

**SEMEN ANALYSIS OF RENAL TRANSPLANT PATIENTS
UNDERGOING IMMUNOSUPPRESSIVE TREATMENT**

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AUTHORS DECLARATION

This study represents original work by the author. It has not been submitted to any other Tertiary Institution. Where use of the work of others was made, it has been duly acknowledged in the text.

The research described in this dissertation was carried out in the Department of Biomedical and Clinical Technology, Faculty of Health Sciences, Durban University of Technology under the supervision of Professor J.K. Adam (Associate Director) and Durban Fertility Clinic, Kingsway Hospital, Amanzimtoti, South Africa under the supervision of Dr S Naidu (Clinical Director, Durban Fertility Clinic).

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DEDICATION

I dedicate this work to:

My omnipotent and omnipresent, **Lord Ranganathar**, who has guided and protected me throughout my life and career. He has taught me to become fearless against all adversity and to always have faith facing all challenges with humility.

My **father and mother**, for providing their children with a platform to succeed in all facets of life. For being our greatest supporters and biggest critics whilst showering us with an abundance of love and support.

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ABSTRACT

Introduction

The prevalence of infertility is increasing at an alarming rate globally. Many couples are afflicted with infertility due to an array of diseases, trauma and psychological stresses. Renal disease is one such pathophysiological condition which is increasing amongst the younger age group. Often the progression of chronic renal disease leads to end stage renal failure that requires a renal transplantation. Post renal transplant, immunosuppressive agents are routinely prescribed to prevent allograft rejection. Immunosuppressive agents are potent drugs that can have deleterious side effects on semen parameters. However, the effects of the immunosuppressive agents on semen parameters in the literature are unclear and require further investigation. It is, therefore, important to assess the effects of immunosuppressive agents on semen, especially the three vital aspects of sperm concentration, motility and morphology which form the basis of male reproduction.

Aim and objectives of study

This was a prospective observational study evaluating the effects of different immunosuppressive regimens on sperm parameters in post renal transplant male patients. The main aspects of semen parameters such as sperm concentration, motility and morphology that determine reproductive potential were assessed in the study patients and compared to the gold standard of semen analysis according to the World Health Organisation (WHO) reference values.

Methodology

Thirty-four renal transplant patients were recruited from the databases of both private nephrologists in the greater Durban area and the academic renal unit at Inkosi Albert Luthuli Central Hospital. Following bioethical approval and informed consent, patients were required to produce a semen sample by masturbation. A questionnaire documenting the patient's lifestyle, aetiology of renal disease, transplant date and immunosuppressive duration and regimen were recorded. The semen samples were analysed comprehensively according to the protocol on semen analysis recommended by the WHO. This included the macroscopic investigation (volume, appearance, colour, viscosity, liquefaction time and pH) and microscopic evaluation (sperm concentration, total motility, morphology, IgG/IgA and vitality). Sperm concentration, total motility, morphology and vitality were examined and recorded in duplicate to strengthen the validity of the results. A biostatistician analysed the data and determined the statistical analysis. Descriptive statistics determined values of semen parameters in renal transplanted males and in each race demographic. The one sample t-test analysed the statistical significance between the mean study values and the WHO reference values. The effect of the immunosuppressive agent on semen parameters was determined using multiple linear regressions whilst ROC analysis determined the sensitivity and specificity of sperm concentration, total motility and morphology in predicting pregnancy from the patients that fathered children post renal transplant.

Results

The mean sperm concentration and morphology in the study patients were 14.0 mill/ml (95% Confidence Interval (CI) 10.2 – 17.7) and 3.3% (95% CI 2.7 – 3.9), respectively. Although values obtained were minimally lower than the WHO reference values, these results were within the 95% CI of the WHO guidelines.

Motility evaluation revealed higher values of 43.2% (95% CI 36.6 – 49.7). In contrast, sperm vitality was considerably decreased, 47.5% (95% CI 40.6 – 54.4). All semen parameters exhibited no statistical significance (one sample t-test) when analysed against the WHO reference values except for sperm morphology, ($p = 0.025$; $p < 0.05$) which showed decreased morphology irrespective of immunosuppressive regimen. Semen volume 1.7 ml (95% CI 1.3 – 2.0) and pH 7.7 (95% CI 7.6 – 7.9) were both within the WHO guidelines. Descriptive statistics according to racial demographics showed no differences in semen values. An almost perfect linear relationship existed between total sperm motility and vitality ($r = 0.967$). Multiple linear regressions of duration and dosages of immunosuppressive drugs tacrolimus and mycophenolate mofetil, could not predict the effect of the immunosuppressive agents on sperm concentration, total motility and morphology. There was a significant difference in morphology between those with and without children post renal transplant. Those with children post renal transplant exhibited a higher morphology value, ($p = 0.001$; $p < 0.05$). Sensitivity and specificity analysis of the patients with children post renal transplant concluded that morphology is the most optimal indicator and predictor of pregnancy (AUC = 0.854). Tacrolimus was the common immunosuppressive agent used in the four patients that fathered children. This was more evident in patients that underwent therapy with Sirolimus followed by Cyclosporin A (CsA) and changed to Tacrolimus as the last immunosuppressive agent used for maintenance therapy.

Conclusion

The ability to procreate in renal transplanted males has become increasingly difficult and emotionally challenging. In this study sperm concentration and morphology of renal transplanted males exhibited parameters similar to the general fertile population. Total motility possessed a higher range of values in contrast to sperm vitality which showed a significant decrease from the WHO reference values. The

effect of immunosuppressive treatment on semen parameters could not be clearly defined due to the number of immunosuppressive regimens that patients were subjected to intermittently resulting in small sample sizes within each immunosuppressive regimen grouping. The majority of patients underwent a triple maintenance therapy of tacrolimus, MMF and prednisone. The dosage and duration of these tacrolimus and MMF was inconclusive in determining a beneficial or detrimental relationship on semen parameters. Morphology was shown to be the most significant indicator in predicting pregnancy in patients that fathered children. Tacrolimus was a common immunosuppressive agent used in the majority of patients that fathered children. It may have protective effects on sperm parameters as shown in patients that fathered children. This was a study with a small sample size and further investigations are required in a larger cohort of patients to assess individualized effects of the different immunosuppressive agents on sperm parameters.

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LIST OF ABBREVIATIONS

%	-	percentage
±	-	approximately
°C	-	degree celsius
tac	-	greater than
≤	-	less than and equal to
μl	-	microliter
μm	-	micrometre
CI	-	confidence interval
cm	-	centimetre
CsA	-	Cyclosporin A
DNA	-	deoxyribose nucleic acid
DNAse	-	deoxyribonuclease
e.g.	-	example
ESRD	-	end stage renal disease
<i>et al</i>	-	<i>et alia</i> (and others)
FSH	-	follicle stimulating hormone
g	-	gram
GFR	-	glomerular filtration rate
GnRH	-	gonadotropic releasing hormone
HOS	-	hypo-osmotic swelling test

Na,K-ATPase	-	sodium-potassium adenosine triphosphatase
IgG	-	Immunoglobulin G
IgA	-	Immunoglobulin A
IM	-	immotile
LH	-	luteinizing hormone
LV	-	lumbar vertebrae
mill/ml	-	million per millilitre
ml	-	millilitre
ml/min per 1.73 m²	-	millilitre per minute 1.73 metre squared
MMF	-	Mycophenolate mofetil
mTOR	-	mammalian target of rapamycin
NP	-	non progressive motility
NTPR	-	National Transplantation Registry
PR	-	rapid progressive motility
VAP	-	velocity of average path
VCL	-	curve line velocity
VSL	-	straight line velocity
WHO	-	World Health Organization
IALCH	-	Inkosi Albert Luthuli Central Hospital
ROC	-	receiver operating characteristics
GCP	-	Good Clinical Practice
DFC	-	Durban Fertility Clinic

SLE	-	systemic lupus erythematosus
MVA	-	motor vehicle accident

CHAPTER 1

INTRODUCTION

Infertility is defined as the inability of a couple to conceive after a year of unprotected intercourse (Brindsen, 1999). The prevalence of infertility over the last decade has become increasingly alarming and demanding (Mascarenhas *et al.*, 2012). Compounded by the many pathophysiological and physical demands, a variety of associated diseases further prevent couples from falling pregnant (Oakley *et al.*, 2008).

Chronic renal disease is a common pathophysiological condition, with an increased incidence worldwide, especially in the younger age group (Li Zhang and Rothenbacher, 2008). The incidence of chronic renal disease has doubled over the past decade reaching approximately 100 new patients per million of population (El Nahas & Bello, 2005). This increase is attributed to the increase in type 2 diabetes mellitus with a consequent increase in diabetic nephropathy and hypertension (King *et al.*, 1998). Chronic renal failure often progresses to end stage renal disease (ESRD) requiring renal dialysis until renal transplantation is done (Iglesias *et al.*, 2010).

During ESRD, there is abnormal gonadotropin releasing hormone (GnRH) pulsatility (Palmer, 1999) leading to high levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH), low testosterone levels and an increase in prolactin levels which impairs spermatogenesis (Talbot *et al.*, 1990; De Celis & Pedron-Nuevo, 1999). Subsequently impaired spermatogenesis has resulted in semen samples presenting with oligozoospermia and azoospermia (Tauchmanova *et al.*, 2004). Chronic renal failure has been associated with loss of libido and impotence in men

due to decreased testosterone levels (Toorians *et al.*, 1997; Palmer, 2003). The progression of advanced renal disease leads to testicular damage and impaired spermatogenesis (Holdsworth, 1978). Histological findings include germinal cell aplasia, atrophy of sertoli cells and interstitial fibrosis and calcification (Lessan-Pezeshki & Ghazizadeh, 2008).

Patients with chronic renal failure undergo dialysis treatment (haemodialysis and/or peritoneal dialysis) in maintaining waste and fluid removal and homeostatic balance; however, dialysis treatment has shown to impair gonadal functionality (Kokot and Wiecek, 1996). Progression of chronic renal failure to ESRD requires a renal allograft in sustaining the patient's longevity (Levey *et al.*, 2003). The success of a renal transplant is the efficiency at which the allograft assumes function and whether there is organ rejection or not (McKay & Josephson, 2008). Patients, post-transplant are exposed to immunosuppressive agents to inhibit T cell differentiation and apoptosis, thereby, promoting the development of self-tolerance and preventing organ rejection (Tauchmanova *et al.*, 2004). These immunosuppressive agents may have potentially harmful effects on spermatogenesis. The literature is unclear on the effect of immunosuppressive agents on sperm parameters (Kaczmarek *et al.*, 2004; Xu *et al.*, 2009; Abou-Shabaan *et al.*, 2011).

Previous immunosuppressive treatment regimens post renal transplant, included mild steroids, azathioprine and cyclosporin A (CsA) (Rodriguez-Rodriguez *et al.*, 1996). Recent treatment regimens incorporated tacrolimus, sirolimus and mycophenolate mofetil (MMF) active agents (Vincenti, 2003). The general movement or shift of regimens from one to another should be incorporated without influencing or affecting the capacity and functionality of the renal graft whilst having little effect on semen parameters. Gonadal toxicity is the fundamental concern related to the use of immunosuppressive agents (Tondolo *et al.*, 2005). Trompeter (1986), reported that the use of immunosuppressive agents was dose related. A single limited course for eight weeks did not show disturbances or changes in sperm concentration and

motility. However, multiple courses of treatment at the same or increased dosage presented with oligozoospermia. It is imperative that assessment of semen parameters are measured against the clinically diagnostic reference values of the World Health Organization (WHO) in predicting reproductive potential (WHO, 1999).

Male sexual ability and infertility are adversely affected by chronic renal failure (Iglesias *et al.*, 2010). Inadequate androgen steroidogenesis has been responsible for both impaired sexuality and spermatogenesis. However, reproductive function and fertility has been significantly improved following renal transplantation outweighing dialysis treatment (McKay and Josephson, 2008). Immunosuppressive agents are solely administered post transplantation to prevent graft rejection; however, few studies have evaluated the effect of immunosuppressives on male fertility extensively. Most studies focussed on CsA based regimens versus CsA free regimens. Later on, a few studies with small sample sizes analysed the effects of other immunosuppressive agents on male fertility and gonadal toxicity (Holmgren *et al.*, 2004; Ostensen *et al.*, 2008; Boobes *et al.*, 2009). Further studies have shown that immunosuppressive treatment post-transplant yielded a poor prognosis for males desiring children of their own genotype owing to major alterations on the hypophyseal gonadal axis (Baumgarten *et al.*, 1977; Akbhari *et al.*, 2003; Tauchmanova *et al.*, 2004).

The desired maintenance treatment post-transplant requires the use of a triple therapy regimen i.e. two immunosuppressive agents with support using a corticosteroid. Continuous administration of immunosuppressive drugs can cause desensitization and adverse events leading to a change of immunosuppressive regimen. Further, the change from one immunosuppressive drug to another has shown to alter the spermatogenesis cycle and degree of sexual functionality (Deutsch *et al.*, 2007; Xu *et al.*, 2009; Chen *et al.*, 2013). Therefore, this study was designed to assess the effects of immunosuppressive therapy on semen parameters of male renal patients post-transplant.

CHAPTER 2

LITERATURE REVIEW AND STUDY BACKGROUND

2.1. Semen analysis

Male fertility is affected by a multitude of external and internal stimuli and stresses. The effect of these stresses and stimuli can be measured and examined by means of a semen analysis. Semen analysis is a comprehensive assessment of semen parameters that is used to investigate and establish the fertility potential of males (Menkveld *et al.*, 2001). A correlation of these results with regards to normality, subfertility or infertility is measured according to the parameters as prescribed by the World Health Organization (WHO, 2010). These criteria form the basis of normozoospermia as extrapolated from data taken from fertile males globally. The cohort of data represents the male fecundity potential and is also unbiased in its region/area/country and continent of origin. There is no single factor that solely reflects the fertilization capacity of any population of spermatozoa. However, analysis of semen provides vital information of the pathophysiological condition and anomalies of the reproductive organs and associated structures. Therefore, a semen analysis aims at evaluating descriptive parameters of the ejaculate (De Jonge and Barratt, 2002; NAFA, 2002) and assess the semen samples from a cohort of renal transplanted patients in this study. Semen analysis is grossly performed as a two-fold investigation which includes assessments macroscopically and microscopically (WHO, 2010).

2.1.1. Macroscopic Investigation

The native sample produced exhibits characteristics that can be verified and

inspected post ejaculation. Simple inspection should be performed within thirty minutes and not longer than one hour preventing dehydration or changes in temperature affecting the semen quality. These include:

2.1.1.1. Volume

Semen in its entirety theoretically comprises of four secretions. The major contributors are the prostate gland and seminal vesicle with smaller secretions by the bulbourethral gland and epididymides (WHO, 2010). This homogenous mixture acts as a buffer and provides the necessary metabolites needed for capacitation and motility activation (Hart *et al.*, 2015). The normal range of semen volume is 1.5 ml to 6 ml. Abnormal indices reflecting hypospermia (volume <1.5 ml) is indicative of congenital bilateral absence of the vas deferens, obstruction of the ejaculatory duct, seminal dysfunction or poor development, spillage during masturbation, partial or complete retrograde ejaculation and androgen deficiency (Robin *et al.*, 2008). Hyperspermia (volume >6 ml) may account for exudation in cases of active inflammation of the accessory organs (Parnham and Serefoglu, 2016).

2.1.1.2. Appearance/Colour

The appearance of the ejaculate is assessed according to the colour, opacity, translucence, mucus streak presence and aggregation of cellular material (Mortimer, 1994). The degree of these secretions and sperm density accounts for the colour of the semen. Human semen exhibits an off-white greyish-yellowish opalescent fluid (WHO, 2010). A less opaque appearance can be attributed to a lower sperm concentration. Abnormalities of semen colour includes a red–brown appearance (haemospermia) which is indicative of red blood cell prevalence in the ejaculate (Papp *et al.*, 2003) or a yellow tinge (pyospermia) in males that present with jaundice or have been subjected to certain vitamins or drugs (Pentyala *et al.*, 2007).

2.1.1.3. Liquefaction and viscosity

The native gelatinous mass ejaculated is indicative of the complexity of the reproductive system and its ingenious design in accumulation and aggregation of the ejaculate prior to its liquefaction to allow for the release of the motile spermatozoa into the cervical canal. Liquefaction is a measure of the semen samples potential to convert from a gelatinous mass into a liquid phase (Lwaleed *et al.*, 2005). Incomplete liquefaction results in the appearance of gelatinous material in a fluid base (Mortimer, 1994). Liquefaction time assessment is most accurate at 37 degree celsius (°C) incubation and analysis. The time documented for liquefaction to occur is within 15 – 30 minutes rendering the heterogenous mixture homogenous. Gentle shaking or rotation of the sample container assists with the liquefaction; however, sample liquefaction can sometimes be longer than 30 – 60 minutes and renders the sample with a high viscosity (Agarwal *et al.*, 2008). Viscosity measure is determined by the thread formation from a graduated pipette, i.e., < 2 centimetre (cm) (WHO, 2010). Abnormal pathologies including benign prostatic hyperplasia, prostatic carcinomas and prostate dysfunction diminish the secretion potential of the prostate in providing the fundamental prostatic proteases that are required for liquefaction and viscosity (Intasqui *et al.*, 2016).

2.1.1.4. pH

The acidic and alkaline secretions of the accessory glands reflect the pH in semen. The prostate produces an acidic secretion which is stabilized by the alkaline seminal vesicle secretions. Classification of normal semen pH values is 7.2 to 8.2. Abnormal pH values may account for ejaculatory duct obstruction, inflammatory disorders, seminal vesicle dysfunction or congenital bilateral obstruction of the vas deferens (Haugen and Grotmol, 1998; Harraway *et al.*, 2000).

2.1.2. Microscopic investigation

2.1.2.1. Sperm concentration

The basic and most important building block in the male reproductive system is the sperm cell. The sperm cell morphology comprises of the mid-piece, neck, tail and head which enclose the haploid expression of deoxyribose nucleic acid (DNA) and a pair of XX or XY sex chromosomes. Spermatozoa represent a small contingency within the semen. The measure of this contingency per millilitre quantifies the amount of sperm that is produced by the seminiferous tubules of the testis. This production is dependent on the secretion of testosterone and activity of the primordial germ cells (Auger *et al.*, 2015).

Total count of sperm is not synonymous with sperm count as total count of sperm refers to the amount of spermatozoa counted multiplied by the volume of the ejaculate; whilst in contrast the sperm concentration represents the number of sperm evaluated per millilitre of semen. The value of normal sperm concentration has changed with the introduction of the revised WHO laboratory manual for the examination of processing human semen. As of the fifth edition; the revised normal sperm concentration is 15 million per millilitre (mill/ml) whilst total sperm concentration is 39 mill/ml as represented in the fifth centile in the 95 % confidence interval (CI) (WHO, 2010).

The WHO (2010), range for sperm concentration is 15 - 250 mill/ml. Concentrations below 15 mill/ml is regarded as being oligozoospermic whilst the complete absence of spermatozoa in an ejaculate is termed azoospermia. A cause of concern in fertility treatments in males is the common diagnosis of oligozoospermia and azoospermia (Grimes and Lopez, 2007). Reijo and colleagues (1996), examined obstructive and non-obstructive azoospermia and ascertained that there was a vast increase in non-obstructive azoospermia from the deletion of the azoospermia factor, i.e., a gene

located on the long arm of the Y chromosome. Further micro-deletions on the long arm of the Y chromosome has been associated with spermatogenic failure (Dada *et al.*, 2003). The cause of spermatogenic failure can be attributed to various pre-testicular, testicular and post-testicular disorders that inhibits the progressive hypophyseal pituitary axis secretion of the gonadotrophins and compromises the blood testis barrier (Bahrke and Yesalis, 2004; Maravelias *et al.*, 2005; De Souza and Hallak, 2011).

Ferlin and colleagues (2013), examined the relationship between telomere lengths in leucocytes and sperm including the sperm concentration and age of the participants. Their results concluded that sperm telomere length is related to sperm concentration in which the sperm telomere length was lower in oligozoospermia as compared to normozoospermic males and was also directly related to the age of the parent at conception.

Calculation and estimation of sperm concentration is performed using many different counting chambers. The Makler (Figure 1) is a 10 micron deep counting chamber that is used most widely in fertility laboratories. The easy design together with a laborious free loading technique makes concentration evaluation rapid whilst ensuring that there is reliability, accuracy and validity of the results (Makler, 1980). Dilution and correction errors are not needed since the native sample, once homogenous, can be evaluated with a 10 microlitre (μl) droplet (Mortimer, 1994).



Figure 1: Makler counting chamber used for the estimation of sperm concentration (Makler, 1980).

A more accurate counting chamber is the Neubauer haemocytometer which was initially designed as an improved method in counting blood platelets (Rees and Ecker, 1923) and later adapted for sperm concentration (Mahadevan and Baker, 1984). Most recently plastic Neubauer counting chambers were designed in an attempt to decrease the cost of the standard Neubauer haemocytometer. However, it was concluded that these plastic devices were not suitable in determining sperm concentration as the results were variable, inconsistent and required an increased time for accurate assessment (Kirkman-Brown and Bjorndahl, 2009). Other chambers that have been or are still used include Horwells, Thoma and Burkner – Turk haemocytometers (Christensen *et al.*, 2005).

2.1.2.2. Sperm motility

The forward progression of spermatozoa and functional capacity is imperative in assisting the sperm cell to initiate the fertilization process with the oocyte. Progressive sperm motility is vital in evaluating the fertility potential of spermatozoa and is a pre-requisite for fertilization in humans (Esfandiari *et al.*, 2002).

The presence of motile spermatozoa in semen is critical to ensure adequate spermatozoa transport to the oocyte and the occurrence of fertilization. The movement pattern of the spermatozoa can be attributed to an array of endogenous and exogenous stimuli that can either hinder or enhance this progression (Said and Land, 2011).

Pathophysiological diseases, e.g., diabetes mellitus (a common cause of renal failure); have shown to greatly reduce the forward potential of the spermatozoa rendering the movement erratic, dis-orientated and sluggish (Vignera *et al.*, 2012). The aforementioned anomalies of motility can also be exacerbated by lifestyle and exposure to external vices including cigarette and cannabis smoking (Forrest, 2001), exposure to endocrine disruptors (Safe, 2000) and occupational exposure to pesticides (Juhler *et al.*, 1999), metals (Figa-Talamanca *et al.*, 2001) and organic compounds (Jouannet *et al.*, 2001).

Motility is one of the most important pre-requisites to achieve fertilization and pregnancy. The head of the sperm must be delivered a great distance, *in vivo*, at a speed of greater than 25 micrometre (μm) per second at 37°C, through the barriers of the reproductive tract to the site of the egg. The sperm must have sufficient forward progression to penetrate the layers of coronal cells surrounding the egg, the zona pellucida and the oolemma of the oocyte (Silverberg and Turner, 2008).

Sperm motility is the first investigation done on a wet preparation before there is cooling or drying of the specimen. This test should be done 30 minutes post ejaculation but not longer than 1 hour. Examination after a prolonged period causes pH or temperature changes and deleterious effects of dehydration (Mortimer, 1994). Cognisance of the patients age, duration of last ejaculate, exposure to external influences such as excessive heat and toxins, method of collection and interval duration taken to analyse the specimen from collection; must be considered when validating the motility results (Silverberg and Turner, 2008). Sperm motility is a

measure of the quantitative parameter whereas the forward progression represents the quality of the movement.

Spermatozoal motility can be classified into three categories, namely:

- Progressive motility (PR) – spermatozoa moving actively, either in a linear motion or in a large circle, regardless of speed.
- Non–progressive motility (NP) – all other patterns of movement with an absence of progression e.g. swimming in small circles, inadequate flagella force depicting no displacement of the sperm head or when the tail exhibits only a flagella beat and
- Immotile (IM) – no movement (WHO, 2010).

Total motility, i.e., NP and PR, should be equal to or greater than 40% whereas the PR should be > 32%. These values represent the lower reference limits as per the fifth centile in the 95% CI (WHO, 2010).

2.1.2.3. Sperm morphology

One of the most important predictors in fertilization capacity is the shape of the sperm or sperm morphology. This quantifiable measure is descriptive of the structure of the sperm cell meticulously examining the head, neck, mid-piece and tail of the spermatozoa. The ergonomics and specific known lengths and diameters of these specific areas is analysed as a basis of diagnostically explaining probable failures in pregnancy (Auger *et al.*, 2015). The oocyte is specific of the sperm cell that is required for fertilization as the sperm morphology acts as an indicator of the general stability and normality of the spermatozoa. Testicular injury, infections, prolonged exposure to heat, occupational hazards and the use of hazardous recreational drugs including cigarette smoking accounts for altered morphology in the spermatozoa (Ong *et al.*, 2002).

The use of the strict criteria as adopted by WHO forms the bench mark of sperm morphology evaluation. The important aspect of morphology evaluation is that the range of the normal biological variations with repeatable analysis should be kept as small as possible thereby deeming it strict (Menkveld, 1987).

In order for spermatozoa to exhibit normal morphological features:

- The sperm head must be a smooth ovoid structure with 40 – 70% of the anterior sperm head having a well-defined acrosome region.
- The sperm must be devoid of any neck, midpiece and tail defects.
- The midpiece must be slender attached axially approximately 1.5 times the head length and $\leq 1 \mu\text{m}$ in width.
- Cytoplasmic remnants or droplets that comprise more than half the head of the sperm are classified as abnormal.
- The tail should be $\pm 45 \mu\text{m}$ long with a straight, slightly thinner and uniform appearance (Menkveld and Kruger, 1995).

The World Health Organization, as a basis of standardization, identified that each abnormal spermatozoon can have one to four abnormalities, i.e., head, neck/midpiece, tail abnormalities or a cytoplasmic residue (Figure 2) (WHO, 1999).

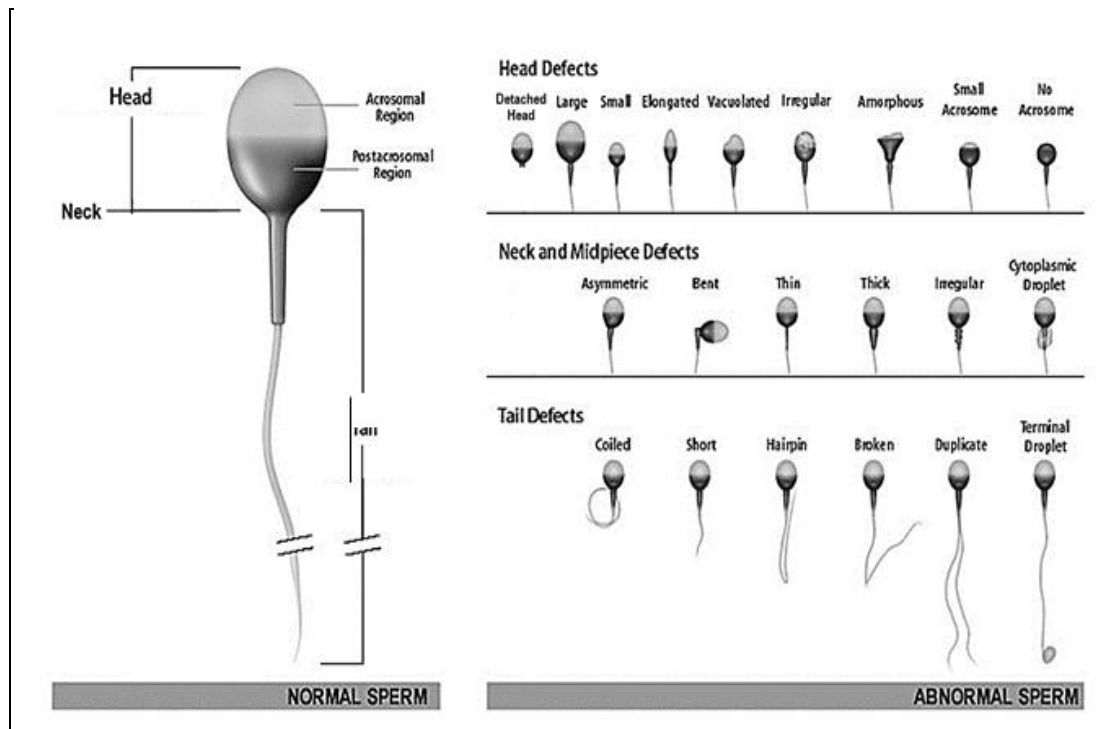


Figure 2: Normal spermatozoon versus abnormal spermatozoa (Cooper, 2016).

It is known that abnormally shaped sperm can fertilize an oocyte, whilst, the increased prevalence of abnormally shaped spermatozoa lead to poor implantation and recurrent miscarriages (Macklon *et al.*, 2002; Zidi-Jrah *et al.*, 2016). The head of the spermatozoa consists of the acrosomal and post acrosomal regions. The acrosomal region contains essential enzymes including acrosin that digests the glycoprotein based zona pellucida. The post acrosomal region contains the paternal haploid DNA and swells to present itself as the paternal pronucleus during fertilization and zygote formation. Head defects can affect the quality of the DNA including the maturity of the chromatin packaging that is essential in delivering the genetic material within the cytoplasm of the oocyte and initiating the fertilization and development potential of the gametes and embryo (Esterhuizen *et al.*, 2000; Nasr-Esfahani *et al.*, 2004). *In situ* nick translations of the human DNA can affect the fertilization potential in which poor chromatin packaging can be correlated with poor

fertilization outcomes *in vitro* (Sakkas *et al.*, 1996; Franken *et al.*, 1999; Razavi *et al.*, 2003).

Rocca and colleagues (2016), introduced the evaluation of sperm telomere length as a new biomarker of sperm quality and concluded that there was a negative correlation with sperm DNA fragmentation ($p = 0.001$) and a positive correlation with protamination ($p = 0.002$) signifying that abnormal morphology can indicate increase levels of DNA fragmentation and protamination.

2.1.2.4. Sperm vitality

Inspection of the native sample on a wet preparation will reveal the forward progression and movement of the spermatozoa. According to WHO (2010), the motility can be expressed as PR, NP and IM. The presence of an immotile sperm does not construe that the cell is necrotic. Sperm cells may exhibit immobility but possess viability (Aitken *et al.*, 2010). The measure of this viability is performed through a sperm vitality test. The most common sperm vitality test is the eosin - nigrosin test (Figure 3) (Bjorndahl *et al.*, 2003). The dye exclusion basis of this technique tests the cellular integrity by assessing the ability of the sperm head plasma membrane in excluding extracellular substances such as dyes thereby distinguishing viable and immotile spermatozoa from dead spermatozoa (Mortimer, 1994).

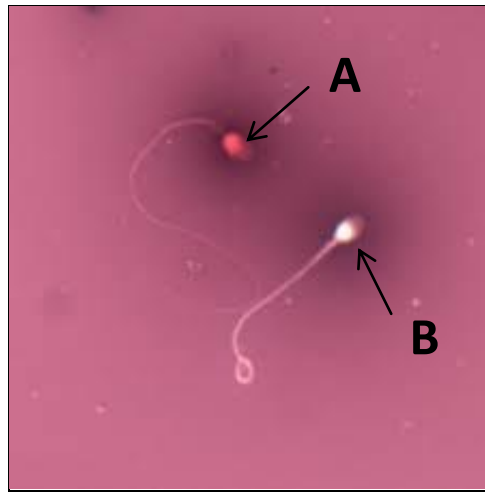


Figure 3: Sperm vitality Eosin – Nigrosin stain, A – pink stained spermatozoa denoting a compromised plasma membrane and necrosis. B – representative of an intact plasma membrane and viable spermatozoa (RBL Image library, 2007 reprinted with permission by Prof. Carin Huyser 2016.10.12).

The vitality also forms the basis of correlating the percentage and accuracy of total progression calculated from the semen sample in which the live spermatozoa quantity should exceed the total percentage of motile spermatozoa (Bjorndahl *et al.*, 2003). There are several staining techniques and tests that are used to measure the sperm vitality including eosin Y (Lin *et al.*, 1998), trypan blue, Hoechst fluorochrome (Mortimer *et al.*, 1990) and the hypo-osmotic swelling (HOS) test (Casper *et al.*, 1996). The HOS test was initially designed as a sperm function test; however, it assesses the osmoregulatory ability of the spermatozoa with regards to membrane integrity, thereby, correlating the vitality of the spermatozoa (Buckett *et al.*, 1997; Agarwal *et al.*, 2016).

2.1.2.5. Sperm MAR IgG and IgA

The presence of sperm antibodies reacting with antigens on the spermatozoa is

considered as typical and specific for immunological infertility. Anti-sperm antibodies belong to different immunological classes; however, of clinical relevance in male fertility is IgG and IgA. The presence of antisperm antibodies can interfere with sperm function, zona binding and the acrosome reaction. The antibodies of each rarely occur independently but the significance of their association is pivotal in infertility. Patients that present with a combination of IgG and IgA or antibodies of IgA only present with a diminished prognosis of falling pregnant and fundamentally are pivotal in the diagnosis and prognosis of infertility and subsequent fertility treatment. Accessory glands produce bulk of the antibodies of IgG and IgA and are present on the spermatozoa and sometimes in seminal plasma (Friberg, 1980; Comhaire and Kunnen, 1985).

2.2. Anatomical structure of the spermatozoon

The spermatozoon is composed of a sperm head, neck, midpiece, tail or flagellum. Almost 50% of sperm ejaculated are abnormal in form either via their morphology, motility or chromatin packaging (Mortimer, 1994; Buffone, 2016). The typical normal spermatozoon is described as possessing an oval head with a clear anterior and darkened (when stained) posterior area, a tail or flagellum of approximately 50 μm in length that inserts into the head symmetrically and axially together with a thickened midpiece region which is situated inferiorly to the base of the head (Table 1) (Mortimer, 1994).

Table 1: Morphometric dimensions of the spermatozoon (Mortimer, 1994).

Description	Length (µm)	Width (µm)	Thickness (µm)
Head	3.0 – 5.0	2.0 – 3.0	Max 1.5
Midpiece	3.0 – 5.0	1.0	-
Tail – Principal piece	45 – 50	-	± 0.5
Terminal piece	4.0 – 6.0	-	-
Overall Tail length	50 – 60	-	-

Of utmost importance is the sperm head which contains the acrosomal and post-acrosomal regions (Figure 4). The post-acrosomal area includes the nucleus that contains the haploid progeny of DNA of the spermatozoa. The DNA is supported by linker proteins which are replaced by protamines during spermatogenesis. These positively charged DNA proteins relay the hypercondensation of the sperm nucleus into a compact, hydrodynamic shape. This compaction is further supported and stabilized by the intra-inter disulphide bonds amalgamated with various other protein molecules (Sutovsky and Manandhar, 2006).

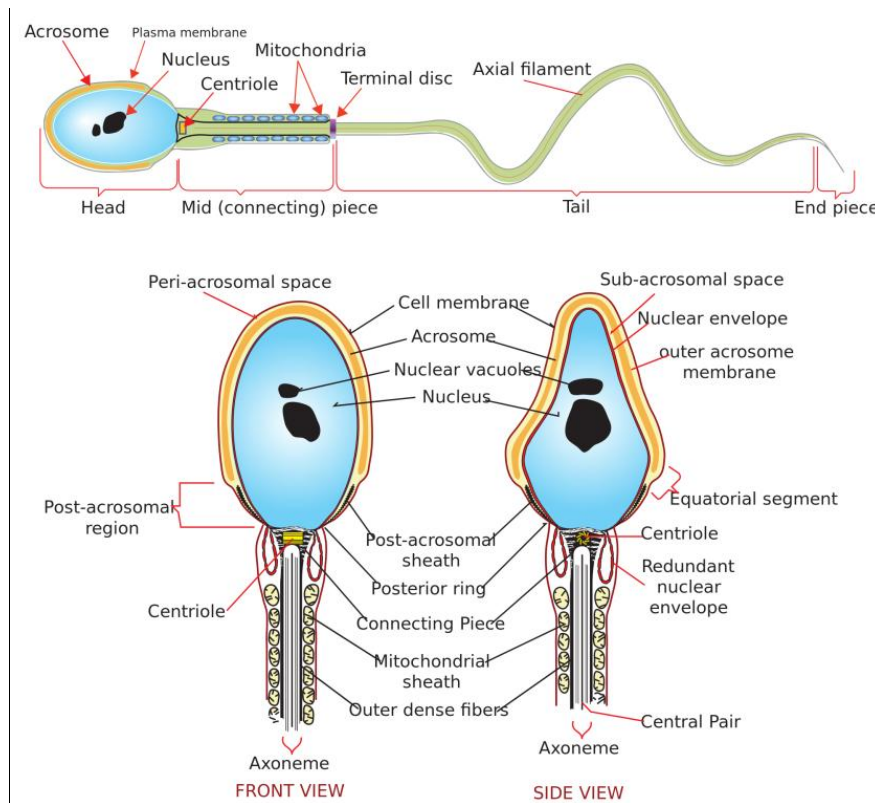


Figure 4: Sagittal section of spermatozoon (Hall, 2015).

Protection of the sperm nucleus is given by the perinuclear theca or matrix. The sperm perinuclear theca can be divided into three different segments that have a major role in fertilization with the oocyte.

These include the following:

- **Sub-acrosomal layer:** anchors the acrosome and harbours proteases and receptors required for sperm interaction with the oocyte's zona pellucida.
- **Equatorial segment:** contains receptor molecules which are pivotal in the initial binding of the spermatozoa with the oolemma of the oocyte, once the sperm penetrates through the zona pellucida and the perivitelline space.
- **Postacrosomal sheath:** contains the sperm borne oocyte activating factors which initiates the signalling pathway that leads to the activation of the oocyte and initiation of zygote development (Sutovsky and Manandhar, 2006).

2.3. Spermatogenesis

Spermatogenesis (Figure 5) is the process of spermatozoa production that takes place in the seminiferous tubules of the testis. The length of sperm production from the germinal cell to a mature spermatozoon takes a period of 70 – 74 days (Vander *et al.*, 2001). The production of spermatozoa is a complex yet truly remarkable process that follows three typical stages, i.e., spermatogonial, spermiogenesis/spermatocyte and spermatid phases.

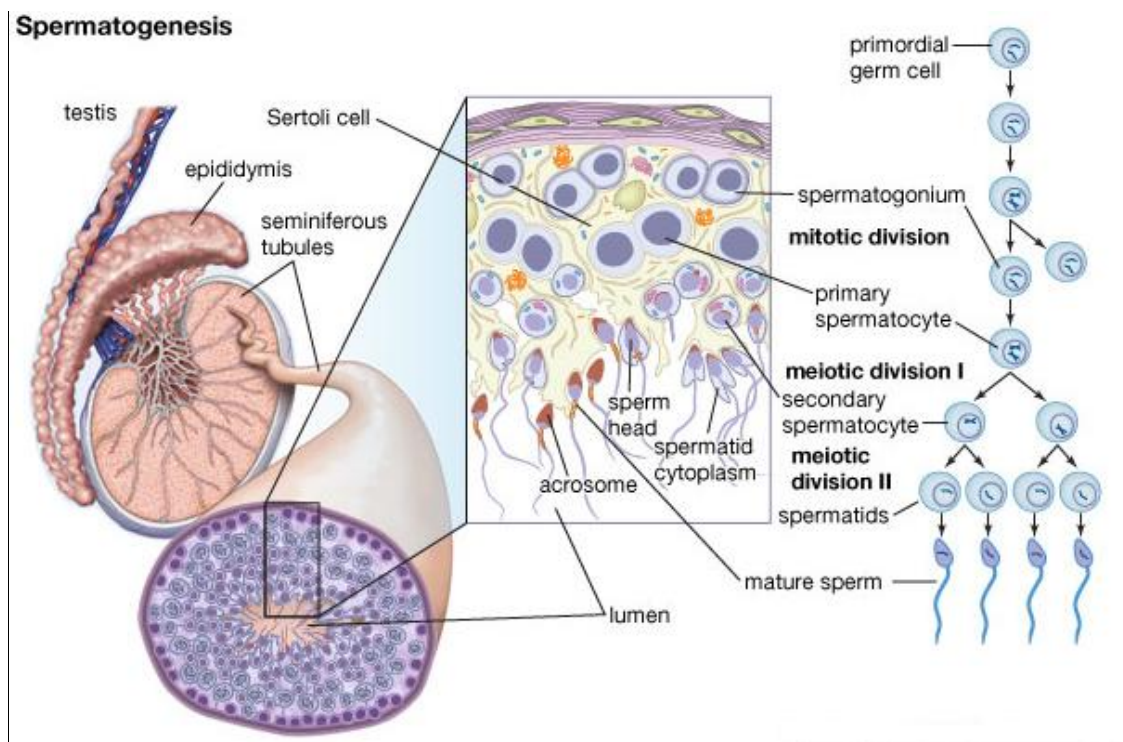


Figure 5: Cross section of seminiferous tubule depicting the spermatogenesis process (Britannica, 2016)

2.3.1. Spermatogonial phase

The spermatogonium or immature germ cell forms the basic building block for the process of spermatogenesis. The spermatogonia undergo mitotic division to produce daughter cells that will either proceed to spermatogenesis, replace cells for future spermatogenesis to occur or degenerate. The human testis contains three types of spermatogonia, i.e.:

1. Type Ad (dark) that develop into
2. Type Ap (pale) which differentiates into
3. Type B differentiating spermatogonia which are the immediate precursors of the primary spermatocytes transforming before the first meiotic division to establish the secondary spermatocytes (Mortimer, 1994; Li *et al.*, 2016).

2.3.2. Spermiogenesis/spermatocyte phase

There is production of the primary spermatocytes via mitotic division of the Type B spermatogonia. These primary spermatocytes undergo their first meiotic division with the production of the secondary spermatocytes. The secondary spermatocytes undergo the second meiotic division with the production of spermatids that have the haploid constituent of DNA and a pair of either XX or XY sex chromosomes. The histones are replaced by the protamines, whilst the chromatin condenses and the nucleus assumes an eccentric position close to the cell membrane in which the golgi complex further defines the acrosome area of the nucleus in proximity of the cell membrane. The chromatin condensing is further supported by the disulphide bond cross-linking of the protamines and is resistant to DNase. The axial filament develops from one of the centrioles that attaches to the pole of the nucleus opposite the developing acrosome together with the axoneme (simple 9 + 2 structure) which develops to form the axial filament complex (Walker, 2011).

The spermatid cytoplasm undergoes reduction and the mitochondria transform into

less ovoid and more tubular structures with an arrangement around the proximal part of the axial filament complex and the emerging tail. The mature spermatid's neck region sheds the residual cytoplasm as it releases from the seminiferous tubules into the lumen of the tubule. The process is referred to as spermiation. Post separation of the spermatozoon into the tubule lumen, a small remnant of the residual cytoplasm is still evident on the spermatozoon. During epididymal transport, further cell maturation takes place in which the residual droplet, migrates along the tail and devoids itself from the spermatozoa (Griswold, 2016).

2.3.3. Spermatid Phase

Consists of 4 sub-phases, i.e., golgi, cap, acrosome and maturation phases, respectively. The golgi phase involves the incorporation of the pro-acrosomal granules to form the acrosomal vesicles which is then re-shaped with the formation of the acrosomal cap (cap phase). The acrosome phase yields the production of the neck, midpiece, principle and end piece formation whist the spermatid re-orientates and the flagellum extends into the lumen. The acrosome phase is proceeded by the maturation phase in which the excess cytoplasm is pinched off and the residual body is phagocytised together with the release of the spermatids of the sertoli cells (Robles *et al.*, 2016; Vedelek *et al.*, 2016).

2.4. Infertility

Infertility as defined by Olmeda (2000), refers primarily to the biological inability of a man or a woman to conceive. It is also defined as the failure to conceive after 12 months of unprotected intercourse (Brindsen, 1999). Infertility affects about 15 - 20% of couples with a demarcated third comprising of male factor aetiology, a third of female and a third of combined male and female reproductive tract disorders (McPhee *et al.*, 2003). The word “infertility” on its own literally translates to “not

fertile.” However, there are many cases where women and men are fertile but have been diagnosed with other pathophysiological conditions which impact on their reproductive capacity. Regarding this, one can term these individuals with subfertility as constituting partial infertility (Bayer *et al.*, 2007).

Infertility diagnosis can be ascertained by determining the level of the infertility, i.e., primary or secondary infertility. Primary infertility refers to couples that have never conceived, whilst in contrast, secondary infertility is concerned with couples who have conceived including miscarriages and ectopic pregnancies; however, presently are experiencing difficulty in conceiving. Secondary infertility is not regarded if there has been a change in the sexual partner of the individual (De Jong and Barratt, 2002).

2.4.1. Male Infertility

Male infertility is due to a variety or combination of disorders. Recognizable causes account for 30 – 50% of individuals with the remaining representation of idiopathic nature (Lammorrone *et al.*, 2003). Male Infertility can be classified into three aetiological classes, i.e., pre-testicular, testicular and post-testicular (Table 2). Subfertility affects one in twenty men worldwide of which abnormal semen quality or sexual dysfunctions are contributing factors in about half of subfertile couples (Hirsh, 2003).

2.4.1.1. Pre-testicular causes

This is an occurrence of endocrine disorders focussed primarily on hypothalamic and commonly pituitary disorders (McPhee *et al.*, 2003). The impairment of infertility is secondary to either an excess or deficiency of the hormones secreted. Fertility in men requires a normal functioning of the hypothalamus, pituitary gland and testis together with complete germ cell proliferation which is dependent on the endocrine

secretion of these glands. Hypogonadotropic hypogonadism is the common aetiology in males. There is pituitary gland failure of producing sufficient and adequate secretions of FSH and LH, thereby, inhibiting the spermatogenesis process and producing decreased sperm counts (Hussein and Al-Faisal, 2010). Hyperprolactinemia also accounts for about 11% of males afflicted by oligozoospermia. It is an endocrine condition that inhibits the pulsatile activity of producing gonadotrophin releasing hormone which in turn decreases FSH, LH and testosterone production. This leads to an arrest of the spermatogonia, impairment of the sperm motility and quality (Al-Daghistani and Abdel-Dayem, 2002).

2.4.1.2. Testicular causes

Male factor infertility precipitated by testicular causes includes varicocele, hydrocele, infection, drugs and toxins including medications, ingestants and environmental exposure. Chromosomal abnormalities such as Klinefelter's syndrome and developmental abnormalities including cryptorchidism also impact greatly on male infertility. From the afore-mentioned, chromosomal causes account for 2% of all male infertility related cases correlating with the 15% of azoospermic males. Testicular atrophy also leads to the failure of the organ (testis) and infertility (Vander *et al.*, 2001).

2.4.1.3. Post-testicular causes

Azoospermia is the most common prognosis made due to bilateral obstruction of the vas deferens. The bilateral obstruction accounts for 50% of male related infertility. Other post-testicular causes include retrograde ejaculation, absence of seminal emission, antibodies to sperm and seminal plasma, developmental anomalies, sexual dysfunction and poor coital techniques (Cooper and Yeung, 1999).

Table 2: Causes of male infertility (Ayaz *et al.*, 2012).

Pre-testicular	Testicular	Post-testicular
1.Hypothalamic pituitary disorders Panhypopituitarism Gonadotropin deficiency Isolated LH deficiency Biologically inactive LH Combined LH and FSH deficiency e.g. Kallmann's Syndrome Prader-Willi Syndrome Laurence-Moon-Biedl syndrome Cerebellar ataxia Pituitary Tumours e.g. Prolactinoma Systemic illness e.g. Cirrhosis, uraemia 2. Thyroid disorders e.g. Hyperthyroidism, hypothyroidism	1.Varicocele, Hydrocele, Spermatocele, Testicular tumours 2.Trauma e.g. Testicular torsion, orchiopexy 3.Infection e.g. Mumps Orchitis Drugs and Toxins 4.Medications e.g. Sulfasalazine, cimetidine, nitrofurantoin, cyclophosphamide, chlorambucil, vincristine, methotrexate, procarbazine 5. Ingestants e.g. Alcohol, alcohol, marijuana, cocaine, anabolic steroids Environmental exposures e.g. Pesticides, radiation, Thermal exposure 6.Chromosomal abnormalities e.g.	1. Ductular obstruction, scarring Pelvic retroperitoneal. Inguinal or scrotal surgery (e.g. Retroperitoneal lymphadenectomy, herniorrhaphy, Y-V plasty, transurethral resection of prostate, vasectomy) Genital tract infections (e.g. Venereal diseases. Prostatitis, tuberculosis) Cystic fibrosis, Bilharzia 2. Retrograde ejaculation (e.g. Diabetic autonomic neuropathy, postsurgical, medications) 3. Antibodies to sperm or seminal plasma 4. Developmental abnormalities Penile anatomic defects (e.g. Hypospadias, epispadias, chordee) Congenital absence (bi- or unilateral) of vas

<p>3. Adrenal disorders e.g. Adrenal insufficiency</p> <p>Congenital adrenal hyperplasia</p> <p>4. Drugs e.g. Phenytoin, androgens</p>	<p>Klinefelter's syndrome, XXY seminiferous tubule dysgenesis, Y chromosome microdeletions</p> <p>7. Developmental abnormalities</p> <p>Cryptorchidism</p> <p>Congenital absence of vas deferens, seminal vesicles</p> <p>Immotile cilia syndrome</p> <p>Bilateral anorchia *(vanishing testis syndrome)</p> <p>Leydig cell aplasia</p> <p>Noonan's syndrome (male Turner's syndrome)</p> <p>Myotonic dystrophy</p> <p>Defective androgen biosynthesis eg. 5 α-reductase deficiency</p>	<p>deferens, bilateral ejaculatory duct obstruction; or bilateral obstructions within the epididymides – all associated with mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene</p> <p>5. Androgen insufficiency (e.g. Androgen receptor deficiency, testicular feminization syndrome)</p> <p>6. Poor coital technique</p> <p>7. Sexual dysfunction, impotence</p> <p>8. Idiopathic</p>
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2.5. Renal anatomy and physiology

2.5.1. General

The kidneys are a major vital organ system that processes the plasma of blood by removing substances and substrates that are waste products. The kidneys are a pair of bean shaped organs that have a fibromuscular capsule. The kidney plays an important role in homeostatic and regulatory function, i.e.:

- Central role of regulating the water concentration, inorganic-ion composition and the volume of the internal environment.
- Excreting metabolic waste products (urea, creatinine, uric acid etc.) into the urine in a rapid time as compared to intake.
- Excretion of foreign chemicals (drugs, pesticides, food additives, metabolites etc.) into the urine.
- Gluconeogenesis, i.e. the synthesis of glucose from amino acids in times of prolonged fasting into the blood.
- Act as an endocrine gland in the synthesis of erythropoietin, renin and 1,25-dihydroxyvitamin D₃ (Table 3) (Bagga, 2016).

Table 3: Functions of the kidney (Fine, 2014).

- | |
|---|
| <ol style="list-style-type: none">1. Regulation of water and inorganic-ion balance.2. Removal of metabolic waste products from the blood and their excretion in the urine.3. Removal of foreign chemicals from the blood and their excretion4. Gluconeogenesis5. Hormone Secretion<ol style="list-style-type: none">a. Erythropoietin – Controls erythrocyte formationb. Renin – Regulates angiotensin formationc. 1,25 dihydroxyvitamin D₃ – Influences calcium balance |
|---|

2.5.2. Renal orientation and relations

The kidneys are a pair of encapsulated organs located on the posterior abdominal wall on each side of the vertebral column extending from the level of lumbar vertebrae (LV) 1 to LV 4. Each retroperitoneal kidney has anterior and posterior surfaces; lateral and medial borders and upper and lower poles. The length of each kidney is approximately 11 - 13 cm in length of which the left kidney is larger and longer than the right kidney (Webb, 2016).

The kidneys are related superiorly to the suprarenal glands on the upper pole and inferiorly to the lumbar triangle. The right kidney has anterior relations to the liver, second part of the duodenum, small intestine and ascending colon. The left kidney is related anteriorly to the descending colon, pancreas, stomach, small intestine and spleen. The posterior relations of the kidneys include the psoas major muscle, pelvic diaphragm, quadratus lumborum, branches of the lumbar plexus, twelfth rib and lateral edge of the erector spinae (Marroig *et al.*, 2015).

2.5.3. Kidney structure

The kidney is covered by a thin, fibromuscular capsule. There are 2 distinct layers of the kidney that is the outer, pale cortex and the inner darker medulla. Sagittally, the kidney contains a vertical fissure called the hilum. The hilum transmits renal nerves and vessels and the upper part of the ureter. The hilum drains into a recess termed the renal sinus. Within the sinus, the renal pelvis consists of two or three wide tubes short in stature called the major calices. Each of the major calices divide into seven to fourteen minor tubes called minor calices. The minor calices contain millions of renal tubules or nephrons. The nephrons are the functional unit of the kidney (Figure 6). Each kidney has approximately one million nephrons. The nephrons contain an excretory duct end that allows for the conduction of urine to the minor calices. The blind end of the nephron forms the glomerular capsule which is double structured

and invaginated by capillaries. The convoluted expansion or tuft of capillaries is defined as the glomerulus whilst the glomerulus and capsule together are termed the renal corpuscle (Lierse, 1986; Mompeo *et al.*, 2016).

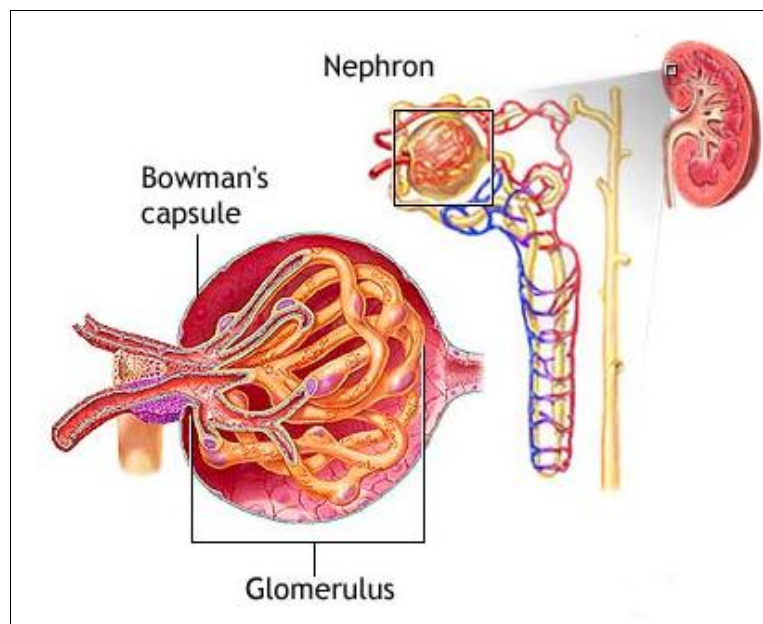


Figure 6: Anatomical sagittal section of the kidney, nephron and Bowman's capsule (Hall, 2015).

2.5.4. Renal Corpuscle

The renal corpuscle is also termed as the filters. They are derived from a compact tuft of interconnected capillary loops termed the glomerulus. Each glomerulus receives arterial supply from the afferent arteriole. The glomerulus protrudes into a fluid filled capsule called the Bowman's capsule. The space between the capillaries of the glomerulus is called the mesangium which are modified smooth muscle cells. Filtration of the blood plasma occurs whilst it traverses the Bowman's capsule and the remainder exits the glomerulus via the efferent arteriole. Located between the capillaries and the epithelial cells is the basement membrane. The glomerular capillary endothelium is fenestrated and has a coat of negatively charged

glycoproteins and glycoaminoglycans. This arrangement allows for the exclusion of plasma proteins such as albumin. The glomerular basement membrane consists of epithelial cells termed podocytes due to their foot like appearance and are connected to each other via desmosomes. The intrinsic glomerular cells and tissue macrophages are two distinct cell types that are located on the mesangium which is an extension of the glomerular basement membrane (Vander *et al.*, 2001; Hall, 2015).

2.6. Renal Failure

The filtration process of the kidneys is vital in regulation and homeostatic control. However, the occurrence of many debilitating factors including chronic diabetes, hypertension, cardiovascular disease and obesity compound the ability of this filtration mechanism in functioning optimally rendering it diseased and termed renal disease (Joannes-Boyau *et al.*, 2013). The incidence of renal disease has increased alarmingly worldwide, with approximately two hundred cases per million worldwide. The prevalence of renal disease exceeds 1800 cases per million (Levey and Coresh, 2012; Ladia *et al.*, 2016).

Bacteria, allergies, congenital defects, kidney stones, tumours and toxic chemicals contribute towards the aetiology of renal disease (Vander *et al.*, 2001). Proteinuria is a common diagnostic factor prevalent in kidney disease (Nadkarni *et al.*, 2016). The membrane permeability of the low molecular proteins allows for a small fraction of protein present in the glomerular filtrate, however, the tubular lumen allows for the complete absorption of this protein devolving the urine of any protein (Hall, 2015). In diseased kidneys, there is an abnormal enlargement of the podocyte spaces increasing the permeability of protein in which the tubular lumen cannot absorb all the protein as a secondary absorption mechanism. The combination of the site and cause of renal disease demarcates if the disease is pre-renal, intra-renal or post-

renal categorically and then further sub divided into categories related to the specific cause and anatomic locations (Table 4) (Ladia *et al.*, 2016).

Pre-renal causes relate to the inadequate blood flow to the kidney including structural lesions of the renal arteries, drug effects on renal blood flow, intravascular volume depletion and hypotension as a precursor to renal hyperfusion. Direct damage to the nephron results in intra-renal causes whilst a secondary effect of nephron damage includes obstruction and inadequate perfusion. The manifestations of these causes include systemic diseases and glomerular injury. Post renal causes are related directly to the obstruction of the urinary tract (Fagundes *et al.*, 2013; Lameire *et al.*, 2013).

Table 4: Major causes of renal disease (McPhee *et al.*, 2003).

Pre-renal
True volume depletion
Gastrointestinal, renal, bleeding and sweat loss
Heart Failure
Hepatic cirrhosis
Nephrotic syndrome
Hypotension
Non-steroidal anti-inflammatory drugs
Bilateral renal artery stenosis
Intra-renal
Vascular disease
Acute
Vasculitis
Malignant Hypertension
Scleroderma
Thromboembolic disease
Chronic
Nephrosclerosis
Glomerular disease
Glomerular nephritis
Nephrotic syndrome

<p>Tubular disease</p> <ul style="list-style-type: none"> Acute <ul style="list-style-type: none"> Acute tubular necrosis Multiple myeloma Hypercalcemia Uric acid nephropathy Chronic <ul style="list-style-type: none"> Polycystic kidney disease Medullary sponge kidney <p>Interstitial disease</p> <ul style="list-style-type: none"> Acute <ul style="list-style-type: none"> Pyelonephritis Interstitial nephritis Chronic <ul style="list-style-type: none"> Pyelonephritis Analgesic abuse
<p>Post-renal</p> <ul style="list-style-type: none"> Obstructive uropathy Prostatic disease Malignancy Calculi Congenital abnormalities

2.6.1. Chronic renal failure

The term chronic renal failure is used as general terminology depicting a variety of heterogenous diseases in a long standing and progressive nature of renal impairment. The similarity of disease variation is expressed by the aetiology, severity and most importantly the rate of progression of the disease. The severity of the disease is attributed to the loss of more than 70% of nephrons which is determined from the glomerular filtration rate (GFR) of < 60 millilitres per minute per 1.73 m^2 and albuminuria which also defines the chronic renal failure. The normal filtration rate in young adults is $125 \text{ ml/min per } 1.73 \text{ m}^2$. Glomerular filtration rate plays a central role in the pathophysiology and allows for

the disease to be categorized into 5 stages:

- **Stage 1:** > 90 ml/min per 1.73 m²
- **Stage 2:** 60 - 89 ml/min per 1.73 m²
- **Stage 3:** 30 – 59 ml/min per 1.73 m²
- **Stage 4:** 15 – 29 ml/min per 1.73 m²
- **Stage 5:** > 15 ml/min per 1.73 m² (Gaston and Wadstrom, 2005).

The most common cause of chronic renal failure is diabetes mellitus followed closely by hypertension and glomerulonephritis. Polycystic kidney diseases, obstruction and infection are amongst the least causes of chronic renal failure (Parmar, 2002). Patients with chronic renal failure present with vascular calcification almost in all localizations, from high calibre arteries such as the aorta, where the prevalence is high to medium and in small sized vessels, including the coronary arteries. This calcification increases the risk of cardiovascular disease and atherosclerotic plaque formations causing major blockages and loss of arterial expansive/contractive ability (Parrinello *et al.*, 2015).

There have been many attempts to evaluate the prevalence of single gene kidney diseases progressing to chronic renal failure. Mutation of genes occur in all societies/hierarchies and is the basis of adaptation, survival and progeny succession; however, in many instances these mutations can be deleterious affecting major physiological systems independently and in most cases as a collage of symptomatic incidences (Sveinbjornsson *et al.*, 2014).

Altered renal functionality due to genotype impairment is demonstrated in diseases such as:

- Autosomal dominant and recessive polycystic kidney disease
- Alport syndrome (progressive renal failure if untreated leading to chronic

renal failure)

- Nail-patella syndrome (autosomal dominant disorder leading to nephropathy)
- Congenital nephrotic syndrome
- Juvenile nephronophritis (autosomal recessive tubulo-interstitial nephritis)
- Bardet-Biedl syndrome (autosomal recessive disorder)
- Nephropathic cystinosis
- Primary hyperoxaluria
- Fabry disease
- Tuberous sclerosis
- Von Hippel-Lindsay disease (Levy and Feingold, 2000; Schrier *et al.*, 2014).

Chronic renal failure as a precursor of ESRD requires an allograft to be transplanted for survival of the individual. The kidneys are a set of extremely potent endocrine organs and a target for hormonal modulation of endocrine function. As a result of the alterations of the signalling and feedback of the hypothalamic–pituitary system, there is altered synthesis of the triggers required for hormone production. Decreased testosterone production (vital hormone for sperm production) impairs sperm production, affects libido and masculine makeup and further leads to hypergonadotropic hypogonadism. Peritoneal and haemodialysis rarely improves the hypothalamic–pituitary axis and renders the process even more detrimental. Renal transplant is said to be the best and most preferred method of reversing this effect, however, the condition of hypogonadism is still very evident and unchanged in the first 2 – 3 years post-transplant (Iglesias *et al.*, 2010).

2.6.1.1 Symptoms of chronic renal disease

From the onset of chronic renal disease, there is a marked change in the urination. Frequent urination of larger volumes with a more pale urine colour is a classic symptom; however, a decrease in urination frequency or urination of a lower dark

coloured volume is also a symptom. A more concerning factor would be the presence of blood in the urine (Jha *et al.*, 2013).

The kidneys main function is filtration and maintenance of the osmolality and osmolarity. Failure of the kidneys in excretion of the extra fluid leads to oedema in the legs, ankles, feet, face and/or hands (Bansal *et al.*, 2015). Erythropoietin is the necessary hormone that is produced by the kidney in stimulating the production of erythrocytes. It is imperative that there is sufficient erythrocyte production in allowing these oxygen carrying red blood cells to transport oxygen to the muscles and brain. As the kidney fails, there is decreased production of erythrocytes leading to anaemia including trouble concentrating, dizziness, cold sensations, fatigue and shortness of breath including pleurisy (Stevens and Levin, 2013).

Failure of the excretion process in eliminating toxins in the blood including by-products (urea and uric acid) leads to an accumulation of waste products in the blood resulting in uraemia. This in turn leads to skin rashes/itching, a metallic taste in the mouth, nausea and vomiting. The distaste produced in the mouth leads to a loss of appetite and severe weight loss (Levey and Coresh, 2012).

2.6.1.2. Confirmation of chronic renal failure using laboratory tests

Correlation of symptomatic diagnosis and laboratory blood tests provides a clear diagnosis of the extent and stage of disease progression. The laboratory tests (Table 5) provide a basis for the evaluation and treatment to follow.

Table 5: Normal levels of various functional laboratory tests used chronic renal disease diagnosis (Levey and Coresh, 2012).

Laboratory test	Normal levels	
Serum creatinine	Male: 0.5 – 1.5 mg/dL	Female: 0.6 – 1.2 mg/dL
Creatinine clearance	Male: 97 -137 mL/min	Female: 88 – 128 mL/min
GFR	> 140 mL/min/1.73 m ²	
Urine albumin	< 30 mg/day	
Microalbuminuria	< 150 mg/L albumin	
Blood urea nitrogen	7 – 20 mg/dL in adults 5 – 18 mg/dL in children	
Haematocrit	Male: 40 – 50%	Female: 36 – 44%
Haemoglobin	Male: 14 – 18 g/dL	Female: 12 – 16 g/dL

2.7. Renal transplant

The concept of transplantation in curing illnesses can be dated back to ancient Greek and Roman mythology. It was the pioneering of Alexis Carrel's canine allografting that demonstrated that a kidney could be transplanted into a distant location, re-vascularised and still perform its main function of filtration producing urine (Gaston & Diethelm, 2005).

Major pharmacological advances and breakthroughs in understanding the pharmacokinetics and pharmacodynamics of various drugs enabled physicians to

treat patients with acute renal failure thus sustaining and prolonging the quality of life of renal failure patients. This attempt was to preserve and save as much of the functional units of the kidney thus delaying the onset of chronic renal failure. However, the degree of damage to the organ in conjunction with compounding diseases including diabetes mellitus and hypertension exacerbated the progression of the renal failure leading to ESRD requiring a renal allograft (Wanner *et al.*, 2016).

From the onset of modern transplantation, cadaveric organs were used; however, religious beliefs and the prolonged wait for a mortality to occur rendered patients extremely frustrated and further increasingly worsened the functionality of the remaining kidneys. Dialysis (haemodialysis and peritoneal dialysis) are instrumental in providing the necessary *in vitro* filtration that regulates the homeostatic status of the body increasing the life expectancy of the patient (Zal *et al.*, 2015). Men that are on haemodialysis have been reported to have a high prevalence of infertility and sexual dysfunction due to an androgen deficiency in which the serum testosterone levels are low. The aetiology of the androgen deficiency is due to multitude of factors including disturbances of the hypothalamic–pituitary-testicular axis. A large degree of the testosterone is cleared by the haemodialysis further impacting of sexual function and capacity (Singh *et al.*, 2001).

2.8. Immunosuppressive therapy

Immunosuppressive therapy is imperative post renal transplant to prevent renal allograft rejection (Vincenti *et al.*, 1998). The transplantation of an immunological non-identical patient can lead to loss or rejection of the allograft, especially in the first 3 months post-transplant, if the patient is not undergoing immunosuppressive treatment. Therefore, the immunosuppressive is required to diminish the immune response to the transplanted kidney and prevent acute rejection of the organ. There are many immunosuppressive agents that are used in various combination regimens

including CsA, sirolimus, tacrolimus and MMF in conjunction with corticosteroids, e.g., prednisone. Initial dosages post-transplant (first 3 months) include higher dosages of drugs commonly administered in a triple immunosuppression therapy e.g. tacrolimus, MMF and prednisone. This is followed by maintenance levels of drug intake for approximately 6 to 12 months preventing adverse events of the immunosuppressive therapy including infections and malignancy (Chon *et al.*, 2014).

2.8.1. Cyclosporin A

Cyclosporin A is a cyclic polypeptide that inhibits calcineurin which is associated with nephrotoxicity and chronic renal damage (Safwenberg *et al.*, 1976). Cyclosporin engages with cyclophilin which is an intracellular protein of the immunophilin family and then forms a complex that interacts with calcineurin (Halloran, 2004).

In a cohort of 34 patients, assessment of sperm parameters using the computer aided sperm analysis have demonstrated a significant decrease in sperm concentration and forward progression of the spermatozoa including straight line velocity in patients undergoing either the triple immunosuppressive therapy or conventional CsA with prednisone treatment (Eid *et al.*, 1996). Previously studies in rats concluded that dose dependant CsA empirically causes decreased fertility, testicular atrophy, testicular dysfunction and decreased weight of the epididymis, seminal vesicle and prostate, desquamation of round spermatids, decrease in the number of spermatocytes and decrease in the diameter of the seminiferous tubules (Seethalakshmi *et al.*, 1987, 1988, 1990; Srinivas *et al.*, 1998).

Groth and colleagues (2010), analysed the exposure of CsA in mice during pregnancy in which the mice were subjected to intrauterine exposure. Cyclosporin A exposure significantly impacted on the ability of the mice in reproducing; however, offspring conceived post exposure were not affected anatomically or physiologically. Of special interest, male offspring produced from CsA exposed mothers were

tracked to adolescence and mated with unexposed females yielding a 100% positive pregnancy outcome.

The effect of immunosuppressive drugs on male fertility was also described in a study of 164 renal transplanted males (Xu *et al.*, 2009). A triple combination therapy of CsA, azathioprine and prednisone was administered to patients over a long term treatment plan. From the 164 patients, 167 children were born of which three patients fathered two children each. The analysis concluded that there was no obvious effect of CsA on male fertility post-transplant.

Further, an evaluation of the reproductive functions in male adolescents following renal transplant was conducted to analyse the semen variables and hormone profiles in eight post pubertal adolescents ($n = 8$). The study sample size was demarcated into 2 groups, i.e., group 1: underwent transplant before 14 years of age and group 2: underwent transplant after 14 years of age. The mean age at the time of transplant was 12 years in group 1 and 17.8 in group 2. The study participants in group 1 received CsA, azathioprine and prednisone with a longer duration of treatment as in comparison to group 2. Sperm concentration {15.5 +/- 15.7 (group 1) versus 82.3 +/- 64.2 mill/ml (group 2)} and sperm motility (37.8 +/- 30.9 versus 57.8 +/- 22.1%) were much lower in group 1 than in group 2. Further, only one patient in group 2 was normozoospermic. This indicated that early onset and longer duration of uraemia impairs reproductive function. The study concluded that introduction of CsA in combination with azathioprine and prednisone is a major contributing factor to sperm anomalies and dysfunction in peri-pubertal transplanted adolescents (Koyun *et al.*, 2009). Testicular function is often impaired even years after transplantation and poor semen quality decreases the prospect of fertility (Tainio *et al.*, 2014).

Long term effects of administered CsA with subsequent conversion to sirolimus were assessed in testicular function and morphology of renal transplanted rats against continuous administration of CsA. The testosterone levels were lower in the

converted group whilst the FSH and LH levels increased in the same group; however, these did not have any statistical significance. Histologically the converted group also showed a high degree of testicular ultrastructure damage concluding that CsA replacement to sirolimus causes severe damage to testicular function than the continuous administration of CsA (Chen *et al.*, 2013).

2.8.2. Sirolimus

Sirolimus is an immunosuppressive agent that modulates immune responses at key points in the cell cycle. It is derived from the macrolytic lactone in *Streptomyces hygroscopicus* (Sehgal *et al.*, 1975; Vezina *et al.*, 1975). The targets of mammalian inhibitors inhibit the cell proliferation signal specifically the endothelial and mammalian cells by deterring DNA and protein synthesis, thus causing cell arrest (Saunders *et al.*, 2001; Dupont and Warren, 2003).

Sirolimus binds to FK BP12 which causes this to bind to the mammalian target of rapamycin (mTOR) (Figure 7). The mTOR is a key regulatory kinase. This newly formed complex now consisting of the mTOR-FKBP12-sirolimus functions via two pathways:

1. Inhibits the kinase p70 S6 (major molecule in cytokine-induced proliferation)
2. Impedes the enzymatic activity of the kinase dependant on cyclin (cdk2-cyclin E complex – functioning in a regulatory capacity of the G1/S conversion (Wicker *et al.*, 1990).

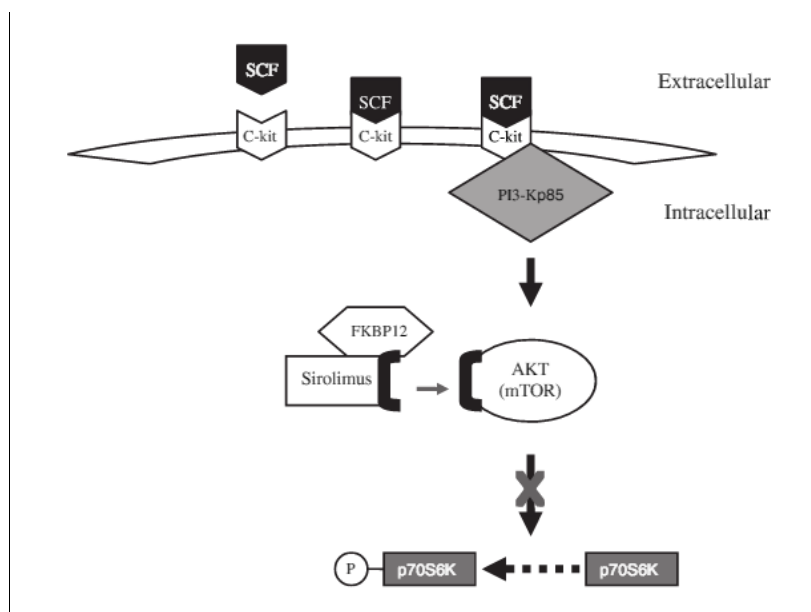


Figure 7: Mechanism of sirolimus action (Huyghe *et al.*, 2007).

Kaczmarek and colleagues (2004), were the first to study the effects of sirolimus on the male hormone profile. They examined the profiles of a 132 heart transplant recipients on sirolimus treatment ($n = 66$) with a case control pair matching ($n = 66$). The results showed a significant decrease in serum testosterone with an increase in FSH and LH. Subsequent to this many other studies evaluated the effects of sirolimus on hormone profile. These investigations included case controls, cross sectional studies and a prospective randomized trial either as a combination of sirolimus with a calcineurin inhibitor or sirolimus with everolimus (mTOR inhibitor). Of significance, levels of free testosterone in patients with sirolimus treatment were much lower. Follicle stimulating hormone and LH levels were markedly increased correlating a direct link between sirolimus and gonadal dysfunction (Fritsche *et al.*, 2004; Tondolo *et al.*, 2005). Sirolimus treatment is also associated with erectile dysfunction and decreased libido (Lee *et al.*, 2005).

In a case report by Bererhi and co-workers (2003), a 36 year old male (post-transplant) was investigated for infertility. Upon investigation there was a marked decrease in sperm concentration, motility and vitality including poor morphology. Sirolimus administration was then interrupted and replaced with a regimen of tacrolimus and further investigated 6 and 12 months later revealing increases in all parameters.

The c-kit-induced activation of phosphatidylinositol 3 kinase is essential for proliferation of the spermatogonia whereas sirolimus has a central inhibitory role in the stem cell factor/ c-kit dependant process in the spermatogonia. The c-kit receptor tyrosine kinase is a key regulator in spermatogenesis and is articulated in the type A spermatogonial cells of which the ligand of the c-kit is the stem cell factor. The sertoli cells produce the stem cell factor and in response to the stem cell factor; the c-kit triggers a multitude of signalling pathways that regulates the proliferation and differentiation of the primordial germ cells. Sirolimus inhibits the cyclin D3 production and phosphorylation that is essential and necessary to induce the stem cell factor in spermatogonia and promote the cell cycle proliferation (Feng *et al.*, 2000; Huyghe *et al.*, 2007). Defective spermatogenesis was also noted as a result of the defective binding of the P13-K to the c-kit in which the males were rendered sterile due to an inhibition in the early stages of spermatogenesis (Kissel *et al.*, 2000; Rostaing and Kamar, 2010).

Analysis of the pituitary-gonadotrophic axis which is essential for the production of gonadotropin releasing hormone (GnRH) receptor showed that when the cells were treated with sirolimus, in contrast to the untreated cells, there was an almost 50% decreased activation of the treated cells following GnRH stimulation (Sosnowski *et al.*, 2000).

Spermatogonial proliferation and differentiation requires signalling through phosphatidylinositol 3 kinase/AKT pathway, which is inhibited by sirolimus and also

decreases the sensitivity of the GnRH receptors (Gonzalez *et al.*, 2012). Pallet and Legendre (2013), further reviewed that spontaneous pregnancy rates appeared to be decreased by 15-fold with mTOR treatment compared with a sirolimus free regimen. They also pointed out the importance of the abolition of spermatogonial cell proliferation by blocking the PI3/AKT pathway by sirolimus.

Zuber and colleagues (2008), conducted an observational study to evaluate the frequency and severity of sirolimus associated alterations in sperm parameters and their impact on fathered pregnancy rate. A complete history was obtained from 95 recipients. A comparison of the patients on sirolimus and those not on sirolimus revealed that the sirolimus group had a significantly reduced total sperm count (28.6 ± 31.2 mill versus 292.2 ± 271.2 mill; $p = 0.006$) and a decreased proportion of motile spermatozoa ($22.2 \pm 12.3\%$ versus $41.0 \pm 14.5\%$; $p = 0.01$). The fathered pregnancy rate was also much lower in the sirolimus group compared to the control group, i.e., approximately 6% of the sirolimus patients fathered children in contrast to 92% in the control group. These results were indicative of the association that sirolimus has on impaired spermatogenesis and in reducing fertility in male renal transplanted patients.

In a case report of a 26 year old male who underwent heart and lung transplantation; a triple combination of sirolimus, tacrolimus and corticosteroids were administered to the patient post operatively (Deutsch *et al.*, 2007). Three years later, the patient was diagnosed with a metastatic testicular lesion and underwent a hemicastration of the left testis. The histological findings revealed a high degree of monomorphic leydig cells and leydig cell neoplasia whilst the rest of the testicular parenchyma exhibited testicular atrophy with severely reduced spermatogenesis as a result of vacuolization of the seminiferous epithelium and loss of differentiating spermatogonia. Furthermore, the semen analysis presented with severe oligoasthenozoospermia with a subsequent increase in sperm concentration and motility after withdrawal of sirolimus (Deutsch *et al.*, 2007).

The testicular toxicity of sirolimus has shown to cause sexual hormone dysfunction, seminiferous tubule dystrophy and altered spermatogenesis; however, withdrawal of sirolimus causes the spermatogenesis blockade to be reversed (Rovira *et al.*, 2012). Gonadal toxicity has also been shown in a study in which the gonadal dysfunction and infertility was assessed in nine patients on sirolimus treatment (6 males and 3 females). The study concluded that there was a high rate of sirolimus-related gonadal dysfunction and infertility amongst the examined cohort of patients. Of note, the authors concluded that serum creatinine levels increased after discontinuation of sirolimus indicating nephrotoxicity of sirolimus, in contrast to previous documented research (Boobes *et al.*, 2009). However, this study sample size was extremely small and statistical significance of the sample was biased and did not represent a true population of the demographic being investigated. The FKBP12, which is the immunophilin 12-kDa FK 506 binding protein, stabilizes the calcium release channel activity in different tissues. The disruption of the FKBP12 and calcium release channel showed a marked decrease in calcium content of sarcoplasmic reticulum by increasing the calcium leakage through the ryanodine receptor. This further concluded that the disruption of the FKBP12-ryanodine receptor complex may lead to modifications of rat vas deferens physiology and compromise male fertility (Scaramello *et al.*, 2009).

2.8.3. Tacrolimus

Tacrolimus is a macrolide immunosuppressant which inhibits calcineurin. It is produced from *Streptomyces tsukubaensis* and is most commonly used in renal transplantation due to its diminished side effects and relative adverse events with other organ systems. It has shown to have a better drug compliance and lower pill burden together with much more improved outcomes in graft survival, lipid profile and glucose tolerance (Ma *et al.*, 2016).

Tacrolimus modulates cell mediated and humoral immune responses associated

with allograft rejection via several pathways. The pivotal mechanism of action is the inhibition of the signal transduction pathway leading to T cell activation due to the formation of a drug complex with the immunophilin FK506 binding protein 12 (Garnock-Jones, 2015). This blocks the phosphatase activity of the calcineurin and production of interleukin 2. It also inhibits cellular activities such as nitric oxide synthetase activation and apoptosis further potentiating the action of the corticosteroids (Scotleveyt *et al.*, 2003). Therefore, tacrolimus inhibits the activation of the T helper cells and cytotoxic T cells and further inhibits the T helper cell dependent B cell proliferation whilst contrastingly preventing inhibition of the secondary proliferation of activated T cells in response to interleukin 2. It also does not modify the function of the mononuclear phagocytes or natural killer cells or interferes with antigen presentation (McCormack, 2014).

Caneguim and co-workers (2009), examined structural alterations in the seminiferous tubules of rats treated with tacrolimus. Noticeably there were decreased weights of the testis of the exposed group of rats in contrast to the control group. Histological sections of the rats treated with tacrolimus yielded displaced sertoli cells to the adluminal compartments together with dis-orientated round and elongated spermatids positioned in the basal compartment and intra-epithelial spaces suggestive of germ cell loss (Figure 8). This also suggested that there was a significant decrease in the quantity of germ cells, spermatocytes and spermatids being produced after tacrolimus treatment. The exposure of tacrolimus in mice could also, be linked to germ cell mutation in which there were aberrations in meiotic chromosome and the formation of abnormal spermatozoa in the epididymis and vas deferens. This in turn led to decreased fertility due to the poor morphology of the spermatozoa and subsequent implantation failure and high incidence of miscarriages. However, no significant decreases or deleterious effects on the sperm concentration were noted (Abou-Shabaan *et al.*, 2011).

End stage renal disease is characterized by hypogonadism and low testosterone

levels together with alterations of the hypogonadal-pituitary axis which leads to oligozoospermia together with libido loss, erectile dysfunction and infertility. The sexual function of patients post-transplant has shown to increase, especially levels of testosterone production, following tacrolimus treatment indicative of improved libido and an increase in sexual frequency (Anantharaman and Schmidt, 2007).

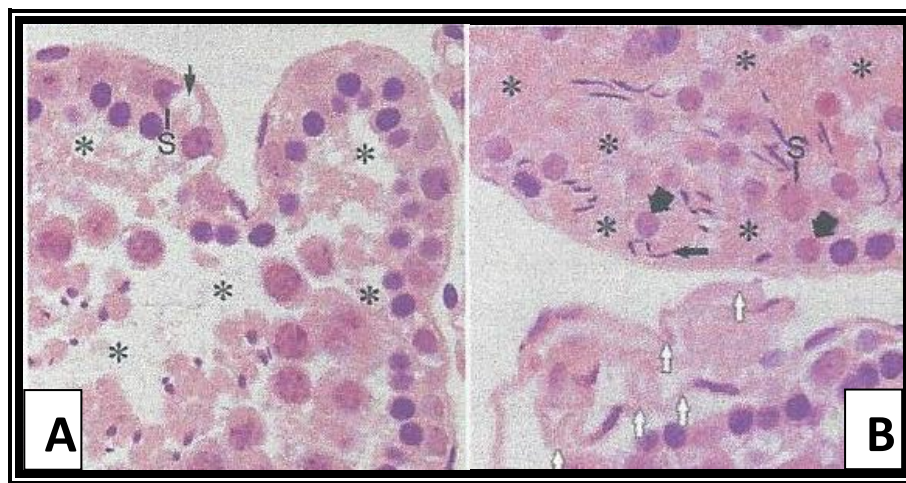


Figure 8: A – Intraepithelial spaces (asterisks) due to lack of spermatocytes. B – Lack of germ cells (asterisks) is noted in the basal and adluminal portions. Round (thick arrows) and elongate (thin arrow) spermatids are abnormally positioned. S denotes a single sertoli cell with a dislocated nucleus (Caneguim *et al.*, 2009).

Researchers have debated the effect of tacrolimus on semen parameters post renal transplant in human and rat models. In a case report by Skrzypek and Krause (2007), a young patient presented with azoospermia during treatment with sirolimus post renal transplantation. After switching to tacrolimus, spermatogenesis of the patient recovered and post inspection after five months cessation of sirolimus treatment yielded a sperm concentration of 8 mill/ml.

The effect of tacrolimus (administered subcutaneously) on spermatogenesis in male

rats were reported by Hisatomi and colleagues (1996). The results indicated that tacrolimus reduced sperm counts and motility through direct action on the sperm in the epididymis, but not sperm production in the testis. Sperm counts and motility returned to normal on discontinuation of tacrolimus. Copulation and fertility index was not affected by tacrolimus, but a decrease in number of live fetuses was observed in the high dose group (3 mg). At the dosage schedule of 1 mg, no modification of the fertility parameter (implantation index) was observed.

The results of a study by Chen and co-workers (2013), compared the effect of long term, orally administered therapeutic doses of tacrolimus (n=8), CsA (n=8) and sirolimus (n=8) on the male reproductive system in unilateral nephrectomised rats. In contrast with CsA and sirolimus, tacrolimus did not affect sperm motility or morphology, body weight gain and testicular injury or development. Tacrolimus induced a mild, statistically insignificant, decrease in sperm count. Testosterone levels were reduced whilst LH, FSH and prolactin levels were increased. The study also concluded that mild impairment of spermatogenesis in tacrolimus treated rats may have been secondary to its effect on the hormonal axis rather than a direct injury to the testis and therefore, tacrolimus induced mild changes in spermatogenesis without histological evidence of testicular injury.

The effect of immunosuppressant therapies on semen parameters was assessed in 37 kidney transplanted on which 20 subjects were on tacrolimus and 17 on CsA based therapies against 15 healthy fertile men. The sperm viability and motility, i.e., total percentage motility; straight line velocity (VSL), curve line velocity (VCL), velocity of average path (VAP) and morphology were assessed. There were no significant differences in sperm viability rate, VCL and VAP between the groups. The rate of anomaly, total motility and VSL were significantly lower and higher, respectively, in the tacrolimus versus CsA group. The study also concluded that tacrolimus combined with MMF could help recover the motility and morphology of sperm in kidney transplanted patients (Cao *et al.*, 2006).

The National Transplantation Registry (NTPR) was established in 1991 in North America and is a registry of female transplant recipients who have had post-transplant pregnancies or currently pregnant and male transplant recipients who have fathered pregnancies (Coscia and Armenti, 2010). Hasani and colleagues (2006), analysed 148 outcomes of 142 pregnancies fathered by 114 kidney transplant recipients (reported to NTPR) on immunosuppressive medications (including tacrolimus, CsA, MMF and sirolimus) and corticosteroids (azathioprine and prednisone). Of the 148 outcomes, there were 137 live-births and 11 spontaneous miscarriages. Within the group of fathers who were on tacrolimus based regimen (n = 40), there was one report of an undescended testis and one case of spina bifida. All remaining children were healthy and developing well, concluding that kidney transplant recipients on immunosuppressive medication appear similar to the general population. Further studies confirmed these results and findings and also emphasised that post renal transplant, the fertility potential of male renal recipients was similar to the general population (Armenti *et al.*, 2000; Holmgren *et al.*, 2004; Coscia and Armenti, 2010).

In a retrospective analysis of 111 males, the reproductive fecundity of males who received a liver transplant between the ages of 5 and 19 was investigated. These recipients were tracked into their reproductive years. Two of the males fathered children four and six years post-transplant, respectively. One of the patients that fathered a child was on tacrolimus therapy (Ecevit *et al.*, 2012).

2.8.4. Mycophenolate mofetil

Mycophenolate mofetil is a lymphocyte selective anti-proliferative agent which is an ester pro-drug of mycophenolic acid. It acts as a reversible non-competitive inhibitor of inosine monophosphate dehydrogenase (Karim *et al.*, 2002; Mak *et al.*, 2009). It reduces the concentration of guanine nucleotides in T and B lymphocytes, thereby, hindering the DNA synthesis and metabolic actions facilitated by guanine

triphosphate (Ransom, 1995). There are very few studies that have evaluated the use of MMF solely and its effects on male fertility potential. Presently, MMF is contraindicated in pregnancy due to its adverse effects in increased foetal loss and congenital malformations (Ostensen *et al.*, 2008; Perez-Aytes *et al.*, 2008).

Jones and colleagues (2013), examined the outcomes of pregnancies in males who received a renal transplant and were undergoing MMF treatment. Study participants were enrolled from the NTPR database and required to answer a questionnaire telephonically together with a telephonic follow up every 1 – 2 years. A total of 152 participants were identified as being treated with MMF products of which 205 pregnancies were achieved. This concluded that male transplant recipients that underwent and are still being treated with MMF have the same capability and potential of fathering children as the general population; however, a limitation of this study was that additional studies designed specifically on MMF need investigation in assessing potential risks or benefits that MMF may have on male infertility.

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1. Patient recruitment

Patients were recruited from the databases of nephrologists in private practice (greater Durban area) and the academic renal unit at Inkosi Albert Luthuli Central Hospital (IALCH), South Africa. Permission for accessing the respective databases and patient recruitment were granted by the private practitioners, medical manager – IALCH (Appendix A) and head of the academic Renal department – IALCH (Appendix B). Patients were recruited according to the inclusion and exclusion criteria of the study.

3.1.1. Inclusion criteria

- Male patients between the ages of 18-45 years.
- Patients on immunosuppressive treatment following a renal transplant.

3.1.2. Exclusion criteria

- Male patients younger than 18 years of age and older than 45 years.
- Male patients on psychological treatment or psychologically challenged.
- Male patients currently or previously exposed to radiotherapy or chemotherapy.
- Male patients that have undergone a vasectomy or clinical diagnosis of obstruction of the vas deferens and/or cryptorchidism.

3.1.3. Informed Consent

Prospective patients from private nephrologist's and the renal unit at IALCH were contacted by the investigator telephonically and at the weekly renal clinic at IALCH. Patients that met the inclusion criteria were interviewed by the investigator and given an information leaflet with attached informed consent to peruse in their desired language of English (Appendix C) or Isizulu (Appendix D). Patients were informed of the nature of the study, risks and benefits as prescribed by good clinical practice (GCP) guidelines (Department of Health, 2008). The investigator encouraged patients to express any concerns pertaining to study participation and for further clarification. Patients were also made aware that study participation is voluntary and participation can be withdrawn/ discontinued at any time without prejudice. Patient's content with the nature of the study and participation, signed the informed consent in the presence of the investigator and countersigned by the principal investigator. A copy of the informed consent and information leaflet were given to the patient as per GCP guidelines.

3.1.4. Medical history

All study patients had a detailed interview with the investigator. A medical history was assessed as an information tool for the study (Appendix E). The history included the following:

- Race demographics
- Age
- Diagnosis and aetiology of renal failure
- Type of dialysis (if any) used pre renal transplant
- Date of transplant
- Commencement of immunosuppressive therapy
- Regimen, dosage and duration of immunosuppressive therapy including

corticosteroid therapy, dose and duration

- Number of children fathered (if any) pre- and post-transplant
- Diabetes
- Hypertension
- Erectile dysfunction.

3.2. Sample collection

Patients were requested to abstain from sexual intimacy for a period of 3 – 5 days prior to sample collection. The semen samples were produced in the masturbatorium at Durban Fertility Clinic (DFC) and a private designated room in the renal clinic at IALCH, South Africa. Patients were counselled on the proper washing guidelines prior to sample production as prescribed by the standard operating procedure at DFC. The washing guidelines included cleansing of genitalia and hands with Bioscrub (*Transpharm 722159#001*) followed by rinsing (distilled water) and drying of the aforementioned areas. Emphasis was placed on proper rinsing and drying to prevent contamination and sperm necrosis due to water contact. Proper delivery of the sample by masturbation, in accordance with aseptic techniques, is pivotal in preventing contaminants including genital epithelium, pubic hair and other artefacts.

3.3. Equipment

The following materials as illustrated in Table 6 were used in the analysis of the collected samples. In accordance with good laboratory practice (Gianaroli *et al.*, 2000) and best practices of andrology (Agarwal *et al.*, 2016), equipment and disposables as prescribed by WHO (2010), were employed in the analysis of the semen samples (Figure 9). Mechanical equipment including light microscopes,

Gilson pipettes and serological pipette aspirator were of sound mechanical operation. All andrology equipment possessed a detailed service history whilst strict quality control was maintained on the light microscopes using the Kohler illumination.

Table 6: Equipment list of disposables and non-disposables.

Disposables	Non-disposables
Eosin B (<i>Sigma-Aldrich 861006-10G</i>)	Schott bottles (50 ml)
Nigrosin (<i>Merck 1.15924.0025</i>)	Light microscope (Nikon)
Hemacolor (<i>Merck 1.11661.0001</i>)	Light microscope (Lasec)
IgG and IgA (<i>ALSACS SPMG_S/SPMA_S</i>)	Laminar Flow Hood (Vivid Air)
10 ml serological pipette (<i>Scientific group 357551</i>)	Centrifuge (Heraeus)
Glass pasteur pipette (<i>Lasec GLAS2P20M150</i>)	Gilson pipette 10 – 100 µl (Scientific Group)
Double frosted slides (<i>Lasec 1000F-02-1009</i>)	
100 µl pipette tips (Lasec)	
pH strips (<i>Merck 1.09535.0001</i>)	
Coverslips (<i>Lasec 0101050</i>)	



Figure 9: Study equipment and consumables (DFC, 2016).

3.4. Study design and method

3.4.1. Study design

This study is a prospective, observational investigation with ethical approval from the DUT ethics committee (Appendix F) and Department of Health ethics board (Appendix G). Recruitment was based on voluntary participation and adherence to the inclusion and exclusion criteria. Study patients were assigned a study number beginning with the prefixes DUTSA and followed by the study number e.g. 01. Patient details including medical history and patient demographics were recorded on a questionnaire (Appendix E) with strict confidentiality maintained by the investigator. Reliability and validity of the results were maintained by duplicate analysis of the macroscopic and microscopic investigations in adherence to WHO guidelines on semen analysis (WHO, 2010) and good laboratory practice (Gianaroli *et al.*, 2000).

3.4.2. Semen analysis

3.4.2.1. Macroscopic investigation

Patients were required to produce a single semen sample that was analysed according to the WHO guidelines on semen analysis (WHO, 2010). Post semen collection, the sample was allowed to rest at room temperature (approximately 23°C) for 20 minutes to allow for liquefaction. The results of the semen parameters were documented on a semen analysis template (Appendix H).

Procedure:

- The sample's volume was measured using a graduated serological pipette with a wide bore of 1.5 mm diameter and range of 1 – 10 ml.
- Appearance and colour of the semen were noted.
- Viscosity was measured by allowing the semen sample to drop from the opening of the pipette by gravity which should form a thread length not more than 2 cm. Observation of thread formation longer than 2 cm was noted as abnormal viscosity whilst liquefaction time would be documented as > 20 minutes.
- Litmus paper with a range of 6 – 10 was used to analyse the pH of the sample.
- An 11 µl drop of semen was aspirated using the Gilson pipette together with a 1 – 100 µl tip and spread evenly on the litmus part of the pH paper. The analysis was performed after there was uniformity of the colour on the litmus paper. This was compared to the reference chart of the pH box.

3.4.2.2. Microscopic investigation

The microscopic investigations analysed the spermatozoa in detail and its viability

using a phase contrast microscope. An initial microscopic examination was performed on the native sample to provide an overview of the sample including mucus strand formation, sperm agglutination and the presence of other cells i.e. epithelial, round cells (leucocytes and immature germ cells) and sperm motility.

3.4.2.2.1. Step 1 – Semen Overview

- Fresh sample was mixed thoroughly by gentle swirling of the sample in the original container avoiding rapid movement in order to prevent formation of air bubbles.
- An aliquot of 10 µl homogenously mixed semen was added to a double frosted slide and covered with a 22 x 22 mm coverslip.
- Once the sample was devoid of air bubbles and stopped drifting, assessment of agglutination and aggregation of spermatozoa were performed noting the degree of aggregation and the grade of agglutination (Table 7) together with other cellular elements including round cells and epithelial cells.

Table 7: Grade and degree of agglutination (WHO, 2010).

	Grade 1	Grade 2	Grade 3	Grade 4
Degree of Agglutination	Isolated <10 sperm/agglutinate, many free sperm	Moderate 10 – 50 sperm/agglutinate, free sperm	Large agglutinates >50 sperm, some sperm still free	Gross all sperm agglutinated and interconnected

3.4.2.2.2. Step 2 – Motility assessment

- The semen sample was mixed to create a homogenous mixture.
- Ten µl of semen was added on a slide and covered with a coverslip (22 x 22

mm).

- The sample was observed under phase contrast microscopy at 200X and 400X magnification.
- Two hundred spermatozoa were analysed according to categories of sperm movement, i.e., progressive motility (PR), non-progressive motility (NPR) and immotile (IM).
- Motility assessment for the study encompassed total motility. Total motility was calculated as follows:

$$\text{Total motility} = \frac{\text{PR} + \text{NPR}}{(\text{PR} + \text{NPR} + \text{IM})} \times 100\%$$

3.4.2.2.3. Step 3 – Concentration evaluation

- The spermatozoa were immobilized by transferring an aliquot of mixed semen into a test tube and subjected to hot water ($\pm 45^{\circ}\text{C}$) for 5 minutes.
- A five μl drop of semen was added using a Gilson pipette onto the Makler chamber and covered with the Makler glass coverslip.
- All sperm within the ten squares (left to right) were counted (Figure 10).
- In duplicate, all sperm within the ten squares (top to bottom) were also counted.
- Both values were added and an average obtained denoting the concentration of sperm mill/ml.
- The Makler chamber and coverslip were then cleansed with water and dried thoroughly with a soft tissue/lint free lens paper.
- The loading and analysis steps were repeated for a duplicate unbiased result.

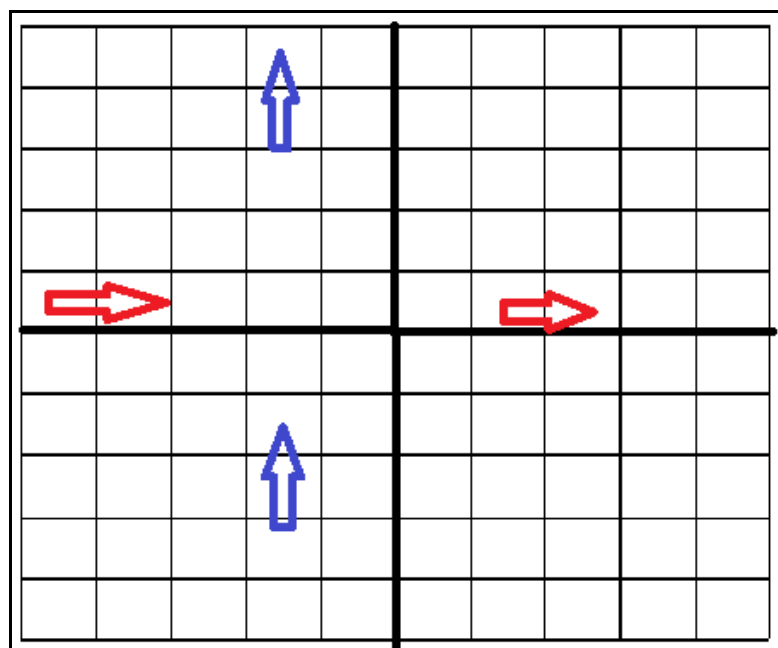


Figure 10: Illustration of sperm counting depicting the Makler grid. Red and blue arrows denote the direction of analysis and counting.

3.4.2.2.4. Step 4 – Slides, smear, vitality stain and antibody preparation

- Two slides each were labelled as morphology, vitality and immunoglobulin with the date, study number and the slide category as previously mentioned.
- An 11.5 µl of homogenous semen was added onto each of the morphology slides. A clean new slide was used to make a feather smear, whilst smears for viscous samples were made using the pipette smear (Figure 11).
- Two drops of semen were expelled in a round bottom test tube followed by 2 drops of eosin. The sample was gently mixed and allowed to stand for 30 seconds. Two drops of nigrosin was then added into the same test tube and the entire sample was mixed and allowed to rest for 30 seconds. A drop each of eosin – nigrosin mixture was added onto the duplicate slides marked vitality; using either a pipette or feather smear and left to air dry.
- On each slide marked immuno, the area of the slide were demarcated into

right and left hemispheres. On the left area, 5 μ l of IgG (white lid solution) in conjunction with an adjacent 5 μ l of IgG (blue lid solution) were added.

On the right hemisphere 5 μ l of IgA solution was added. A 5 μ l drop of homogenous semen was then added next to each droplet complex. The edge of each new coverslip (22 x 22 mm) was used to mix each complex thoroughly and placed over each mixed area.

- The immune slides were placed in a humid box for 10 - 15 minutes to allow for homogenous mixing, attachment and activation.

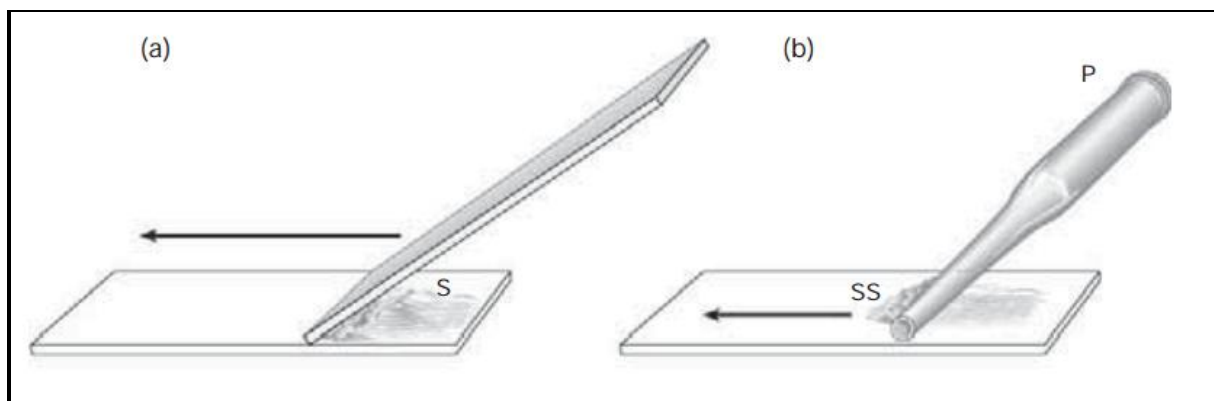


Figure 11: Schematic illustration of slide smear techniques; (a) – Feathering method, (b) – Pipette method (WHO, 2010).

3.4.2.2.5. Step 5 - Staining of morphology slides and evaluation of IgG and IgA.

- The Hemacolor staining technique was used for morphology staining. Four Schott bottles were labelled with H1, H2, H3 and rinse. Each solution of Hemacolor was added into corresponding marked bottles, respectively. Distilled water was used for the rinse. Each morphology slide was placed individually into H1 for 20 seconds, H2 for 20 seconds, H3 for 20 seconds, rinsed thoroughly (dipped 5 – 8 times) and allowed to air dry.

- After 10 -15 minutes had elapsed for the immuno slides; the slides were examined under phase contrast microscopy at 200X magnification. Positive IgG or IgA was observed if a free swimming sperm had immunobeads attached to the head, neck or tail.
- No attachments implied a negative test for IgG and IgA. An aggregation of the beads indicated that the mixing of the complex was not complete and new slides were made and examined.
- If attachments of the immunobeads were noted, 200 spermatozoa were counted as either positive or negative denoting attached and non-attached, respectively.

3.4.2.2.6. Step 6 - Morphology and vitality evaluation

- An analysis of 200 spermatozoa was performed at random points of each slide using phase contrast microscopy with a 100X oil immersion objective (Figure 12).
- The sperm head (acrosome and post acrosome regions) was initially evaluated followed by the neck and tail.
- Sperm head dimensions or abnormalities as either small, megalo, double heads, abnormally shaped and elongated heads were examined.
- The insertion of the neck with the head and attachments of residual bodies on the neck, i.e., precursor cells including the thickness of the neck region were evaluated.
- The length of the sperm tail was examined for curvatures, spiralled tails including the distal end which was also regarded as abnormal including double tails.
- For vitality evaluation, 200 spermatozoa were analysed also on a phase contrast microscope using a 100X oil immersion objective.
- The number of pink stained spermatozoa versus white stained spermatozoa

- was counted. The pink stained spermatozoa denoted that the sperm was necrotic and the sperm head membrane became permeable to the eosin stain. The white spermatozoa showed intact membrane integrity indicative of viable spermatozoa.
- Sperm stained with a small band at the base of the head and in junction with the neck were deemed as viable.

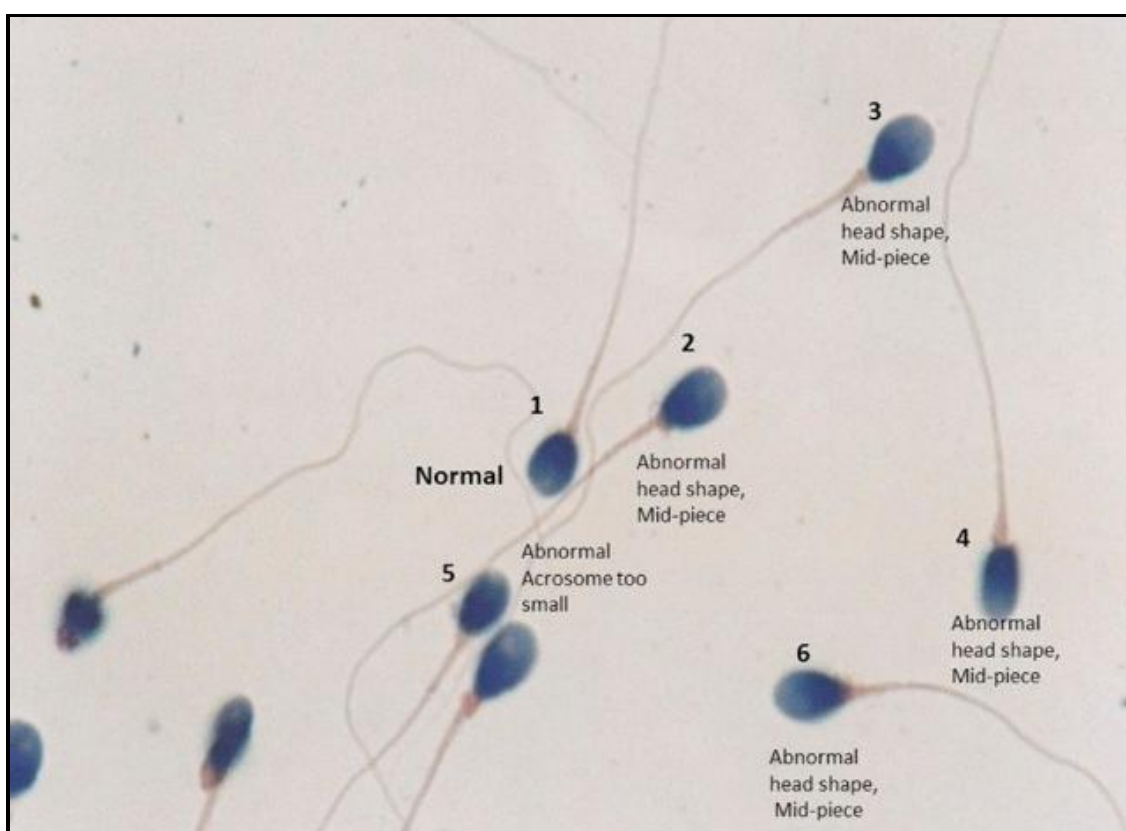
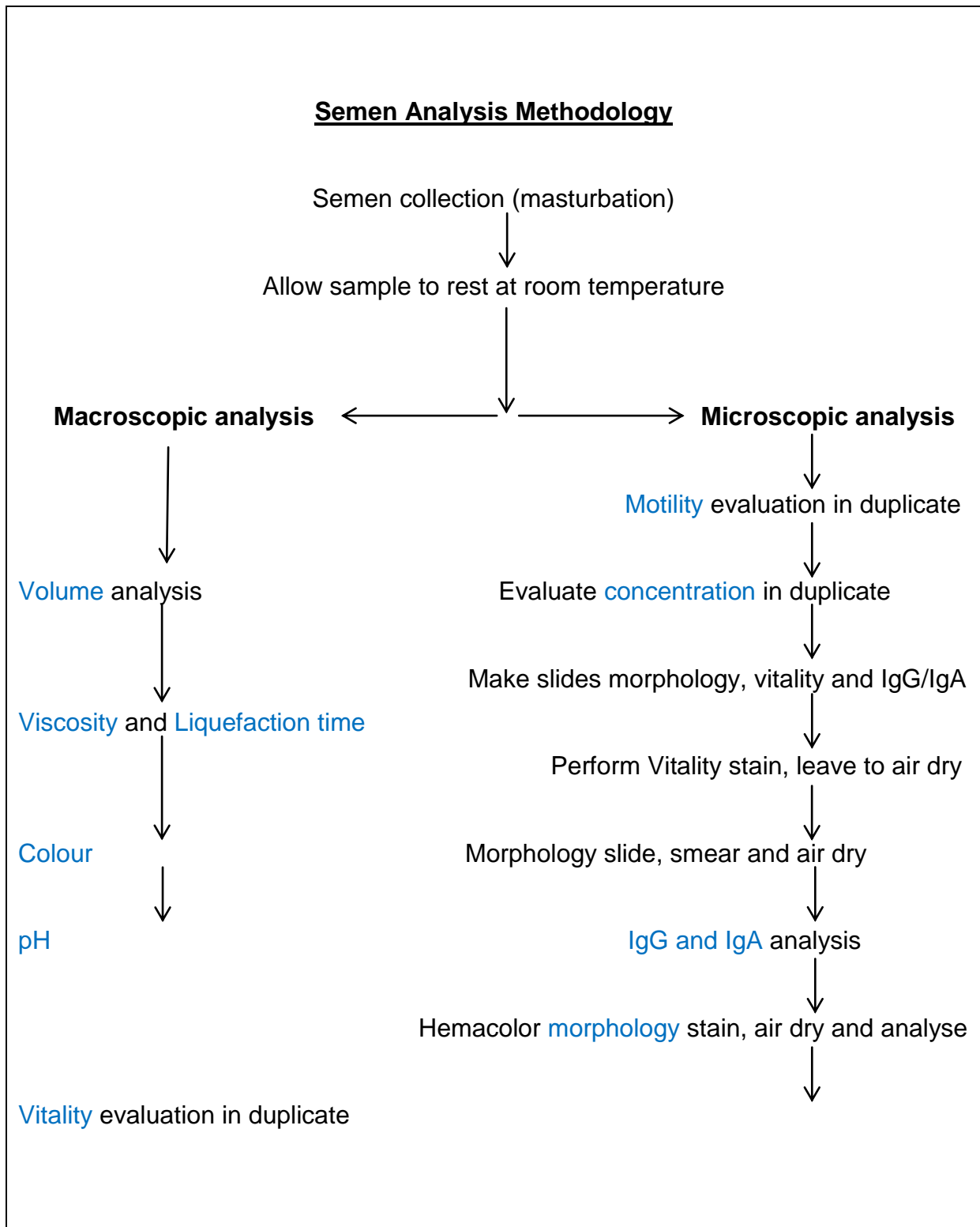


Figure 12: Hemacolor morphology stain with analysis of normal and abnormal sperm morphology (Franken, 2015).



3.5. Statistics

3.5.1. Statistician

Consultation, analysis and interpretation of the statistics were performed with the assistance of a biostatistician, Dr Tonya Esterhuizen, University of Stellenbosch, Cape Town.

3.5.2. Software and methodology

The statistical significance of the data was assessed and evaluated using the IBM SPSS version 23.0 (SPSS Inc, Chicago, Illinois, USA). A p value of < 0.05 was considered statistically significant. Parametric statistical tests were used since the continuous outcomes were approximately normally distributed. Descriptive statistics were presented as mean, standard deviation, range and 95% confidence intervals for quantitative distributed variables. One sample t-tests were used to compare mean values with the WHO reference values. Two sample t-tests were used to compare independent groups. Pearson's correlation was used to determine the direction, strength and significance of the correlation between X and Y variables between the different semen parameters. Multiple linear regression analysis was used to assess the relationship between dose and duration of selected drugs on the outcomes. ROC curves were used to assess predictive value for fathering children and assessing cut off values to optimise sensitivity and specificity.

3.6. Confidentiality

A strict and high degree of patient anonymity was maintained in the study. Patients were assigned study numbers which were recorded in a patient recruitment list. All patient details were known solely by the investigator and stored in an electronic

format with encryption. Encryption passwords were known only by the investigator. All material and documents related to the study and patient details, i.e., hard copies and electronic backups are stored in a secure restricted lockable cupboard of which access can be obtained by the investigator only. All documents will be stored for a period of 5 years and thereafter disposed of appropriately, i.e., hard copies will be shredded and electronic backups deleted and storage devices formatted.

CHAPTER 4

RESULTS

4.1. Introduction

The aim and objective of the study was to assess the effect of immunosuppressive therapy on semen parameters of male patients post renal transplant. Semen analysis is an optimal diagnostic tool in evaluating male fertility potential (Walker, 2011). The WHO guidelines were derived from semen characteristics of fathers, whose partners were impregnated within 12 months of contraception discontinued (WHO, 2010). Study patients underwent a comprehensive semen analysis as prescribed by the WHO guidelines. The raw data recorded was consistent and compliant with the standard operating procedure of DFC's semen analysis in accordance with the WHO guidelines. Macroscopic and microscopic evaluations were performed in duplicate to strengthen the validity and reliability of the results. Sperm concentration, total motility and morphology were used as a measure of reproductive capacity depicting a quantifiable variable.

4.2. Sample size

Thirty-four patients that met the inclusion criteria were recruited in the study. From the sample size, twenty-nine patients presented with data that could be analysed whilst 5 patients did not have biological material for analysis, i.e., presented with aspermia (no ejaculate) and azoospermia (no spermatozoa in ejaculate). The data from the 5 patients were not included in the study because of the large range and variance (Table 8). Urine of these 5 patients excluded from the study, were

inspected post ejaculation for the presence of spermatozoa and yielded no evidence of retrograde ejaculation.

Table 8: Summary of sample size and data analysed.

	Data analysed	Aspermia	Azoospermia
No of subjects n = 34	29	2	3

4.3. Age

The inclusion criteria of the study included male renal transplant patients between 18 and 45 years of age. The mean age of the study participants was 31.8 years (95% CI 28.8 - 34.7). Figure 14 illustrates the ages of the analysed group (n = 29). The age group 30-34 had the highest participation of patients.

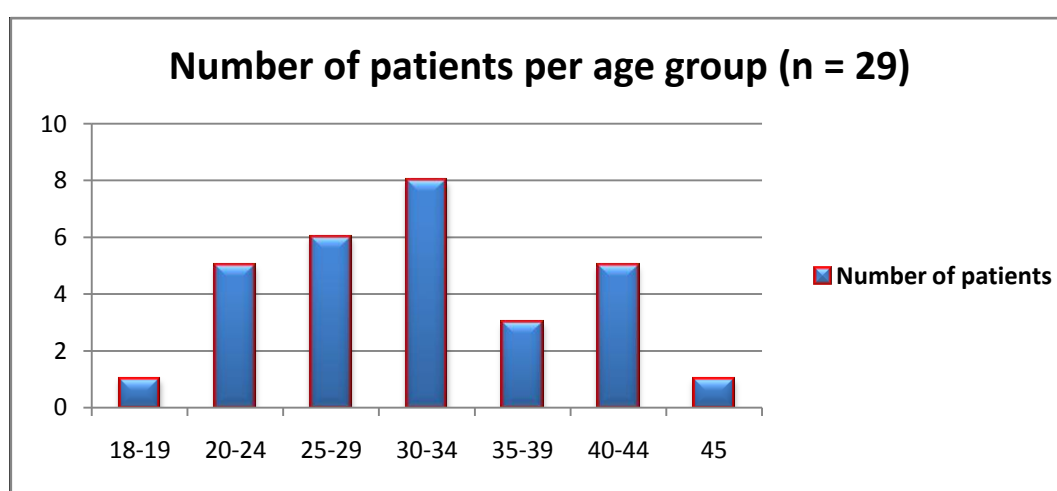


Figure 14: Column representation illustrating the age range and age group demarcation of patients that participated in the study.

4.4. Population demographics

Population demographics of the entire sample size ($n = 34$) were as follows: 21 Indian, 6 Black, 5 White and 2 Coloured males, respectively. Statistical analysis, excluding the 5 patients (Figure 15), was performed on 17 Indian, 6 Black, 4 White and 2 Coloured males, respectively ($n = 29$).

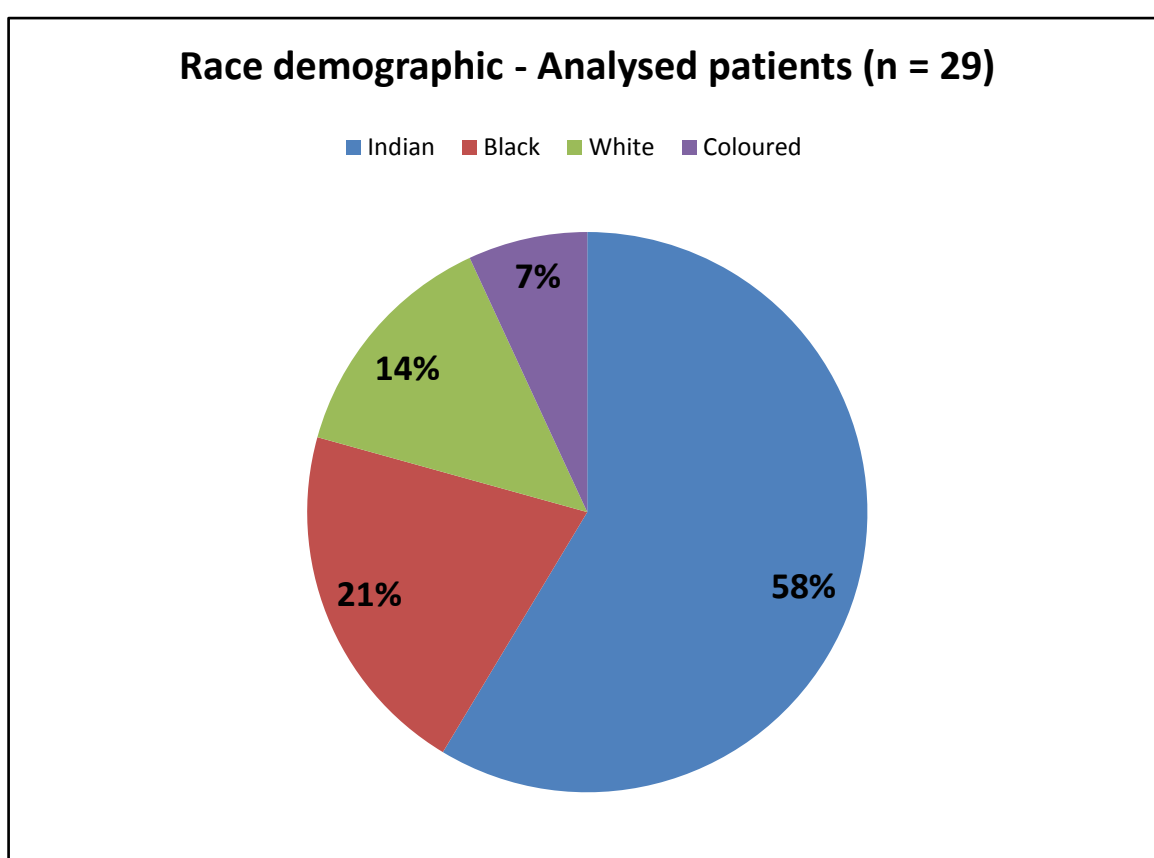


Figure 15: Pie graph of race demographic as per analysed subjects

4.5. Mean sperm concentration

Sperm concentration is a measure of spermatozoa present in a millilitre of native sample. The mean sperm concentration ($n = 29$) was 14.0 mill/ml (95% CI 10.2 – 17.7). Figure 16 denotes a large range of data from severe oligozoospermia (min: 0.5 mill/ml) to normal concentration (max: 36 mill/ml). The median (12.5 mill/ml) was below the WHO guideline of 15 mill/ml.

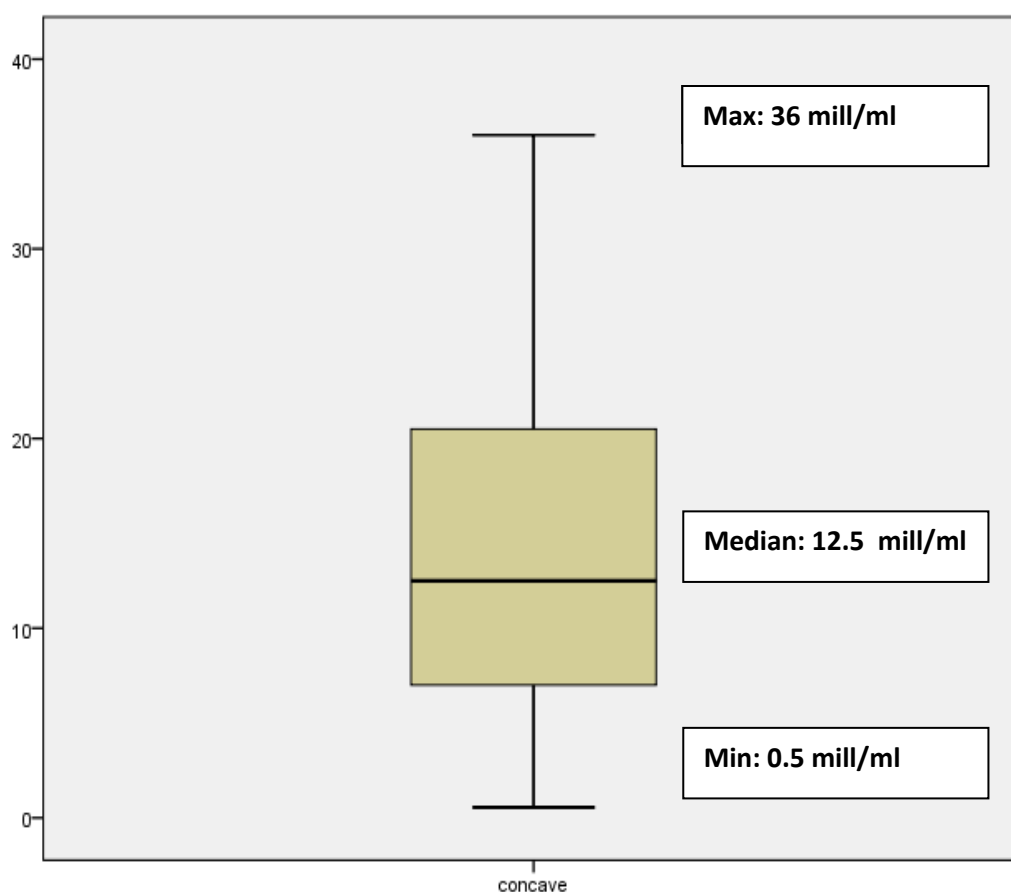


Figure 16: Box and whisker plot depicting the concentration ranges including the min/max and median values of sperm concentration.

4.6. Average total sperm motility

Forward progression of the spermatozoa was assessed quantifying progressive, non-progressive motility and immobility. The average total sperm motility in Figure 17 revealed a mean of 43.2% (95% CI 36.6 – 49.7). A large range was also noted with min/max values at two extremes, i.e., 5.5% (asthenozoospermia) and 75.5%, respectively.

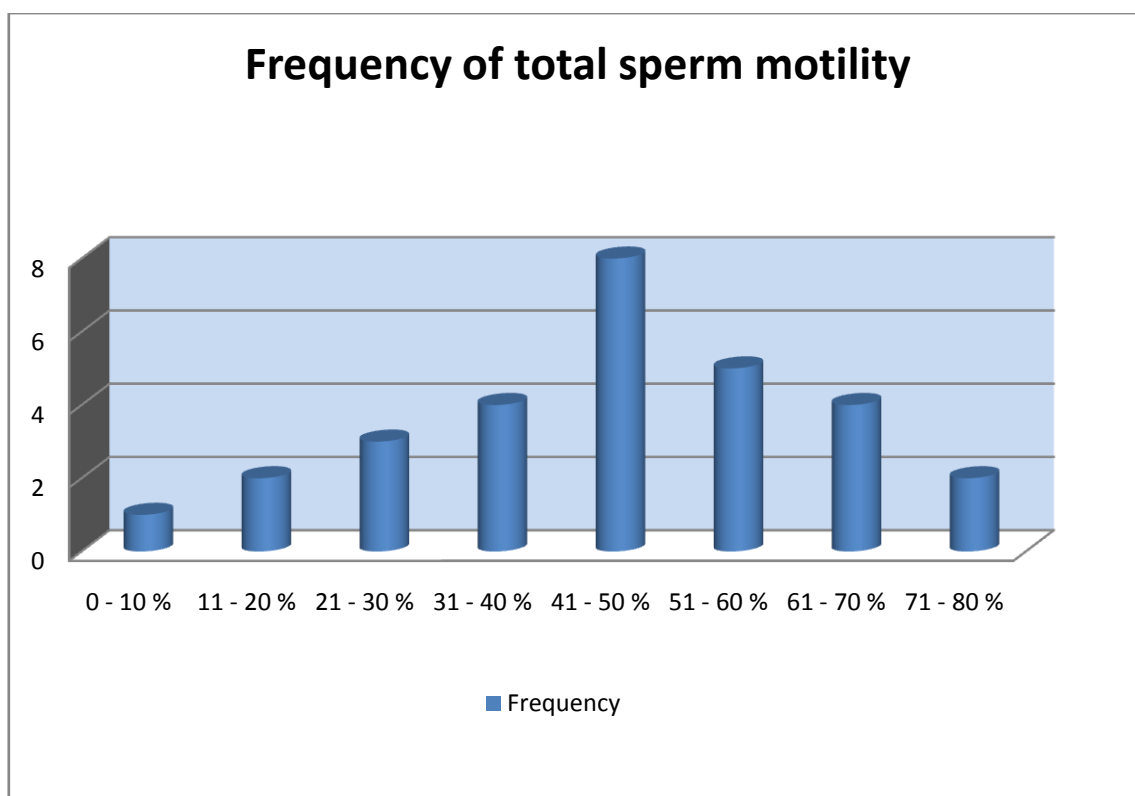


Figure 17: Bar representation denoting the frequency of motility amongst the analysed patients (n = 29). The frequency was higher in the 41 – 50 % group correlating with the mean total motility.

4.7. Mean sperm morphology

Analysis of sperm morphology determined the mean as 3.3% (95% CI 2.7 – 3.9). Sperm morphology analysis revealed a trend towards lower sperm morphology. This is further shown in figure 18 in which the distribution is slightly positively skewed as the median lies closer to the first quartile. Nineteen patients presented with teratozoospermia (morphology < 4%) whilst ten patients were within the WHO normal range (4 – 100%) for morphology.

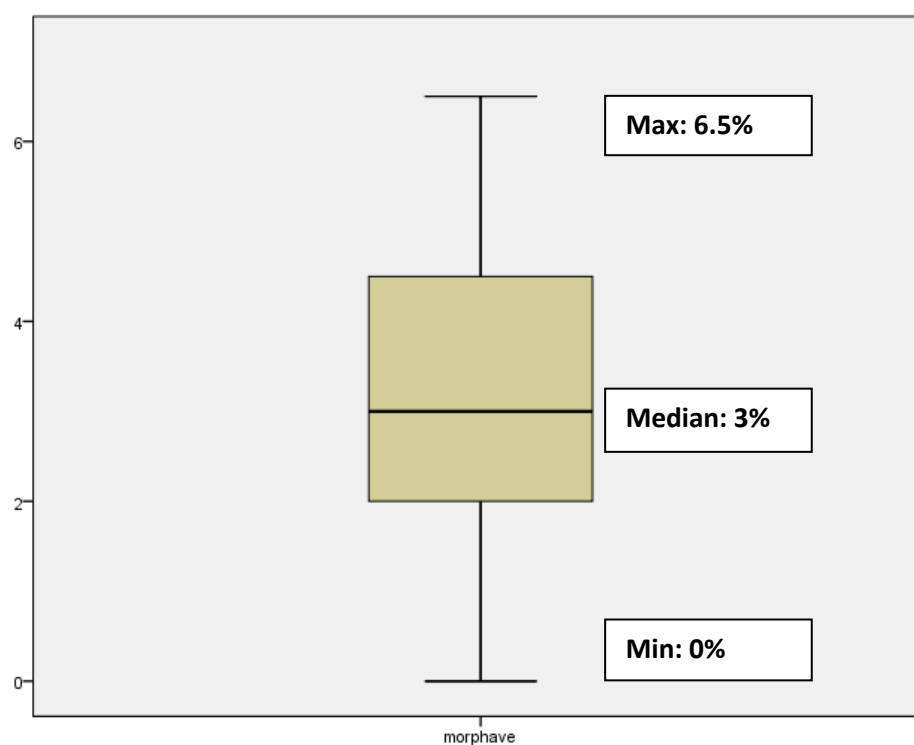


Figure 18: Box and whisker plot depicting min/max and median values of sperm morphology.

4.8. Associated sperm parameters

Semen comprises of secretions from three main accessory glands, i.e., prostate, seminal vesicle and bulbourethral glands, respectively. Each secretion has a unique function and association with the spermatozoa in aiding its ability to fertilize with the oocyte and creating a buffer for the progeny of sperm. The associated semen parameters recorded included volume 1.67 ml (95% CI 1.34 – 2.00) and pH 7.7 (95% CI 7.6 – 7.9) which showed no variation from the WHO guidelines. However, there was a marked decrease in mean vitality 47.5 (95% CI 40.6 – 54.4) (Table 9).

Table 9: Summary of study results including WHO values and t-stats (n = 29).

Variable	Sample Mean	Standard Deviation	Sample 95% CI		WHO Value	WHO 95% CI		t-stat	p value
			Min	Max		Min	Max		
Volume	1.7	0.9	1.3	2.0	1.5	1.4	1.7	1.06	0.299
Concentration	14.0	9.9	10.2	17.7	15	12	16	-0.57	0.576
Total Motility	43.2	17.2	36.6	49.7	40	38	41	0.99	0.330
Morphology	3.3	1.6	2.7	3.9	4	3.0	4.0	-2.36	0.025
Vitality	47.5	18.1	40.6	54.4	58	55	63	-3.13	0.004

The frequency of subjects with a normal viscosity and liquefaction time; n = 32 (2 subjects presented with aspermia) was seventeen (53%) whilst the remaining fifteen patients (47%) presented with increased viscosities and longer liquefaction periods. Four subjects (13%) presented with round cells greater than 1 mill/ml whilst all patients statistically analysed (n = 29) tested negative for IgG and IgA.

Appearance and colour examination of the semen for the thirty-two subjects revealed all samples presenting with a white grey appearance except for one patient who presented with haemospermia.

4.9. Hypothesis

The three main parameters assessed in the study included sperm concentration, total motility and morphology. From the one sample t-tests; the study values obtained were evaluated against the WHO reference values in determining if there existed a statistical significance (if any), i.e., deviation from the WHO reference values of the fertile male population. The one sample t-test for mean sperm concentration $t = 1.06$, $p = 0.299$ ($p > 0.05$) and total motility $t = 0.99$, $p = 0.330$ ($p > 0.05$) indicated mean sperm concentration and total motility of renal transplanted patients are not significantly different from the WHO reference values supporting the null hypothesis, $\mu = 15$ and $\mu = 4$, respectively. A large standard deviation was noted for the above-mentioned parameters of 9.9 mill/ml and 17.2%, respectively denoting the insignificance of the correlation. The t-test for sperm morphology $t = -2.36$, $p = 0.025$ ($p < 0.05$) revealed a significant difference from the WHO value supporting the alternate hypothesis, $\mu \neq 4$. A smaller standard deviation was noted for morphology, i.e., 1.6% correlating the statistical significance (Table 10).

Table 10: One sample t-tests of concentration, total motility and morphology tested against the WHO reference values (n =29).

One-Sample Test						
Concentration	Test Value = 15					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
	-.588	28	.576	-1.03793	-4.7971	2.7212
Motility	Test Value = 40					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
	.991	28	.330	3.15517	-3.3898	9.6799
Morphology	Test Value = 4					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
	-2.382	28	.025	-.68968	-1.2877	-.0918

4.10. Supplementary statistics

Table 11 represents the age and body mass index (BMI) of the study patients (n = 29). The mean values of sperm concentration, total motility, morphology and vitality determined no significant correlation of age and BMI with the semen parameters investigated. However, there existed an almost perfect linear correlation of motility with vitality, $r = 0.967$ (Figure 19).

Table 11: Correlations of Age and BMI with sperm concentration, total motility and morphology.

		Age			BMI
concentration_average	Pearson Correlation	-.088	concentration_average	Pearson Correlation	.121
	Sig. (2-tailed)	.649		Sig. (2-tailed)	.531
	N	29		N	29
motility_average	Pearson Correlation	.093	motility_average	Pearson Correlation	.126
	Sig. (2-tailed)	.630		Sig. (2-tailed)	.515
	N	29		N	29
morphology_average	Pearson Correlation	.167	morphology_average	Pearson Correlation	.164
	Sig. (2-tailed)	.385		Sig. (2-tailed)	.396
	N	29		N	29

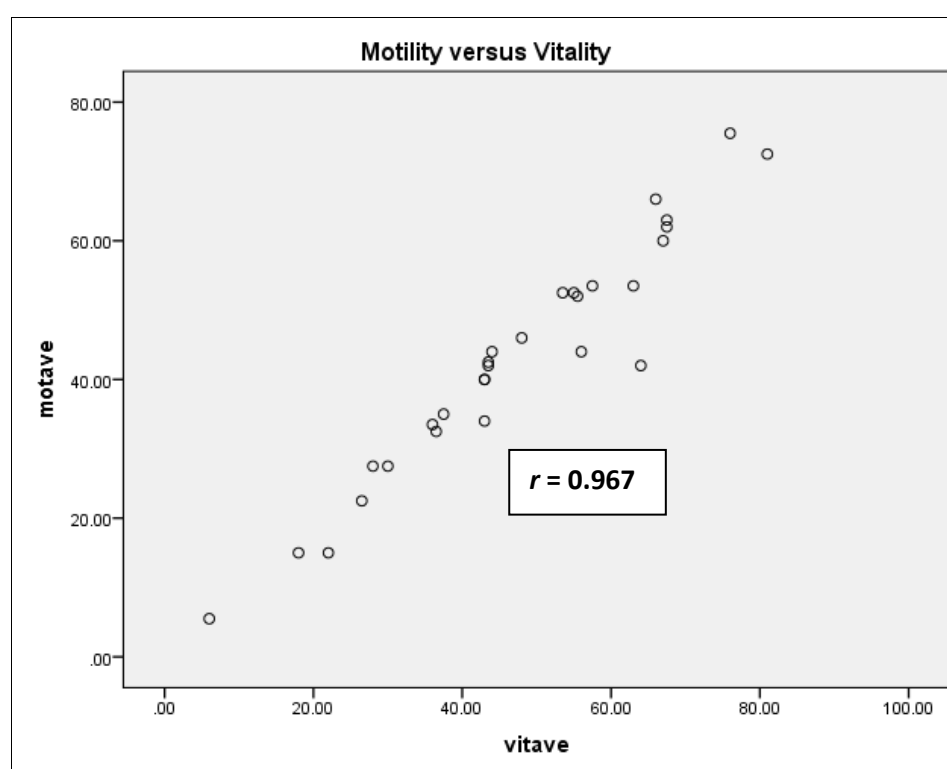


Figure 19: Scatter plot graph illustrating a significant linear relationship between motility and vitality ($r = 0.967$).

Descriptive statistics according to the different age groups in table 12 revealed no differences in sperm concentration, total motility and morphology amongst renal transplanted males from different race groups.

Table 12: Descriptive statistics per race demographic depicts the mean values of sperm concentration, motility and morphology.

		Race			
		Black	Coloured	Indian	White
Concentration	Count	6	2	17	4
	Mean	11.1	7.5	15.5	15.0
	Standard Deviation	9.6	2.1	11.3	4.3
	Minimum	1.20	6.00	.55	10.00
	Maximum	28.00	9.00	36.00	20.50
Motility	Count	6	2	17	4
	Mean	46.1	50.0	41.9	40.8
	Standard Deviation	11.8	31.8	19.8	4.3
	Minimum	32.50	27.50	5.50	35.00
	Maximum	62.00	72.50	75.50	44.00
Morphology	Count	6	2	17	4
	Mean	3.3	2.8	3.4	3.3
	Standard Deviation	1.3	1.1	1.9	.5
	Minimum	2.00	2.00	.00	3.00
	Maximum	5.00	3.50	6.50	4.00

4.11. Immunosuppressive treatment (IST) and sperm parameters

The IST regimens used in the thirty-four patients were categorized into eight groups according to their grouping of the IST namely;

Group 1 – Tacrolimus + MMF

Group 2 – CsA + Tacrolimus + MMF

Group 3 – Sirolimus + MMF

Group 4 – CsA + Sirolimus + MMF

Group 5 – CsA + Sirolimus + Tacrolimus + MMF

Group 6 – CsA + Tacrolimus

Group 7 – CsA + MMF

Group 8 – MMF

Each group represented the number of patients pertaining to their specific IST regimen (Figure 20). From the data obtained majority of patients were undergoing maintenance immunosuppressive therapy with tacrolimus, MMF and a corticosteroid (group 1).

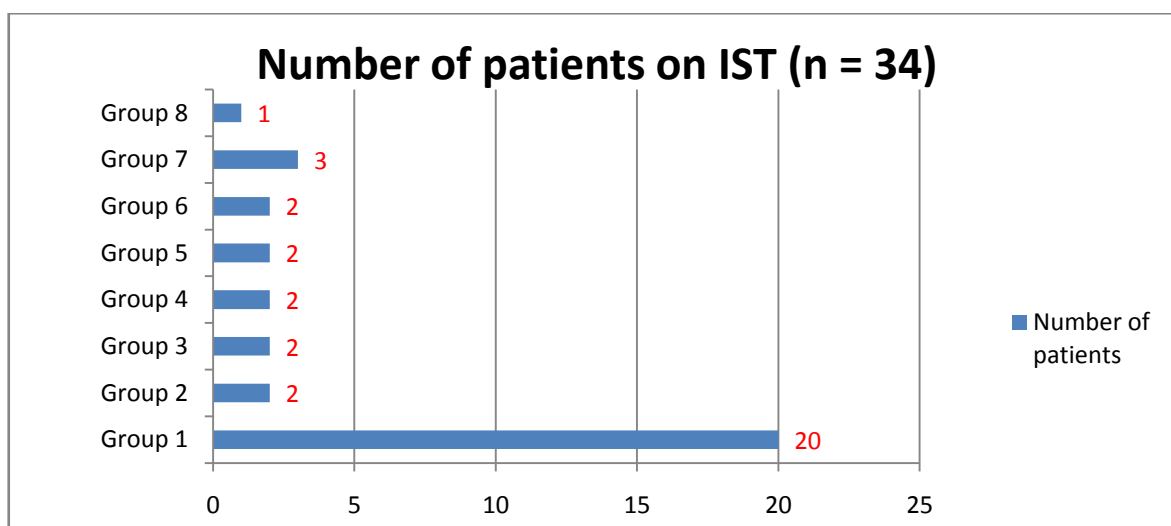


Figure 20: Illustration of the number of patients on the differing IST regimens.

Azoospermia and aspermia were present in 3 patients (Groups 1, 5 and 6) and 2 patients (Group 1 and 7), respectively. The effect of immunosuppressive therapy on semen parameters was evaluated in group 1. Two study patients in group 1 that presented with azoospermia and aspermia respectively were excluded from the analysis because of the large range and variance ($n = 18$). Multiple linear regressions for sperm concentration, total motility and morphology were investigated and included in the regression analyses. The predictors used included tacrolimus duration and dose and MMF duration and dose. Tacrolimus duration was dropped from the model due to co-linearity, i.e., there was a perfect correlation between MMF duration and tacrolimus duration. Statistical analysis revealed that none of the independent variables significantly predicted concentration, total motility and morphology (Table 13).

Table 13: Multiple linear regressions of concentration, total motility and morphology of the Tacrolimus + MMF cohort of patients (n = 18).

Coefficients						
Model		Unstandardized Coefficients		Standardized Coefficients	T	Sig.
		Beta	Std. Error	Beta		
1	(Constant)	11.509	8.885		1.295	.216
	MMF duration	.043	.055	.194	.794	.440
	Tacrolimus dose	.776	.613	.315	1.265	.226
	MMF dose	-.003	.005	-.132	-.537	.599
Dependent variable: concentration						
1	(Constant)	32.091	16.579		1.936	.073
	MMF duration	-.068	.102	-.167	-.666	.517
	Tacrolimus dose	.215	1.145	.048	.188	.854
	MMF dose	.012	.009	.357	1.410	.180
Dependent variable: motility						
1	(Constant)	3.624	1.154		3.139	.007
	MMF duration	.012	.007	.424	1.732	.105
	Tacrolimus dose	-.050	.080	-.155	-.623	.543
	MMF dose	.000	.001	-.152	-.614	.549
Dependent variable: morphology						

The number of children fathered by renal transplanted patients was obtained from the medical history. Ten patients had children before transplant whilst nine males had children post renal transplant reporting between 1 – 3 children fathered.

However, two patients who reported 1 – 3 children after transplant presented with aspermia and azoospermia, respectively. The two-sided t-test was performed against the WHO reference values for the seven subjects that fathered children post-transplant and those that did not ($n = 22$). The results yielded no significant differences in sperm concentration $p = 0.290$ ($p > 0.05$) and total motility $p = 0.146$ ($p > 0.05$). Comparison of morphology in the group with children ($n = 7$) and group with no children ($n = 22$) revealed a high significance between morphology and number of children fathered; $p = 0.001$ ($p < 0.05$). The trend of morphology in this group revealed decreased values of morphology in patients without children whilst patients with children revealed higher values of morphology $> 4\%$ (Table 14).

Table 14: Group statistics indicating the difference in morphology between males with and without children post renal transplant.

Group Statistics						P value
	Children post	N	Mean	Std. Deviation	Std. Error Mean	
Concentration	No	22	12.8477	9.92464	2.11594	0.290
	Yes	7	17.4643	9.60081	3.62877	
Motility	No	22	40.5227	16.88087	3.59901	0.146
	Yes	7	51.4286	16.44544	6.21579	
Morphology	No	22	2.8182	1.28680	.27435	0.001
	Yes	7	4.8571	1.43510	.54242	

Sensitivity and specificity analysis was performed to determine which of the three semen parameters (sperm concentration, total motility or morphology) optimally predicted pregnancy in patients that fathered children post renal transplant. The area

under the curve was not significantly different from 0.5, indicating that concentration (AUC = 0.662) and motility (AUC = 0.685) are not good predictors of pregnancy in renal transplanted patients. The area under the curve was statistically significant for morphology (AUC = 0.854) and concluded that morphology is the best predictor of pregnancy in renal transplanted patients (Figure 21).

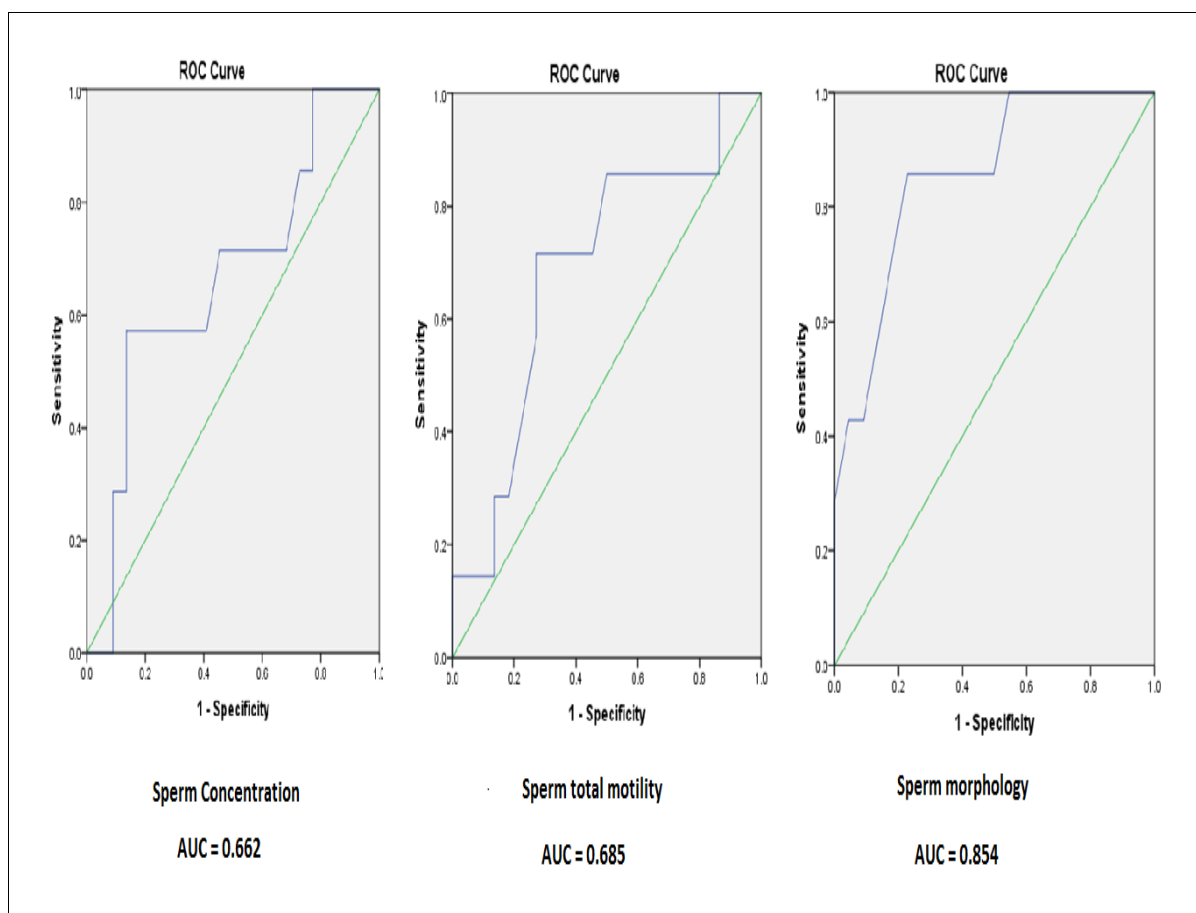


Figure 21: Sensitivity and specificity of sperm concentration, motility and morphology in predicting pregnancy. ROC analysis prediction revealed morphology as the best indicator of pregnancy in renal transplanted patients (AUC = 0.854).

Data analysis of five patients that fathered children pre- and post-transplant indicated that sperm concentration, total motility and morphology from these patients are well within the WHO reference values except for DUTSA013 (Table 15). Further, the degree of fertility impairment is miniscule or not prevalent.

Table 15: Sperm parameters of five patients with children pre- and post-transplant and associated immunosuppressive therapy.

Study no	Child Pre	Child Post	IST regimen	Sperm Parameters		
				Conc. (mill/ml)	Motility (%)	Morph (%)
DUTSA006	1	1	Tac + MMF	28	62	4.5
DUTSA007	1	2	CsA + Tac + MMF	27.5	75.5	6.5
DUTSA011	2	1	Tac + MMF	21.5	22.5	4.5
DUTSA013	2	1	Tac + MMF	12.5	52	2.5
DUTSA022	1	2	CsA + Tac	21.5	42	5

CHAPTER 5

DISCUSSION

Semen analysis is a comprehensive examination of semen parameters according to the WHO guidelines (WHO, 2010). It is the gold standard of measuring male fertility and the fertilizing potential of the sperm cell (Hallak, 2016). Studies have shown that sperm concentration, motility and morphology are the fundamental contributing factors of fertility in males (Guzick *et al.*, 2001; Slama *et al.*, 2002; Hofny *et al.*, 2010; Sofimajidpour *et al.*, 2016). Deterioration of these parameters are compounded by the many social iniquities including cigarette smoking (Kunzle *et al.*, 2003), alcohol abuse (Muthusami and Chinnaswamy, 2005) and recreational drugs (Whan *et al.*, 2006; Fronczak *et al.*, 2012).

Lifestyle iniquities have led to an increase in comorbid diseases including diabetes mellitus and hypertension (Joannes-Boyau *et al.*, 2013). These pathophysiological diseases are the most common causes of chronic renal disease leading to end stage renal failure in which a renal allograft is required (Iglesias *et al.*, 2010). The efficacy of a transplanted kidney is determined by its ability to assume filtration function whilst deterring organ rejection (Singh *et al.*, 2001). Immunosuppressive therapy is an important tool in suppression of immunity and in preventing organ rejection (Vincenti *et al.*, 1998). The literature is unclear on the effect of immunosuppressive therapy on semen parameters. Studies with small sample sizes have shown deleterious effects of immunosuppressive therapy on semen parameters (Kaczmarek *et al.*, 2004; Xu *et al.*, 2009; Abou-Shabaan *et al.*, 2011; Rovira *et al.*, 2012; Chen *et al.*, 2013; Tainio *et al.*, 2014).

The main parameters assessed in this study included sperm concentration, total

motility and morphology. Patients in this study underwent a comprehensive semen analysis as per the standard operating procedure adapted from the WHO procedural guidelines (refer to Figure 13).

The mean sperm concentration of this study was 14.0 mill/ml (95% CI: 10.2 - 17.7) which is within the 95% CI of the WHO guidelines. This is a surprising finding. Analysis of sperm concentration revealed that 66% of the twenty-nine analysed patients were below the WHO reference value. Nine patients presented with sperm concentrations ranging from 0.5 to 5 mill/ml spermatozoa indicative of severe oligozoospermia. However, this unexpected finding can be explained by the large range of data of minimum and maximum values with a higher standard deviation of 9.9 mill/ml. Sperm concentration is dependent on the level of testosterone production and hormone profile of the patient (Zuber *et al.*, 2008). Immunosuppressive usage has been shown to cause decreased testosterone levels in renal transplanted males (Bererhi *et al.*, 2003; Kaczmarek *et al.*, 2004; Koyun *et al.*, 2009; Chen *et al.*, 2013). Hence immunosuppressive therapy in itself, post renal transplant, may account for the reduced sperm concentrations due to decreased testosterone levels. In this study 66% of patients presented with oligozoospermia. This finding could be explained by the long standing renal failure leading to low testosterone levels and further aggravated by the use of immunosuppressive therapy.

Motility is a measure of sperm forward progression which is required in aiding sperm transport of the sperm head to the mature oocyte for fertilization to occur (Esfandiari *et al.*, 2009). In this study, motility (43.2%) was higher than the WHO 95% confidence interval (95% CI 38 – 41). Renal transplantation is essential in assuming filtration function of the kidney and in excretion of waste products or excess substrates e.g. sodium and potassium. The Na,K-ATPase isoform has shown to have an important role in sperm motility due to the presence of the alpha 4 isoform of Na,K-ATPase which is unique to the testis (Woo *et al.*, 2000). Therefore, increased

sperm motility is indicative of optimal renal graft functionality of transplanted males in this study which is evident as only seven patients presented with asthenozoospermia (motility < 40%) in this study.

Morphology is an important predictor of fertilization capacity (De Vos *et al.*, 2003; Oehninger *et al.*, 2014; Lemmens *et al.*, 2016). The mean value of morphology was 3.3% (95% CI 2.7 – 3.9). The two sided one sample t-test of these parameters exhibited statistically significant differences of morphology in which morphology values were decreased ($p = 0.025$; $p < 0.05$). In this study, 62% (eighteen patients) presented with teratozoospermia (morphology < 4%). Several factors can affect sperm morphology including environmental factors, temperature changes, and testicular tumours such as varicocele, spermatoceles and extraneous causes that could have potentially toxic effects (Auger *et al.*, 2015). In this study, the effect of immunosuppressive drugs on sperm morphology was evaluated. This study showed that sperm morphology was significantly decreased which could be attributed to the prolonged usage of immunosuppressive drugs. Immunosuppressive usage over a prolonged period and at higher dosages has shown to cause gonadal toxicity (Kaczmarek *et al.*, 2004; Boobes *et al.*, 2009; Groth *et al.*, 2010). Therefore, gonadal toxicity due to immunosuppressive therapy may account for decreased morphology values dependent on the dosage and duration of immunosuppressive use which has shown previously to have deleterious effects on sperm morphology (Ostensen *et al.*, 2006).

The proliferation and differentiation of spermatogonia requires signalling of the phosphatidylinositol 3 kinase/AKT pathway. Sirolimus has shown to inhibit this signalling and decreases the sensitivity of the GnRH receptors (Gonzalez *et al.*, 2012). This in turn alters the hypophyseal gonadal axis and leads to poor sperm production and quality of male gametes. Cyclosporin A and tacrolimus are calcineurin inhibitors which inhibit the signal transduction pathway leading to T cell activation deterring the production of interleukin 2. Interleukin 2 has a pivotal role in

spermatogonia production. However, prolonged immunosuppressive usage has shown to inhibit interleukin 2 production adversely affecting sperm morphology (Garnock-Jones, 2015).

Idiopathic, comorbid diseases (hypertension and diabetes), nephrotic syndrome, glomerulosclerosis, SLE and trauma accounted for cause of renal failure in this study. The relation of renal failure aetiology with male infertility was not examined since the focus of this study was to determine the effects of the immunosuppressive therapy on semen parameters. Therefore, the cause of renal failure in this study could not account for poor morphology. Although a small sampling size was used, the ROC analysis of the seven study patients that fathered children showed that sensitivity and specificity of morphology is the most optimal indicator and predictor of pregnancy (refer to Figure 21). This correlation further advocates the importance of morphology in fertility assessment and as key factor in assisted reproductive success.

In a natural cycle, females produce a single oocyte either on the right or left ovary alternating each month with their menstrual cycle (Vander *et al.*, 2001). Unlike the female, the male continually produces sperm which is excited upon ejaculation (Giacone *et al.*, 2016). However, like all cells in the human body, a life span which determines viability of the spermatozoa exists *in vivo*. Sperm vitality measures the viability of the spermatozoa in a sample (Bjorndahl *et al.*, 2003). In this study, there was a marked decrease in the vitality mean 47.5% (95% CI 40.7 – 54.4) in comparison with the WHO value of 58% (95% CI 55 – 63). The two-sided t-test indicated that $t = -3.13$, $p = 0.004$ ($p < 0.05$) denoting a significant difference in the vitality values. However, linear correlation of motility and vitality revealed an almost perfect correlation, $r = 0.967$, as shown in previous studies (Auger *et al.*, 2000; Koca *et al.*, 2003; Taha *et al.*, 2012). Sexual abstinence of the patients was standardized between 2 to 3 days before a semen sample was produced. Therefore, increased sexual abstinence was excluded for low vitality as a cause of decreased vitality.

However, toxicity of immunosuppressive therapy on sperm viability and vitality requires investigation to ascertain potential toxic effects. The latter has been demonstrated by Baykalir and colleagues (2016), in which immunosuppressive therapy with CsA presented with increased toxicity and decreased vitality of sperm.

The ability of the native sample to transit from a gelatinous mass to a liquid phase is termed liquefaction and is measured by the viscosity of the sample (Jiang *et al.*, 2012). The prostatic proteases are responsible for this process in ensuring that the sperm have a non-viscous medium allowing forward progression and more especially rapid progression (Du Plessis *et al.*, 2013). The results presented in this study revealed that 53% of the analysed samples ($n = 32$) had normal liquefaction times and viscosity whilst 47% presented with abnormal viscosity and liquefaction times (> 20 minutes). Samples that presented with a higher viscosity were left in an oven at 37°C and periodically assessed at 30, 45 and 60 minutes, respectively, showing no change in the viscosity. Increased viscosity has shown to affect spermatozoa motility and the sperm's ability to interact with the oocyte (Suarez, 2016). All study patients were not examined by a specialist urologist. This limitation could not exclude pre-existing prostate and seminal vesicle dysfunction including chronic prostatitis, seminal vesicle dysfunction, tumours and benign prostatic hyperplasia. Effects of immunosuppressive therapy on prostate function and morphology in rats have shown to cause atrophy of the prostate with altered morphology and subsequent prostate dysfunction (Grabowska *et al.*, 2016). However, further human studies are required to determine the possible effects of immunosuppressive therapy on prostate function.

The native sample produced comprises of secretions of the prostate, bulbo-urethral and seminal vesicle glands together with the sperm thus making up the volume of the ejaculate. These secretions act as a buffer and provide the necessary fructose that is needed for capacitation and the acrosome reaction to occur (Gubbels *et al.*, 2013). The mean volume in the study was 1.67 ml (95% CI 1.3 – 2.0). Eleven (34%)

of the thirty-two analysed patients presented with hypospermia and one presented with hyperspermia (6.6 ml). Volume in this study was within the WHO guidelines. The pH for all samples are within range of 7.2 to 8.2 with the mean pH at 7.7 (95% CI 7.6 – 7.9).

The mean age of the study was 31.8 years (95% CI 28.9 - 34.7). In this cohort, there were no statistical correlations of age and BMI ($p < 0.5$) with sperm concentration, total motility and morphology. The results showed no association of obesity, measured by BMI, with deteriorating semen parameters. The mean values of sperm concentration, total motility and morphology of each race demographic were evaluated and compared against each other. No differences were noted in sperm concentration, motility and morphology across the differing race populations.

The use of immunosuppressive agents post-transplant is pivotal in ensuring the survival of the renal graft and imperative in preventing renal allograft rejection (Vincenti *et al.*, 1998). Tacrolimus, sirolimus and CsA are the most commonly used immunosuppressive agents in conjunction with MMF and corticosteroids. Patients are subjected to high dosages of immunosuppressive agents post-transplant especially during the initial three months and are gradually weaned to maintenance dosages indefinitely (Brunet *et al.*, 2016). Differing active ingredients of immunosuppressive agents require varying administration dosages as per the desired pharmacokinetics and pharmacodynamics, respectively. Although the efficacy of the drug is quality assured and clinically tried, adverse events still exist due to immunological and non-immunological factors. These events may lead to deleterious effects especially on the functioning of the renal allograft. To prevent possible renal rejection due to the adverse events, the regimen of immunosuppressive therapy may be discontinued and replaced with an alternative regimen e.g. CsA with tacrolimus (Scandling *et al.*, 2015).

In this study, patients presented with a combination of immunosuppressive agents

administered immediately after transplant and during their respective maintenance therapies. Adverse events in eight patients accounted for more than one immunosuppressive agent being administered. Most patients were solely on the tacrolimus–MMF–prednisone therapy (62%). The remaining patients underwent sirolimus or CsA therapy exclusively. In cases of toxicity or adverse events, the choice of immunosuppressive agent was changed. Only two patients reported the use of CsA and MMF exclusively. Statistical analysis on the effects of immunosuppressive therapy on each group was extremely difficult due to the small sample sizes, i.e., 1 – 2 patients in each therapy group in contrast to the tacrolimus-MMF group (n = 18). Secondly, the combination of immunosuppressive agents created too many variables including varying dosages and durations for analysis. This was further compounded by insufficient data of semen parameters, at the time of discontinuing one immunosuppressive and the change to an alternate regimen.

Analysis was performed on the tacrolimus-MMF–prednisone group of patients (n = 18). Four patients presented with pregnancies and live births (22%) whilst 78% patients undergoing the same maintenance therapy were unable to procreate. Analysis of the four patients that fathered children revealed that the morphology and motility values of all four patients were greater or equal to 4% and 40%, respectively, i.e., within the WHO guidelines. Decreased sperm concentrations were noted in two of the four patients. Multiple linear regressions of sperm concentration, total motility and morphology (n = 18) revealed that none of the independent variables (tacrolimus, MMF dosage and duration) significantly predicted concentration, total motility and morphology. Although 22% pregnancies have been reported in this group; the concern is that 78% of patients on the tacrolimus-MMF–prednisone group did not father any children post renal transplantation. These results represent patients that are presently or have previously tried to father children with no success. There has been major debate on the effect of tacrolimus on semen parameters. An initial study on rats showed that tacrolimus usage reported decreased sperm concentrations and motility. This was attributed to testicular calcifications and not

alterations in the spermatogenesis axis (Hisatomi *et al.*, 1996). Latter studies have shown no effects of tacrolimus usage on sperm parameters (Armenti *et al.*, 2000; Bozzini *et al.*, 2013; Chen *et al.*, 2013). Further studies have also shown that tacrolimus usage could assist in recovering the motility and morphology of renal transplanted patients (Cao *et al.*, 2006).

The number of patients that fathered children post-transplant based on immunosuppressive usage was 9 patients. This included Tacrolimus – MMF, 4 patients; CsA – tacrolimus – MMF, 1 patient; CsA – sirolimus – tacrolimus – MMF, 1 patient; CsA – tacrolimus, 1 patient and CsA – MMF, 1 patient. Analysis of the sub group of patients who fathered children post-transplant on immunosuppressive therapy was scrutinized in detail. The most number of pregnancies was in the tacrolimus - MMF group as compared to the other single and combination regimens. Interestingly, three of the pregnancies were achieved with tacrolimus as the last immunosuppressive being used. Pregnancies were achieved in eight of the nine patients (89%) with tacrolimus administered. This indicates that tacrolimus does have the potential in increasing sperm parameters and achieving pregnancy and may have a protective role (Skryzpek *et al.*, 2007).

Ten males fathered children before transplant in comparison to the nine males with children post renal transplant. However, two patients (with children post-transplant) presented with azoospermia and aspermia, respectively. Examination of the medical history revealed that the last live birth of each child was in 2008 and 2013, respectively. The afore-mentioned patients did not present with a history of testicular injury, trauma, infections or carcinomas post renal transplant whilst still on immunosuppressive therapy. Testosterone levels and physical examinations were not investigated in these two patients to determine if the drastic change of semen parameters were attributed to physiological and anatomical aberrations or immunosuppressive dosage and duration.

The number of patients on immunosuppressive usage with no children included CsA – sirolimus – MMF, 1 patient; Sirolimus – MMF, 2 patients; Sirolimus – CsA – MMF, 1 patient; Tacrolimus – sirolimus – CsA – MMF, 1 patient; MMF only, 1 patient; CsA – tacrolimus – MMF, 1 patient; CsA – tacrolimus, 1 patient and CsA – MMF, 3 patients. The above-mentioned results indicate that patients on CsA and sirolimus therapy have decreased fertility potential in comparison to patients on tacrolimus therapy. These results agree with previous studies on the effect on CsA and sirolimus on semen parameters (Eid *et al.*, 1996; Kissel *et al.*, 2000; Koyun *et al.*, 2009; Rostaing and Kamar, 2010; Rovira *et al.*, 2012; Tanio *et al.*, 2014). Furthermore, patients on CsA and sirolimus presented with oligozoospermia (sperm concentration < 15 mill/ml), teratozoospermia (morphology < 4%), asthenozoospermia (motility < 40%) or a combination of these conditions.

In this study, tacrolimus was shown to be the common contributing and protective immunosuppressive agent in fathering children post renal transplant. Interestingly, one patient that initially began treatment with tacrolimus and subsequently changed to sirolimus and CsA, respectively, presented with severe oligoasthenoteratozoospermia (decreased sperm concentration, motility and morphology) and did not father children post renal transplant. Tacrolimus, in this study, has shown to have decreased effects on the fertility of male renal transplanted patients. Further, it has shown to recover spermatogenesis of renal transplanted patients that have been previously on sirolimus and CsA (Bererhi *et al.*, 2003).

LIMITATIONS OF STUDY

There were many limitations in the study which were unforeseen. The recruitment of patients in the study was extremely difficult due to its sensitivity. The recruitment process was laborious and tedious and done over a period of eighteen months.

Patients were apprehensive of producing a sample by masturbation. This was more

prevalent amongst Black patients due to their cultural beliefs. Non-compliance of the patients was a major stumbling block in which patients would not attend the renal clinics as per the said documented appointment date. Furthermore, transplanted patients from private practitioners were very reluctant to travel to Amanzimtoti to participate in the study.

Due to the heterogeneity of the study group, the sample size recruited did not have an even spectrum of patients on the different immunosuppressive agents. Therefore, statistical analysis was not investigated for CsA and sirolimus patients due to the small sample size. A major limitation of the study is that semen analysis data pre-transplant was not available to compare reproductive indices pre- and post- renal transplant. This comparison would have served as a reference for semen parameters pre- and post- renal transplant, especially since dialysis has shown to impair spermatogenesis and sexual function (Hamdi *et al.*, 2014).

Patients were not assessed by a specialist urologist to exclude anatomical abnormalities e.g. varicoceles, hydroceles, spermatoceles and testicular tumours. This was critical, especially, in patients with severe oligoasthenoteratozoospermia, azoospermia and aspermia in determining if the aetiology of poor fertility indices was attributed to physiological, anatomical or immunosuppressive administration.

CHAPTER 6

CONCLUSION

Infertility affects 1 in 5 couples in South Africa with an alarming incidence due to male factor infertility (Dyer *et al.*, 2002). This is further compounded by the many pathophysiological conditions and diseases that are prevalent in society. One such condition is chronic renal failure which often progresses to end stage renal failure requiring a renal transplant. Post renal transplant immunosuppressive therapy is necessary to prevent allograft rejection. In infertility clinics, the semen analysis based on the WHO guidelines (WHO, 2010) is used as a basis to evaluate the fertility potential of the male individual and secondly in determining the assisted reproductive technique i.e. intrauterine insemination, *in vitro* fertilization or intracytoplasmic sperm injection, to be employed in assisting the couple in procreation.

The data represented in this study has enabled debate and discussion into the types of immunosuppressive agents administered and its potential effect on semen parameters. Whilst this study could not effectively define the effects of each of the immunosuppressive drugs on semen analysis, it did show the trend of semen parameters in renal transplanted patients. This positive trend is reassuring in the fecundity process and correlates the validity of the WHO reference values in predicting pregnancy. Furthermore, the study has also shown that with a small cohort of patients' tacrolimus may be a protective immunosuppressive agent that has minimal deleterious effects on sperm parameters. Tacrolimus has shown to improve reproductive indices of renal transplanted males that have been on previous CsA and sirolimus therapy. A larger study pre- and post-transplant needs to be investigated to elucidate the effects of individual immunosuppressive agents whilst

also, taking into consideration the effects of dialysis treatment on semen parameters. There should be clear policies in place from nephrology societies and departments ensuring that holistic evaluation of the patients are assessed to ensure survival of the patient whilst taking into consideration the impact of immunosuppressive therapy on procreation. All males of reproductive age that have been diagnosed with chronic renal failure should be counselled on the potential deleterious effects of immunosuppressive treatment on semen parameters. Pro-active measures should ensure that the relevant stakeholders, i.e. patient, fertility specialist, embryologist and urologist are consulted to preserve the fertility of these patients as the time for spermatogenic recovery is unknown post-transplant. Therefore, emphasis should be placed on sperm cryopreservation and banking, in the event of possible testicular atrophy, gonadal toxicity or testicular failure.

CHAPTER 7

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APPENDIX A



health

Department:
Health
PROVINCE OF KWAZULU-NATAL

Inkosi Albert Luthuli Central Hospital
Ethekwini Health District
Office of the Medical Manager
Private Bag X 03, Mayville, 4058
800 Bellair Road, Mayville, 4058
Tel.: 031 240 1059,
Fax.: 031 240 1050
Email: ursulanun@ialch.co.za
www.kznhealth.gov.za

Reference: REC 60/14
Enquiries: Medical Management

30 April 2015

Mr N Moodley
Department of Renal
IALCH

Dear Dr Moodley


RE: PERMISSION TO CONDUCT RESEARCH AT IALCH

I have pleasure in informing you that permission has been granted to you by the Medical Manager to conduct research on: **Semen analysis of renal transplant patients undergoing immunosuppressive treatment.**

Kindly take note of the following information before you continue:

1. Please ensure that you adhere to all the policies, procedures, protocols and guidelines of the Department of Health with regards to this research.
2. This research will only commence once this office has received confirmation from the Provincial Health Research Committee in the KZN Department of Health.
3. Kindly ensure that this office is informed before you commence your research.
4. The hospital will not provide any resources for this research.
5. You will be expected to provide feedback once your research is complete to the Medical Manager.

Yours faithfully


Dr M Letebele
Medical Manager

uMnyango Wezempilo . Departement van Gesondheid

Fighting Disease, Fighting Poverty, Giving Hope

APPENDIX B



INKOSI ALBERT LUTHULI CENTRAL HOSPITAL

DEPARTMENT OF NEPHROLOGY
800 Bellair Road, Mayville, 4091
Private Bag X03, Mayville, 4058
Tel: 031 - 240 1325
Fax: 0865217163
e-mail address: janepad@ialch.co.za

20 April 2015

Dr Letebele
Medical management
IALCH Hospital

Dear Dr Letebele

I hereby, wish to support the research to be conducted by Mr. Neville Moodley for Master's Degree in Clinical Technology.

The research is entitled "*Semen analysis of renal transplant patients undergoing immunosuppressive treatment*" was approved by DUT ethics committee (attached is the ethics approval letter).

The research could give us insight in the reproductive status of our transplant population.

The department of nephrology has the capacity to support the above research.

Yours sincerely



PROF A ASSOUNGA
HEAD: NEPHROLOGY

APPENDIX C



LETTER OF INFORMATION

Title of the Research Study: Semen analysis study of renal transplant patients undergoing immunosuppressive treatment

Principal Investigator/s/researcher: Neville S Moodley

Co-Investigator/s/supervisor/s: Supervisor: Prof JK Adam

Co-Supervisor: Dr S Naidu

Brief Introduction and Purpose of the Study: Hi my name is Neville Moodley and I am studying for a Masters degree at the Durban University of Technology. Many couples are affected by infertility. There are many causes of infertility both in men and women. Of note, there has been an increase in infertility amongst patients that have had renal transplants. To prevent the transplanted organ from failing, patients are given drugs called immunosuppressive's which could lead to infertility. I would greatly appreciate it if you would take part in my research by providing a semen sample via masturbation (self pleasure) for analysis. The intention is analyse the effect of the immunosuppressive drug, given to you after the kidney transplant, on your semen parameters.

Outline of the Procedures: The study requires 34 patients who have had kidney transplants. These patients also have to be on immunosuppressive treatment. There will be no changes to your treatment. The study requires you to produce a semen sample by masturbation or self pleasure. This semen sample will then be analysed according to the study design and protocol. Total privacy will be given to the subjects when they are producing the sample in a clean, secured and private room at Durban Fertility Clinic designed for this purpose.

Risks or Discomforts to the Participant: There will be no risks or discomfort to you as there will be no change to your treatment.

Benefits: The results of the semen analysis will be given to each subject. This analysis is done according to the World Health Organization guidelines and can be used as information for other medical specialists. You will also have personal information regarding your semen fertility status.

Reason/s why the Participant May Be Withdrawn from the Study: Your participation in this research is completely voluntary. You may withdraw at any time and this will not affect your treatment.

Remuneration: There will be no form of remuneration. Participation is voluntary.

Costs of the Study: You will not be asked to cover any cost relating to the study.

Confidentiality: All the information collected will be kept confidential. You will be allocated a number and all your details will be recorded under that number. This means that anyone who looks at my records will not be able to trace it to you. This is done to protect your privacy. In addition, a statement of confidentiality will be signed by both my supervisors and me.

Research-related Injury: There will be no research-related injury as there will be no alterations made to your dialysis treatment.

Persons to Contact in the Event of Any Problems or Queries:

Please contact the researcher (031 904 3980), my supervisor (031 373 5291) or the Institutional Research Ethics administrator on 031 373 2900. Complaints can be reported to the DVC: TIP, Prof F. Otieno on 031 373 2382 or dvctip@dut.ac.za.



CONSENT

Statement of Agreement to Participate in the Research Study:

- I hereby confirm that I have been informed by the researcher, **NS MOODLEY** (name of researcher), about the nature, conduct, benefits and risks of this study - Research Ethics Clearance Number: **REC 60/14**,
- I have also received, read and understood the above written information (Participant Letter of Information) regarding the study.
- I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report.
- In view of the requirements of research, I agree that the data collected during this study can be processed in a computerised system by the researcher.
- I may, at any stage, without prejudice, withdraw my consent and participation in the study.
- I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.
- I understand that significant new findings developed during the course of this research which may relate to my participation will be made available to me.

Full Name of Participant **Date** **Time** **Signature / Right Thumbprint**
 I, _____ (name of researcher) herewith confirm that the above participant has been fully informed about the nature, conduct and risks of the above study.

Full Name of Researcher **Date** **Signature**

Full Name of Witness (If applicable) **Date** **Signature**

Full Name of Legal Guardian (If applicable) **Date** **Signature**

APPENDIX D



Incwadi yolwazi ngocwaningo kanye nesivumelwano

Isihloko socwaningo: Isifundo ngocwaningo lwesidoda kwiziguli ezifakelwe izinso ezintsha zibezelashelwa ingcindezi kumasosha omzimba.

Umcwaningi omkhulu: Neville Moodley

Abaqaphi bocwaningo: Omkhulu - Prof Adam

Omncane – Dr S Naidu

Isingeniso kanye nenhloso yocwaningo: Sawubona, Igamalamingingu -Neville Moodley ofundela iziqu zeMasters degree esikhungweni sase Durban University of Technology. Ngingathanda kakhulu uma ungaba yingxenywe yocwaningo lami lapho ngizodinga khona ukuba unginike isidoda sakho esincane khona sizosicwaninga.Injongo yalolucwaninga ukubheka ukuthi amaphilisi owatholayo engcindezi kumasosha omzimba wakho akanamthelela omubi yini kwisidoda sakho.

Uhlelo locwaningo Iziguli ezingamashumi amathathu nane ezikade zifakelwe izinso ezintsha zibe zelashwa ngamaphilisi engcindezi kumasosha zizokhethwa ukyingenela lolucwaningo. Akuzukushintshwa ukwelashwa kwakho.Okuzodingeka kuwena ukuba unikezele ngesidoda sakho ngendlela yokuzijabulisa wena.Loluketshezi lwesidoda sakho luzohlolwa ehleliwe yalolucwaningo.

Ubungozi nokungaphatheki kahle komngeneli Abukho ubungozi noma ukungaphatheki kahle okuzokwenzeka ngokungenela kwakho lolucwaningo njengoba nokwelashwa kwakho kungezokushintshwa.

Inzuzo Imiphumela yalolucwaningo ngokuhlolwa kwesidoda nesidoda luzokunika ulwazi mayelana nezinga lesidoda sakho siphinde sikunike nezindlela yokuzigcina nesidoda esiphilile kanye nokwelashwa.

Ilungelo leziguli lokungenela lolucwaningo: Ukungenela lolucwaningo akuphoqelekile, unganqaba ukuba ukungenele noma usimise noma yinini uma usungenelile. Ukuyeka kwakho kulolucwaningo ngeke kuvimbele ukuthola usizo lokelashwa ngokujwayelekileyo. Umcwaningi omkhulu unalo ilungelo lokukukhipha kulolucwaningo uma ebona kunesidingo.

Inkokhelo Ayikho inkokhelo ozoyithola ngokungenela lolucwaningo. Ukungenela akuphoqiwe.

Izindleko zocwaningo Akukho zindleko ezikhokhwa nguwe kulolucwaningo.

Imfihlo Imininingwane yakho yonke yalolucwaningo ngeke idalulwe kumuntu izogcinwa iyimfihlo. Kuzoba khona inombolo ozonikwa yona ekuzofakwa kuyo yonke imininingwane yakho. Ulwazi lonke kanye nemiphumela etholwe emva kwalolucwaningo ezofakwa kuzincwadi zochwepheshe noma ezincwadini zokuqhakambisa ngeke idalule noma iveze ukuthi isiguli esithize besizimbandakanye nalolucwaningo.

Ukulimala ngesikhathi socwaningo Angeke kube khona ukulimala ngesikhathi socwaningo njengoba kungezoshintshwa ukwelashwa kokukhuculula igazi lakho.

Abantu ongaxhumana nabo uma unenkinga noma unemibuzo:

Ungathintana nomcwaningi (031 904 3980), umcwaningi omkhulu (031 373 5291) noma umqaphi wocwaningo (0313732900). uma unesikhalo ungabika ku DVC: TIP, prof I Otieno ku 031 373 2382 noma dvctip@dut.ac.za.



Kwemvume


Isitatimende sesivumelwano ngocwaningo:

- Ngqiqinisekisa ukuthi ucwaningo olwenziwe u Neville Moodley ngesimo, ukuziphaha, inzuzo nobungozi balo ucwaningo – Inombolo yokucacisa: _____
- Ngibuye ngamukela, ngaqonda lolulwazi olungenhla (participant incwadi yokwaziswa) mayelana nocwaningo.
- Ngityaqondo ngemiphumela yocwaningo, kuhlenganise neminingwane ngobulili, iminyaka yobudala, usuku lokuzalwa, iziqalo zamagama ami kanye nemiphumela yami ngeke idalulwe makuqhutshwa lolucwaningo.
- Ngenxa yezidingo zalolucwaningo, ngiyavuma ukuthi iminingwane ethokale idluliswe ngohlelo lwe khomputha ngumcwaningi.
- Uma kungenzeka, nginoxise ucwaningo kuyobe kungahlangene nokucwasa.
- Ngibe nethuba elenele ukubuza imibuzo, ngendlela engithanda ngayo. Ngazivumela ukuba ingxenywe yalolucwaningo.
- Ngityaqondo ukuthi kukhona okubalulekile okusha okutholakele khathi kuqhubeka lolucwaningo, ekuhlanganyeleni kwami.

_____	_____	_____	_____
Igama eligcele umhlanyeli Isithupha sokudla	Date	Isikhathi	Isignesha /
I - _____ (igama umcwaningi) lapha ziqinisekisa ukuthi iqhaza ngenhla azisiwe ngokuphelele ngesimo, ukuziphatha kanye nezingozi ze-ncwaningo ngenhla.			
_____	_____	_____	_____
Igama eligcwele umcwaningi	Date	Isignesha	
_____	_____	_____	
_____	_____	_____	_____
Igama eligcwele lafakazi (Uma kufanele)	Date	Isignesha	
_____	_____	_____	
_____	_____	_____	_____
Igama eligcwele umbheki (uma kufanele)	Date	Isignesha	
_____	_____	_____	

APPENDIX E

Clinical analysis of renal transplant patients under long-term immunosuppressive treatment



HISTORY				
Participant Name: _____				
Date of Birth: _____				
Study ID no: _____				
No	Content	Notes		
1.	Age			
2.	Race	Indian	coloured	Black
		Other: _____		
3.	Occupation			
4.	Place of residence			
Renal Disease/Transplant History				
5.	Initial date of renal diagnosis/failure			
6.	Cause of renal failure			
Pre Transplant				
7.	Treatment	Hemodialysis	Peritoneal	Other
8.	Duration of treatment			
9.	Corticosteroids Name/dosage/duration			
10.	Transplant date			
11.	ISP start date			
Post Transplant				
12.	Immunosuppressive Regimen			
13.	Active Ingredient			
14.	Dosage			
15.	Duration			
16.	Corticosteroids Name/dosage/duration			
17.	Height			
18.	Weight			
Fertility				
19.	Does participant have children?			
	If Yes, how many?			
	If Yes, was this pre or post transplant			
	Date of children being born			
20.	Smoking	Yes	No	
	No of cigarettes			
	No of years			

Screening survey of renal transplant patients undergoing immunosuppressive treatment

2013

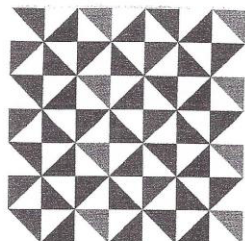
21.	Diabetic Treatment	Yes	No
22.	Hypertensive Treatment	Yes	No
23.	Other Medical Conditions		

Notes:

NS Moodley

Date

APPENDIX F



Institutional Research Ethics Committee
Faculty of Health Sciences
Room MS 49, Mansfield School Site
Gate 8, Ritson Campus
Durban University of Technology

P O Box 1334, Durban, South Africa, 4001

Tel: 031 373 2900

Fax: 031 373 2407

Email: lavishad@dut.ac.za

http://www.dut.ac.za/research/institutional_research_ethics

www.dut.ac.za

30 September 2014

IREC Reference Number: **REC 60/14**

Mr N S Moodley
53 Limeclay Lane
Clayfield
Phoenix
4068

Dear Mr Moodley

Semen analysis of renal transplant patients undergoing immunosuppressive treatment

I am pleased to inform you that Full Approval has been granted to your proposal REC 60/14.

The Proposal has been allocated the following Ethical Clearance number **IREC 068/14**. Please use this number in all communication with this office.

Approval has been granted for a period of one year, before the expiry of which you are required to apply for safety monitoring and annual recertification. Please use the Safety Monitoring and Annual Recertification Report form which can be found in the Standard Operating Procedures [SOP's] of the IREC. This form must be submitted to the IREC at least 3 months before the ethics approval for the study expires.

Any adverse events [serious or minor] which occur in connection with this study and/or which may alter its ethical consideration must be reported to the IREC according to the IREC SOP's. In addition, you will be responsible to ensure gatekeeper permission.

Please note that any deviations from the approved proposal require the approval of the IREC as outlined in the IREC SOP's.

Yours Sincerely

Prof C Napier
Chairperson: IREC (Acting)

APPENDIX G



health

Department:
Health
PROVINCE OF KWAZULU-NATAL

Health Research & Knowledge Management sub-component
10 – 103 Natalia Building, 330 Langalibalele Street
Private Bag x9051
Pietermaritzburg
3200
Tel.: 033 – 3953189
Fax.: 033 – 394 3782
Email.: hrkm@kznhealth.gov.za
www.kznhealth.gov.za

Reference : HRKM 113/15
NHRD: KZ_2015RP10_270
Enquiries : Mr X Xaba
Tel : 033 – 395 2805

Dear Mr N.S. Moodley

Subject: Approval of a Research Proposal

1. The research proposal titled 'Semen analysis of renal transplant patients undergoing immunosuppressive treatment' was reviewed by the KwaZulu-Natal Department of Health.

The proposal is hereby **approved** for research to be undertaken at Inkosi Albert Luthuli Central Hospital.

2. You are requested to take note of the following:
 - a. Make the necessary arrangement with the identified facility before commencing with your research project.
 - b. Provide an interim progress report and final report (electronic and hard copies) when your research is complete.
3. Your final report must be posted to **HEALTH RESEARCH AND KNOWLEDGE MANAGEMENT, 10-102, PRIVATE BAG X9051, PIETERMARITZBURG, 3200** and e-mail an electronic copy to hrkm@kznhealth.gov.za

For any additional information please contact Mr X. Xaba on 033-395 2805.

Yours Sincerely

Chairperson, Health Research Committee

Date: 28/07/15

uMnyango Wezempilo . Departement van Gesondheid

Fighting Disease, Fighting Poverty, Giving Hope

APPENDIX H



Semen Analysis Report

Date:

Male	
ID No.	
Tel:	
Study No:	

Semen Parameters	Result	Reference range	Unit
Method of collection	Masturbation		
Time produced			
Time tested			
Days Abstinence		2 - 7	Days
Volume (ml)		1.5 - 6.0	ml
Liquefaction		<20	min
Colour		White grey	
pH		7.2 - 8.0	
Viscosity		1 - 30	mm
Agglutination		0	%
Sperm Parameters	Result	Reference range	Unit
Count (million/ml)		15 - 250	million/ml
Motility (%)		40 - 100	%
Vitality (%)		40 - 100	%
IgG		<40	%
IgA		<40	%
Progression		0: No Motility 1: Non Forward 1+: One Forward 2: Directionless 2+: Forward sluggish 3: Fast not direct 3+: Straight good FP	
Morphology	Result	Reference range	Unit
Normal		14 - 100	%
Abnormal			%
Megalo			%
Small			%
Elongated			%
Duplicate			%
Neck			%
Tail			%
Precursors			%
Other cells	Result	Reference range	Unit
WBC		0	/HPF
Epithelial		0	/HPF
Comments			
Embryologist: Mr NS Moodley			