history of ART, presumably because it was not documented by clinicians or laboratory tests were not done. This could have been because patients were confused and could not give details or permission or died before tests were done. We could not check the accuracy of data. Data did not include clinical presentation or development of complications such as seizures or focal signs, nor did it include the presence of neurological sequelae in those who survived, such as hearing impairment and cognitive dysfunction, or the use of adjunctive treatment like corticosteroids. The Malawi study which was used to compare our findings covered all bacterial meningitis, but PM accounted for 85% of CSF culture positive patients.

Conclusion

In this 3-year study, adults with PM had a high mortality similar to that in other African countries. There was a very high rate of immunosuppressive predisposing disease, mostly HIV infected patients, over half of whom had low CD4 counts. There was a high number of patients with decreased level of consciousness. The CSF neutrophil count was generally much lower than in comparative studies for other countries. Penicillin resistance was found in 26% of patients. There was no dominant pneumococcal serotype or serogroup, but 66% of patients had non-PCV 13 serotypes. This may reflect vaccine serotypes/serogroup replacement. Although 7 factors were statistically significant on univariate analysis, only a low GCS was significant on multivariate analysis. A prospective clinical study is warranted to better evaluate clinical predictive factors for mortality.

Table 1: Clinical and laboratory parameters as predictors of mortality using univariate analysis

		Final outcome of patient's hospital stay				Univariate analysis P-value
		Survived N=77	Died N=64	Total N=141	Missing Cases N	
Gender	Female	46 (59.7%)	28 (43.8%)	74 (52.5%)	0	0.059
	Male	31 (40.3%)	36 (56.3%)	67 (47.5%)		
Age years (median, IQR)		38 (33-44)	41 (33-49)	40 (33-47)	0	0.313
Mental status	Alert	25 (44.6%)	2 (4.9%)	27 (27.8%)	44	**<0.001**
	Disorientated	22 (39.3%)	21 (51.2%)	43 (44.3%)		
	Stupor / comatose	9 (16.1%)	18 (43.8%)	27 (27.9%)		
Glasgow coma scale: total score (/15) (median, IQR)		14 (14-15)	13 (12-14)	14 (12-15)	48	**<0.001**
Predisposing risk factors	Yes	63 (81.8%)	42 (65.6%)	105 (74.5%)	0	**0.030**
	No	14 (18.2%)	22 (34.4%)	36 (25.5%)		
HIV-serostatus	Positive	62 (87.3%)	41 (91.1%)	103 (88.8%)	25	0.531
	Negative	9 (12.7%)	4 (8.9%)	13 (11.2%)		
CD4 count categories/mm^3	0 - 50	6 (12.8%)	3 (14.3%)	9 (13.2%)	73	0.393
	51 - 100	9 (19.1%)	2 (9.5%)	11 (16.2%)		
	101 - 200	9 (19.1%)	7 (33.3%)	16 (23.5%)		
	201 - 350	11 (23.4%)	7 (33.3%)	18 (26.5%)		
	351 - 500	7 (14.9%)	2 (9.5%)	9 (13.2%)		
	>500	5 (10.6%)	0 (0%)	5 (7.4%)		
CD4 count cell/µL (median, IQR)		210 (85-370)	160 (77-269)	180 (85-329)	73	0.144
Viral load copies/ml (median, IQR)		2 574 (127-73 437)	79 820 (213-305 036)	4 485 (213-121 000)	98	0.166
Ceftriaxone	Yes	62 (98.4%)	40 (85.1%)	102 (92.7%)	31	**0.028**
	No	1 (1.6%)	7 (14.9%)	8 (7.3%)		
Days in hospital (median, IQR)		15 (11-17)	4 (2-8)	11 (4-16)	1	**<0.001**
Haemoglobiin (g/dL) (median, IQR)		11.1 (9.1-13.0)	12.3 (10.6-14.1)	11.5 (9.9-13.3)	16	**0.014**
Platelet count (x10^9/L) (median, IQR)		227 (179-296)	190 (123-249)	211 (152-281)	16	**0.010**
White cell count (x10^9/L) (mean, SD)		14.78±7.48	14.74±7.55	14.76±7.48	16	0.975
Albumin g/L (median, IQR)		29 (24-35)	30 (26-35)	29 (24-35)	58	0.543
CRP IU (mean, SD)		216.47±111.65	275.42±115.66	243.54±116.72	43	**0.015**

Sodium mmol/L (mean, SD)		133.25±6.10	133.75±7.67	133.48±6.83	17	0.684
Creatinine mmol/L (median, IQR)		80 (66-98)	87 (68-112)	83 (66-102)	17	0.270
CSF neutrophils (per uL) (median, IQR)		78 (8-480)	86 (12-410)	84 (11-450)	8	0.768
CSF lymphocytes (per uL) (median, IQR)		13 (0-80)	4 (0-50)	8 (0-68)	8	0.131
CSF glucose (mmol/L) (median, IQR)		<0.1 (<0.1-0.8)	<0.1 (<0.1-0.2)	<0.1 (<0.1-0.3)	14	0.121
CSF glucose (MMO/L)	< 0.5	47 (71.2%)	53 (86.9%)	100 (78.7%)	14	0.089
	0.5 - 2.2	10 (15.2%)	5 (8.2%)	15 (11.8%)		
	≥ 2.3	9 (13.6%)	3 (4.9%)	12 (9.4%)		
CSF protein (g/L)		4.25±3.03	5.31±3.13	4.75±3.11	11	0.068
CSF chloride (mmol/L)		111.39±8.89	109.85±8.35	110.62±8.59	75	0.466
Bacteraemic	Both CSF & BC +ve	15 (10.6%)	9 (6.4%)	24 (17.0%)	0	.0396
Serotype or serogroup	PCV 13 Vaccine Serotype	15 (27.8%)	19 (40.4%)	34 (33.7%)	40	0.209
	Non-Vaccine Serotype	39 (72.2%)	28 (59.6%)	67 (66.3%)		

Table 2: Descriptive characteristics of patients with known HIV serostatus and pneumococcal meningitis (n=116)

Variables	*HIV infected*	*n*	*Non-HIV infected*	*n*
Age years (median, IQR)	38 (33-47)	103	41 (33-54)	13
Females' n (%)	55 (53.4%)	103	4 (30.8%)	13
Died	41 (39.8%)	103	4 (30.8%)	13
Disorientated on presentation	38 (46.9%)	81	2 (33.3%)	6
GCS (mean, SD) (range)	13.4 ±1.8 (5-15)	78	13.8 ±1.3 (12-15)	5
Predisposing risk factors other than HIV	6	103	2	13
Received antibiotics in this admission	82	88	8	8
Streptococcus pneumoniae bacteraemia	18 of 103	103	2 of 13	13
CSF neutrophils (median, IQR)	90.5 (11.5-435.5)	96	211.5 (60.5-939.0)	12
CSF protein (mean, SD) (range)	4.4±3.0 (0.2-11.0)	93	4.6±3.2 (1.3-10.4)	12
CSF glucose (median, IQR)	<0.1 (<0.1-0.3)	91	<0.1 (<0.1-0.9)	12
Serum white cell count (mean, SD) (range)	14.9 ±7.6 (2.3-39.7)	94	17.9 ±6.0 (7.8-6.0)	11
Haemoglobin (mean, SD)	11.3 ±2.6	94	12.7 ±2.8	11
Platelets (mean, SD)	217.9 ±108.0	94	244.2 ±116.4	11
Sodium (mean, SD)	132.9 ±6.2	92	134.9 ±6.1	11
Albumin (mean, SD) (range)	29.5 ±7.2 (16-48)	62	36.1 ±8.8 (22-47)	8
CRP (median, IQR)	272 (174-328)	75	241 (148-290)	7
Creatinine (median, IQR))	82 (67-102)	92	89 (62-95)	11
Penicillin resistant pneumococci	19 of 67	67	1 of 8	8

Table 3 Multivariate Logistic regression model

Variable	Multivariate P Value	Odds ratio	95% CI
Male Gender	0.678	1.500	0.221-10.171
Age	0.276	0.950	0.867-1.041
Glasgow coma scale	**0.013**	0.309	0.121-0.781
Total hospital stay	**0.008**	0.771	0.636-0.934
Ceftriaxone use	0.980	1.047	0.028-38.680
Risk factors present	0.133	0.130	0.009-1.856
Haemoglobin	0.712	1.094	0.678-1.765
Platelet count	0.567	1.003	0.992-1.014
CRP	0.139	0.994	0.986-1.002
CSF neutrophils	0.803	0.999	0.990-1.007
CSF glucose	0.394	0.673	0.271-1.673
CSF protein	0.877	0.971	0.667-1.413

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APPENDIX A: ETHICS CLEARANCE CERTIFICATE

R14/49 Dr Omphemetse Moedi

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M170964

NAME: (Principal Investigator)	Dr Omphemetse Moedi
DEPARTMENT:	Internal Medicine Chris Hani Baragwanath Academic Hospital
PROJECT TITLE:	In Patient Mortaliy Associated with Pneumococcal Meningitis in Adults in Soweto
DATE CONSIDERED:	29/09/2017
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Alan Karstaedt
APPROVED BY:	Prof A Woodiwiss, Co-Chairperson, HREC (Medical)
DATE OF APPROVAL:	02/10/2017

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 301, Third floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting the study was initially reviewed. In this case, the study was initially reviewed in September and will therefore be due in the month of September each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX B: MMED PROTOCOL AND EXTENDED LITERATURE REVIEW

In-Patient Mortality Associated with Pneumococcal Meningitis in Adults in Soweto

Dr. Omphemetse Moedi

MB BcH BaO (University of Ireland in Galway)

Registrar, Department of Medicine

University of the Witwatersrand (Student # 883167)

Supervisor: Professor A. Karstaedt

Division of Infectious Diseases, Department of Medicine

Chris Hani Baragwanath Academic Hospital

Faculty of Health Sciences

University of the Witwatersrand

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1.1 INTRODUCTION

Acute bacterial meningitis (ABM) is a suppurative infection of the central nervous system (CNS) that affects the meninges, subarachnoid and brain parenchyma collectively known as meningoencephalitis. It has an annual incidence of >2.5 cases per 100 000 population in the United States. The organisms most commonly implicated in community acquired meningitis are *Streptococcus pneumoniae* (50%), *Neisseria meningitidis* (25%), *Group B streptococcus* (15%), and *Listeria monocytogenes*. *Haemophilus influenzae* accounts for less than 10%. (Dennis Kasper, 2015)

Invasive pneumococcal disease occurs in the extremes of age (less than 2 years or more than 50 years). It is defined as isolation of pneumococcus from normally sterile sites such as cerebrospinal fluid, joint aspirate and blood. Human immunodeficiency virus (HIV), splenectomy, diabetes mellitus, alcoholism, use of immunosuppressive drugs is found in 20% of adults with pneumococcal meningitis. Other predisposing conditions to invasive disease is seen in patients with underlying conditions, such as splenectomy or asplenic states, sickle cell disease, multiple myeloma, hypo-gammaglobulinaemiae, alcoholism, chronic liver or kidney disease, malignancy, malnutrition, Wiskott-Aldrich syndrome, thalassemia major, diabetes mellitus, and basilar skull fracture with leakage of cerebrospinal fluid (CSF); and in children with cochlear implants. (Mook-Kanamori et al., 2011)

In the Dutch Meningitis Cohort study, an observational prospective study it was found that 245 out of 343 cases (70%) had underlying disorders. They also found that the mortality rate for *S. pneumoniae* was 20%, 3-7% for *H. influenzae*, *N. meningitidis*, or *Group B streptococcus*; and 15% due to *L. monocytogenes*. Determinants and risk factors for mortality were related directly to decreased level of consciousness on admission, onset of seizures within 24 hrs of admission, signs of increased intracranial pressure (ICP), younger age (infancy) and age >50 years, the presence of comorbid factors including shock or need for mechanical ventilation and delay in initiation of treatment and markedly raised CSF protein of >3g/L. Common sequelae included decreased intellectual function, memory impairment, seizures, hearing loss, dizziness and gait disturbances. (Weisfelt et al., 2006b)

1.2 PATHOGENESIS WHICH PUT *S. PNEUMONIAE* TOP IN ACUTE BACTERIAL MENINGITIS

S. pneumoniae is the most common cause of meningitis in adults and accounts for almost half of the reported cases (1.1 per 100 000 persons per year). (Dennis Kasper, 2015). The human nasopharynx is the main reservoir for *S. pneumoniae*, where it usually leads to asymptomatic colonisation. Carriage rates of *S. pneumoniae* are highest among young children (37%) and may rise to 58% in crowded situations such as day care centres. In adults, crowding may also lead to increased carriage rates, specifically in hospitals, long term care facilities, shelters, and prisons, where carriage rates of up to 40% have been reported, compared to 4% in the general adult population. The bacterium is transferred between people mainly by coughing and sneezing. During colonisation, adherence, nutrition, and replication are the pneumococcus' main priorities. (Mook-KanamoriGeldhoffvan der Poll et al., 2011)

S. pneumoniae colonise the nasopharynx by attaching to the epithelial cells. The bacteria are then able to enter the blood stream by one of two ways: they can be transported across epithelial cells in membrane-bound vacuoles to the intravascular space, or invade the space directly by creating separations in the apical tight junctions of columnar epithelial cells. Once in the bloodstream, the bacteria are able to avoid phagocytosis by neutrophils and classic complement-mediated bactericidal activity because of the presence of a polysaccharide capsule. Blood borne bacteria can reach the intraventricular choroid plexus, directly infect choroid plexus epithelial cells, and gain access to the CSF. *S. pneumoniae* can adhere to cerebral capillary endothelial cells and subsequently migrate through or between these cells to reach the CSF. Bacteria can multiply in the CSF because of the paucity of effective host immune defences in the CSF. Normal CSF contains few white blood cells (WBCs) and relatively small amounts of complement proteins and immunoglobulins. The paucity of the latter two prevents effective opsonisation of bacteria, an essential prerequisite for bacterial phagocytosis by neutrophils. Phagocytosis of bacteria is further impaired by the fluid nature of CSF, which is less conducive to phagocytosis than a solid tissue substrate. (Dennis Kasper, 2015)

A critical event in the pathogenesis of bacterial meningitis is the inflammatory reaction induced by the invading bacteria. Many of the neurological manifestations and complications of bacterial meningitis result from the immune response to the invading pathogen rather than from direct bacteria induced tissue injury. The lysis of bacteria and subsequent release of cell-wall components into the subarachnoid space is the initial step in the induction of the inflammatory response and the formation of a purulent exudate in the subarachnoid space. Bacterial cell-wall components of *S. pneumoniae* (such as peptidoglycan), induce meningeal inflammation by stimulating the production of inflammatory cytokines and chemokines by microglia, astrocytes, monocytes, microvascular endothelial cells, and CSF leukocytes. (Dennis Kasper, 2015)

Invasive pneumococcal disease may take place when two situations coincide: first, the host is colonised with a pneumococcal strain that it has not yet established immunity to, and second, an alteration of the natural barriers or host immune system has occurred. Furthermore, damage to the naso- and oropharyngeal mucosae may be elicited by local pneumococcal infection, such as sinusitis or otitis, by viral respiratory infections (especially by influenza virus), by smoking, or by allergy. (Mook-KanamoriGeldhoffvan der Poll et al., 2011)

Human observational studies have repeatedly found long term sequelae after pneumococcal meningitis, including sensorimotor deficit, hearing loss, and cognitive impairment, which may occur in up to 30% of surviving patients. (Weisfelt et al., 2006a). Human histopathological data showed that the parenchymal damage was caused by increased ICP, cytotoxic and vasogenic oedema, herniation and local leukocyte infiltration or abscess formation, as well as by cortical necrosis and hippocampal neuronal loss. Cerebrovascular complications are very common during pneumococcal meningitis: Arterial strokes occur in up to 30% of patients, cerebral venous thrombosis in 9%, and intracerebral haemorrhage in up to 9%. Autopsy studies in the1930s through 1960s showed inflammatory infiltrations of cerebral arteries and veins. Taking these together with angiographic descriptions of segmental arterial narrowing in patients with ischemic stroke complicating pneumococcal meningitis,

the general assumption has been that infarctions during ABM are caused by vasculitis. (Mervyn D.I. Vergouwen, 2010)

In a recent human histopathological analysis of patients with pneumococcal meningitis, among whom half of patients had evidence of cerebral infarctions and 67% showed microhaemorrhages, there was no evidence of large-vessel vasculitis. Moreover, the observed small-vessel vasculitis did not localize with areas of infarction, and in this series, no evidence of disseminated intravascular coagulation in the systemic compartment was observed. (Mervyn D.I. Vergouwen, 2010). There is the possibility of cerebral intravascular coagulation, independent of systemic coagulopathy or cerebral vasculitis, as the cause for both cerebral infarctions and haemorrhages. The pathogenesis of cerebral infarction remains unclear and is the subject of ongoing research, which has focused largely on two areas: first, the dysregulation of the coagulation and fibrinolytic pathways, not only systemically but also locally, as exemplified by the upregulation of PAI-1 and elevated levels of prothrombin fragments F1 and -2 and soluble tissue factor in the CSF of patients with pneumococcal meningitis; and second, endothelial cell dysfunction, which may lead to localized swelling and release of procoagulant factors and proinflammatory cytokines. (Pfister et al., 2000)

1.3 VACCINATION AND SEROTYPES

Community acquired bacterial meningitis continues to exact a heavy toll, even in developed countries, despite the implementation of childhood vaccination programmes and effective antimicrobial agents. Despite advances in medical care, mortality from pneumococcal meningitis ranges from 16 to 37%, and neurological sequelae, including hearing loss, focal neurological deficits, and cognitive impairment, are estimated to occur in 30 to 52% of surviving patients. (Mook-KanamoriGeldhoffvan der Poll et al., 2011)

In 2009 South Africa (SA) became the first African country to adopt the 7-valent pneumococcal vaccine (PCV7) into its immunisation programme. (Madhi et al., 2012). The novel three-dose regimen is given to infants at six and fourteen weeks, and a booster at nine months. The 13-valent pneumococcal vaccine (PCV13) replaced the PCV7 in April 2011. Laboratory monitoring of incidents of invasive pneumococcal disease began in SA in 1999. (MadhiCohen and von Gottberg, 2012). The Group for Enteric, Respiratory, and Meningeal Disease Surveillance in South Africa (GERMS-SA) has used observational data as part of a surveillance study to examine the effect of PCV7 introduction in both the HIV infected and non-infected. The period of study was from January 2005 to December 2012. 24 sentinel hospitals were enrolled from all 9 provinces. The laboratory-based surveillance system showed reduction of 89% incidence of invasive pneumococcal disease caused by PCV7 serotypes and 82% reduction in those caused by penicillin non-susceptible serotypes within four-years of introduction in children less than 2years of age. In the same period of study, they managed to show that invasive pneumococcal disease decreased by more than 50% in unvaccinated adults of 25-44 years and more than 30% decrease in unvaccinated children, highlighting the herd effect. (von Gottberg et al., 2014)

1.4 RESISTANCE AND TREATMENT

The classical triad of fever, nuchal rigidity, and altered mental status is found in 60% of patients. The increase of drug-resistant pneumococci has become an emerging problem worldwide, with a reported prevalence of penicillin-resistant strains of up to 35% in some regions of the United States. Penicillin resistance in pneumococci often coincides with a decreased susceptibility to other antimicrobial agents, and multidrug-resistant bacteria have been reported to result in treatment failures for patients with pneumococcal meningitis. Although pneumococci with low to intermediate susceptibility to penicillin may respond well to monotherapy with penicillin in adequate dosages in non-meningitic infections, levels in CSF are expected to be insufficient to kill highly resistant organisms. Therefore, empirical therapy for pneumococcal meningitis should consist of an expanded-spectrum cephalosporin (cefotaxime or ceftriaxone), and vancomycin if there is ceftriaxone resistance in the area, until *in vitro* susceptibility is known. (Brouwer et al., 2010)

The roles of newer B-lactam antibiotics (cefepime, meropenem, and ertapenem), quinolones (garenoxacin, gemifloxacin, gatifloxacin, and moxifloxacin), and lipopeptides (daptomycin) are being explored in experimental meningitis studies, with a special emphasis on the treatment of infection with highly resistant pneumococcal strains. The efficacy of antibiotics could be enhanced by combining synergistically acting agents (e.g., cephalosporins, vancomycin, and rifampin). (BrouwerTunkel and van de Beek, 2010)

Since 2002, three large trials have been performed to evaluate the role of adjunctive dexamethasone therapy for adults with community-acquired bacterial meningitis. A European trial showed a clear reduction in mortality for all suspected bacterial meningitis patients, while trials in Malawi and Vietnam did not. The Vietnam trial, however, did show a decreased rate of mortality for patients with confirmed bacterial meningitis. Currently, adjunctive dexamethasone is advised for patients with suspected bacterial meningitis in high-income countries. Activated protein C has been shown to decrease mortality for patients with severe sepsis but cannot be advised for those with a low risk of death due to increased bleeding complications. (BrouwerTunkel and van de Beek, 2010)

Adjunctive glycerol (glycerine 1-2-3-propanetriol), a hyperosmolar compound, is an essential component of cell membranes. It has been used in neurosurgery, neurology, and ophthalmology to decrease raised tissue pressures. Toxicological data show that it is safe and is associated with few, rare, mild, mostly gastrointestinal side effects. Furthermore, glycerol is inexpensive and readily available, facilitating widespread implementation if it is effective. The effects of glycerol in the neurological/neurosurgical setting have been hypothesized to lie in the resulting increase of plasma osmolality, which was shown to reduce the excretion of CSF by some 20 to 30%, leading to increased cerebral blood flow and improvement of brain oxygenation. Though glycerol seems attractive as a potential adjuvant treatment for bacterial meningitis, the lack of effectiveness in experimental models, combined with its harmful effects in the Malawi trial, question the value of additional studies on adjunctive glycerol for the treatment of adult meningitis. (BrouwerTunkel and van de Beek, 2010)

A retrospective study from Turkey investigated independent predictors of the outcome in pneumococcal meningitis with special emphasis on therapeutic implications. Following the diagnoses of bacterial meningitis on admission, 243 patients received ceftriaxone, five patients received cefotaxime, and 58 patients received ceftriaxone plus vancomycin

empirically. The mean time elapsed between hospitalization and the institution of antibiotics was 3 h (SD 7, range 0.5–72 h) and did not differ significantly between antibiotic regimens. Therapy was de-escalated in 28 patients; of these patients, two (7.1%) died. Therapy was escalated in 28 patients. In 13 of these patients, vancomycin was added to the therapy following the isolation of penicillin-resistant pneumococcus (PenRP). Three of these 13 (23.7%) patients died, and six out of the 10 remaining patients had one of the therapeutic failure parameters other than penicillin resistance. Neither escalating nor de-escalating treatment was found to have a significant effect in terms of the outcome. All 306 isolates had been screened with oxacillin. A total of 241 (78.7%) isolates were susceptible to oxacillin, while 65 (21.2%) isolates were found to be resistant. Among oxacillin-resistant isolates, 43 (14%) were further tested for penicillin MICs and 38 were found to be penicillin-resistant. The remaining 22 oxacillin-resistant isolates were not tested for the penicillin minimum inhibitory concentration (MIC), hence they were not confirmed as penicillin-resistant. Eventually, 246 (79.1%) isolates were assigned as penicillin-sensitive pneumococcus (PenSP) and 60 isolates were assigned as Oxacillin-resistant pneumococcus, of which 38 (12.2%) were PenRP; 22 (7.1%) remained undetermined in terms of penicillin susceptibility. A total of 42 out of the 306 patients died of pneumococcal meningitis within the 30-day follow-up period. The crude mortality rate (CFR) was 13.7%. (Erdem et al., 2014)

1.5 MORTALITY RATES IN PNEUMOCOCCAL AND OR BACTERIAL MENINGITIS

The associated rate of mortality of pneumococcal meningitis is high. For adults with pneumococcal meningitis, reported case-fatality rates vary between 20 and 37% in high-income countries and up to 51% in resource-poor areas (e.g., Malawi). Most common causes of death among patients with pneumococcal meningitis are cardiorespiratory failure, stroke, status epilepticus, and brain herniation. (BrouwerTunkel and van de Beek, 2010). Following the introduction of penicillin in the 1950s mortality rates from ABM in resource rich settings have improved from 45–50% to 11–25% in the last decade. This improvement is associated with early institution of antibiotic therapy and better supportive care. In contrast, adult ABM mortality rates in sub-Saharan Africa have been reported to vary between 54–70% without any change over time and survivors experience significantly higher rates of disabling neurological sequelae compared to European patients (Table 1). (Wall et al., 2013).

Three studies have detailed prognostic factors for mortality/outcome in acute bacterial meningitis. The studies have derived and validated some bed side risk scores for adverse outcome. These are depicted in Table 2, 3 and 4 below.

Table 1. Mortality rates from African countries versus Europe & United States.

MAIN AUTHOR	TYPE / STUDY YEAR	COUNTRY / REGION	ABM/PM	STUDY SIZE	MEDIAN AGE	MORTALITY (%)	ANNUAL INCIDENCE OF PM Per 100 000
(Gordon et al., 2000)	Prospective 1998-1999	Blantyre, Malawi	ABM	996	Not stated	41% in patient hospital mortality. PM 23.8%	
(WallCartwrightScarborough et al., 2013)	Retrospective 1990-2012	Blantyre, Malawi	ABM	715	31	45% AT DAY 10 54% AT DAY 40	12 per 100 000
(Traore et al., 2009)	Prospective 2002-2006	Burkina Faso and Togo	ABM	2689	>15yrs	47% for PM	14 cases per 100 000, 17% of specimens were due to *S. pneumoniae*
(Aronin et al., 1998)	Retrospective 1970-1995	Connecticut, United States	ABM	269	57	Hospital mortality rate was 27%	48% of specimens were due to *S. pneumoniae*
(Kastenbauer and Pfister, 2003)	Retrospective 1984-2002	Munich, Germany	PM	87	50	24.1% in hospital mortality	
(Weisfeltvan de BeekSpanjaard et al., 2006b)	Prospective 1998-2002	Amsterdam, Neitherlands	PM	352	58	30% (107) died in this cohort study. Most of them 79% (85) of them died within 2 weeks of admission	
(ErdemElaldiOztoprak et al., 2014)	Retrospective 1998-2012	Turkey	PM	306	45	13.7% died within a 30-day follow up	
(Johnson et al., 2007)	Retrospective 1998-2005	England	PM	3840	56	Case fatality rates increasing from 5% in <15 years and 30% in >64 years	1 case per 100 000

ABM acute bacterial meningitis, PM pneumococcal meningitis.

Table 2. Independent Risk factors for an unfavourable outcome in adults with acute bacterial meningitis in Amsterdam, Netherlands. *(n=694).* (Weisfelt et al., 2008)

CHARACTERISTIC	OR (95% CI)
Age, yr*	1.24 (1.1-1.38)
Heart rate >120 beats/min	2.97 (1.67-5.28)
GCS score**	0.87 (0.82-0.93)
Cranial nerve palsies	2.48 (1.45-4.24)
White-cell count < 1000/mm3	3.91 (2.50-6.11)
Gram stain	
Gram-positive cocci	3.63 (2.18-6.04)
Gram-negative cocci	1.00 (reference)
Other bacteria	1.27 (0.40-4.09)
Negative	1.21 (0.59-2.49)

**odds ratios (ORs) were calculated in 10-year increments for age*

***Glascow coma scale (GCS) scores range from 3-14, with 14 indicating a normal level of consciousness (abnormal flexion was omitted from the scale). The GCS score was obtained in 694 episodes. CI is confidence interval*

Table 3. Independent Risk factors for an unfavourable outcome in adults with community-acquired bacterial meningitis in Connecticut, United States. *(n=176).* (AroninPeduzzi and Quagliarello, 1998)

Dichotomous Variable	Episodes	Adverse Clinical Outcomes	Odds Ratio (95% CI)	*P* Value
	n	*n* (%)		
Hypotension			2.39 (1.18–4.83)	0.015
Yes	41	23 (56)		
No	135	47 (35)		
Altered mental status			7.26 (2.42–21.8)	0.001
Yes	147	67 (46)		
No	29	3 (10)		
Seizures			4.94 (1.95–12.51)	0.001
Yes	22	16 (73)		
No	154	54 (35)		
Ethnicity			0.74 (0.37–1.50)	>0.2
White	134	51 (38)		
Nonwhite	42	19 (45)		
Immunocompetence			1.32 (0.67–2.60)	>0.2
Yes	47	21 (45)		
No	129	49 (38)		
Positive cerebrospinal fluid Gram stain			1.09 (0.58–2.05)	>0.2
Yes	111	45 (41)		
No	65	25 (38)		
Charlson comorbidity score			1.49 (0.81–2.74)	0.2
≥1	85	47 (55)		
0	91	59 (65)		

Table 4. Malawian Adult Meningitis Score (MAMS) nomogram

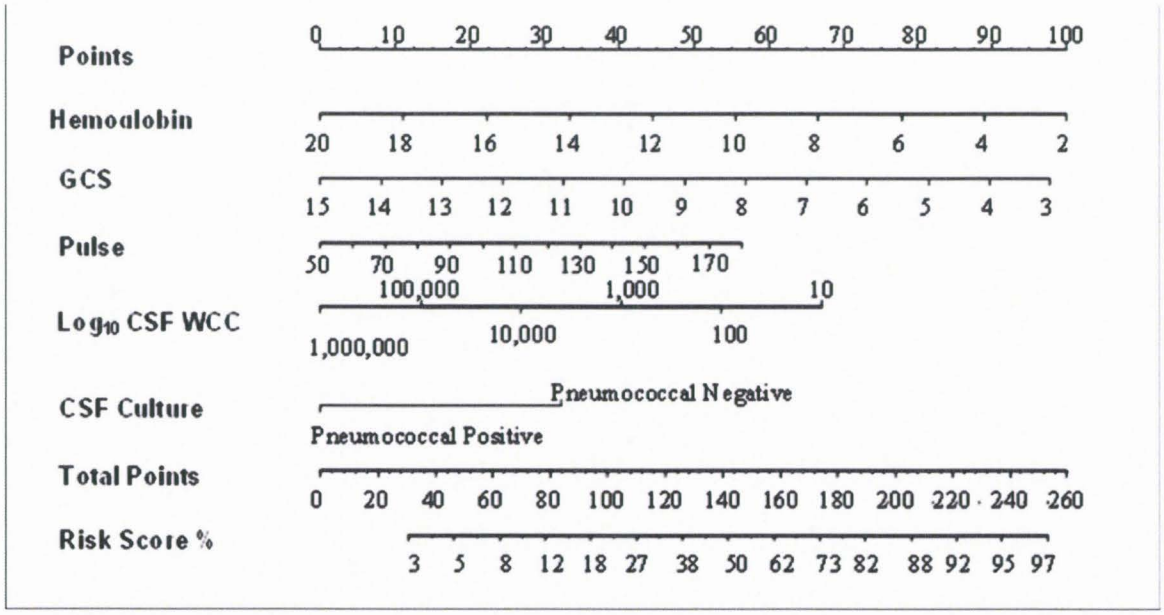


Table 5. Malawian Adult Meningitis Score (MAMS) nomogram, Points table.

Group	Probability Centile of Death	Observed Deaths, No. (%)	Predicted Deaths, No. (%)	Total
1	0.234	6 (11.3)	9 (17.0)	53
2	0.326	14 (26.9)	15 (28.8)	52
3	0.389	23 (44.2)	19 (36.5)	52
4	0.460	24 (45.3)	22 (41.5)	53
5	0.540	25 (48.1)	26 (50.0)	52
6	0.604	30 (57.7)	30 (57.7)	52
7	0.677	30 (56.6)	34 (64.2)	53
8	0.763	40 (76.9)	37 (71.2)	52
9	0.836	42 (80.8)	42 (80.8)	52
10	0.962	45 (86.5)	46 (88.5)	52
Total		279	279	523

Hosmer-Lemeshow C-statistic. X2=5.48, df=8, P=0.705

Table 4 & 5: A variable is measured, and its value is matched to the corresponding point on the first row of the nomogram. The total points are matched to the corresponding risk score percentage at the bottom. (Wall et al., 2017)

2.1 Study aims

There are contrasting mortality rates reported for Europe/United States (13-30%) as compared to Africa (e.g. in Malawi, Burkina Faso) where the reported rates are higher 41-54%. I want to determine prognostic markers and mortality rates in South Africa, which have not been evaluated before.

2.2 Study objectives

1. To describe a cohort of patients looking at demographics, immunocompromised state, current tuberculosis or cotrimoxazole therapy, laboratory parameters and cerebrospinal fluid cell counts. To evaluate these parameters as prognostic indicators for in-hospital death.
2. To determine the in-patient mortality rates in patients with pneumococcal meningitis in the adult population admitted to Chris Hani Baragwanath hospital.
3. To evaluate selected clinical and laboratory parameters as prognostic indicators for in-patient mortality in pneumococcal meningitis.

2.3 Study site

The research will be based at Chris Hani Baragwanath Academic Hospital (CHBAH). The hospital is the third largest hospital in the world. It has a bed occupancy of more than 3 400 beds. It is one of the teaching academic hospitals of the University of Witwatersrand. CHBAH serves the population of Soweto area in Johannesburg, South Africa. Soweto is a township area with a population more than 2 million.

2.4 Study design

- This will be a retrospective study
- I will do a secondary analysis of a database from GERMS-SA, with additional selected laboratory data (CSF and blood) from the National Health Laboratory Service (NHLS) Trakcare online website.

2.5 Patient Population

- A study of patients aged ≥14 years with pneumococcal meningitis confirmed by CSF culture.
- A review of the cases from August 2013 to July 2017 admitted at Chris Hani Baragwanath Academic hospital.
- The patients will be obtained from the database of GERMS-SA.

The Group for Enteric, Respiratory, and Meningeal Disease Surveillance in South Africa (GERMS-SA) is a nationwide network of clinical microbiology laboratories (both in the public and private sector) which participate in laboratory-based surveillance programme for pathogens of public importance. The SA population under surveillance is estimated to be about 53 million.

GERMS-SA has enhanced surveillance sites at 25 public sector hospitals (CHBAH is one of them) in all 9 provinces of South Africa. The Respiratory and Meningeal Pathogens Reference Unit (RMPRU), a subdivision of GERMS-SA, functions as a national reference centre for pathogenic respiratory and meningeal bacteria invasive disease.

2.6 Cerebrospinal fluid culture technique at CHBAH

CSF is centrifuged upon receipt and the sediment is placed into 3 culture media: 5% blood agar, chocolate agar and brain heart infusion broth. These culture media are then incubated in carbon dioxide incubators for 18-24 hours. The morphology of pneumococci is that they are alpha haemolytic colonies and have draughtsmen shaped colonies on blood and chocolate agar. A gram stain is simultaneously prepared on the CSF. Optochin antibiotic disc is used for identification of S. pneumoniae only, of which they have sensitivity to Penicillin and cefotaxime E- Test strips (minimum inhibitory concentrations) are also placed on the media to check for sensitivities. Routinely, if the pneumococci are grown on CSF, it will be sent across to the NHLS for serotyping by polymerase chain reaction (PCR). If there no bacterial growth on the CSF on the above protocol, but the chemistry is suggestive of bacterial infection, upon request by physician the laboratory will then do a pneumococcal latex test and if it is positive it will be transferred to the NHLS for confirmation by PCR.

2.7 Inclusion criteria

- Patients aged ≥14 years admitted to CHBAH between August 2013 and July 2017.
- Pneumococcal meningitis confirmed by CSF culture.

2.8 Exclusion criteria

- Any duplicate cultures of pneumococcus grown on the same patient with 21 days of the previous one.
- Co-infection in the CSF with another bacterium, fungus or virus.
- CSF evidence of meningitis but pneumococcus cultured in blood and not in CSF.

2.9 Data collection

- Please see sample data collection sheet from GERMS-SA under appendices. The list includes patient details and demographics (age, gender), admission date and outcome (including death), course of hospital admission, severity of illness, prior cotrimoxazole or tuberculosis treatment, vaccinations, HIV data (CD4 count, anti-retroviral therapy), other immunocompromised risk factors, CSF culture, serotype, and antibiotic sensitivities of pneumococcus. The data will be supplied in an excel sheet.
- I will personally collect additional laboratory data from the NHLS lab track. Please see the sample data collection sheet which comprises of CSF chemistry, cell count, protein and glucose; and blood tests such as haemoglobin, platelet count, white cell, albumin, alanine transaminase (ALT), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), sodium (Na), urea, creatinine and estimated glomerular filtration rate (eGFR). These will be added to the excel sheet.

2.10 Data analysis

- I will use the data collected to test for prognostic variables for mortality using univariate analysis and enter them into a multivariate analysis to test the association. I will employ logistic regression to relate the parameters to mortality.
- Categorical variables (such as gender, cotrimoxazole or TB therapy, immunocompromised risk factors and pneumococcus serotypes) will be compared by Pearson chi square test, or where necessary by Fisher's exact test. Continuous variables (such as age, CD4 counts, laboratory values) will be compared by the Student T test, or by unequal variances t-test with Welch's approximation if they are normally distributed. Where continuous variables are not normally distributed, the Mann Whitney's U test will be applied.

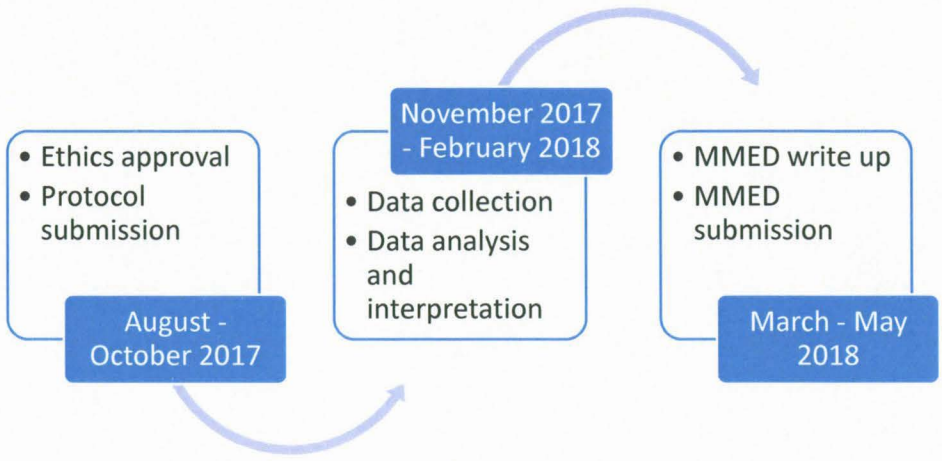
2.11 Limitations

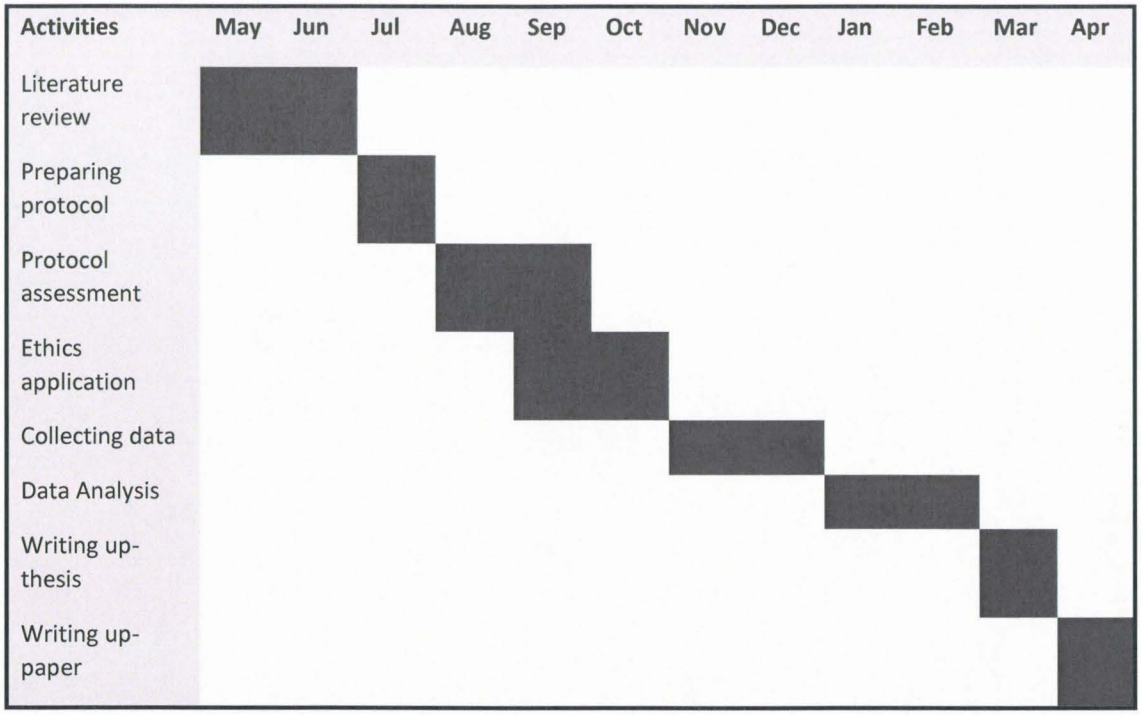
Data may be missing in the database and laboratory tests may not have been performed. The GERMS-SA surveillance officers were not involved in the care of the patients and may have misinterpreted data documented by the doctors. I will not be performing a record review, so that clinical data not captured by GERMS-SA will not be analysed. This will include such criteria as seizures and focal neurological signs.

2.12 Ethics

I will apply for permission to conduct this study to the Human Research Ethics Committee, University of the Witwatersrand. I will get the permission of the chief executive officer of CHBAH. This is a retrospective study comprising secondary analysis of an existing database and laboratory results, so consent from patients will not be required.

2.13 Chronology of research completion





2.14 Funding

No funding for this MMED project is necessary. All miscellaneous costs will be sustained by myself.

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APPENDIX

SAMPLE DATA COLLECTION SHEET FOR ADDITIONAL DATA *(NOT IN GERMS-DATA)*

Demographics

INDEX CASE NUMBER:

Laboratory parameters

Haemoglobin count___, Platelet count___, White cell count___ (N__, L__).
Albumin ____, ALT ____.
Inflammatory markers: CRP___, ESR__.
Electrolyte disturbance Na___, Urea___ Creatinine___, eGFR ____.
CSF chemistry cell count (N___, L___, E___), protein___, glucose ___.