USMANU DANFODIYU UNIVERSITY, SOKOTO
(POSTGRADUATE SCHOOL)

EVALUATION OF SERUM SEX HORMONES LEVELS AMONG HIV POSITIVE ON HAART, HAART NAÏVE AND APPARENTLY HEALTHY INDIVIDUALS IN SOKOTO METROPOLIS

A Dissertation

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BY

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DEDICATION

This research work is dedicated to Almighty Allah, all praises and gratitude are due to Him, for sustaining my life up to this moment. It is also dedicated to my beloved wife (Sadiya A), and child (Khalid A).

CERTIFICATION

This Dissertation by AHMED Armiya'u Yelwa (Adm. No. **15211227018**) has met the requirements for the award of the Master of Science Degree in Medical Laboratory Sciences (Chemical Pathology) of the Usmanu Danfodiyo University, Sokoto, and is approved for its contribution to knowledge.

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ACRONYMS/DEFINITION OF TERMS

AIDS	Acquired Immunodeficiency Syndrome
Ag	Antigen
ANOVA	Analysis of Variance
ART	Antiretroviral Therapy
CARVT	Combination Active Retroviral Therapy
CCRS	Chemokine Co-Receptor CCR5
CD_4^+	Cluster of Differentiation Type 4
CD_8^+	Cluster of Differentiation Type 8
CXCR4	Chemokine Co-Receptor CXCR4
DNA	Deoxyribonucleic Acid
E_1	Oestrone
E_2	Oestradiol
E_3	Oestriol
E_4	Oestetrol
ED	Erectile Dysfunction
EDTA	Ethylene Diamine Tetra Acetic Acid

ELISA	Enzyme Linked Immunosorbent Assay
ERs	Oestrogen Receptors
FCM	Flow Cytometry
FMOH	Federal Ministry of Health
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
GPER	G Protein-Coupled Oestrogen Receptor 1
GPR30	G Protein-Coupled Receptor 30
HAART	Highly Active Antiretroviral Therapy
HCG	Human Chorionic Gonadotropin
HIV	Human Immunodeficiency Virus
HR-QOL	Health Related Quality of Life
LH	Luteinizing Hormone
LHB	Luteinizing Hormone Beta Subunit
MAO	Monoamine Oxidase
mERs	Membrane Oestrogen Receptors
NACA	National Agency for The Control of AIDS
PIs	Protein Inhibitors

PLHIV	People Living with HIV
RNA	Ribonucleic Acid
SD	Sexual Dysfunction
SSA	Sub Saharan Africa
SEM	Standard Error of Mean
SSBD	Sokoto State Business Directory
SMOH	Sokoto Ministry of Health
STD	Standard
TSH	Thyroid Stimulating Hormone
UNAIDS	Joint United Nations Programme on HIV/AIDS
WHO	World Health Organization

ABSTRACT

Acquired immunodeficiency syndrome (AIDS) is a chronic disease associated with human immunodeficiency virus (HIV) induced progressive depletion of $CD4^+$ T cells, increased vulnerability to opportunistic infections, which may lead to many complications. This study estimated the serum sex hormones [testosterone, oestrogen, follicle stimulating hormone (FSH) and luteinizing hormone (LH)] and $CD4^+$ count among HIV patients on HAART, HAART naïve patients and controls. A total of (90) HIV seropositive patients (45 on HAART and 45 HAART naive), and forty-five (45) age- and sex- matched apparently healthy controls were enrolled. $CD4^+$ cell count was measured in accordance with flow cytometry method using cyflow counter. Testosterone, Oestrogen, LH and FSH were estimated using method of competitive enzyme immunoassay technique. There were significantly ($p< 0.05$) lower Testosterone and $CD4^+$ levels among HIV positive on HAART and HAART naïve men when compared with controls. LH and FSH showed significant ($p< 0.05$) increase among HIV positive on HAART, both men and women. HIV is associated with changes in sex hormones which may lead to sexual dysfunction in infected individuals and probably antiretroviral therapy may improve sexual functions. Further study is needed to evaluate its effect on other sexual functions.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Acquired immunodeficiency syndrome (AIDS) is a chronic disease associated with human immunodeficiency virus (HIV) induced progressive depletion of $CD4^+$ T cells, increased vulnerability to opportunistic infections, which may lead to many complications and even death (Doitsh *et al*., 2014).

HIV infects vital cells in the human immune system such as helper T cells (specifically $CD4^+$ T cells), macrophages and dendritic cells. HIV infection leads to low levels of $CD4^+$ T cells through a number of mechanisms, including pyroptosis of abortively infected T cells, apoptosis of uninfected bystander cells, direct viral killing of infected cells and killing of infected $CD4^+$ T cells by CD8 cytotoxic lymphocytes that recognize infected cells. When $CD4^+$ T cell numbers decline below a critical level, cell-mediated immunity is lost (Doitsh *et al.*, 2014).

HIV infection is a chronic disease with potential mental, psychological and physical effects on infected individuals. These effects may be temporary (short-term), medium-term or long-term depending on the individual. The drugs used to treat the infection (antiretroviral drugs), co-morbidities or other related or no-related conditions may sometimes worsen the situation (Kamali *et al.*, 2010).

Antiretroviral drugs are medications for the treatment of infection by retroviruses, primarily HIV. When several of such drugs, typically three or four are taken in combination, the approach is known as highly active antiretroviral therapy (HAART). HAART use is associated with sexual dysfunction and this indirectly affects people living with HIV/AIDS (PLWHAs) health-related quality of life (HR-QoL) and by extension, their adherence (Panos *et al*., 2008).

Sexuality is an intrinsic part of a person‘s wellbeing, knowledge gaps exist on our understanding of issues of sexuality outside the risky behaviours paradigm among PLWHA on HAART in Sokoto and Nigeria at large. A bidirectional relationship between PLWHA sexual dysfunction and adherence to HAART underscore the need for this study. Risky sexual behaviours among PLWHA on HAART could potentially result in the transmission of the drug resistant virus creating another epidemiological nightmare.

UNAIDS (2010) report indicated that the world was beginning to register significant reduction in HIV-related morbidity and mortality in many of the heavily burdened countries. There was also a reduction in the number of new infections in several countries including those heavily burdened in sub-Saharan Africa (SSA) (UNAIDS, 2010). These achievements have been attributed to changes in sexual behaviour with increased knowledge and awareness, credited for reversal of the epidemic and expanded access to HAART credited for improved health and prolongation of lives (Kamali *et al.*, 2010).

The sub-Saharan Africa (SSA) region, which has only 12% of the total world population, remains the epicentre of the HIV epidemic with 68.0% of the global total in 2010 (WHO, 2011). The same UNAIDS report (2010) showed that of the 33 countries where the incidence of HIV had dropped between 2001 and 2009 globally, 22 were in SSA. However, majority of new infections continue to occur in SSA, with 31.0% of them being in only 10 countries (UNAIDS, 2010). Heterosexual intercourse remains the main mode of HIV transmission in SSA (WHO, 2011). Most HIV-infected individuals are young (<30 years old) and majority are in long-term heterosexual relationships, 62.0% in Kenya and 78.0% in Malawi (WHO, 2011).

The advent of the increased access to combination/highly-active antiretroviral therapy (cARVT/HAART) globally has led to great optimism for more efficient and long-term management of HIV infection. There is also great expectation among programme workers, service providers and patients that more infected individuals especially in the developing countries will live healthier and longer than hitherto. Many patients who previously would have viewed the diagnosis of HIV infection as a death-sentence have a new lease of life (WHO, 2011).

Evidence from published literature shows that HIV has a myriad of effects on the individual's sexuality and reproduction. While the majority were able to continue to have regular and enjoyable sexual relationships (WHO, 2011), there is a growing body of literature mainly from the developed world indicating higher prevalence of sexual problems including sexual dysfunction among infected individuals than the general population (Wilson *et al*., 2010). The exact mechanisms or pathogenesis thereof is not clearly understood and studies on associated/risk factors have yielded conflicting findings.

Sexual dysfunction may impact on the individual's quality of life, interpersonal relationships, health, uptake and adherence to cARVT (Trotta *et al.*, 2008), with the risk of increased infectivity and worsening of HIV disease itself. These sequelae of events will invariably pose new challenges to the HIV programming and service delivery in SSA countries whose health care systems are already overstretched (Trotta *et al.*, 2008).

1.2 Statement of Research PROBLEM

HAART has proven to have therapeutic success in significantly suppressing viral replication, improving immune system function, decreasing susceptibility to life-threatening opportunistic infections. It is associated with the general improvement in physical functioning among people living with HIV (Panos *et al*., 2008).

As HIV-infected individuals live longer now than before the introduction of HAART, age-related factors such as hormonal, vascular and neurological disorders may complicate the picture as well (Kamali *et al*., 2010).

An increasing number of individuals with HIV infection treated with HAART have complained about sexual dysfunction (SD). However, in clinical trials, SD has not been reported as a potential side-effect associated with HAART, but this is likely to be due to reporting bias, because patients are not routinely or systematically questioned about SD and may be unwilling to admit to such difficulties unless directly challenged. Many reasons why individuals with HIV may suffer from SD can be suggested, but to date only a few studies have addressed the question of whether SD may be caused by HAART (Panos *et al.*, 2008).

1.3 Justification of the Study

HAART intervention in HIV patients has not only reduced the morbidity and mortality rate but also said to cause sexual dysfunction. The extent to which HAART causes SD has not been well documented. Many reasons why individuals with HIV may suffer from SD can be suggested, but to date only a few studies have addressed the question of whether SD may be caused by HAART (Panos *et al.*, 2008).

Evaluation of sex hormone profile would be beneficial for assessing and determining the effect of HAART on sex hormones. Whether the use of HAART in PLWHA has any beneficial/harmful effect on sexual function is the rationale behind this research.

1.4 Research Hypothesis

Null hypothesis (H_O): Treatment with HAART will not have significant effect on the level of sex hormone in PLWHA.

Alternate hypothesis (H_A): HAART treatment will have significant effect on the level of sex hormone in PLWHA.

1.5 Aim and Objectives

1.5.1 Aim

The aim of this study was to evaluate serum sex hormones levels among HIV positive on HAART, HAART-naïve and apparently healthy individuals in Sokoto metropolis.

1.5.2 Objectives

The objectives of this study were to:

1. Estimate serum levels of sex hormones (testosterone, oestrogen, follicle stimulating hormone (FSH) and luteinizing hormone (LH)) and $CD4^+$ count

among male and female HIV patients on HAART, HAART naïve patients and controls.

2. Compare the results of serum sex hormone levels and $CD4^+$ count obtained from three different groups of the study population (HIV patients on HAART, HAART-naïve patients and controls).
3. Establish the relationship between $CD4^+$ count and each of serum levels of sex hormones among male HIV patients on HAART, HAART-naïve patients and controls.
4. Establish the relationship between $CD4^+$ count and each of serum levels of sex hormones among female HIV patients on HAART, HAART-naïve patients and controls.

1.6 Research Questions

1. What is the pattern of serum sex hormones in people living with HIV infection?
2. Does HAART have any effect on sex hormones profile in people living with HIV infection?
3. What is the pattern of $CD4^+$ count and sex hormone profile in people living with HIV infection?
4. Is there any relationship between the mean $CD4^+$ count and sex hormone levels in people living with HIV infection?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 HIV Infection/Acquired Immunodeficiency Syndrome (AIDS)

Human Immunodeficiency Virus (HIV) is a member of Lent virus sub-family of retroviruses. There are two (2) types of HIV designated as HIV-1 and HIV-2, both of which have been documented as the causative agents of acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi *et al.*, 1983).

HIV-1 and HIV-2 are approximately 50% related to each other at the nucleotide level (Kanki *et al.*, 1997). However, they differ in their genetic reconstitutions. Evidences have shown that an accessory gene vpu is unique to HIV-1 (Cohen *et al*., 1998), while the vpx gene is found only in HIV-2 (Kappers *et al*., 1988).

HIV-1 is more widely distributed accounting for approximately 95% of all HIV infections worldwide (Meloni *et al*., 2006). HIV-2 is also endemic in West Africa; however, its prevalence rate has been relatively low accounting for about 2% in Cote D'ivoire, Ghana, Burkina Faso, Guinea Bissau and Senegal (Nkengasong, 2004). Studies focused on the differences between HIV-1 and HIV-2 has shown evidences for genetic diversity on the biological phenotype of the viruses. While HIV-2 is transmitted through the same modes as HIV-1 and HIV-2 transmission rates are significantly lower with the most common modes being perinatal and heterosexual (Kanki *et al*., 1997). HIV-2 has also been shown to be less pathogenic than HIV-1 with a progression to AIDS significantly longer than that with HIV-1 infection (Marlink *et al*., 1994). In addition, viral load levels are lower with HIV-2 as compared to HIV-1 infection (Popper *et al.*, 1999) which likely contributes to the lower pathogenicity of HIV-2.

The unique characteristics of HIV is due to the possession of a special enzyme, reverse transcriptase which in the course of HIV replication enables the viral genetic material

transcribed from ribonucleic acid (RNA) to deoxy nucleic acid (DNA). Both HIV-1 and HIV-2 infect mainly the $CD4^+$ lymphocytes, monocytes and macrophages and are regarded as pathogenic in infected persons. Generally, $CD4^+$ bearing cells are infected after binding of the glycosylated envelope proteins of HIV gp 120 to both the $CD4^+$ receptor proteins and chemokine receptors including CCR5 and CXCR4 and co-receptor which are found on the surface of the target cells (Mc Farland, 2005).

Progression of HIV infections associated with depletion of $CD4^+$ count and consequent increased risk for the development of opportunistic AIDS-defining diseases (Mansur *et al.*, 1989). The depletion of $CD4^+$ count is mediated through mechanisms that may involve; direct viral killing of infected cells; increased rates of apoptosis in infected cells and killing of infected cells (Cohen *et al.*, 1999).

Acquired immunodeficiency syndrome (AIDS) is a clinical diagnosis that represents the late stages of HIV disease progression. HIV-infected people classified as having progressed to AIDS either have $CD4^+$ count lower than 200 cells/µl or one or more AIDS-defining conditions (Akinsete *et al.*, 1998). In Nigeria, the most common AIDS-defining conditions includes severe weight loss, pulmonary tuberculosis, candidiasis, herpes zoster infection, recurrent bacterial pneumonia and kaposis sarcoma (Anteyi *et al*., 1996; Akinsete *et al.*, 1998).

2.1.1 Epidemiology of HIV/AIDS

2.1.1.1 Global HIV/AIDS epidemic

HIV remains a global health problem of unprecedented dimensions. Globally, there were an estimated 36.9 million people living with HIV in 2014 (UNAIDS, 2014). Adults constitute 34.3 million, women account for 17.4 million and children less than 15 years' age constitute 2.6 million.

An estimated 200,000 new HIV infections occurred among children under the age of15 and 1.8 million among adults bringing the total to 2.0 million people newly infected with HIV, while a total 1.2 million AIDS-related deaths occurred in 2014 (UNAIDS, 2014). Sub-Saharan Africa remains the most heavily affected region accounting for 70% of all HIV infections in 2014. An estimated 1.4 million people living in Sub-Saharan Africa became newly infected with HIV, bringing the total number of people living with HIV to 25.8million. An estimated 790,000 AIDS-related deaths also occurred in sub- Saharan Africa in 2014 (UNAIDS, 2014). This number represents a 16% decline in annual HIV-related mortality in the region since 2004.

2.1.1.2 Nigerian HIV/AIDS epidemic

The spread of HIV has increased significantly in Nigeria since the first case of AIDS reported in 1986, thereby establishing the occurrence of HIV infection in the country. Consequently, and in line with the WHO guidelines, the Government adopted ANC sentinel surveillance as the system for monitoring the epidemic. The first HIV sentinel survey in 1991 showed a prevalence of 1.8%. Subsequent sentinel surveys showed prevalence of 3.8% (1993), 4.5% (1996) and 5.8% (2001) (FMOH, 2006). In 2007, the National

Population-based survey showed an overall HIV prevalence of 3.6% (4.0% among females and 3.2% males) slightly higher in the urban areas (3.8%) compared with the rural areas (3.5%). It was highest in the North central zone (5.7%) and lowest in the south west (2.1%) (FMOH, 2007).

In 2008, the overall HIV prevalence among women attending ANC during the period was 4.6%. The highest zonal prevalence (7.0%) was in south-south and the lowest in south west (2.0%); prevalence in Sokoto stood at (6.0%) (FMOH, 2008). The HIV and AIDS epidemic has continued to grow largely through heterosexual relationships,

mother-to-child transmission and contaminated blood and blood products (FMOH, 2008).

2.2 Sex Hormones

Sex steroids, also known as gonadal steroids, are steroid hormones that interact with vertebrate androgen or estrogen receptors. Their effects are mediated by slow genomic mechanisms through nuclear receptors as well as by fast non-genomic mechanisms through membrane-associated receptors and signaling cascades (Seftel *et al*., 2004). The term sex hormone is nearly always synonymous with sex steroid. The non-steroid hormones luteinizing hormone, follicle-stimulating hormone and gonadotropin-releasing hormone are usually not regarded as sex hormones, although they play major sex-related roles (Seftel *et al.*, 2004).

Natural sex steroids are made by the gonads (ovaries or testes), by adrenal glands or by conversion from other sex steroids in other tissue such as liver or fat (Seftel *et al*., 2004).

2.2.1 Types of sex hormones

In many contexts, the two main classes of sex hormones are androgens and oestrogens, of which the most important human derivatives are testosterone and oestradiol respectively. Others include progestogens as a third class of sex steroids, distinct from androgens and estrogens. Progesterone is the most important and only naturally-occurring human progestogen. In general, androgens are considered "male sex hormones", since they have masculinizing effects, while oestrogens and progestogens are considered "female sex hormones" (ElAttar and Hugoson, 1974); although all types are present in each sex, albeit at different levels.

The sex hormones include (1) androgens: anabolic steroids, androstenedione, dehydroepiandrosterone, dihydrotestosteroneand testosterone; (2) oestrogens: oestradiol, oestriol and oestrone and (3) progestogens: progesterone.

2.2.1.1 Testosterone

Testosterone is the primary male sex hormone and an anabolic steroid. In men, testosterone plays a key role in the development of male reproductive tissues such as the testis and prostate, as well as promoting secondary sexual characteristics such as increased muscle and bone mass and the growth of body hair (Mooradian *et al.*, 1987). In addition, testosterone is involved in health and well-being and prevention of osteoporosis (Bassil *et al.*, 2009). Insufficient levels of testosterone in men may lead to abnormalities including weakness and bone loss (Tuck and Francis, 2009).

Testosterone is also used as a medication to treat male hypogonadism and certain types of breast cancer. Since testosterone levels gradually decrease as men age, synthetic testosterone is sometimes prescribed to older men to counteract this deficiency (Luetjens and Weinbauer, 2012).

Testosterone is a steroid from the androstane class containing a keto and hydroxyl groups at the three and seventeen positions respectively. It is biosynthesized in several steps from cholesterol and is converted in the liver to inactive metabolites. It exerts its action through binding to and activation of the androgen receptor (Luetjens and Weinbauer, 2012).

In humans and most other vertebrates, testosterone is secreted primarily by the testicles of males and, to a lesser extent, the ovaries of females. Small amounts are also secreted by the adrenal glands. On average, in adult males, levels of testosterone are about 7–8 times as great as in adult females (Torjesen and Sandness, 2004). As the metabolic

consumption of testosterone in males is greater, the daily production is about 20 times greater in men. Females are also more sensitive to the hormone (Southren *et al*., 1965).

2.2.1.2 Oestrogen

Oestrogen is the primary female sex hormone as well as a medication. It is responsible for the development and regulation of the female reproductive system and secondary sex characteristics. Oestrogen may also refer to any substance, natural or synthetic that mimics the effects of the natural hormone (Baker, 2013). The oestrane steroid oestradiol is the most potent and prevalent endogenous oestrogen, although several metabolites of this hormone also have oestrogenic hormonal activity. Oestrogens are used as medications as part of some oral contraceptives, in hormone replacement therapy for postmenopausal, hypogonadal and transgender women and in the treatment of certain hormone-sensitive cancers like prostate and breast cancers. They constitute one of three types of sex hormones, the others being androgens/anabolic steroids such as testosterone and progestogens like progesterone (Baker, 2013).

Oestrogens are synthesized in all vertebrates as well as some insects (Mechoular *et al*., 2005). Their presence in both vertebrates and insects suggests that these hormones have an ancient evolutionary history (Mechoular *et al*., 2005). The three major naturally occurring forms of oestrogen in women are oestrone (E_1), oestradiol (E_2), and oestriol (E_3). Another type of oestrogen called oestetrol (E_4) is produced only during pregnancy. Quantitatively, estrogens circulate at lower levels than androgens in both men and women (Burger, 2002). While estrogen levels are significantly lower in males compared to females, oestrogens nevertheless also have important physiological roles in males (Lombardi *et al*., 2001).

Like all steroid hormones, oestrogens readily diffuse across the cell membrane. Once inside the cell, they bind to and activate oestrogen receptors (ERs) which in turn

modulate the expression of many genes (Whitehead and Nussey, 2001). Additionally, oestrogens bind to and activate rapid-signaling membrane oestrogen receptors (mERs) such as GPER (GPR30) (Soltysik and Czekaj, 2013).

2.2.1.2.1 Biological function of oestrogen

The actions of oestrogen are mediated by the oestrogen receptor (ER), a dimeric nuclear protein that binds to DNA and controls gene expression. Like other steroid hormones, oestrogen enters passively into the cell where it binds to and activates the oestrogen receptor. The oestrogen-ER complex binds to specific DNA sequences called a hormone response element to activate the transcription of target genes (in a study using an oestrogen-dependent breast cancer cell line as model, 89 such genes were identified) (Soltysik and Czekaj, 2013). Since oestrogen enters all cells, its actions are dependent on the presence of the ER in the cell. The ER is expressed in specific tissues including the ovary, uterus and breast. The metabolic effects of oestrogen in post - menopausal women have been linked to the genetic polymorphism of the ER (Soltysik and Czekaj, 2013).

While oestrogens are present in both men and women, they are usually present at significantly higher levels in women of reproductive age. They promote the development of female secondary sexual characteristics such as breasts and are also involved in the thickening of the endometrium and other aspects of regulating the menstrual cycle. In males, oestrogen regulates certain functions of the reproductive system important to the maturation of sperm and may be necessary for a healthy libido. Furthermore, there are several other structural changes induced by oestrogen in addition to other functions (Soltysik and Czekaj 2013).

2.2.1.2 Follicle stimulating hormone

Follicle-stimulating hormone (FSH) is a gonadotropin, a glycoprotein polypeptide hormone. FSH is synthesized and secreted by the gonadotropic cells of the anterior pituitary gland (Pierce and Parsons, 1981) and regulates the development, growth, pubertal maturation and reproductive processes of the body. FSH and luteinizing hormone (LH) work together in the reproductive system.

FSH is a 35.5 kDa glycoprotein heterodimer, consisting of two polypeptide units, alpha and beta. Its structure is similar to those of luteinizing hormone (LH), thyroid-stimulating hormone (TSH) and human chorionic gonadotropin (hCG). The alpha subunits of the glycoproteins LH, FSH, TSH and hCG are identical and consist of about 96 amino acids, while the beta subunits vary (Pierce and Parsons, 1981). Both subunits are required for biological activity. FSH has a beta subunit of 111 amino acids (FSH β) which confers its specific biologic action and is responsible for interaction with FSH receptor (Jiang *et al*., 2012). The sugar portion of the hormone is covalently bonded to asparagines and is composed of N-acetylgalactosamine, mannose, N-acetylglucosamine, galactose and sialic acid.

2.2.1.3 Luteinizing hormone

Luteinizing hormone (LH), also known as lutropin and sometimes lutrophin (Ujihara *et al.*, 1992) is a hormone produced by gonadotropic cells in the anterior pituitary gland. In females, an acute rise of LH (LH surge) triggers ovulation and development of the corpus luteum. In males, where LH had also been called interstitial cell–stimulating hormone (ICSH), it stimulates Leydig cell production of testosterone (Louvet *et al.*, 1975). It acts synergistically with FSH.

LH is a heterodimeric glycoprotein in which each monomeric unit is a glycoprotein molecule where one alpha and one beta subunit make the full functional protein. Its

structure is similar to that of the other glycoprotein hormones. The protein dimer contains 2 glycopeptidic subunits, labeled alpha and beta subunits that are non-covalently associated (that is, without any disulfide bridge linking them) (Louvet *et al.*, 1975).

The alpha subunits of LH, FSH, TSH and hCG are identical, and contain 92 amino acids in human but 96 amino acids in almost all other vertebrate species (glycoprotein hormones do not exist in invertebrates), while the beta subunits vary. LH has a beta subunit of 120 amino acids (LHB) that confers its specific biologic action and is responsible for the specificity of the interaction with the LH receptor. This beta subunit contains an amino acid sequence that exhibits large homologies with that of the beta subunit of hCG and both stimulate the same receptor. However, the hCG beta subunit contains an additional 24 amino acids and the two hormones differ in the composition of their sugar moieties (Louvet *et al.*, 1975). The different composition of these oligosaccharides affects bioactivity and speed of degradation. The biologic half-life of LH is 20 minutes shorter than that of FSH (3–4 hours) and hCG (24 hours).

2.2.1.3.1 Effects of LH in females

LH supports theca cells in the ovaries that provide androgens and hormonal precursors for oestradiol production. At the time of menstruation, FSH initiates follicular growth, specifically affecting granulosa cells (Bowen, 2004). With the rise in oestrogens, LH receptors are also expressed on the maturing follicle, which causes it to produce more oestradiol. Eventually, when the follicle has fully matured, a spike in 17α-hydroxyprogesterone production by the follicle inhibits the production of oestrogens, leading to a decrease in oestrogen-mediated negative feedback of gonadotrophic-releasing hormone (GnRH) in the hypothalamus, which then stimulates the release of LH from the anterior pituitary (Mahesh, 2011). However, another theory of the LH peak

is a positive feedback mechanism from oestradiol. The levels keep rising through the follicular phase and when they reach an unknown threshold, this results in the peak of the LH (Guyton and Hall, 2006). This effect is opposite from the usual negative feedback mechanism presented at lower levels. In other words, the mechanism(s) are not yet clear. The increase in LH production only lasts for 24 to 48 hours. This "LH surge" triggers ovulation, thereby not only releasing the egg from the follicle but also initiating the conversion of the residual follicle into a corpus luteum that in turn, produces progesterone to prepare the endometrium for a possible implantation. LH is necessary to maintain luteal function for the second two weeks of the menstrual cycle. If pregnancy occurs, LH levels will decrease and luteal function will instead be maintained by the action of hCG (human chorionic gonadotropin), a hormone very similar to LH but secreted from the new placenta (Guyton and Hall, 2006).

Rupture of the ovarian follicle at ovulation causes a drastic reduction in oestrogen synthesis and a marked increase in secretion of progesterone by the corpus luteum in the ovary, reinstating a predominantly negative feedback on hypothalamic secretion of GnRH-1 (Norris and Carr, 2013).

2.2.1.3.2 Effects of LH in males

LH acts upon the Leydig cells of the testis and is regulated by GnRH (Bowen, 2004). The Leydig cells produce testosterone (T) under the control of LH which regulates the expression of the enzyme 17β-hydroxysteroid dehydrogenase that is used to convert androstenedione, the hormone produced by the testes to testosterone, an androgen that exerts both endocrine and intratesticular activities on spermatogenesis (Bowen, 2004).

LH is released from the pituitary gland and is controlled by pulses of GnRH. When testosterone levels are low, GnRH is released by the hypothalamus, stimulating the pituitary gland to release LH. As the levels of testosterone increase, it acts on the

hypothalamus and pituitary through a negative feedback loop and inhibit the release of GnRH and LH consequently (Mahesh, 2011). Androgens inhibit monoamine oxidase (MAO) in pineal, leading to increased melatonin and reduced LH and FSH by melatonin-induced increase of GnIH synthesis and secretion. Testosterone can also be aromatized into oestradiol (E_2) to inhibit LH. E_2 decreases pulse amplitude and responsiveness to GnRH from the hypothalamus onto the pituitary (Pitteloud *et al*., 2008).

Changes in LH and testosterone blood levels and pulse secretions are induced by changes in sexual arousal in human males (Storelu *et al*., 1993).

2.2.2 Sex hormone and libido

Testosterone appears to be a major contributing factor to sexual reproduction motivation in male primates including humans. The elimination of testosterone in adulthood has been shown to reduce sexual reproduction motivation in both male humans and male primates (Waller, 2001). Male humans who had their testicular function suppressed with a GnRH antagonist displayed decreases in sexual desire and masturbation two weeks following the procedure (Waller, 2001). Study from male rhesus monkeys suggests testosterone functions to increase sexual reproduction motivation, thereby motivating males to compete for access to sexual partners. It is postulated that the motivating effects of testosterone in male rhesus monkeys promotes successful sexual competition and may be particularly important motivating tools for low ranking males (Waller, 2001). It is important to note that elimination of testosterone in primates does not reduce the ability to copulate but rather, it reduces the motivation to copulate.

Testosterone levels in males have been shown to vary according to the ovulating state of females. Males who were exposed to scents of ovulating women recorded a higher testosterone level than males who were exposed to scents of non-ovulating women

(Miller and Maner, 2009). Being exposed to female ovulating cues may increase testosterone, which in turn may increase males' motivation to engage in and initiate sexual behaviour. Ultimately, these higher levels of testosterone may increase the reproductive success of males exposed to female ovulation cues.

The relationship between testosterone and female sexual reproduction motivation is somewhat ambiguous. Study suggests that androgens such as testosterone are not sufficient by themselves to prompt sexual reproduction motivation in females (Waller, 2001). In particular, studies on rhesus macaques have observed that testosterone was not significantly associated with variations in level of sexual reproduction motivation in females (Waller, 2001). However, study with non-human primates suggests a role for androgens in female sexual reproduction behaviour (Johnson and phoenix, 1976). Adrenalectomized female rhesus monkeys displayed diminished female sexual receptivity (Johnson and phoenix, 1976). Later studies revealed that this diminished sexual receptivity was specific to the elimination of androgens that can be converted to oestrogen (Veney and Rissman, 2000).

It was suggested that levels of testosterone are related to the type of relationship in which one is involved. Men involved in polyamorous relationships display higher levels of testosterone than men involved in either a single partner relationship or single men (Van Anders *et al.*, 2007). Polyamorous women have both higher levels of testosterone and score higher on measures of sexual desire than women who are single or women who are in single-partner relationships (Van Anders *et al.*, 2007).

Oestrogen and progesterone typically regulate motivation to engage in sexual reproduction behaviour for females in mammalian species, though the relationship between hormones and female sexual reproduction motivation is not well understood. In particular, oestrogens have been shown to correlate positively with increases in female

sexual reproduction motivation, and progesterone has been associated with decreases in female sexual reproduction motivation (Ziegler, 2007).

The periovulatory period of the female menstrual cycle is often associated with increased female receptivity and sexual reproduction motivation (Ziegler, 2007). During this stage in the cycle, oestrogens are elevated in the female and progesterone levels are low. At this time, mating can result in female pregnancy.

Females at different stages of their menstrual cycle have been shown to display differences in sexual attraction. Heterosexual females not using birth control pills who are ovulating (high levels of oestrogens) have a preference for the scent of males with low levels of fluctuating asymmetry (Gangestad and Thornhill, 2005). Ovulating heterosexual females also display preferences toward masculine faces and report greater sexual attraction to males other than their current partner (Gangestad *et al.*, 2005). From an evolutionary perspective, increases in oestrogens during fertile periods in females may direct sexual reproduction motivation toward males with preferential genes (the good genes hypothesis).

Following natural or surgically induced menopause, many women experience declines in sexual reproduction motivation (Giles, 2008). Menopause is associated with a rapid decline of oestrogen, as well as a steady rate of decline of androgens (Jones, 2010). The decline of oestrogen and androgen levels is believed to account for the lowered levels of sexual reproduction desire and motivation in post-menopausal women, although the direct relationship is not well understood.

2.3 Sexual Dysfunction and HIV/AIDS

A prospective study in Nairobi on 25 patients (7 with asymptomatic HIV, 8 with AIDS-related complex and 10 with AIDS) and 25 age- and sex-matched controls was evaluated for its autonomic functioning. Substantial autonomic dysfunctions were seen

in AIDS patients relative to controls and mild abnormalities in the majority of HIV-infected patients were found (Rogstad *et al*., 1999). This finding could explain the association between delayed ejaculation and peripheral neuropathy (Bell *et al.*, 2006). The physiological process of ejaculation is under autonomic control via the hypogastric (sympathetic) and pudendal (parasympathetic) nerves (Bell *et al.*, 2006). Another relevant point regarding the pathophysiology of the sexual dysfunctions in this population is that many patients who are on HAART for the management of their HIV infection who usually have lipodystrophy also have elevated oestrogen levels and complain of low sexual desire. As a possible pathological mechanism, one can consider an increase in the number of fibroblasts and macrophages present in lipoatrophic areas that could convert testosterone to oestrogen by intracellular aromatization. This process is known to be enhanced by increased levels of tumour necrosis factor, interleukin 6 (IL- 6) and hydroxycorticosteroids present in many patients with HIV lipodystrophy (Goldmeier *et al.*, 2002).

In addition, a study on rabbits has found oestrogen receptors in the cavernous body of the penis and pathophysiological changes in erectile function when rabbits were treated with continuous oestrogen (Srilatha and Adaika, 2004). Another study of older men found that the balance between testosterone (decreased) and oestradiol (increased) is associated with erectile dysfunction (ED) (Srilatha *et al.*, 2007). Anecdotal reports suggest an association between protease inhibitor use and sexual dysfunctions, but only a few studies have found specific evidence for the link. These studies have found a possible effect of the protease inhibitors on the binding of steroids hormones with their receptors (Yang *et al*., 2005) or causing the blockade of androgen receptor (Baker *et al.*, 1978), both which could be related to sexual difficulties associated with protease inhibitors.

Hormonal changes such as changes in serum levels of testosterone and estradiol have been implicated in SD but findings from some studies are conflicting (Colson *et al*., 2002; Collazos *et al*., 2002; Lamba *et al*., 2004; Schrooten *et al*., 2011). Lamba *et al.* (2004) observed that low libido and ED among HIV infected individual men on HAART were associated with elevated oestradiol levels. Hypogonadism is a well-known cause of ED (Morale *et al*., 2004). It has been shown to be common among HIV infected men with incidence of between 29 and 50% before HAART and between 20 and 30% after HAART intervention (Crum *et al*., 2005). Moreno-Perez *et al.* (2010) observed that 53.4% of their study population had ED and although there was no correlation between the total serum testosterone levels and degree of ED, all patients who had hypogonadism had ED (Moreno-Perez *et al*., 2010). Bancrofti (2005) stated that a direct effect of oestrogen on sexual desire remains unproven in the general population, while Dennerstein *et al*. (1999) and Motofei *et al*. (2005) opined that desire, arousal and other aspects of sexuality are closely linked to the woman's sexual experiences, duration and quality of relationships rather than hormonal deficiencies. Collazos *et al*. (2002) reported that anti-retroviral therapies (ARVTs) were associated with elevated testosterone and 17b-oestradiol but sexual dysfuction (SD) was not related to alterations in the sex hormone pattern.

Elevation of prolactin is commonly observed in HIV infected individuals on HAART and reported to occur in about 20% of them (Collazos *et al.*, 2002). Prolactin acts centrally by inhibiting gonadotrophin release through which it is thought to cause SD. However, it was reported that high prolactin levels do not appear to lead to SD as no inhibition of gonadotrophin has been found and patients with SD did not have higher prolactin values than those who did not complain of SD (Collazos *et al*., 2002).

There has been a lot of debate on the role of ARVT in SD. whereas some studies support the role of ARVT (Lamba *et al*., 2004), others did not find an association (Lallemand *et al*., 2002). Colson *et al.* (2002) reported that HIV infected men were about 3.4 times more likely to complain of ED after a protease inhibiting (PI) drug was added, while Colebunders *et al.* (2001) observed that the SD regressed or disappeared completely after switching to non-PI containing regimen. A large study on men in 10 European countries revealed that ED and diminished desire were more common in patients on PIs than the PI-naive patients (Schroter *et al*., 2001). Another large multi-country study found an association between ARVT and ED but no association with specific ARV drug (Asboe *et al*., 2007). With regards to specific ARV drugs PIs in general (indinavir and ritonavir) have been the most commonly involved drugs (Colson *et al.*, 2002). A prospective study involving men did not find any relationship between ED and PIs or ARVT in multivariate analysis despite the highly significant association observed with the duration of HAART and PIs in the univariate analysis (Schroter *et al*., 2001). In a large European cohort study, Florence *et al*. (2004) did not observe differences on any sexual domains between those on ARVT and those not. Bassil *et al*. (2009) in their study in Spain showed an "unexpected" improvement of sexual function during the use of atazanavir, a new PI which metabolic pathway is different from that of others. The role of HAART seems to be observed more in men than women and women seem to be overrepresented in studies which did not find an association. It is not clear though whether this is a true observation or that the effect of ARVT in women was masked by the high prevalence of psychological disorders observed in them. It however, appears that there is a direct or indirect causal effect of ARVT on SD. This is supported by the fact that the prevalence of SD has not reduced with introduction of HAART

despite the significant improvement in the two main factors responsible for SD before HAART that is, advanced disease and hypogonadism (Colson *et al*., 2002).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was carried out at the Specialist Hospital, Sokoto. Sokoto State is located at the extreme part of North-Western Nigeria between longitude 3 and 7^{o} East and between latitude 10 and 14^{o} North of the equator. It shares borders with Niger Republic to the north, Kebbi State to the south-west and Zamfara State to the east (SSBD, 2007). The state covers a total land area of about 32,000 square kilometres with a population of 4.6 million based on 2013 census projection (UNFPA, 2013). Sokoto State has semi-arid climate and vegetation is largely Sudan Savannah with an annual rainfall of between 500 and 1300 mm and temperature range between 15 and over 40^{0}C during warm days (SSBD, 2007).

3.2 Study Population

A total of 135 subjects were recruited for this study. These consisted of 45 HIV patients on HAART (tenofovir, lamivudine and efavirenz), 45 HAART-naïve HIV patients and 45 apparently healthy individuals as controls.

The targeted age-group were adults in the range of 15 to 60 years who presented to the Antiretroviral Therapy (ART) Clinic of the Specialist Hospitals Sokoto.

3.2.1 Inclusion criteria

HIV-positive on HAART and HAART-naïve HIV patients, subjects within the age range of 15 to 60 years both males and females who had no clinical conditions likely to affect serum concentrations of sex hormones such as diabetes mellitus, cardiovascular disease and other related conditions were included in this study.

3.2.2 Exclusion criteria

1. HIV-positive patients who had clinical conditions likely to affect serum sex hormones levels such as diabetes mellitus, cardiovascular disease and other related conditions were excluded from this study.
2. Subjects with established cases of sexual dysfunction and/or infertility before commencement of HAART therapy were excluded from the study.
3. Subjects who declined to give consent for inclusion were excluded from the study.

3.2.3 Sample size determination

The sample size for the study was calculated using the formula below (Cochran, 1977)

$$n = \frac{z^2 p \times q}{d^2}$$

Where:

n= the desired sample when the population is greater than 10,000

Z= the desired normal deviate, usually set at 1.96 which corresponds to the 95% confidence level.

P= the current prevalence rate of HIV in Sokoto which is 5.6% (NACA, 2016)

q =1-p

d=degree of accuracy desired, usually set at 0.05.

Therefore, the minimum sample size was calculated as follows:

$$n= \frac{1.96^2 \times 0.56 \times (1-0.056)}{0.05^2}$$

$$n= \frac{1.96^2 \times 0.56 \times 0.944}{0.0025}$$

$$n= \frac{3.8416 \times 0.56 \times 0.944}{0.0025}$$

$$n= \frac{0.2030823424}{0.0025}$$

$$n= 81.232 \approx 81$$

The calculated sample size was 81. However, 10% (≈9 patients) was added as attrition rate. Therefore, the final calculated sample size was 90 subjects.

3.2.4 Informed consent

Informed consent for inclusion was obtained from the selected participants using a standard protocol (Appendices I and II).

3.2.5 Ethical consideration

Ethical approval of the study was obtained from the Ethical and Research Committee of the Specialist Hospital, Sokoto.

Adequate information about the study was explained to each subject to enable him/ her take independent decision about whether or not to participate in the study and informed consent for inclusion was obtained from these subjects before enrolment into the study. Strict confidentiality on patient's record was maintained at all levels in the conduct of the study.

3.3 Study Design

This is a cross-sectional descriptive study. The study subjects were grouped as follows:

Group I (n=45): HIV patients on HAART (Tenofovir, Lamivudine and Efavirenz)

Group II (n=45): HAART-naïve HIV patients

Group III (n=45): Apparently healthy subjects (controls)

3.4 Sampling Techniques

3.4.1 Subjects selection.

Simple random sampling technique was used to recruit 45 HIV patients on HAART and equally 45 HIV patients that were yet to commence HAART attending the Antiretroviral Therapy (ART) Clinic of Specialist Hospital, Sokoto and 45control subjects were also randomly selected from staff of Specialist Hospital, Sokoto.

An informed consent was obtained from each subject prior to the recruitment.

3.4.2 Blood sample collection and processing

From each selected subject, 5 ml of venous blood sample was collected at the clinic using a sterile disposable syringe and needle. The tourniquet was tied above the elbow and the prominent vein at the antecubital fossa was located. The site was cleaned with 70% alcohol and allowed to air dry. A needle was inserted into the vein with the bevel facing up and the blood was carefully withdrawn. Four milliliter (4 ml) of blood was transferred to plain tubes, allowed to clot at room temperature and centrifuged at 4000 rpm for 5 minutes. The sera were removed and placed into another plain tube and stored at -20^0C until the time of analysis.

The remaining 1 ml was transferred into a sterile EDTA specimen bottle and used for the estimation of $CD4^+$ count within 3 hours of the blood collection.

3.5 Reagents

i. Sex hormones were estimated using ACCUBIND ELISA assay kit which was obtained from Monobind Inc (lake forest USA) Through Nums Diagnostic.
ii. Determine HIV 1 and 2 test kit manufactured by Abbort Laboratories (Japan) was used for HIV Screening 1.
iii. Uni-Gold TM HIV test kit manufactured by Trinity Biotech Plc. Bray, Co. (Wicklow, Ireland) was used for HIV Screening 2.
iv. STAT- PAK HIV 1 and2 assay test kit manufactured by Chembio Diagnostic System Inc, (USA) was used for HIV screening 3 or tie breaker.

3.6 Equipment

The equipment used for the study include the following:

i. ALC 4235 Centrifuge anno Di fabric Milano (Italy) was used to spin the blood specimens

ii. Spectrophotometer Human 300 (Germany) and Microplate reader RT-2100 C Ray were used for the measurements of the serum sex hormones.

iii. Cyflow $CD4^+$ counter by Partec Munster (Germany) was used to count the $CD4^+$ cells.

3.7 Laboratory Analytical Methods

3.7.1 Estimation of serum testosterone

This was based on competitive enzyme immunoassay technique described by Horton and Tait (1966).

3.7.1.1 Principle

Competitive enzyme immunoassay (Type 7)

The essential reagents required for an enzyme immunoassay include antibody, enzyme-antigen conjugate and native antigen. Upon mixing biotinylated antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competitive reaction results between the native antigen and the enzyme-antigen conjugate for a limited number of antibody binding sites. The interaction is illustrated by the followed equation:

$$^{Enz}Ag + Ag + Ab_{Btm} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{Btn} + {}^{Enz}AgAb_{Btn}$$

Ab_{Btm}= Biotinylated Antibody (Constant Quantity)

Ag = Native Antigen (Variable Quantity)

^{Enz}Ag = Enzyme-Antigen Conjugate (Constant Quantity)

$AgAb_{Btn}$= Antigen Antibody Complex

$^{Enz}AgAb_{Btn}$= Enzyme-Antigen Conjugate-Antibody Complex

k_a= Rate Constant Association

k_{-a}= Rate Constant of Disassociation

$k = k_a/k_{-a}$ = Equilibrium Constant

A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurs. This affects the separation of the antibody bound fraction after decantation.

$AgAb_{Btn} + {}^{Enz}AgAb_{Btn} + streptavidin_{cw}$ = Immobilized Complex

$Streptavidin_{cw}$ = Streptavidin Immobilized on well

Immobilized complex = Sandwich complex bound to the solid surface.

The enzyme activity in the antibody bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

3.7.1.2 Procedure

Before proceeding with assay all reagents, serum and control were brought to room temperature. Serum and control (10 µl) each were pipetted into the assigned wells and 50 µl of working testosterone enzyme reagent was added to all wells. The microplate was swirled for 30 seconds to mix. Fifty (50) µl of testosterone biotin reagent was added to all wells. The microplate was swirled gently for 20-30 seconds to mix and then covered and incubated at room temperature for 60 seconds. The content was discarded by decantation. The plate was blot dried with absorbent. Wash buffer, 350 µl was added, decanted and blot dried. This was repeated for additional 2 times for a total of three washes.

Working substrate solution, 100µl was added to all wells and incubated at room temperature for 15 minutes. Stop solution, 50 µl was added to each well and gently mixed for 20 seconds.

The absorbance in each well was read at 450nm using a reference wavelength of 630nm to minimize well's imperfection in a microplate reader.

3.7.1.3 Calculation

A dose-response curve was used to determine concentration of testosterone in the unknown specimen.

3.7.2 Estimation of serum oestrogen

This was based on competitive enzyme immunoassay technique described by Abraham (1981).

3.7.2.1 Principle

Competitive enzyme immunoassay (type 9)

The essential reagents required for an enzyme immunoassay include antibody, enzyme-antigen conjugate and native antigen. Upon mixing biotinylated antibody with a serum containing the antigen, a reaction results between the antigen and the antibody. The interaction is illustrated by the followed equation:

$$Ag + Ab_{Btn} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{Btn}$$

Ab_{Btm}= Biotinylated Antibody (Constant Quantity)

Ag = Native Antigen (Variable Quantity)

$AgAb_{Btn}$= Immune Complex

After a short incubation, the enzyme conjugate was added (this delayed addition permits an increase in sensitivity for low concentration samples). Upon the addition of the enzyme conjugate, competition reaction results between enzyme analog and the antigen in the sample for a limited number of antibody binding sites (not consumed in the first incubation).

$$^{Enz}Ag + Ag + rAb_{Btm} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{Btn} + {}^{enz}AgAb_{Btn}$$

^{Enz}Ag = Enzyme-antigen conjugate (constant quantity)

$^{Enz}AgAb_{btn}$= Enzyme-Antigen Conjugate-Antibody Complex

rAb_{Btm} = Biotinylated antibody not reacted in the first incubation

k_a= Rate constant association

k_{-a}= Rate constant of disassociation

$k = k_a/k_{-a}$ = Equilibrium constant

A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurs. This affects the separation of the antibody bound fraction after decantation.

$AgAb_{Btn} + {}^{Enz}AgAb_{Btn} + Streptavidin_{cw}$= Immobilized Complex

$Streptavidin_{cw}$= Streptavidin Immobilized on well

Immobilized Complex = Sandwich complex bound to the solid surface.

The enzyme activity in the antibody bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

3.7.2.2 Procedure

Before proceeding with assay all reagents, serum and control were brought to room temperature (25^0C). Serum and control (25 µl) were pipetted into the assigned wells and 50 µl of oestradiol biotin reagent was added to all wells. The microplate was swirled for 30 seconds to mix. The microplate was covered and incubated at room temperature for 30 minutes. Fifty (50) µl of oestradiol enzyme reagent was added to all wells. The microplate was swirled gently for 30 seconds to mix and then covered and incubated at room temperature for 90 minutes. The content was discarded by decantation. The plate was blot dried with absorbent. Wash buffer, 350 µl was added and then decanted and blot dried. This was repeated for additional 2 times for a total of three washes.

Working substrate solution, 100µl was added to all wells and incubated at room temperature for 20 minutes. Stop solution, 50 µl was added to each well and gently mixed for 20 seconds.

The absorbance in each well was read at 450nm using a reference wavelength of 630nm to minimize well's imperfection in a microplate reader.

3.7.2.3 Calculation

A dose-response curve was used to determine the concentrations of testosterone in the unknown specimen.

3.7.3 Estimation of serum luteinizing hormone

This was based on competitive enzyme immunoassay technique described by Kosasa (1981).

3.7.3.1 Principle

Immunenzymometry assay (Type 3)

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibody (enzyme and immobilized) with different and distinct epitope recognition in excess and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal and anti-LH antibody.

Upon mixing monoclonal biotinylated antibody, the enzyme-labeled antibody and a serum containing the native antigen reaction results between the native antigen and the antibodies without competition or steric hindrance to form a soluble sandwich complex. The interaction is illustrated by the following equation:

$$^{Enz}Ab_{(p)} + Ag_{LH} + {}^{Btm}Ab_{(m)} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} {}^{Enz}Ab_{(p)}\text{-}Ag_{LH}\text{-}{}^{Btn}Ab_{(m)}$$

$^{Btm}Ab_{(m)}$= Biotinylated monoclonal antibody (excess quantity)

Ag_{LH} = Native antigen (variable quantity)

$^{Enz}Ab_{(p)}$ = Enzyme labeled antibody (excess quantity)

$^{Enz}Ab_{(p)}$-Ag_{LH}-$^{Btn}Ab_{(m)}$= Antigen-antibody sandwich complex

k_a= Rate Constant association

k_{-a}= Rate Constant of disassociation

$k = k_a/k_{-a}$ = Equilibrium constant

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. The interaction is illustrated below:

$^{Enz}Ab_{(p)}$-Ag_{LH}-$^{Btn}Ab_{(m)}$ + $streptavidin_{cw}$ = Immobilized complex

$Streptavidin_{c.w.}$= Streptavidin immobilized on well

Immobilized complex = Antibodies-antigens sandwich bound.

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose-response curve can be generated from which the antigen concentration of an unknown can be determined.

3.7.3.2 Procedure

Before proceeding with assay all reagents, serum and control were brought to room temperature. Serum and control (50 µl) each were pipetted into the assigned wells and 100 µl of LH-Enzyme reagent was added to all wells. The microplate was swirled for 30 seconds to mix and covered to incubate for 60 minutes at room temperature. The content was discarded by decantation. The plate was blot dried with absorbent. Wash buffer, 350 µl was added and then decanted and blot dried. This was repeated for additional 2 times for a total of three washes.

Working substrate solution, 100µl was added to all wells and incubated at room temperature for 15 minutes. Stop solution, 50 µl was added to each well and gently mixed for 20 seconds.

The absorbance in each well was read at 450nm using a reference wavelength of 630nm to minimize well's imperfection in a microplate reader.

3.7.3.3 Calculation

A dose-response curve was used to determine the concentrations of luteinizing hormone (LH) in the unknown specimen.

3.7.4 Estimation of serum follicle stimulating hormone

This was based on competitive enzyme immunoassay technique described by Wennink *et al*., (1990).

3.7.4.1 Principle

Immunoenzymometry (type3)

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibody (enzyme and immobilized) with different and distinct epitope recognition in excess and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-FSH antibody.

Upon mixing monoclonal biotinylated antibody the enzyme-labeled antibody and a serum containing the native antigen, reaction results between the native antigen and the antibodies without competition or steric hindrance to form a soluble sandwich complex. The interaction is illustrated by the following equation:

$$^{Enz}Ab_{(p)} + Ag_{FSH} + {}^{Btm}Ab_{(m)} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} {}^{Enz}Ab_{(p)}\text{-}Ag_{FSH}\text{-}{}^{Btn}Ab_{(m)}$$

$^{Btm}Ab_{(m)}$ = Biotinylated monoclonal antibody (excess quantity)

Ag_{FSH} = Native antigen (variable quantity)

$^{Enz}Ab_{(p)}$ = Enzyme labeled antibody (excess quantity)

$^{Enz}Ab_{(p)}$-Ag_{LH}-$^{Btn}Ab_{(m)}$ = Antigen-antibody sandwich complex

k_a= Rate Constant association

k_{-a}= Rate constant of disassociation

$k = k_a/k_{-a}$ = Equilibrium constant

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. The interaction is illustrated below:

$^{Enz}Ab_{(p)}$-Ag_{FSH}-$^{Btn}Ab_{(m)}$ + $streptavidin_{cw}$ = Immobilized complex

$Streptavidin_{c.w.}$= Streptavidin immobilized on well

Immobilized complex = Sandwich complex bound to the solid surface.

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose-response curve can be generated from which the antigen concentration of an unknown can be determined.

3.7.4.2 Procedure

Before proceeding with assay all reagents, serum and control were brought to room temperature. Serum and control (50) µleach were pipetted into the assigned wells and 100 µl of FSH-Enzyme reagent was added to all wells. The microplate was swirled for 30 seconds to mix and covered to incubate for 60 minutes at room temperature. The content was discarded by decantation. The plate was blot dried with absorbent. Wash buffer, 350 µl was added and then decanted and blot dried. This was repeated for additional 2 times for a total of three washes. Working substrate solution, 100µl was added to all wells and incubated at room temperature for 15 minutes. Stop solution, 50 µl was added to each well and gently mixed for 20 seconds. The absorbance in each well was read at 450nm using a reference wavelength of 630nm to minimize well's imperfection in a microplate reader.

3.7.4.3 Calculation

A dose-response curve was used to determine the concentrations of FSH in the unknown specimen.

3.7.5 $CD4^+$ count using cyflow counter

The Cyflow counter (Partec Munster, Germany) is highly precise portable/mobile flow cytometry system (FCM) which allows highly precise and affordable routine $CD4^+$ count enumeration (Cassens *et al*., 2004).

3.7.5.1 Principle

For flow cytometric analysis cells are separated in an aqueous suspension and stained with fluorescent dyes. While passing through a flow cuvette, the cells are individually illuminated by an excitation light source of the laser (488nm). Due to excitation, the dye molecules emit fluorescence of characteristic colour or emission wavelength spectrum which is scattered by the cells. The fluorescent signals are then displayed and analysed in histograms.

3.7.5.2 Procedure

To a Partec test-tube, 1ml of well-mixed EDTA anti-coagulated blood was added. Twenty microliters (20 µl) of $CD4^+$ Ab PE was added into the tube. The content was mixed gently and incubated for 15 minutes at room temperature, protected from light (in the dark). Eight hundred microliters (800 µl) of no-lyse buffer was then added and shacked gently. Following incubation, the contents of the sample tube was mixed gently for 5 seconds to re-suspend cells immediately before counting. The sample tube was then connected on a Partec cyflow counter for analysis.

3.8 Statistical Analysis

The data generated were analyzed using Statistical Package for Social Sciences (SPSS) version 22.0.The results of serum sex hormones were analyzed and expressed as mean ±

SEM. The results obtained were compared between different groups using one-way analysis of variance (ANOVA). A p-value of $p < 0.05$ was considered significant.

3.9 Quality Control

Assay kits (commercially prepared) have standard reagents which were used as a guide in the analysis. The quality control measures that were taken to ensure the reliability of the results include the following:

i. The serum was separated and kept at -20^0C immediately and the reagents used were kept at appropriate temperature (5^0C) until the time of analysis.

ii. Correct sample collection and processing was carried out to ensure its suitability for the intended use.

iii. During the analysis, the sample and reagents used were thawed at room temperature before the analysis and multiple thawing was avoided.

iv. During the analysis correct incubation time and wavelength were ensured for accurate results.

v. The machine used was calibrated before use and also controls were ran along with the test to ensure reliability of the results.

CHAPTER FOUR

4.0 RESULTS

The demographic characteristics of the study population was shown in Table 4.1. Majority of the HIV infected patients are married (66.7%) while the unmarried (single) (25.2%).

Table 4.2 shows age and gender distribution of HIV-positive patients on HAART, HAART-naive HIV patients and HIV-negative controls.

Table 4.3 and 4.4, show the results of serum sex hormones (testosterone, oestrogen, LH, FSH) and $CD4^+$ count for HIV positive on HAART, HAART naive and controls both men and women respectively. The results indicated significant decrease in the level of serum testosterone and $CD4^+$ count among HIV positive on HAART and HAART-naive when compared with controls. The results also show significant increase in the levels of FSH among HIV positive on HAART when compared with controls. The results were summarized in Fig. 4.1, 4.2, 4.3 and 4.4.

The $CD4^+$ count of HIV positive on HAART, HAART-naive and control were correlated with the serum sex hormones and the findings are presented in Table 4.5 and 4.6.

Table 4.1: Demographic characteristics of the study population

Characteristics	N	Percentage (%)
Marital status		
Married	90	66.7
Single	34	25.2
Widowed	9	6.6
Divorce	2	1.5
Total	**135**	**100**
Ethnicity		
Hausa	99	73.3
Fulani	5	3.7
Yoruba	11	8.1
Igbo	14	10.5
Others	6	4.4
Total	**135**	**100**

Table 4.2: Age (years) and gender distribution of HIV-positive on HAART, HIV-positive HAART-naive and HIV-negative controls

	Group I		Group II		Group III		Total
Age group (years)	Male	Female	Male	Female	Male	Female	Total
15-24	12	9	5	6	2	5	**39**
25-34	5	10	4	6	7	9	**41**
35-44	5	3	5	5	8	6	**32**
45-54	1	0	6	4	5	1	**17**
55-64	0	0	3	1	1	1	**6**
Total	**23**	**22**	**23**	**22**	**23**	**22**	**135**

Group I= HIV on HAART, GROUP II= HIV-positive HAART-naive, Group III= controls, HAART= Highly active antiretroviral therapy, HIV= Human immune virus

Table 4.3: **Comparison of serum sex hormones and $CD4^+$ count among male subjects**

Parameters	Group I n=23	Group II n=23	Group III n=23	P-value Group I vs II	P -value Group II vs III
Testosterone (ng/ml)	3.40 ± 0.70*	3.43 ± 0.59*	10.34 ± 0.56*	0.001	0.001
Oestrogen (pg/ml)	83.84 ± 7.82	104.15 ± 10.32	102.32 ± 6.25	0.356	0.632
LH (MIU/ml)	10.79 ± 2.93*	7.03 ± 0.88*	3.94 ± 0.54	0.032	0.015
FSH (MIU/ml)	12.05 ± 2.86*	5.51 ± 1.00	4.05 ± 0.78	0.036	0.391
$CD4^+(cell/mm^3)$	280.63 ± 42.41*	245.79 ± 45.48*	790.32 ± 36.50*	0.001	0.001

Values are mean ± SEM, n= number of subjects, *= statistically significant at $p < 0.05$, using ANOVA. Group I= HIV-positive on HAART, Group II = HIV-positive HAART-naïve and Group III = HIV-negative control

Table 4.4: Comparison of serum sex hormones and $CD4^+$ count among female subjects

Parameters	Group I n=22	Group II n=22	Group III n=22	P -value Group I vs II
Testosterone (ng/ml)	0.43 ± 0.18	0.38 ± 0.21	0.93 ± 0.10	0.059
Oestrogen (pg/ml)	86.88 ± 4.79*	116.66 ± 18.69	148.18 ± 17.00	0.012
LH (MIU/ml)	10.43 ± 2.26	5.26 ± 1.39	10.34 ± 3.81	0.998
FSH (MIU/ml)	10.01 ± 1.54*	5.05 ± 0.70*	3.68 ± 0.63	0.036
$CD4^+$ (cell/mm^3)	294.95 ± 40.38*	268.10 ± 45.34*	867.15 ± 45.09*	0.001

Values are mean ± SEM, n= number of subjects, *= statistically significant at $p < 0.05$ using ANOVA, Group I= HIV-positive on HAART, Group II = HIV-positive HAART-naive and Group III = HIV-negative control

Table 4.5: Correlation of $CD4^+$ count with sex hormones among HIV positive men on HAART, HAART-naïve and HIV negative controls

Parameters	r-value	p-value
Testosterone (ng/ml)	0.682**	<0.001
Oestrogen (pg/ml)	0.025	0.140
LH (MIU/ml)	-0.181	0.025
FSH (MIU/ml)	-0.271*	0.027

**=correlation is significant at 0.01 level (2-tailed), LH= Leutinizing hormone, FSH= Follicular stimulating hormone.

Table 4.6: Correlation of $CD4^+$ count with sex hormones among HIV positive women on HAART, HAART-naïve and HIV negative (controls)

Parameters	r-value	p-value
Testosterone (ng/ml)	0.230	0.061
Oestrogen (pg/ml)	0.222	0.071
LH (MIU/ml)	0.027	0.829
FSH (MIU/ml)	-0.138	0.264

LH= Leutinizing hormone, FSH= Follicular stimulating hormone

CHAPTER FIVE

5.0 DISCUSSION

The introduction of highly active antiretroviral therapy (HAART) in the mid-1990s resulted in a dramatic benefit in the management of HIV infection, but this therapy was reported to be associated with some adverse events (Martinez *et al*., 2014). Shortly after the generalization of HAART as a standard of care, a report suggested a possible association between protease inhibitors and HIV in the development of sexual dysfunction (Martinez *et al*., 2014).

The finding of significantly decreased ($p<0.05$) serum levels of testosterone in HIV on HAART and HAART-naïve among male subjects when compared with controls was in agreement with previous study of Lamba *et al*. (2004). This may be due to the fact that hypogonadism is common in HIV infected men with incidence of between 29 and 50% before HAART and between 20 and 30% after HAART as reported by Crum *et al*., 2005. There were several debates on the role of HAART in the derangement of sex hormones. However, the possible role remains controversial. Whereas, some studies supported the role of HAART (Colson *et al*., 2002; Collazos *et al*., 2002; Lamba *et al*., 2004; Schrooten *et al*., 2011), others did not find any association (Lallemand *et al*., 2002; Florence *et al*., 2004; Crum *et al*., 2005; Guaraldi *et al*., 2007). Colson *et al*. (2002) reported that HIV infected individuals were about 3.4 times more likely to complain about sexual dysfunction due to sex hormone changes after HAART administration, while Colebunders *et al*. (2001) observed that the sexual dysfunction regressed or disappeared completely after HAART regimen. It has been shown that decrease levels of testosterone and elevated oestradiol were observed among HIV infected individuals on HAART (Lamba *et al*., 2004). Though, other

studies by Moreno-Perez *et al*. (2010) showed no relationship between testosterone levels and the degree of sexual dysfunction among HIV infected individuals on HAART.

There was significant increase in the levels of FSH among HIV positive men and women on HAART and HIV positive HAART-naïve when compared with controls. LH also showed significant increase among HIV positive men on HAART and HAART-naïve when compared with controls. The significant differences might be due to the relationship between LH, FSH and testosterone whereby LH and FSH regulate the secretion and release of testosterone. Leydig cells produce testosterone under the control of LH which regulates the expression of the enzyme 17β-hydroxysteroid dehydrogenase that is used to convert androstenedione, the hormone produced by the testis to testosterone, an androgen that exerts both endocrine and intratesticular activities on spermatogenesis (Bowen, 2004).
When testosterone levels are low, GnRH is released by the hypothalamus, stimulating the pituitary gland to release LH. As the levels of testosterone increase, it acts on the hypothalamus and pituitary through a negative feedback loop and inhibit the release of GnRH and LH consequently (Mahesh, 2011). Changes in LH and testosterone blood levels and pulse secretions are induced by changes in sexual arousal in human males (Storelu *et al*., 1993).

The findings of the present study also demonstrated significant decrease in the levels of oestrogen in HIV positive women on HAART. This was in agreement with the previous report of Hayes *et al*. (2008) and this could be due to the fact that the effect of this hormone with respect to sexual dysfunction was observed more in women than men. While oestrogens are present in both men and women, they are usually present at significantly

higher levels in women of reproductive age. In males, oestrogen regulates certain functions of the reproductive system important to the maturation of sperm and may be necessary for a healthy libido (Soltysik and Czekaj 2013).

The finding of the present study demonstrated significant decreased ($p<0.05$) $CD4^+$ count among HIV on HAART and HAART-naïve male and female subjects and this is in agreement with previous reports of Tindall *et al*. (2004). This may be due to the fact that HIV infection itself and its stages of progression with decreased $CD4^+$ count have been shown to be associated with changes in serum sex hormones levels (Dobs *et al*., 2008).

The finding of significant positive correlation between $CD4^+$ count and serum levels of testosterone in men suggests that a decrease in $CD4^+$ count in HIV positive on HAART and HAART-naïve is accompanied with a decrease in the serum testosterone in men. The finding also demonstrated significant negative correlation between $CD4^+$ and serum levels of FSH but did not show significant correlation between $CD4^+$ count and serum levels of oestrogen and LH. There was no significant correlation between $CD4^+$ count and sex hormones levels in women. These findings were consistent with the previous reports of Tindall *et al*. (2004).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

From this study we can conclude that**:**

1. HIV infection is responsible for the decrease in the serum levels of Testosterone and Oestrogen in men and women, respectively.

2. Treatment with HAART has no adverse effect on serum levels of Testosterone and Oestrogen. Hence, it is not likely to cause sexual dysfunction.

6.2 Recommendations

Considering the findings of the present study, it is recommended that:

1. Evaluation of serum sex hormones be included in the Management of HIV patients
2. Periodic assessment of $CD4^+$ count and sex hormones should be done to enhance effective professional counseling of HIV patients at the risk of sexual dysfunction.
3. Other hormones such as progesterone and prolactin should be evaluated as additional parameters for the assessment of gonadal functions in both HIV patients on HAART and HIV positive HAART-naive.

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APPENDIX I

CONSENT FORMTO PARTICIPATE IN A STUDY

Serial No …….

Hospital No …….

Age ……

1. This study is about Evaluation of serum sex hormones level among HIV positive on HAART, HAART naïve and apparently healthy individuals in sokoto metropolis. The procedure involves collection of blood specimen.

2. The result from the study will be treated with strict confidentiality.

3. You are free to participate or decline in this study without any consequence

4. The study will hopefully improve your present health status and that of others in the future.

If you wish to participate in this study kindly sign the attached form.

Thank you.

APPENDIX II

INFORMED WRITTEN CONSENT

I……from

……. (Address)agree to participate in the study of Evaluation of serum sex hormones level among HIV positive on HAART, HAART naïve and apparently healthy individuals in sokoto metropolis. The procedure involves collection of blood specimen. The full procedure and probable benefits were explained to me. I understand that a sample of my blood will be taken for tests and that if I so wish the results will be communicated to me in confidence.

I made this consent willingly without being subjected to any pressure.

Participants Name**.....**……. Signature……

Witness Name……Signature……

Researchers Name……. Signature……

APPENDIX III

QUETIONARE FOR EVALUATION OF…….

SECTION A: RESPONDENT'S DEMOGRAPHIC AND PERSONAL DATA

1. Age (years).....

2. Sex: Male [] Female []

3. Weight (kg).....

4 Height (m).....

5. Body Mass Index.....

5. Marital Status:

Married [] Single [] Widowed []

Divorced [] Separated []

6. Residential Address: .....

…….....

7. Local Government Area: .....

8. State: .....

9. Nationality: .....

10. Tribe:

Hausa [] Yoruba [] Fulani []

Igbo [] others [] (specify)

SECTION B: RESPONDENT'S SOCIO-ECONOMIC DATA

1. Occupation……

2. Educational level attained…….

SECTION C: RESPONDENT'S HIV STATUS

1. Initial HIV Screening Result (Determine HIV 1 & 2 Kit)

a) Negative [] (control group)

b) Positive []

2. Confirmatory HIV screening result

a) Negative []

b) Positive []

3. Duration of HIV infection…….

4. Duration of HAART administration…….

SECTION D: RESPONDENT'S CDC CLASSIFICATION OF HIV INFECTION

1. Stage I []

2. Stage II []

3. Stage III []

SECTION E: RESPONDENT'S BIOCHEMICAL DATA

a) Serum testosterone - - - - - - -

b) Serum oestrogen - - - - - - -

c) Serum follicle stimulating hormone- - - - - - - -

d) Serum luteinizing hormone ----------

d) $CD4^+$ count of control - - - - - - - - - - - - - -

d) $CD4^+$ count before HAART administration- - - - - - - - -

e) $CD4^+$ count after HAART administration- - - - - - - - - - -

APPENDIX IV

Brief Sexual Symptom Checklist: Men's Version

Please answer the following questions about your overall sexual function in the past **3 months** or more.

1. Are you satisfied with your sexual function?

Yes…… No……

If No, please continue.

2. How long have you been dissatisfied with your sexual function?

_____

3a. The problem(s) with your sexual function is: (mark one or more)

1 Problems with little or no interest in sex

2 Problems with erection

3 Problems ejaculating too early during sexual activity

4 Problems taking too long, or not being able to ejaculate or have orgasm

5 Problems with pain during sex

6 Problems with penile curvature during erection

7 Others:

3b Which problem is most bothersome (circle) 1 2 3 4 5 6 7

Brief Sexual Symptom Checklist: Women's Version

Please answer the following questions about your overall sexual function in the past **3 months** or more.

1. Are you satisfied with your sexual function?

Yes _ No_

If No, please continue.

2. How long have you been dissatisfied with your sexual function?

_____

3a. The problem(s) with your sexual function is: (mark one or more)

1 Problems with little or no interest in sex

2 Problems with decreased genital sensation (feeling)

3 Problems with decreased vaginal lubrication (dryness)

4 Problems reaching orgasm

5 Problems with pain during sex

7 Others:

3b. Which problem is most bothersome (circle) 1 2 3 4 5 6 7