

**PLASMA KISSPEPTIN LEVELS IN TYPE 2 DIABETIC MALES AT THE KORLE-BU
TEACHING HOSPITAL**



**THESIS WORK SUBMITTED TO THE DEPARTMENT OF CHEMICAL PATHOLOGY,
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DECLARATION

This thesis work is presented to University of Ghana, School of Graduate Studies through the College of Health Science, School of Basic and Allied Health Sciences, Department of Chemical Pathology. I hereby affirm that not including references to former works by people whom I have properly recognised; this thesis is the work of my own research which took place at the National Diabetes Management and Research Centre (NDMRC) and the Central Laboratory Unit all at Korle-Bu Teaching Hospital under the strict supervision of Professor Henry Asare-Anane and Dr Ben D.R.T. Annan. This thesis work shows evidence of original research undertaken by me and neither all nor parts of this thesis have been presented for another degree.

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DEDICATION

I dedicate this work to my husband David Azuma Agbey.



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I give God all the glory for His faithfulness. I say God is indeed faithful for helping me mightily in this course and bringing helpful people my way.

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ABSTRACT

People living with type 2 diabetes mellitus is on the increase and research has established that there is decreased secretion of testosterone in Type 2 Diabetic Mellitus males which may be linked to decreased levels of kisspeptin secretion that can cause the development of diabetic complications such as erectile dysfunction. Decreased kisspeptin levels have strongly been linked to decreased secretion of gonadotropins from the pituitary gland and this causes type 2 diabetic males to have decreased levels of testosterone resulting in secondary hypogonadism. Kisspeptins, therefore exerts a major influence in the downstream regulations of gonadotropins and testosterone production in men with type 2 diabetes mellitus.

The work aimed to determine the concentrations of plasma kisspeptin levels in type 2 diabetic males in Ghana, to establish a relationship between low kisspeptin and testosterone concentrations in Ghanaian males above age of 30, and also to identify factor(s) that may be linked to low kisspeptin in these men.

One hundred and twenty male subjects above the age of 30 (60 type 2 diabetics and 60 non-diabetic) were selected for this investigation. Diabetic subjects were contacted during outpatient clinic appointments at the National Diabetes Management and Research Centre (NDMRC), Korle-Bu Teaching Hospital. Non-diabetic matched control subjects were recruited by convenience sampling at the same catchment area. Kisspeptin, luteinizing hormone (LH), follicle stimulating hormone (FSH) and testosterone (TT) concentrations in subject's sera were assessed using ELISA assays performed according to manufacturer's instructions. Age, blood pressure, lipid profile, body mass index (BMI), and waist circumference of subjects were assessed. Significant lower levels of kisspeptin (ng/ml) was seen in diabetic males compared to the non-diabetic males (8.34 ± 7.6 vs 16.26 ± 12.72 , $p=0.001$). Serum TT (ng/ml) was similarly significantly lower in the diabetic males compared with non-diabetic group (6.49 ± 9.62 vs 11.38 ± 2.56 , $p=0.001$). Serum LH (mIU/ml) in

subjects (14.67 ± 9.62 vs 10.82 ± 2.37 , $p=0.003$) and FSH (mIU/ml) in subjects (5.83 ± 17.29 vs 10.39 ± 2.65 , $p=0.017$) were both significantly elevated in type 2 diabetic males than in the control group. The results revealed a significant difference in BMI and blood pressure of diabetic group compared to the non-diabetic controls ($p < 0.005$ in both cases). Age was slightly higher in diabetic subjects than non-diabetic group but was not significant ($p > 0.005$). Both FBG and HbA1c were raised in diabetic subjects compared to non-diabetic and the difference was significant ($p = 0.001$ in both cases). Plasma kisspeptin levels in type 2 diabetic males showed significant inversely association with age ($r = -0.590$, $p = 0.0001$), BMI ($r = -0.389$, $p = 0.002$) and T. Chol/HDL ($r = -0.321$, $p = 0.012$) and a significant association with TT ($r = 0.531$, $p = 0.0001$), LH ($r = 0.432$, $p = 0.001$), FSH ($r = 0.366$, $p = 0.004$) and HDL ($r = 0.362$, $p = 0.005$). Blood pressure, waist circumference, FBG, HbA1c, T. Chol, TG, LDL, and VLDL did not show statistical significance ($p > 0.005$) with plasma kisspeptin levels in males having type 2 diabetes mellitus. This investigation showed that low testosterone (OR= 1.473, $p = 0.003$) and increase in age (OR= 0.890, $p = 0.004$) are independent risk factors for low kisspeptin levels in males with type 2 diabetes mellitus.

It can be concluded that low testosterone is a major risk factor for the development of low kisspeptin in Ghanaian adult males living with type 2 diabetes mellitus.

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LIST OF ABBREVIATION

ADA	American Diabetes Association
ADP	Adenosine Diphosphate
ARC	Arcuate Nucleus
ATP	Adenosine Triphosphate
AVPV	Anteroventral Periventricular
BMI	Body Mass Index
CHH	Congenital Hypogonadotropic Hypogonadism
CI	Confidence Interval
CNS	Central Nervous System
CPP	Congenital Precocious Puberty
DBP	Diastolic Blood Pressure
DKA	Diabetes Ketoacidosis
DYN	Dynorphin
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER α	Estrogen Receptor Alpha

FBG	Fasting Blood Glucose
FSH	Follicle Stimulating Hormone
GAD	Glutamic Acid Decarboxylase
GDM	Gestational Diabetes Mellitus
GnRH	Gonadotrophin-Releasing Hormone
GPR54	G-Protein-Coupled Receptor 54
GSIS	Glucose-Stimulated Insulin Secretion
H ₂ O ₂	Hydrogen Peroxide
HbA1c	Glycated Hemoglobin
hCG	Human Chorion Gonadotrophin
HDL	High Density Lipoprotein
HH	Hypogonadotrophic Hypogonadism
HHS	Hyperglycaemic Hyperosmolar State
HLA	Human Leukocyte Antigen
HPG	Hypothalamic- Pituitary Gonadal
HRP	Horseradish Peroxidase
IDDM	Insulin-Dependent Diabetes Mellitus

IDF	International Diabetes Federation
IGT	Impaired Glucose Tolerance
KDN _y	Kisspeptin/Neurokinin B/Dynorphin
KISS1R	Kiss 1 Receptor
LADA	Latent Autoimmune Diabetes of Adult
LDL	Low Density Lipoprotein
LH	Luteinizing Hormone
LHRH	Luteinizing Hormone-Releasing Hormone
Md	Mean Difference
MgCl ₂	Magnesium Chloride
MHC	Major Histocompatibility Complex
ml	Milliliters
Mmol/L	millimole per litre
MODY	Maturity-Onset Diabetes of the Young
MRDM	Malnutrition-Related Diabetes Mellitus
mRNA	Messenger Ribonucleic Acid
NDDG	National Diabetes Data Group

NDMRC	National Diabetes Management and Research Centre
NIDM	Non-Insulin Dependent Diabetes Mellitus
NIRKO	Neuron-Specific deletion of Insulin receptor Knock-o
NKB	Neurokinin B
OR	Odd Ratio
PLC	Phospholipase C
POMC	Pro-Opiomelanocortin
PSA	Prostate-Specific Antigen
PTA	Phosphotungstic Acid
RF	Releasing Family
SBAHS	School of Basic and Allied Health Sciences
SBP	Systolic Blood Pressure
SD	Standard Deviation
SPSS	Statistical Package for the Social Sciences
T. Chol	Total Cholesterol
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus

TAC3	Tachykinin 3
TMB	Tetramethylbenzidine
TRIG	Triglycerides
TT	Total Testosterone
VITROS CHOL	Vitros Cholesterol
VLDL	Very Low Density Lipoprotein
WC	Waist Circumference
WHO	World Health Organization



CHAPTER ONE

INTRODUCTION

1.1 Background

Kisspeptin are hypothalamic neuropeptide hormones, which currently have been proven to influence fertility and reproduction in humans (Clarke *et al.*, 2015). Kisspeptin-54 is located in the hypothalamus and is formed through the differential cleavage of 145-amino acid precursor of the KiSS 1 gene (Lee *et al.*, 1996). Biologically, active Kisspeptin-54 excites the neurons associated in gonadotropin-releasing hormone (GnRH) production and thus regulate pituitary-gonadal-axis (Novaira *et al.*, 2009). The Gonadotrophin-releasing hormone directly acts on the anterior pituitary gland, to stimulate them to produce luteinizing hormone (LH) and follicle-stimulating hormone (FSH) after which stimulates sex organs in humans (Dhillon *et al.*, 2005). Luteinizing hormone (LH) excites Leydig cells located in the male sex organ to produce testosterone whilst the sertoli cells are stimulated by FSH to produce sperms. Male gonadal steroids such as testosterone, estradiol, and dihydrotestosterone are essential in reproductive organs and secondary characteristics developments in males (Anawalt & Grant, 2003).

Kisspeptin hormone is detected in peripheral blood and its receptor has been found in many organs in the body such as testes, ovary, liver, placenta and the pancreas (Katagiri *et al.*, 2007). Adequate levels of circulating kisspeptin are needed to regulate the gonadal-pituitary axis. Ramachandran *et al.*, (2008) recommends plasma kisspeptin concentrations of <0.890ng/ml in healthy males. Loss-of-function or genetic changes in the pathway of kisspeptin signalling have resulted in congenital hypogonadotropic hypogonadism [CHH] and impaired sexual development (Topaloglu *et al.*, 2012). Kisspeptin receptor knockout mice showed hypogonadotropic hypogonadism (HH),

however, administration of endogenous GnRH corrected the levels of GnRH suggesting the role kisspeptin plays in stimulating endogenous GnRH (Seminara *et al.*, 2003).

Diabetes Mellitus is among the metabolic malfunction disease with global prevalence of 8.5% among adults above 18 years in 2014 (WHO, 2016). Diabetes Mellitus belongs to a group of metabolic disorder accompanied with hyperglycemia as a result of defects in beta-cells secretion of insulin (Celik & Zimmet, 2001). Diabetes mellitus affects about 415 million adults worldwide and a projected rise to 645 million is expected in 2040 (IDF, 2016). Diabetes mellitus is categorised into two core class; Type 1 and Type 2 diabetes. In type 1 diabetes, the pancreatic beta cells are completely destroyed and this results in complete loss of insulin secretion (American Diabetes Association, 2016). Type 2 diabetes which prevails more is observed when there is ineffective use or action of insulin by the body.

A key character of type 2 diabetes is insulin resistance. Ninety percent of all diabetes mellitus cases in the world fall under type 2 diabetes mellitus (Mendis *et al.*, 2015). Obesity, commonly caused by lack of leptin receptor, is among the contributing factors that progresses insulin resistance and other heart diseases. Lack of leptin receptors in humans does not only lead to obesity but also causes hypogonadism (Farooqi & O'Rahilly, 2009). Administration of exogenous leptin increases kisspeptin expression indicating the intermediary role kisspeptin plays between metabolic signals of leptin and GnRH function (Smith *et al.*, 2006).

Most studies done in adult males with type 2 diabetes mellitus used testosterone concentrations as the basis for determining hypogonadism (Dhindsa *et al.*, 2004). Al Hayek *et al.*, (2013) showed that about 25% of type 2 diabetic men have reduced testosterone concentration, resulting in diabetes-related dysfunction. Report by Maneesh *et al.*, (2006) indicated considerable low levels of serum testosterone in diabetic men suggesting an abnormal functioning of the cells in the hypothalamus that secretes luteinizing hormone-releasing hormone (LHRH). Low LHRH levels exert its functions in bringing about insulin resistance and consequently type 2 diabetes. A study

by Asare-Anane *et al.*, (2013) on Ghanaian men with T2DM reported a slightly raised luteinizing hormone (LH) and follicle-stimulating hormone (FSH) but decreased testosterone levels, a case of primary hypogonadism. Kisspeptin has been shown to have the ability to increase LH pulsatility and testosterone levels in T2DM males with reproductive disorders such as hypogonadism (George *et al.*, 2013). Also, a novel treatment of kisspeptin administration has been known to treat infertility in these males with T2DM (Hameed *et al.*, 2011).

1.2 Problem Statement

Increasing changes in human lifestyles over the past years have caused tremendous rise in the number of individuals with type 2 diabetes mellitus in Ghana (Amoah *et al.*, 2002). In 2015, Ghana recorded 266,200 cases of diabetes with 1.9% prevalence (International Diabetes Federation [IDF], 2016). The massive rise of people diagnosed yearly of having diabetes majority of which are T2DM makes the disease a health threat in the 21st century. Uncontrolled T2DM in males are likely to cause serious health complications including loss of libido, erectile dysfunction, impaired sperm production, depressive symptoms, loss of energy, irritability and decreased in cognitive abilities, heart diseases, stroke and chronic inflammation than non-diabetic males (Brophy *et al.*, 2007). These complications result in an upsurge in infertility, illness and death rates (Dhindsa *et al.*, 2004).

It has been reported that low kisspeptin and testosterone concentrations increase the susceptibility of people acquiring insulin resistance and type 2 diabetes mellitus (Oh *et al.*, 2002) also may predict acute myocardial fracture in men with T2DM (Daka *et al.*, 2015). Low levels of kisspeptin have been linked to decreased release of gonadotropins in males with T2DM (True *et al.*, 2011).

Managing the health of these individuals with type 2 diabetes mellitus imposes huge and social problem in the area of cost on the patient and also the health care system.

1.3 Justification

Diabetes has been implicated as the cause of death worldwide and this prevalence is on the rise. A person dies from diabetes mellitus every six seconds accounting to five million deaths worldwide (IDF, 2016). Variations in Kisspeptin levels may be implicated in the development of type 2 diabetes and its associated diseases such as erectile dysfunction. A report by Oh *et al.*, (2002) indicated low endogenous kisspeptin secretion as one of the common metabolic and endocrine pathways in the advancement of testosterone deficiency in type 2 diabetic men. However, due to genetic and environmental factors, these results cannot be extrapolated in type 2 diabetic men living in Ghana. A case in point, black African men have higher testosterone than their Caucasian counterpart (Tsai *et al.*, 2006). Treatment of low testosterone with testosterone replacement drugs increases levels of serum Prostate-Specific antigen (PSA), Thus increasing the danger of developing prostate cancer (Bassil *et al.*, 2009). Clinical trials using kisspeptin to treat low testosterone has shown no such effect on PSA secretion (University of Edinburg, 2013).

Low levels of plasma Kisspeptin may serve as a warning sign to the development of low testosterone concentrations in males with type 2 diabetes as these levels have not been established in Ghana. Considering the key role kisspeptin plays as a regulator of gonadotrophin releasing hormone (GnRH) secretion (Jyothis & Seminara, 2012), there is a need to elucidate the plasma kisspeptin levels in type 2 diabetics and their correlation to testosterone level and diabetic complications. Data from this work could be useful evidence for the management and care of men with T2DM.

1.4 Hypotheses

The levels of Kisspeptin will not be low in type 2 diabetic adult males in Ghana.

1.5 Aims

The study aimed to measure plasma Kisspeptin levels in type 2 diabetic adult males in Ghana.

1.6 Specific Objective

- To determine plasma kisspeptin, luteinizing hormone, follicle-stimulating hormone and total testosterone using ELISA kit in study subjects.
- To determine fasting plasma glucose and percentage concentration of glycated hemoglobin among study subjects.
- To establish a relationship between kisspeptin, insulin resistance, and obesity.
- To assess clinical and biochemical risk factors to acquiring low kisspeptin levels in type 2 diabetic adult males in Ghana.



CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of Diabetes Mellitus

Diabetes is a systemic metabolic ailment marked by hyperglycemia which is often accompanied by excessive thirst, polyuria, and polyphagia results from a pancreatic defect of insulin production, its function or both (America Diabetes Association, 2016). The beta cells of the pancreas produces a hormone called insulin which utilizes glucose from food digested to produce energy for the body. A defect in the action of insulin results from insufficient secretion of insulin and/or loss of sensitivity of tissue insulin at different points in insulin's action pathway (America Diabetes Association, 2016). The defect in insulin action or both causes disorders in carbohydrates, lipid and protein metabolism. These activities result in high level of plasma glucose (hyperglycaemia) with the cells deficient in intracellular glucose (Rizza, 2010). The body cells in diabetics have to depend on fats and proteins reserves for energy (Rizza, 2010). In the insulin-deficient state in diabetics, glucagon increases the catabolism of amino acid by the liver and accelerates lipid metabolism of fatty acids and triglycerides in adipose tissues resulting in the increased formation of ketones (Mazzone *et al.*, 2008). These ketones cannot be utilized by the brain and thus results in hypoglycemia and ketoacidosis in diabetics.

The incidence of diabetes is increasing rapidly worldwide with about 450 million people living with diabetes (IDF, 2013). The incidence of diabetes worldwide is 8.3% and this figure is expected to rise to 17.6% by 2030 in the absence of better control or cure (Guariguata *et al.*, 2014). In Ghana, approximately 6% of the populace (mostly in the urban areas) are living with diabetes and this figure is likely to double by the year 2035 (Danquah *et al.*, 2012; IDF, 2013)

2.2 Diagnostic criteria and Classification of types of Diabetes

2.2.1 Diagnostic Criteria

An individual can be classified into a type of diabetes based on current prevailing situations during period of diagnosis. Before the late 1970's, no meaningful classification and prevalence of diabetes mellitus within populations could be made as there were no agreed criteria for the identification of the disorder. The World Health Organisation (WHO) in 1965 presented its initial report on the diagnostic criteria for diabetes mellitus which was later amended by the National Diabetes Data Group (NDDG) of the United States of America also the second World Health Organization Expert Committee on Diabetes Mellitus in 1979, 1980 also 1985 (World Health Organisation, 1980, 1985). The 1999 WHO criteria presented a new diagnostic criterion making major changes to the 1985 recommendations. An individual can be said to have diabetes mellitus when glucose concentration of the fasted plasma is 7.0mmol/l (126mg/dl) or higher than and 6.1mmol/l (110mg/dl) or more for whole blood (WHO, 1999). World Health Organisation recommends 75g oral glucose tolerance test (OGTT) to be performed when casual blood glucose values fall below or above normal values (Alberti & Zimmert, 1998).

2.2.2 Classification

The 1980 NDDG recommendations endorsed by WHO, were based on numerous etiologically and clinically distinct disorders sharing hyperglycemia in common. This resulted in classifying diabetes into; Insulin-dependent diabetes mellitus (IDDM), Non-insulin dependent diabetes mellitus (NIDM), Impaired Glucose tolerance (IGT), Gestational diabetes mellitus (GDM), Malnutrition-related diabetes mellitus (MRDM), and "Other specific types" diabetes mellitus (World Health Organisation, 1980, 1985, 2003). Although minor changes were done by WHO in 1985, not much changed at that time. The term IDDM and NIDM were seen to be misleading as it diagnosed patients on management and not pathological start of the disorder. Therefore, a WHO consultation with the American Diabetes Association Expert Committee made amendments based

on both aetiopathogeneses of diabetes mellitus (Kuzuya & Matsuda, 1997). Therefore, type 1 and type 2 were terms introduced in place of IDDM and NIDM respectively (Alberti & Zimmert, 1998).

Type 1 diabetes is characterised by annihilation of pancreatic beta cell, resulting from autoimmune processes often leads to completely insulin deficiency. The etiology of this type is idiopathic and prone to ketoacidosis (Kumar & Clark, 2002). Specific conditions such as mitochondrial defects, cystic fibrosis that destroys beta-cells do not result in type 1 diabetes (Baynest, 2015). Nearly 90% of all diabetes mellitus cases are made up of type 2 diabetes which is caused from defect(s) in insulin secretion and/or in insulin action (Baynest, 2015). The interaction among genetics, behaviour and environment are contributing factors that results in bringing about type 2 diabetes mellitus (Wild *et al.*, 2004). The class Malnutrition-related diabetes mellitus (MRDM) was removed after an International review workshop that showed that even though protein-deficiency influences the development of different types of diabetes, the evidence was not convincing enough (Tripathy & Samal, 1997). Impaired glucose tolerance (IGT) was seen in any disorders of hyperglycemic control and is itself not diabetes, thus it was seen as a clinical stage in the transition from normality to being diabetics (WHO, 1999). People having impaired glucose tolerance (IGT) stand a greater chance of advancing to type 2 diabetes mellitus. The class GDM was seen as an operational classification including women who develop impaired glucose tolerance during gestation, thus this class was maintained.

Hence the new classification which was based on clinical stage and aetiological type are T1DM, T2DM, Gestational diabetes, and “other specific types” are the four main classes of diabetes mellitus (Alberti & Zimmet, 1998). It is therefore very important to know the origin of the hyperglycemia and effectively treat it.

2.3 Type 1 Diabetes Mellitus (T1DM)

Type 1 diabetes mellitus, formerly known as insulin-dependent diabetes, results from complete damage of the pancreatic beta cells by autoimmune T-cells processes (Baynest, 2015). Type 1 diabetes mellitus is a main class of diabetes and comprises about 5-10% of all diagnosed diabetes mellitus cases (American Diabetes Association, 2016). Profound characteristics of type 1 diabetes are the expression of islet cell specific autoantibodies, insulin autoantibodies, anti-glutamic acid decarboxylase (GAD) autoantibodies, tyrosine phosphate IA-2 and IA-2b autoantibodies, and alterations in the CD4+ cell compartments which are also markers of the autoimmune damage of the pancreatic beta-cells (Holt, 2004). Insulin-producing cells that are destroyed mostly lead to a complete insulin deficiency making these individuals dependent on insulin therapy so as to prevent metabolic disorders such as ketoacidosis (Polychronakos & Li, 2011). About 85% of T1DM individuals exhibit detectable islet cell antibodies and anti-insulin antibodies before starting insulin therapy (Raju & Raju, 2010). Majority of the beta cell antibodies function against glutamic acid carboxylase in the pancreas causing the beta-cells to be destroyed. The rate at which the pancreatic beta-cells is destroyed is not constant; very fast in infants as well as children and at a slower pace in adults, which in adults is occasionally identified as latent autoimmune diabetes [LADA] (American Diabetes Association, 2016). In some children and adolescents, ketoacidosis sometimes show itself as the initial sign of the disorder while in others; in the face of stress have mild hyperglycaemia which easily switches to severe hyperglycaemia and eventually into ketoacidosis (Raju & Raju, 2010). Some adults have very little beta-cell function which prevents them from developing ketoacidosis; however, such people are at risk of developing ketoacidosis and must depend on insulin for survival. The latter stage of T1DM is characterized by low or undetectable C-peptides in plasma as a result of little or no insulin secretion from the beta-cells (American Diabetes Association, 2016).

Also, there is excess secretion of glucagon resulting from the abnormal alpha-cell function of the pancreas in T1DM individuals (Baynest, 2015). Hyperglycaemia normally reduces glucagon secretion but this is contrary in patients with T1DM. Insulin loss in these individuals lead to abnormal lipolysis that raises the plasma's free fatty acid levels and thus, impairs targeted tissues utilization of glucose resulting in major metabolic derangement such as impaired glucose, lipid and protein metabolism (Holt, 2004). Type 1 diabetes mellitus has strong human leukocyte antigen (HLA) linkage with the major histocompatibility complex (MHC) class II genes which are responsible for regulating the immune system in human/ and can be either predisposing or protective (American Diabetes Association, 2016). Common clinical symptoms associated with T1DM are weight loss, polyuria, polydipsia, polyphagia, fatigue and blurred vision. Prolonged term complications are microvascular and microvascular complication diseases (American Diabetes Association, 2016).

The destruction of the beta-cells by autoimmune processes have several genetic factors making these patients easily susceptible to autoimmune disorders such as autoimmune hepatitis, myasthenia, Addison's disease, Graves' disease and Hashimoto's thyroiditis (American Diabetes Association, 2016).

Idiopathic T1DM is a subclass of T1DM with no known aetiologies but likely to be caused by transient beta-cell function and glucose resistance (Giugliano *et al.*, 2008). Some patients with T1DM have permanent inadequate secretion of insulin (insulinopenic) and are highly prone to developing ketoacidosis (Polychronakos & Li, 2011). A small percentage of individuals with T1DM fall into this category with most from Africa and Asian origin. This type of T1DM has a strong genetic influence and thus, no immunological proof of beta-cell autoimmunity is evident (Polychronakos and Li, 2011). A total requirement on insulin therapy is needed in patients with T1DM as glycemic control is well managed (American Diabetes Association, 2016).

2.4 Type 2 Diabetes Mellitus

2.4.1 Epidemiology

Type 2 diabetes mellitus (T2DM) is a common chronic metabolic ailment that happens from complex collaboration amid genetics and environment. The most common contributing factors such as age, family history, obesity and lifestyle, Type 2 diabetes mellitus as well as its complications have emerged as a serious global health crisis (Wu *et al.*, 2014). In 2013, the International Diabetes Federation (IDF) estimated that T2DM causes 5 million deaths per year, predicting T2DM to become the 7th cause of death globally in 2030. High prevalence rate of T2DM have been reported in middle-income and developing countries where urbanisation and modernisation have greatly affected lifestyles causing a major burden on these countries (WHO, 2016).

Epidemiological review done from 1999-2011 by Alberti & Zimmert, (1998), Hall *et al.*, (2011) and the American Diabetes Association, (2016) reported different prevalence rates of T2DM in some sub-Saharan Africa countries [Cameroon 6.06%; Ghana 6.4%; Guinea 6.7% in urban and 5.3% in rural; Kenya 10% in urban and 5% in rural; Nigeria 2.5%; South Africa 3.9%; Uganda 0.6% and Zimbabwe having the highest prevalence rate Of 10%], these results were based on random blood glucose and fasting blood glucose concentrations on either capillary or venous blood. Although low prevalence was recorded in countries such as Uganda the notion still stands that T2DM is not rare in sub-Saharan Africa.

2.4.2 Influencing factors on T2DM

2.4.2.1 Genetic component

Although the pathophysiology of T2DM has not been fully understood, genetic susceptibility is higher in T2DM than in T1DM. There exists enough evidence that shows T2DM strong connection

to genetics. Lyssenko & Markku, (2013) showed that a person having a first degree T2DM family history stands a double-fold increase risk of developing T2DM in the future.

Another study done in twins showed a high concordance rate among monozygotic twins (96%) compared to dizygotic twins (Groop *et al.*, 1999). Offspring with one parent affected with T2DM have 40% risk of developing T2DM especially if the affected parent is the mother, probably from the maternal hyperglycaemic effect on the pregnancy and this increases to about 70% if both parents have T2DM (Groop *et al.*, 1999).

Type 2 diabetes mellitus, being a polygenetic disorder, have several identified loci that affects the secretion and action of insulin. Thus no single major locus explains the susceptibility to the development of the condition and its inheritance (Wu *et al.*, 2014).

2.4.2.2 Environmental factors that influences the origin of diabetes type 2

The progression into type 2 diabetes is greatly affected by the environment one lives in and these factors are mostly behavioural or lifestyle. A large variety of lifestyles such as physical inactivity, smoking, alcohol consumption and sedentary lifestyle greatly affects the growth of type 2 diabetes mellitus. Bassuk & Manson, (2005), reported about obesity being the most ranked key contributing factor for T2DM development, and also greatly influences the progression of insulin resistance. Insulin resistance is the most important feature within individuals with type 2 diabetes mellitus. Although these individuals are not solely dependent on insulin, administration of exogenous insulin is needed when the blood glucose is properly not managed well.

Nearly 90% of individuals that develop T2DM results from excess body weight (WHO, 2016). With obesity, the greater percentage of visceral fat a person possesses, the higher the chance of acquiring diabetes (Carey *et al.*, 1997). A study conducted by Baynest, (2015) found that most T2DM individuals exhibit visceral (intra-abdominal) obesity which is nearly comparative to

insulin resistance in these individuals. Hypertension and dyslipidaemia are also associated in these individuals.

Physical sedentariness is linked to a high threat of diabetes as it increases the deposition of visceral fat in certain body parts including the heart, abdomen and the veins (Bassuk & Manson, 2005). Certain physical activity interventions such as walking, jumping, cycling, swimming and activities done in the gym improves glucose tolerance, helps to reduce weight and reduces the danger of acquiring type 2 diabetes mellitus by about 60% (Bassuk & Manson, 2005). A study conducted in Finland revealed that individuals who engaged in 150 minutes per week of walking experienced 60% fall to the danger of type 2 diabetes mellitus development compared to individuals that that walked 60 minutes per week (Laaksonen *et al.*, 2005).

Alcohol consumption reduces the body's sensitivity to insulin and increases the body's resistance to insulin (Holt, 2004).

2.4.3 Pathogenesis of type 2 diabetes mellitus

Type 2 diabetes mellitus initially titled non-insulin-dependent diabetes is a serious hyperglycaemic condition occurring from the gradual damage of the pancreatic beta cells causing insulin resistance with comparative and not complete deficiency of insulin secretion (American Diabetes Association, 2016). Individuals with this type of diabetes have two main pathophysiological defects: increased resistance to insulin due to insulin action being less effective and less effective insulin production arising from dysfunction in pancreatic beta-cells (American Diabetes Association, 2016).

2.4.4 Insulin resistance

Resistance to insulin is a pathological condition resulting from the failure of the pancreatic islet cells to function regularly through insulin action which is a primary predominant defect in individuals with T2DM (Baynest, 2015). In most of these cases, majority of the beta-cells

undergoes a transformation of raising the supply of insulin to compensate for the excessive demand (Kumar & Clarke, 2002). Increasing insulin supply causes both the fasting and non-fasting plasma insulin concentration to also be raised, but this rise is relative to the severity of the insulin resistance.

2.4.5 Beta-cell dysfunction

Insulin secreted in response to glucose follows a biphasic order: an initial acute phase lasting a few seconds and immediately proceeded by a second phase which is maintained. Abnormalities of beta-cells are marked with loss/reduction of insulin initial-phase secretion and, a weak insulin second-phase secretion in established diabetes (Baynest, 2015). These abnormalities in the beta-cells are also identified in the early medical background of the type 2 diabetics and also in their immediate families, suggesting a strong part in the advancement of T2DM (Pratley & Weyer, 2001).

Although the mechanisms underlying the destruction of the beta-cells remain unclear, a number of environmental factors, hyperlipidaemia and hyperglycaemia may increase the decline of function of the beta-cells (American Diabetes Association, 2016). However, in type 2 diabetes mellitus, the beta cells are not harmed by autoimmune processes. Ketoacidosis rarely occurs in this type of diabetes and is seen in the face of stress (Diabetes Association, 2016).

2.5 Gestational Diabetes Mellitus

The class gestational diabetes is known as slightly intensity of glucose bigotry resulting in different severity of glucose levels which is detected at the onset or during pregnancy (American Diabetes Association, 2016). Gestational diabetes mellitus results in approximately 7% of all complications in pregnancies and 90% of all diabetes mellitus cases in pregnancies (American Diabetes Association, 2016). Adverse pregnancy outcome such as stillbirth, fatal microsoma, neonatal metabolic disturbances, and related problems are conditions associated with GDM

(Dabalkae, 2007). The risk factors for developing this disorder are; maternal obesity, maternal age, ethnic background, previous diagnosis of gestational or pre-diabetes and offspring of mothers with GDM (American Diabetes Association, 2016). Gestational diabetes mellitus is identified between the sixth to ninth months of pregnancy that is not obviously vulnerable diabetes. Women with gestational diabetes mellitus (GDM) and their children stand a greater likelihood of acquiring type 2 diabetes mellitus and other metabolic abnormalities.

2.6 Other specific types of diabetes mellitus

Another specific type of diabetes mellitus arises from various known aetiologies factors. This types of diabetes constitutes about 10% of diabetes mellitus cases and known aetiologies ranges from a defect in the beta cells which occurs before age 25 and is generally known as maturity-onset diabetes of the young (MODY); persons with genetic defect in insulin mechanism (formally known as type A insulin resistance); persons with endocrinopathies such as Cushing's syndrome, acromegaly, and glucagonoma; individuals with defeats in pancreas such as pancreatic cancer, and pancreatitis; individuals having dysfunctional pancreas caused by infection, drug or chemical such as congenital rubella, nicotinic acid and persons with certain genetic syndrome such as Klinefelter syndrome, Down syndrome and Turner syndrome (Baynest, 2015).

2.7 Risk Factors and Complexities of Type 2 Diabetes Mellitus

Type 2 diabetes is a progressive disease with risk factors such as obesity, alcohol consumption, bad dietary habits such as high intake of foods rich in carbohydrates and fats, age (increased insulin resistance at the onset of puberty), genetic conditions and absence of physical activity (Baynest, 2015). It has been documented that about 90% or even more of all diabetes cases are triggered by the collaboration between genetic and environmental influences (American Diabetes Association, 2016). Waist circumference larger than 102cm for adult males and 88cm for adult females also shows a strong link to the development of T2DM which is independent of their body mass index (The InterAct Consortium, 2012).

Complications associated with T2DM can be grouped into two: acute and chronic. Acute complications result in hypoglycemia catastrophes (Diabetes Ketoacidosis, [DKA]) and hyperglycemic crises (Hyperglycaemic Hyperosmolar State, [HHS]). Severe difficulties are as a result of progressive harm, non-function, and even death of certain organs including the kidneys, heart, eyes and blood vessels which result in nephropathy, cardiovascular disease, neuropathy and retinopathy (Baynest, 2015). Also the presence of glycated end products which are evidence of major biochemical abnormalities will result in uncontrolled blood glucose levels causing atherosclerosis when left in the blood for a long time (Basta *et al.*, 2004, Brophy *et al.*, 2007).

Metabolic complications such as low free testosterone, low LH pulse frequency and impaired kisspeptin signalling was reported in most adult males with diabetes type 2 (Costanzo *et al.*, 2014).

2.8 Kisspeptin Structure and Distribution

The gene *Kiss1* encoding Kisspeptin was first found as a suppressing-gene in different malignancies and initially named metastin (Lee *et al.*, 1996). The word “kiss” in kisspeptin was derived from the place of origin, the “Hershey Chocolate Kiss” in Hershey, Pennsylvania (ki) and from its function as a suppressing gene (ss). The gene (*kiss1*) can be found on chromosome 1q32 and has major 145 amino acid peptides which is cut to 54 amino acid peptides through distinctively proteolytic processing of the antecedent (West *et al.*, 1998). The 54-amino acid peptide for its instability can again be cleaved to shorter peptides (14, 13 and 10); all belonging to the RFamide family and share the COOH terminal sequence of arginine-phenylalanine residues (Arg-Phe-NH₂) and now they are all together called Kisspeptin (Muir *et al.*, 2001). Earlier studies done in 2001, identified every kisspeptin type as ligands for the waif G protein-coupled receptor 54(GPR54) initially observed in animal brain and later in humans and was later named *Kiss1r* (Kotani *et al.*, 2001; Muir *et al.*, 2001; Gottsch *et al.*, 2009). The *kiss1r* forms part of the seven membrane rhodopsin group GPR, although it has sequence similar (40%) to that of galanin receptor family it fails to fix to the galanin peptide *in vitro* studies (Lee *et al.*, 1999; Muir *et al.*, 2001). Several molecular

pathways are initiated when kisspeptin binds to the GPR54 system as it exerts its functions in a cell-specific way. The tying of kisspeptin to GPR54 causes activation of phospholipase C (PLC) by kiss1r and in addition mobilises secondary intracellular envoys (inositol triphosphate and diacylglycerol) resulting in mediating the discharge of calcium which in turns activates protein kinase C and brings about signalling of kisspeptin (Constantin *et al.*, 2009). The increase intracellular calcium by kiss1r is very fast in the initial phase and its followed by a more sustained but slower second phase, which is maintained by mobilising kiss 1r to prevent desensitisation (Min *et al.*, 2014).

2.8.1 Kisspeptin Distribution Overview

High levels of kisspeptin was initially established to be expressed in the placenta and later observed in small intestine, pancreas, ovary and testes (de Roux *et al.*, 2003). Within the Central Nervous System (CNS), majority of kiss1 and kiss1r are located within the hypothalamus, mostly expressed in the pituitary stalk and the anterior part of the hypothalamus of the brain (Hrabovszky *et al.*, 2010) with humans having more kisspeptin mRNA mostly expressed at the infundibula (Rometo *et al.*, 2007). The spreading of kisspeptin cell lines varies among species (Colledge, 2009).

2.8.2 Sexual dimorphism of kisspeptin neuron distribution

An earlier study done presented a population of sexual difference in kisspeptin characteristics at the hypothalamus' periventricular region and infundibulum pathway in humans (Hrabovszky *et al.*, 2010). Female hypothalamus has notable kisspeptin fibers in the ventral periventricular zone and infundibulum than in adult males (Hrabovszky *et al.*, 2010) with a noticeable high collection of kisspeptin manifestation in the hypothalamus's periventricular of females. Also, high kisspeptin population is observed in the hypothalamic infundibulum of females with only few seen among males (Hrabovszky *et al.*, 2011).

2.8.3 Kisspeptin and the regulation of GnRH secretion

In primates including humans, kisspeptin neuron is in close apposition with GnRH neurons (Liu *et al.*, 2008). In the hypothalamic preoptic zone, nerve cells of kisspeptin extend to the GnRH neurocytes (Clarkson *et al.*, 2006). Studies done *in vitro* and *in vivo* showed a significant release of GnRH as Kisspeptin receptors are activated and stimulated (d'Angelemont de Tassigny *et al.*, 2008, Liu *et al.*, 2008). This effect is hindered by administration of GnRH antagonists (d'Angelemont de Tassigny *et al.*, 2008). Moreover, treatment with Kisspeptin showed a striking rise in GnRH mRNA concentrations (Novaira *et al.*, 2009). In addition, central and auxiliary kisspeptin administration into discrete hypothalamic regions lead to a marked rise in circulating follicle stimulating hormone (FSH) and luteinizing hormone (LH) in both animal and human studies (Dhillon *et al.*, 2005, Leonor *et al.*, 2012). These data together elicits kisspeptin to excite the hypothalamic GnRH neurocytes to produce GnRH into the vein that carries blood, which afterwards, stimulates the production of gonadotrophs (LH, FSH) from the hypothalamus. This effect is hindered if earlier treatment of GnRH antagonist has being performed (Messenger *et al.*, 2005).

2.8.4 Function of Kisspeptin in the start of Puberty

Mammals including humans develop physical and endocrinal characteristics that bring about reproduction. Kisspeptin neurons exert a key function in starting puberty (Hameed *et al.*, 2011). Kisspeptin regulates the reproductive axis as demonstrated by the disadvantages of genetic change in kisspeptin signalling channel in individuals with congenital hypogonadotropic hypogonadism (CHH) (de Roux *et al.*, 2003). This phenotype (CHH) is also observed among individuals with heterozygous kisspeptin receptor mutations (Topaloglu *et al.*, 2012). The diminished function due to genetic change in kisspeptin receptor or genes have also been noticed in individuals who did not go through puberty and also from those who have hypogonadotropic hypogonadism (Topaloglu *et al.*, 2012), whereas mutations that results in function causes early puberty development (Teles *et*

al., 2008), whilst an activating mutation is associated with early puberty development. Conversely, genetic differences in the *kiss1r* have been found in patients having congenital precocious puberty (CPP) (Rhie *et al.*, 2014). The function of kisspeptin in reproduction goes beyond adolescence but extends into adulthood; as there is elevation in levels of LH, FSH and TT when exogenous kisspeptin is given to healthy men (Dhillon *et al.*, 2005). According to Leonor *et al.*, (2012), kisspeptin administered into the hypothalamus causes a great release of LH; LH then binds to Leydig cells in the testis, stimulating the synthesis and secretion of testosterone.

2.8.5 Function of Kisspeptin in keeping Feedback of Hypothalamic Gonadal Steroid

The sex hormones generated by the sex organs exert feedback signalling into the hypothalamus yielding either a bad or good controlling effect on GnRH secretion. The gonadal steroid feedback pathway was poorly understood before the discovery of kisspeptin; this was because as steroid hormone impacted level of GnRH, the cell lines of GnRH were not seen; GnRH neurons do not define progesterone, androgen receptors and oestrogen receptor alpha (Roseweir *et al.*, 2009). The advent of *kiss1r* showed its association as major mediator of gonadal steroid feedback in the hypothalamus. One type of isoform of oestrogen receptor (ER α) expressed at the Anteroventral Periventricular (AVPV) causes a favourable feedback of oestrogen on GnRH neurons to induce elevation in LH that produced early ovulation while in contrast, ER α expressed on cell lines of kisspeptin at the Arcuate Nucleus (ARC) are part of the undesirable feedback pathway in inhibiting GnRH production and release in response to oestrogen (Wintermantel *et al.*, 2006). An overview of the function of kisspeptin in the hypothalamus is described in Figure 1 from (Hameed *et al.*, 2011).

Figure 2.1: An overview of Hypothalamus -Pituitary-Gonadal Axis

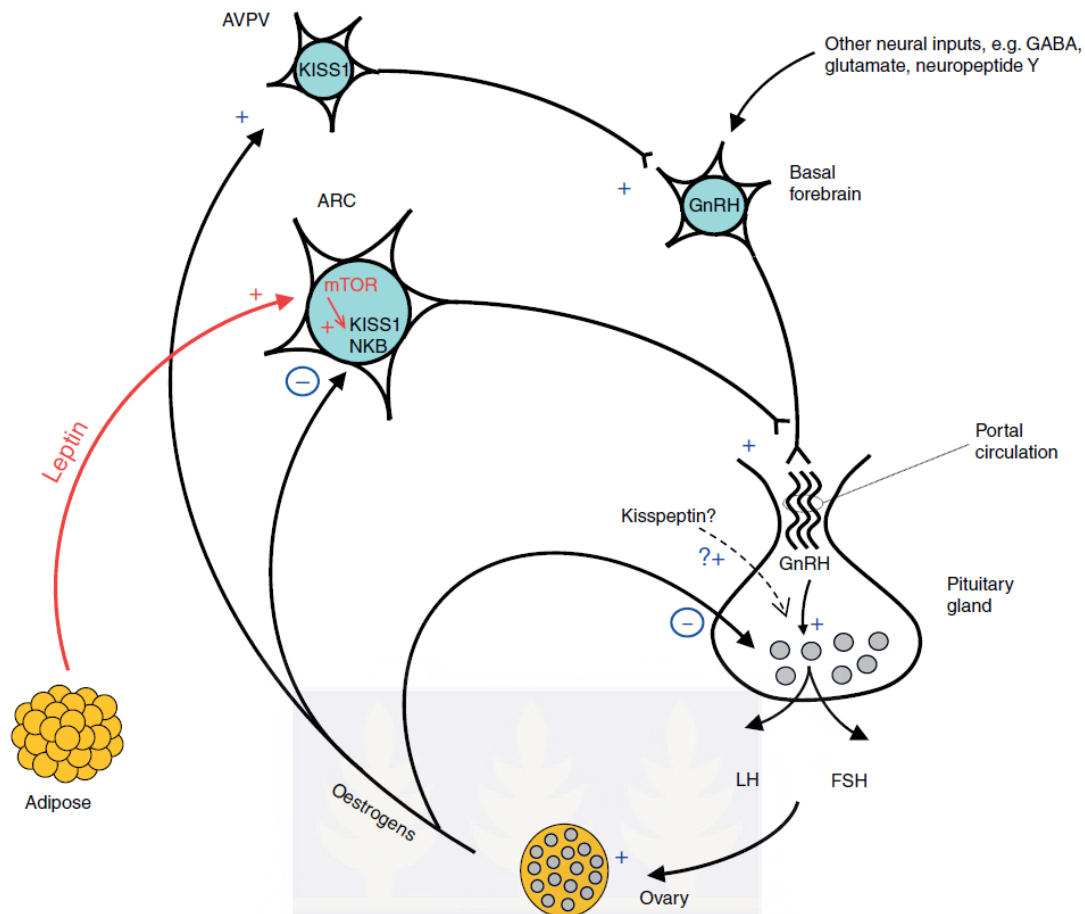


Figure 2.1: An overview of Hypothalamus -Pituitary-Gonadal Axis (Hammed *et al.*, 2011).

2.8.6 Kisspeptin potential direct effect on the gonads

Earlier studies did show kisspeptin ability to exert an effect on the gonadotrophs directly to excite its yield of LH and FSH (Kotani *et al.*, 2001, Richard *et al.*, 2008). The pituitary gonadotrophs which express the *kiss1r* genes yields LH in a measured-dependent manner. Further, Kisspeptin receptors and genes are also seen on human testes and spermatozoa (Kotani *et al.*, 2001; Pinto *et al.*, 2012). A study done in acycline treated monkeys showed enhanced human chorion gonadotrophin (hCG) which stimulated testosterone release upon the administration of kisspeptin but did not have any such effect without kisspeptin administration (Irfan *et al.*, 2014). This finding suggests kisspeptin effect on hCG on the release of testosterone from the gonads. In another study, sperm cells exposed to kisspeptin ended in a double phase elevation of intracellular calcium with a direct rise in sperm cell movement (Pinto *et al.*, 2012). Recent study by Tariq & Shabab (2016)

found no significant production of testosterone by the testis upon the administration of exogenous kisspeptin. This study which was inconsistent with work done by Irfan *et al.*, (2014) could be because it was done *in vitro* in testicular tissues.

2.8.7 Kisspeptin and other neuropeptide's functions together in HPG-axis regulation

The human infundibula nucleus shows that kisspeptin neurons co-concentrate neurokinin B (NKB) and Dynorphin (DYN) and this occurrence is much preserved in many mammals including humans (Hrabovszky *et al.*, 2011). Both NKB and DYN regulate HPG axis through its part in regulating feedback mechanism of GnRH expression (Clarke *et al.*, 2015). Neurokinin B (NKB) belongs to the Peptide-related tachykinin (TAC3) family which is much observed on GnRH cells (Todman *et al.*, 2005) and directly stimulates GnRH neurons to greatly increase LH release (Hameed *et al.*, 2011). In humans, genetic changes in the receptor TAC3 have proven to cause hypogonadism (Topaloglu *et al.*, 2009). The endogenous opioid dynorphin (DYN) also regulates GnRH production through a negative feedback by progesterone (Goodman *et al.*, 2007). These infundibula nucleus neurons that together express all these neuropeptides are identified as KNDy neurons (Cheng *et al.*, 2010) and these neurons are conserved across all species. The KNDy neurons make direct contact with GnRH cells to yield GnRH that is well regulated. Thus, neurokinin B exerts a stimulatory role and Dynorphin plays an inhibitory action all auto-synaptically work together pulsate yield kisspeptin, and in turn, produce a pulsatile yield of GnRH and LH (Dahl *et al.*, 2009) as shown in figure 2.2.

Figure 2.2: Diagram displaying the Kisspeptin-GnRH trail and the association between KNDy neurons and GnRH neurons in human

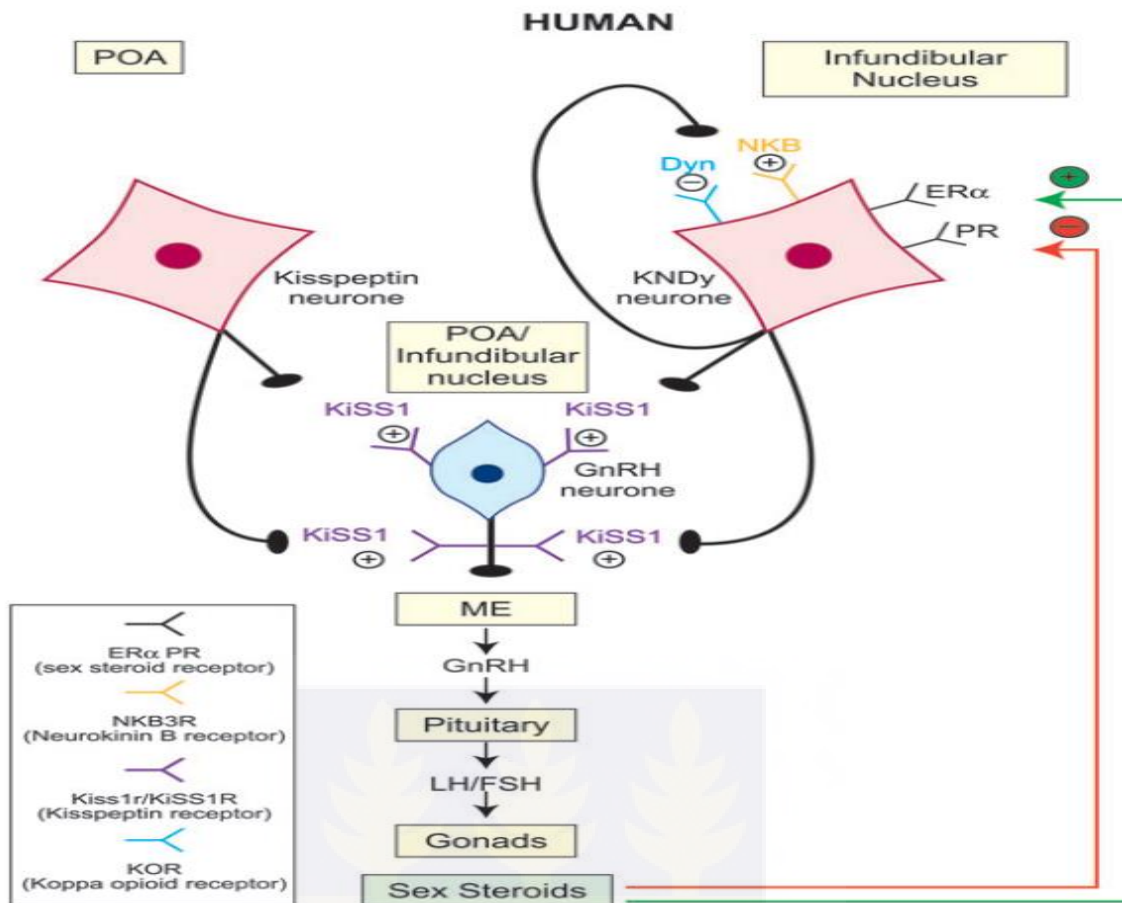


Figure 2.2: Diagram displaying the Kisspeptin-GnRH trail and the association between KNDy neurons and GnRH neurons in humans (Skorupskaite *et al.*, 2014)

2.8.8 Role of Kisspeptin in regulating nutritional signals and fertility

Kisspeptin neurons are able to receive information about metabolic status by recognising energy reserves and carrying this signalling to control production of GnRH. Thus kisspeptin provides a link between nutrition and fecundity. The hormone leptin is produced from white adipose tissues and relays information about body stored energy (Zhang *et al.*, 1994). Individuals with mutations in leptin receptor have late puberty (Farooqi & O’Rahilly, 2009). Gonadotrophin-releasing hormone (GnRH) neurons does not have leptin receptor but about 40% of kisspeptin neurons show leptin receptor, implicating the function kisspeptin plays as an intermediary between the metabolic signals of leptin and GnRH task (Smith *et al.*, 2006, Ojeda *et al.*, 2010).

The availability of energy stores determines the level of KiSS 1r mRNA. Rats with deficient nutrient showed decreased hypothalamic kisspeptin neuron levels (Hammed *et al.*, 2011) and delayed puberty in prolonged nutrient deficient state which can be corrected with kisspeptin peptide administration (Castellano *et al.*, 2005).

A study done investigating the interaction of kisspeptin with glucose metabolism revealed that before beta cells are damaged in the advancement of type 2 diabetes, high levels of glucagon are observed (D'Alessio, 2011). The researchers showed that hepatic kisspeptin production is stimulated by glucagon, and this causes the beta cells to yield small amount of glucose-stimulated insulin secretion [GSIS] (Song *et al.*, 2014). Also, it was shown that individuals having type 2 diabetes had high kisspeptin in serum in addition to high amount of kisspeptin appearing in the liver (Song *et al.*, 2014). These findings show that glucagon levels have an effect on GSIS impairment in the development of type 2 diabetes mellitus.

2.9 Kisspeptin association with diabetes type 2 (T2DM)

Studies by Oh *et al.*, (2002) have shown that low testosterone which arises from insulin resistance and hyperglycemia is a contributing marker to developing type 2 diabetes. Decrease available calories in the body cells disrupt kisspeptin signaling leading to decreased release of gonadotrophin (True *et al.*, 2011). Changes in energy balance as seen in diabetes mellitus (positive energy balance) often leads to impairment of fertility (Wahab *et al.*, 2015) plus it causes hypothalamic hypogonadotropic hypogonadism in diabetic males (Skorupskaite *et al.*, 2014). In type 2 diabetic males, Costanzo *et al.*, (2014) established that there is decreased testosterone (T) and also decreased LH pulsate hypothalamic/pituitary basis. Al Hayek *et al.*, (2013) also established that 36.5% of patients with T2DM had decreased levels of total testosterone and androgen deficiency. Another study done in Ghana pointed an inverse correlation of testosterone with body mass index, blood glucose and triglycerides in T2DM males (Asare-Anane *et al.*, 2013) However, administration of exogenous kisspeptin showed a 2-fold rise in LH secretion in diabetic

men with LH value of 10.7IU/L compared to healthy men with top LH value of 14.5IU/I (George *et al*, 2013). Uninterrupted kisspeptin administration is evident to desensitization of the HPG axis which down-regulates GnRH receptors and desensitizes gonadotrophs (Ramaswamy *et al.*, 2007), however, other study in humans have not given consistent evidence with desensitization (George *et al.*, 2013). In addition, patients with deactivated mutations in the gene encoding NKB had restored LH pulsatility with continuous kisspeptin administration (Young *et al.*, 2013).

Thus, the level of Kisspeptin in plasma will help determine the level of testosterone in men with T2DM as it may help in the assessment of their cardiovascular risk (Daka *et al.*, 2015).



CHAPTER THREE

METHODOLOGY

3.1 Study Design

This was a hospital based comparative cross-sectional study. Sixty (60) T2DM males were age-matched with 60 apparently healthy males.

3.2 Study Site

The study was conducted at the National Diabetes Management and Research Centre (NDMRC), Korle-Bu Teaching Hospital. The Korle-Bu Teaching Hospital is a leading tertiary hospital in Accra with about 2000 bed capacity. The hospital serves the city of Accra (with a population of about three million) and mostly the whole of the southern sector of Ghana. At Korle-Bu Teaching Hospital, there is an average daily out-patient attendance of 1,000 and 100 admissions daily (Personal Communication and Hospital Records). The Diabetic Clinic of the NDMRC holds over 80,000 patient's records with about 160 patients attending the clinic each day (NDMRC Records). The controls were recruited within the same environment.

3.3 Target Population

The study was carried out on subjects established with type 2 diabetes based on American Diabetes Association (2016) standards that are undergoing management of their conditions in the out-patient's clinic at NDMRC from August 2016 to March 2017. Men who served as controls were screened to make sure they were non-diabetic. Questionnaires were given to subjects to fill to obtain information on subject's age, period of start of Type 2 Diabetes and drug usage which served as selection criteria for subjects.

3.4 Inclusion and Exclusion Criteria

3.4.1 Inclusion Criteria

- Cases: Male subjects with type 2 diabetes mellitus (T2DM) within the age of 30-65 years and were on management at the NDMRC
- Controls: Non-diabetics within the age 30-65.

3.4.2 Exclusion Criteria

Type 2 Diabetes Mellitus men

- below the ages of 30 years
- on testosterone, steroids, or opiates
- on medications that can cause hyperprolactinemia

3.5 Sample Size

Minimum sample scope was determined via this formulation;

$$N = Z^2 (p*q)/e^2 \text{ (Spiegel \& Stevens, 1998)}$$

$$N = (1.96)^2 (0.083) (1-0.083) / (0.05)^2$$

$$N = 117$$

Where **Z**, 1.96 is the standard score for the confidence interval of 95%

P, 0.083 is the sample proportion of the 8.3% prevalence of diabetes in Ghana (International Diabetes Federation, 2013).

e is the minimum allowable error (5% or 0.05)

Based on the formula above, a sample size of 120 was estimated for the study, comprising 60 diabetes type 2 subjects and 60 non-diabetic controls allowing for any drop outs.

3.6 Ethical Issues

The work was approved by the Ethical and Protocol Review Committee of University of Ghana School of Biomedical and Allied Health Sciences of College of the Health Sciences CHS-Et/ M.1-p 4.2/2016-2017 on 22nd September, 2016. Consents were also obtained from participants involved in this work.

3.7 Sampling and Sample Collection

3.7.1 Sampling Technique

This study employed the convenience sampling technique to recruit all subjects.

3.7.2 Specimen Collection, Transport, and Storage

Participant's blood (10mls) samples were withdrawn after an overnight fast (10-12 hours) for all assays according to Helsinki protocol declaration (2008). Two millilitres (2mls) of whole blood was put into ethylene diamine tetra-acetic acid (EDTA) tubes for the measurement of glycated hemoglobin (HbA_{1c}). One milliliter (1ml) of whole blood was transferred into sodium fluoride tubes and the plasma was alienated for the measurement of glucose. Two millilitres (2mls) of whole blood was further transferred into lithium heparin containing tubes and plasma was separated and aliquot into plain Eppendorf tubes and was kept at -20°C pending kisspeptin estimation. The remaining five millilitres (5mls) of blood was transferred into serum separator tubes, centrifuged at 4°C for 10 minutes at 3000rpm. Serum samples were aliquot in 1ml portions into 5 sterile Eppendorf tubes and stored at -20°C until needed for the estimation of luteinizing hormone (LH), follicle stimulating hormone (FSH), total testosterone (TT) and lipid parameters.

3.7.3 Anthropometric Measurements

Height and weight of participants were estimated respectively using a standardized weighing balance and a standardized stadiometer. Body mass index (BMI) was then calculated as body weight (in kilograms)/ square of the height (in meters). The subject's waist circumference was measured at the navel region, parallel to the hip bone according to WHO (2003) protocol for waist circumference measurements.

3.7.4. Blood Pressure

Blood pressure (diastolic and systolic) measurements were taken by a qualified nurse from the left upper arm of subjects in the sitting position (after sitting for at least five minutes) with the aid of a mercury sphygmomanometer and stethoscope using the auscultation method.

3.8 Biochemical Analysis

Glycated hemoglobin was determined centred on a latex agglutination inhibition test using Randox Daytona auto analyzer. Fasting glucose and lipid profile parameters were estimated using the VITROS slides method and Calibrator kit on VITROS Chemistry. Low density lipoproteins (LDL) and Very low density lipoprotein (VLDL) were calculated using the Friedewald formula while T. Chol/HDL ratio was also calculated in all subjects. Solid phase enzyme-linked immunosorbent assay (ELISA) was used for plasma kisspeptin, LH, FSH and total testosterone measurements.

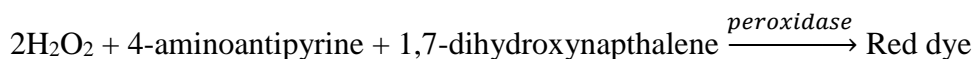
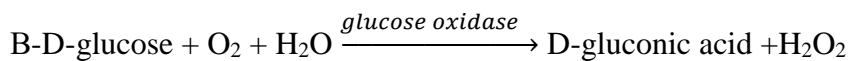
3.8.1 Glucose Measurement

Principles of the procedure

The glucose concentrations were quantitatively analyzed based on the glucose oxidase method using the VITROS GLUCOSE (GLU) slide technique. The VITROS GLU is a multi-layered, diagnostic component that has been layered on a polyester support. Five microliters (5 μ l) of the sample was placed on the slide and was distributed uniformly by the distribution layer to the

fundamental layers. The oxidation of glucose in the sample is sped up by glucose oxidase to form hydrogen peroxide and gluconate. The reaction was then preceded by an oxidative link catalysed by peroxidase in the presence of dye precursors to produce a colour. The intensity of the colour was measured by reflected light (Curme *et al.*, (1978).

Reaction sequence:



3.8.2 Glycated Haemoglobin (HbA_{1c}) Measurement

Principle of measurement

The measurement of HbA_{1c} was based on the latex agglutination inhibition assay. The agglutinator was made up of a synthetic polymer having numerous duplications of the immunoreactive share of HbA_{1c} that caused agglutination of latex covered with HbA_{1c} specific mouse monoclonal antibodies. In the non-appearance of HbA_{1c} in sample, the antibody-layered micro-particles in the HbA_{1c} reagent 1 and the agglutinator in the HbA_{1c} reagent 2 were agglutinated causing a rise in the absorbance. The degree of adhesion of the HbA_{1c} reagent 1 and the agglutinator in the HbA_{1c} reagent 2 slowed down because of the presence of HbA_{1c} in the sample. The rise in absorbance was determine at 700nm was inversely proportional to the concentration of HbA_{1c} in sample.

3.8.3 Plasma Kisspeptin Measurement

Principle of the procedure

The test used the double-antibody sandwich enzyme-linked immunosorbent one-step process assay (ELISA) to analyse level of Kisspeptin-54 in samples. The principle involved the reaction of kisspeptin present in sample simultaneously reacting with antigen conjugate (horseradish

peroxidase) and anti-kisspeptin coated wells. Standards, test samples and horseradish peroxidase (HRP)-labelled kisspeptin-54 antibodies were added to enzyme wells which were pre-layered with kisspeptin-54 and all incubated at 37°C for one hour and then washed to remove uncombined enzyme. Chromogen solution A and B which were blue were used for coloring after the reactant was thoroughly washed to eliminate any unbound enzyme. The reaction with the acid caused the color blue of Chromogen Solution A and B to become yellow. The intensity of the color developed was determined spectrophotometrically at 450nm \pm 10nm and the optical density (O.D) of standard curve was used to determine kisspeptin concentration.

3.8.4 Measurement of Serum Testosterone

Principle of procedure

The test used the solid phase enzyme-linked immunosorbent assay which followed the typical competitive binding schematic to assay levels of testosterone in samples. There was competition between unlabelled antigen (existing in standards, controls and subject samples) and enzyme-linked antigen (conjugate) for a restricted quantity of antibody binding sites on the micro-well plate. The well plates were then washed with buffer and decanted to eliminate any unbound materials. After washing, the enzyme substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB) was added to each well at timed intervals, incubated on a plate for 10-15 minutes at room temperature until desired dark blue color appears. A stop solution was then added to the enzymatic reaction to terminate the reaction. The absorbance was then estimated on a microliter plate reader. The intensity of color developed was inversely proportional to the concentration of testosterone in the samples. A set of standards was used to design a standard curvature to read testosterone concentration from it.

3.8.5 Measurement of Serum Luteinizing Hormone (LH)

Principle of procedure

The test utilised the ‘sandwich’ type enzyme immunoassay that engaged a double extremely specific monoclonal antibody. A monoclonal antibody specific for LH (horseradish peroxidase) was restrained onto the micro-well plate and another monoclonal antibody specific for a dissimilar zone of LH was bond to horse radish peroxidase (HRP). Luteinizing hormone (LH) in standards and samples were permitted to attach to the well plate, cleaned with distilled water and further incubated with the HRP conjugate. The plate was then washed for the second time after which the enzyme substrate was added. A stop solution was then added to stop the enzymatic reaction. The absorbance was read on a microliter plate reader. The intensity of colour developed from the enzymatic reaction was directly proportional to the concentration of LH in the sample. A set of standards was used to design a standard curve from which the amount of LH in subject samples and controls were directly read.

3.8.6 Measurement of Follicle Stimulating Hormone (FSH)

Principle of procedure

The test followed the sandwich type assay which employed the use a double highly specific monoclonal antibodies: A monoclonal antibody specific for FSH restrained onto a micro-well plate and an alternative antibody specific for a dissimilar region of FSH conjugated to horse radish peroxidase (HRP). Follicle stimulating hormone (FSH) in samples and standards were allowed to attach to the plate, washed, and subsequently incubated with the HRP conjugate. After incubation, another washing was repeated and enzyme was then added. The enzymatic response was stopped by addition of the stop solution. The absorbance was read on a microtiter plate reader. The concentration of the dye developed by the enzymatic reaction was directly proportional to the

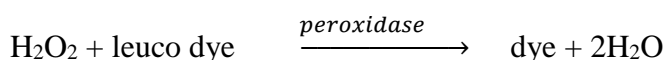
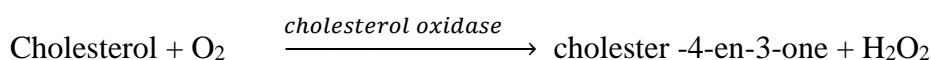
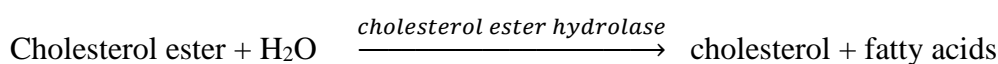
concentration of FSH in the sample. A set of standards was used to design a standard curve from which the quantity of FSH in subject and control samples was directly read.

3.8.7 Measurement of Total Cholesterol (T. CHOL)

Principle of the procedure

The test analysis was based on the VITROS Cholesterol (CHOL) slide technique. Six microliters (6µls) sample was dropped on the slide and was ultimately disseminated by the distribution layer to the fundamental layers. The Triton X-100(TX 100) surfactant present in the distribution layer helped in separating the cholesterol and cholesterol esters from the lipoprotein complexes that were present in the sample. Cholesterol ester hydrolase hydrolyzed the breakdown of cholesterol into free cholesterol. Free cholesterol was then oxidized to form cholestenone and hydrogen peroxide by the help of cholesterol oxidase. Lastly, hydrogen peroxide oxidized a Leuco colorant existing in peroxidase to produce a color which was measured by reflectance spectrophotometry at 540nm wavelength. The concentration of the color developed was proportional to the cholesterol concentration that was available in the sample. The development time for the whole reaction was 300 seconds at 37°C (Allain *et al.*, 1974).

Reaction sequence:

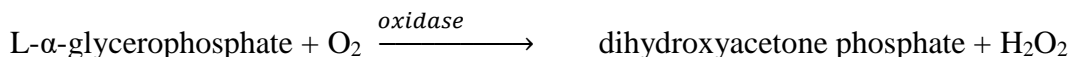
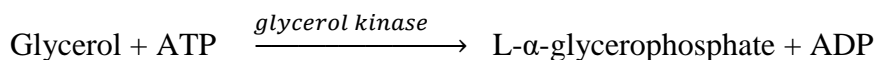
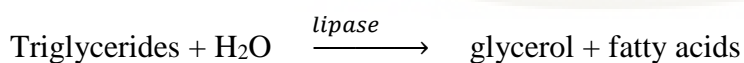
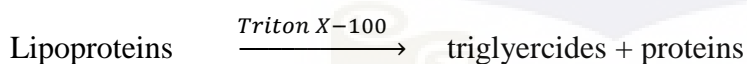


3.8.8. Measurement of Triglyceride (TRIG)

Principles of procedure

The VITROS Triglyceride (TRIG) slide was employed. Six microliters (6 μ ls) of sample was dropped onto the slide and distributed consistently by the distribution layer to the fundamental layers. The Triton X-100 surfactant present in the distribution layer helped in separating the triglycerides from the lipoprotein complexes that were available in the sample. The triglycerides fragments were then hydrolyzed by lipase to give glycerol and fatty acids. Glycerol then dispersed to the reagent layer, where it is phosphorylated by glycerol kinase of adenosine triphosphate (ATP). L- α -glycerophosphate was oxidized in the presence of oxygen to give dihydroxyacetone phosphate and hydrogen peroxide. Lastly, peroxidase catalyzed the oxidation of leuco dye by hydrogen peroxide which produced a colored dye and was measured at a wavelength of 540nm by reflectance spectrophotometry. The density of the dye formed is directly proportional to the amount of triglycerides that was present in sample. The development time for the whole reaction was 300 seconds at 37°C (Spayd *et al.*, 1978).

Reaction sequence:

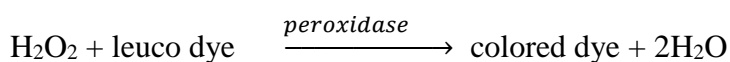
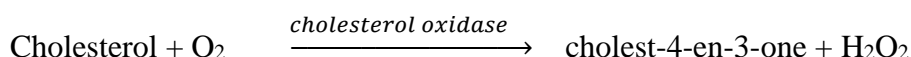
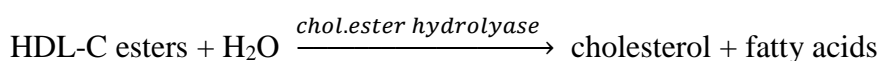
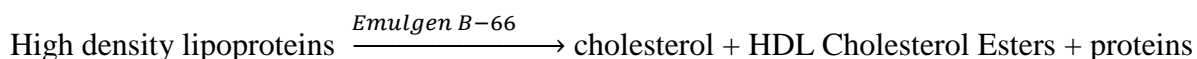


3.8.9 Measurement of High Density Lipoprotein (HDL)

Principle of the procedure

The VITROS cholesterol slide method was. Ten microliters (10µls) of test sample was dropped on the slide and was dispersed evenly by the distribution layer to the fundamental layers. Magnesium chloride (MgCl₂) and phosphotungstic acid (PTA) in the distribution layer dissociated the HDL cholesterol by the precipitation of non-high density lipoproteins (non-HDL). Also, the Emulgen B-66 surfactant in the distribution layer helped in the discerning separation of the cholesterol and cholesterol esters from the HDL cholesterol complexes existing in the sample. Carefully chosen ester hydrolase hydrolyzed the HDL-derived esters into free cholesterol. Free cholesterol was then oxidized into cholestenone and hydrogen peroxide in the company of cholesterol oxidase. The last reaction involved the oxidation of leuco dye by hydrogen peroxide in the presence of peroxidase that resulted in a color formed. The concentration of the color formed is proportional to the amount of HDL cholesterol in test sample and was calculated at a wavelength of 670nm by reflectance spectrophotometry. The development time for the whole reaction was 300 seconds at 37°C (Allain *et al.*, 1974; Burstein *et al.*, 1970)

Reaction sequence:



3.8.10 Determination of Low Density Lipoproteins (LDL) cholesterol

The indirect method equation proposed by Friedwald *et al.*, (1972) was used to estimate LDL cholesterol.

$$\text{LDL cholesterol (mmol/L)} = [\text{Total cholesterol}] - [\text{HDL cholesterol}] - \frac{[\text{Triglyceride}]}{2.22}$$

3.9 Data Handling

The standard safety laboratory protocol for sample collection and handling were strictly adhered to. All data was entered into Microsoft excel spreadsheet for storage and subsequently analyzed. Data was handled confidentially. Codes were assigned to research participants. Only the Principal Investigator was able to match codes to study subjects. Questionnaires were stored in a safety cabinet. Electronic data was stored under a password. Data was presented in the tabular or graphical form. Questionnaires were kept under lock and key and will be shredded after two years.

3.10 Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) version 20.0 was used for the statistical analysis. The Kruskal-Wallis test, Mann-Whitney U- test, and two-tailed unpaired t-test was employed to test the difference in biochemical levels between diabetic and non-diabetic male subjects. Spearman's product moment correlation coefficient (r) analysis was employed to see the association between numeric variables. Multiple logistic regression analysis was used to access the relationship between the hormones analysed in this study with low kisspeptin levels in T2DM males. P-values less than 0.05 were considered significant.

3.11. Dissemination of Results

The outcome of this study will be presented at the School of Biochemical and Health Sciences research seminar. A copy of this study will also be submitted to the Department of Chemical pathology, SBAHS in partial fulfilment for the award of Master of Philosophy in Chemical

Pathology. Copies of the dissertation will also be submitted to the School of Graduate studies and the Diabetic Clinic as well. In addition, copies of this dissertation will be published in recognized scientific journals and also presented at various scientific conferences and seminars.



CHAPTER FOUR

RESULTS

4.1 Demographic and Clinical indices of the study population.

The total number of subjects who took part in this study was one hundred and twenty (120). The subjects comprised sixty (60) type 2 diabetic men whose glucose levels were being managed. These were age-matched with sixty (60) apparently healthy non-diabetics as control group. The mean age for the diabetic subjects and non-diabetic controls were 49.37 ± 10.86 and 48.35 ± 7.91 years respectively. The difference between the means of the ages of the diabetics and non-diabetics was not significant [$p=0.057$] (Table 4.1). The mean for body mass index (BMI) was also seen to be significantly elevated in diabetic subjects compared to the non-diabetic control group ($p=0.013$). The blood pressure and waist circumference were highly significant ($p=0.0001$) between means of both diabetic subjects and control group. The mean duration of diabetes in diabetic subject was 1.83 ± 1.22 was in the range of 1-5 years.

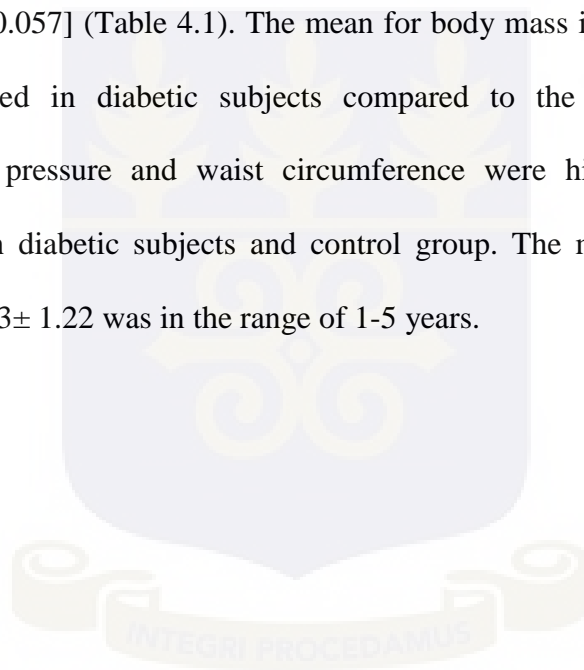


Table 4.1: Demographic and clinical indices of the study population

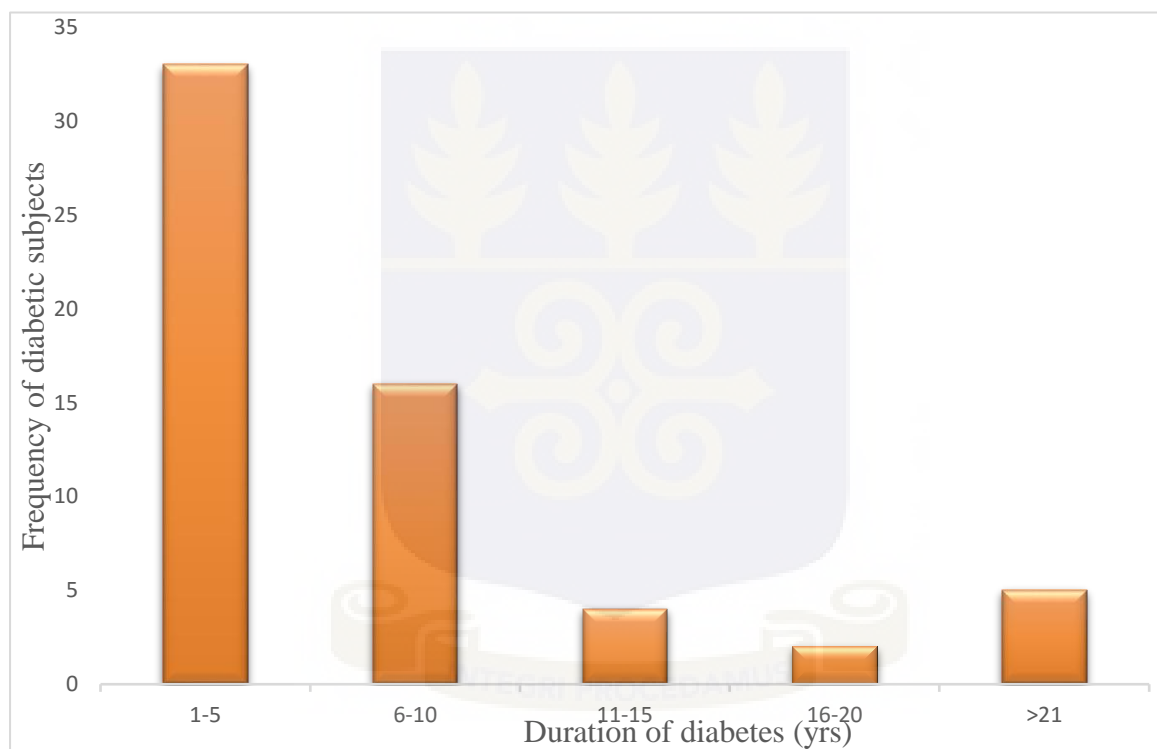
Parameters	Type2 Diabe (N = 60)	Non-Diabetic control (N = 60)	95% CI of m difference	p-value
Age (yrs.)	49.37±10.86	48.35±7.91	1.58-8.45	0.057
BMI (kg/m²)	28.39±3.57	25.21±3.17	1.97-4.41	0.013*
SBP (mmHg)	138.37±15.86	129.32±8.52	4.45-13.65	0.0001**
DBP (mmHg)	81.12±9.58	78.28±8.37	2.82-8.54	0.0005**
Waist circumference (cm)	94.09±7.56	84.15±5.72	7.50-12.34	0.0001**
Duration of diabetes (yrs.)	1.83±1.22 (1-5)	-	-	-

Table 4.1 shows the demographic and medical parameters (Age, BMI, SBP, DBP, Waist circumference and Duration of diabetes). Values for Age, BMI, SBP, DBP, Waist circumference and Duration of diabetes are specified as mean ± standard deviation. *mean difference is significant (p<0.05). **mean difference is highly significant (p<0.01).

4.2 Frequency Distribution of Diabetic Subjects with Duration of type2 Diabetes

Overall number of type 2 diabetic subjects in the cohort study was grouped into duration of type 2 diabetes as shown in figure 4.1. It was observed that the number of diabetics subjects with the condition within 1-5 years were 33, 6-10 years were 16,11-15 were 4, 16-20 were 2 and 5 subjects have been diabetic over 21 years. However, stratification of subjects had nothing to do with a decline of age with increase of duration of type 2 diabetes.

Fig. 4.1: Frequency distribution of type 2 diabetic subjects at various duration of diabetes.



The figure (Fig. 4.1) showed a decline in the number of type 2 diabetic subjects with increase in duration of diabetes in years.

4.3 Body Mass Index Percentage distributions within the study population

The percentage distribution of body mass index (BMI) between type 2 diabetics and non-diabetics were determined in this study. Subjects and controls were grouped into four (4) categories. These were underweight ($<18.5\text{kg/m}^2$), healthy ($18.5\text{-}24.9\text{kg/m}^2$), overweight ($25\text{-}29.9\text{kg/m}^2$) and obese ($>30\text{kg/m}^2$). A total of 1(2%) of the diabetic subjects and 4(7%) of the non-diabetic control group were underweight ($<18.5\text{kg/m}^2$). Ten (10), (16%) of the diabetics and 31(51%) of the control group were in the healthy range ($18.5\text{-}24.9\text{kg/m}^2$). Also, 34(57%) of the diabetic subjects and 21(35%) of the non-diabetics were overweight ($25\text{-}29.9\text{kg/m}^2$). A total of 15(25%) of the diabetic subjects and 4(7%) of the control group were in the obese range ($>30\text{kg/m}^2$) as shown in figure 4.2.

Figure 4.2: Percentage distribution of Body mass index (BMI) across the population

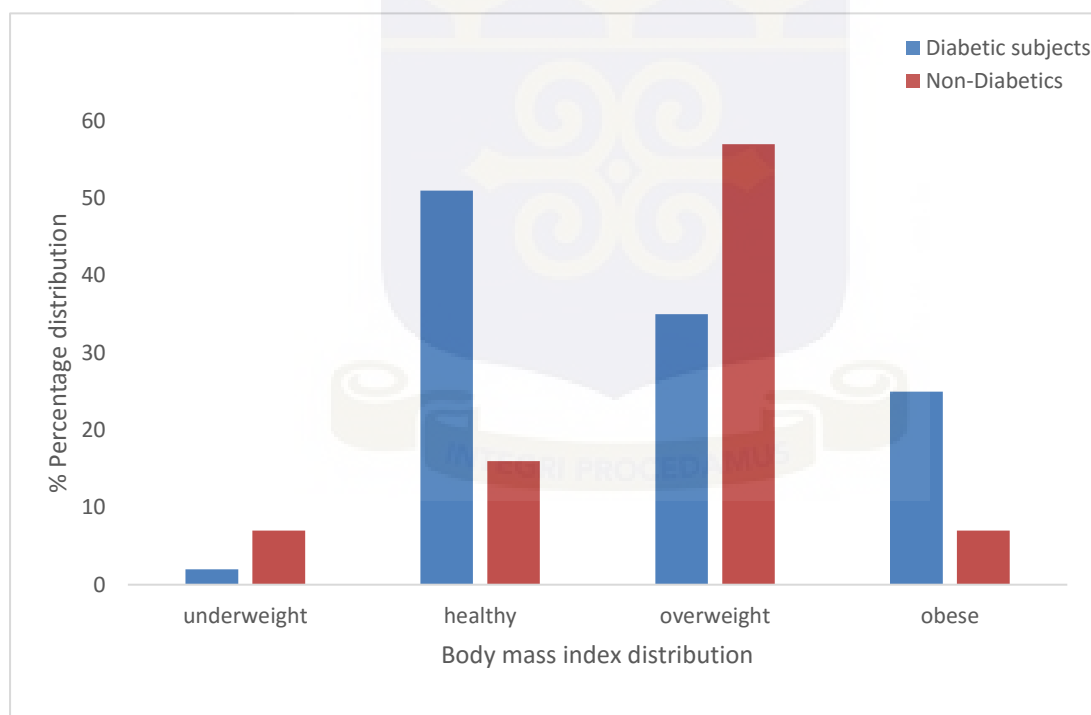


Figure 4.2 shows the percentage dissemination of the body mass index across the group. Body mass index has been categorised into four groups: underweight ($<18\text{kg/m}^2$), healthy ($18.5\text{-}24.9\text{kg/m}^2$), overweight ($25.0\text{-}29.9\text{kg/m}^2$) and obese ($>30\text{kg/m}^2$). A total of 2% (1) of the diabetic subjects and 7% (4) of the control group were underweight. Healthy group comprised of 16% (10) and 51% (31) of diabetic and non-diabetic subjects respectively. Overweight group comprised of 57% (34) diabetic subjects and 35% (21) non-diabetic subjects whilst obese group was made up of 25% (15) diabetics and 7% (4) control subjects.

4.4 Biochemical indices of the study

The means of the measured biochemical factors of the study subjects are shown in table 4.2. Fasting blood glucose (FBG), glycated haemoglobin (HbA1c), total cholesterol (T. Chol), triglycerides (TG), low density lipoproteins (LDL), very low density lipoproteins (VLDL) and cardiovascular risk (T. Chol/HDL ratio) were significantly high ($p < 0.005$) in diabetic subjects compared with non-diabetic control group. However, high density lipoprotein (HDL) was significantly low in diabetics than non-diabetics.

Table 4.2: Biochemical indices of the study population

	Type 2 Diabetes	Non- Diabetics	95% CI of	
	(N = 60)	(N = 60)	Mean difference	p- value
FBG (mmol/L)	9.95 ± 3.60	5.10 ± 0.63	3.91 – 5.75	0.001*
HbA1c (%)	9.78 ± 1.46	5.81 ± 0.68	3.56 – 4.37	0.001*
T. Chol (mmol/L)	4.59±0.39	3.66±1.09	-1.22-(-0.63)	0.0001**
TG (mmol/L)	1.35±0.75	0.80±0.31	0.34-0.76	0.0001**
HDL (mmol/L)	0.95±0.50	1.49±0.43	-0.71-(-0.75)	0.0001**
LDL (mmol/L)	2.74±0.52	2.18±0.88	-0.83-(-0.31)	0.001*
VLDL (mmol/L)	0.62±0.34	0.37±0.14	0.16-0.34	0.0001**
T. Chol/HDL ratio	4.74±2.84	3.28±0.83	0.71-2.22	0.001*

Table 4.2 shows the biochemical factors of the study population. Values are specified as mean ± standard deviation. *mean difference is significant ($p < 0.05$). **mean difference is greatly significant ($p < 0.0001$). FBG is fasting blood glucose, HbA1c is glycated haemoglobin, T. Chol is total cholesterol, TG is triglycerides, HDL is high density lipoproteins, LDL is low density lipoproteins, and VLDL is very low density lipoproteins. Cardiovascular risk is T. Chol/HDL.

4.5 Hormonal levels in diabetic subjects related with non-diabetic controls

Plasma kisspeptin, Total testosterone (TT), Luteinizing hormone (LH) and Follicle stimulating hormone (FSH) levels were investigated in this study. The means of the measured hormonal levels are presented in Table 4.3. The mean concentrations of plasma kisspeptin was significantly higher ($p < 0.001$) in the non-diabetics (16.26ng/ml) than the diabetics (8.34ng/ml). Significant difference was found between diabetic and non-diabetic subjects for Luteinizing hormone ($p = 0.003$) and Follicle stimulating hormone ($p = 0.019$). The mean for Total testosterone (TT) was also found to be significantly higher in non-diabetic than the diabetic subjects ($p = 0.001$).

Table 4.3: Hormonal levels in the study population

Parameters	Type 2 diabetes subjects = 60)	Non-Diabetics (N = 60)	95% CI of difference	p- value
Kisspeptin (ng/ml)	8.34 ± 7.64	16.26 ± 12.72	-11.71- (-4.12)	0.001**
LH (mIU/ml)	14.67 ± 9.62	10.82 ± 2.37	1.31 – 6.38	0.003*
FSH (mIU/ml)	15.83 ± 17.29	10.39 ± 2.65	0.97 – 9.92	0.017*
Testosterone (ng/ml)	6.49 ± 9.62	11.38 ± 2.56	-6.00 – (-3.79)	0.001**

Table 4.3 shows the hormonal levels in diabetic subjects compared with non-diabetic control men. Values are given as mean ± standard deviation. LH = Luteinizing hormone. FSH = Follicle stimulating hormone. *mean difference is significant ($p < 0.05$). **mean difference is greatly significant ($p < 0.001$).

4.6 The percentage distribution of kisspeptin levels in the study population

The percentage distribution of kisspeptin levels in diabetics and non-diabetics was examined in this study. Study subjects were grouped into three (3) categories as shown in figure 4.3. These were below reference range (<6.3ng/ml), within reference range (6.3-200ng/ml) and above reference range (>200ng/ml). A total of 33, (55%) of the diabetic men and 11, (18.33%) of the non-diabetics were below the reference range. Also, 27, (45%) and 49, (81.7%) had kisspeptin levels within reference range for the diabetic subjects and non-diabetic controls respectively. Moreover, none of the study groups had kisspeptin levels above reference range.

Figure 4.3: Percentage distribution of categorised kisspeptin levels in the study population

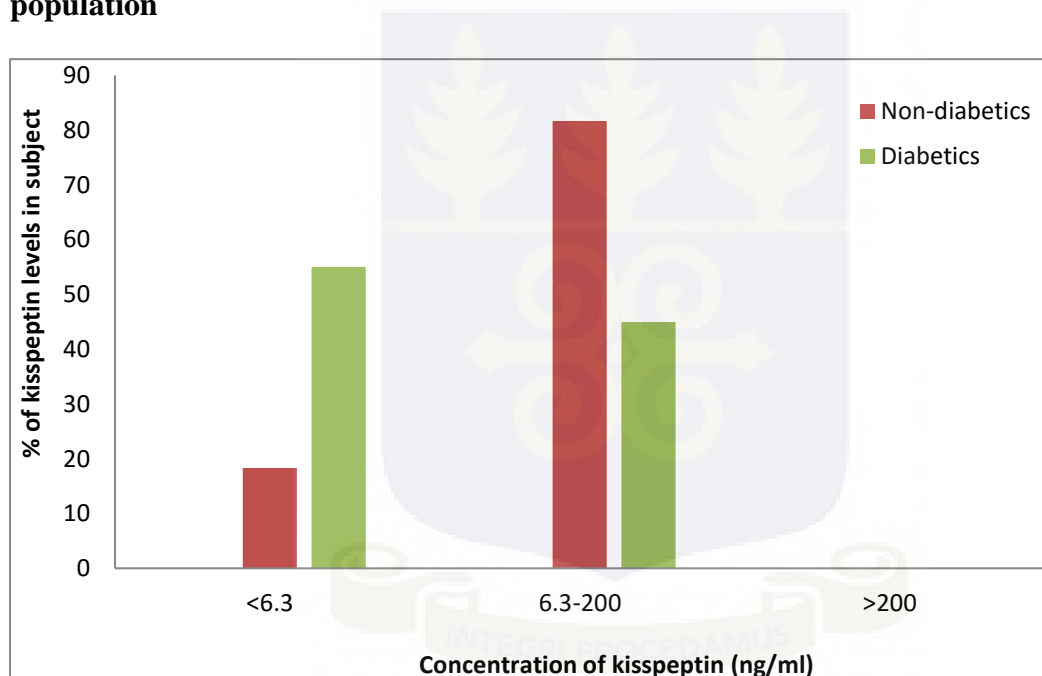


Figure 4.3 shows kisspeptin levels of diabetic and non-diabetic men that have been categorised into below reference range (<6.3ng/ml), within reference range (6.3-200ng/ml) and above reference range (>200ng/ml). Percentage distribution of categorised kisspeptin levels for the diabetic subjects were 55 for below reference range and 45 for within reference range. Percentage dissemination of categorised kisspeptin levels for the non-diabetics was 18.3 for below reference range and 81.7 for within reference range. None of the study groups had kisspeptin levels above reference range.

4.7 Comparison of kisspeptin levels with duration of diabetes

Plasma kisspeptin levels from type 2 diabetic subjects were categorised by the duration of diabetes in years. The mean plus/minus standard deviation (means \pm SD) of the kisspeptin concentration among the duration of type 2 diabetes are shown in table 4.4. Difference between means among group was also investigated. Statistical significance ($p=0.019$) was observed among groups. Statistical significance was seen between mean kisspeptin levels for 1-5 vs >21 ($p=0.016$) and 6-10 vs >21 ($p=0.013$). No statistical significance ($p>0.05$) was however found between mean kisspeptin levels for the rest of the group.



Table 4.4: Comparison of kisspeptin levels with duration of type 2 diabetes

Duration (yrs.)	Mean \pm SD		1-5	6-10	11-15	16-20	>21	p-value
1-5	14.78 \pm 10.28	Md	-	1.52	-0.82	0.34	-7.81*	
		p	-	1.00	1.00	1.00	0.016	
6-10	8.13 \pm 6.67	Md	-1.52	-	-1.15	1.19	-7.47*	
		p	1.00	-	1.00	1.00	0.013	
11-15	7.32 \pm 3.56	Md	0.82	1.15	-	2.34	-6.66	
		p	1.00	1.00	-	1.00	0.35	0.019*
16-20	6.97 \pm 3.63	Md	-0.34	-1.19	-2.34	-	-9.00	
		p	1.00	1.00	1.00	-	0.23	
>21	5.79 \pm 0.26	Md	7.81*	7.47*	6.66	9.00	-	
		p	0.016	0.013	0.35	0.23	-	

Table 4.4 shows the kisspeptin levels in type 2 diabetic subjects at various duration period of diabetes. Values are given as mean \pm standard deviation. md = mean difference within group. *Mean difference is significant ($p < 0.05$).

4.8 Association of kisspeptin levels with Demographic parameters in Type 2 Diabetic Subjects.

Results obtained in this study showed an inversely and highly significant difference between kisspeptin and age ($p < 0.0001$). Also there was inverse significant difference between BMI and kisspeptin ($p = 0.002$). However, no significant association were observed for SBP ($p = 0.325$), DBP ($p = 0.243$) and waist circumference ($p = 0.982$) as shown in Table 4.5.

Table 4.5: Correlation of Kisspeptin levels with Demographic parameters in type 2 diabetic subjects

Kisspeptin levels (ng/ml) in Type 2 Diabetic subjects		
(N = 60)		
Parameters	Coefficient of correlation (r)	p- value
Age (yrs.)	-0.590**	0.0001
BMI (kg/m ²)	-0.389**	0.002
SBP (mmHg)	0.008	0.950
DBP (mmHg)	0.092	0.482
Waist circumference (cm)	-0.116	0.378

Table 4.5 shows data presented as Spearman's correlation coefficient, r. *Correlation is significant at $p < 0.05$; **Correlation is greatly significant at $p < 0.01$. BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure.

4.9 Correlation of kisspeptin levels with Biochemical Parameters in Type 2 Diabetic Subjects

The results of association of kisspeptin levels with biochemical indices of the type 2 diabetic subjects are presented in Table 4.6. Highly significant association with kisspeptin was established for high density lipoproteins ($p=0.005$) and an inverse significant association was found for cardiovascular risk ($p=0.012$). No correlation with kisspeptin was however detected for fasting blood glucose ($p=0.137$), glycated haemoglobin ($p=0.549$), total cholesterol ($p=0.382$), triglycerides ($p=0.216$), low density lipoproteins ($p=0.877$), and very low density lipoproteins ($p=0.254$).



Table 4.6: Correlation of kisspeptin levels with Biochemical parameters in type 2 diabetic subjects

Parameters	Kisspeptin levels (ng/ml) in Type 2 Diabetic subjects (N = 60)	
	Coefficient of correlation (r)	p- value
FBG (mmol/L)	0.194	0.137
HbA1c (%)	0.079	0.549
T. Chol (mmol/L)	0.115	0.382
TG (mmol/L)	-0.162	0.216
HDL (mmol/L)	0.362**	0.005
LDL (mmol/L)	0.020	0.877
VLDL (mmol/L)	-0.150	0.254
T.Chol/HDL	-0.321*	0.012

Table 4.6 shows data presented as Spearman's correlation coefficient, r. *Correlation is significant at $p < 0.05$; **Correlation is greatly significant at $p < 0.01$. FBG: fasting blood glucose; HbA1c: glycated haemoglobin; T. Chol: total cholesterol; TG: triglycerides; HDL: high density lipoprotein; LDL: low density lipoprotein; VLDL: very low density lipoprotein; T. Protein: total protein

4.10 Correlation of kisspeptin levels with pituitary hormones and testosterone in Type 2**Diabetic Subjects**

Association between testosterone, luteinizing hormone and follicle stimulating hormone with kisspeptin was investigated among type 2 diabetic subjects and results shown in Table 4.7. Highly significant relationship with kisspeptin was observed for luteinizing hormone ($p=0.001$), follicle stimulating hormone ($p=0.004$) and testosterone ($p=0.0001$).

Table 4.7: Pituitary Hormones association with kisspeptin levels in Type 2 Diabetic Subjects

Parameters	Kisspeptin levels (ng/ml) in Type 2 Diabetic subjects (N = 60)	
	Coefficient of correlation (r)	p- value
LH (mIU/ml)	0.432**	0.001
FSH (mIU/ml)	0.366**	0.004
Testosterone (ng/ml)	0.531**	0.0001

Table 4.7 shows data presented as Spearman's correlation coefficient, r. *Correlation is significant at $p<0.05$; **Correlation is greatly significant at $p<0.001$. LH: luteinizing hormone; FSH: follicle stimulating hormone.

4.11 Risk factors for low kisspeptin levels in type 2 diabetic males

Parameters that showed a significant correlate with kisspeptin levels in diabetic subjects were further investigated to see if they can be used as a predictive parameter for low testosterone in diabetic subjects. Results in Table 4.8 showed that the risk of developing low kisspeptin levels (<6.3ng/ml) in type 2 diabetic subjects is more likely to be predicted using their testosterone levels (OR-1.47, p=0.003) and age (OR-0.89, p=0.004). The rest of the parameters showed no significant predictive value with low kisspeptin in the diabetic subjects.

Table 4.8: Possible factors for low kisspeptin in type 2 diabetic males

Risk factors	OR	95% CI	p-value
Testosterone	1.473	1.143-1.901	0.003*
LH (mIU/ml)	1.055	0.930-1.159	0.434
FSH (mIU/ml)	1.039	0.930-1.159	0.500
HDL (mmol/L)	1.886	0.464-7.662	0.375
T. Chol/HDL	0.802	0.574-1.119	0.194
Age (yrs.)	0.890	0.823-0.963	0.004*
BMI (kg/m²)	0.938	0.758-1.160	0.554

Table 4.8 showed a relationship between significant correlates (testosterone, LH, FSH, HDL, T. Chol/HDL, age and BMI) and low kisspeptin levels in diabetic subjects. Results are given as odd ratio (OR). LH=luteinizing hormone, FSH=follicle stimulating hormone, HDL= high density lipoproteins, T. Chol/HDL=cardiovascular risk, BMI= body mass index. *Odd ratio is significant (p<0.05)

CHAPTER FIVE

DISCUSSION

Diabetes mellitus has been associated with many complications including neuropathy, retinopathy as well as low reproductive hormone levels resulting in infertility in these subjects (George *et al.*, 2013). Low levels of the reproductive hormones cause a series of symptoms including low GnRH secretion as a result of decreased kisspeptin secretion (Jyothis & Seminara, 2012). These decreased levels cause an adverse effect on the LH and FSH secretion and ultimately testosterone levels in men (Oh *et al.*, 2002). Kisspeptin has been implicated in the signalling of reproductive hormones however; kisspeptin levels have not been studied in T2DM males in Ghana.

In this study, the demographic characteristic indicated no significant difference in mean age between the diabetics and non-diabetic males (Table 4.1). This could be because subjects were age-matched. Also a significantly higher body mass index was found in the diabetic subjects than the non-diabetic males (Table 4.1). This supported report by WHO, (2011) that predicted 90% of individuals that developed type 2 diabetes was as a result of excess body weight. The study also showed a significant difference in systolic and diastolic blood pressure between diabetics and non-diabetics (Table 4.1). This was in agreement with Amoah *et al.*, (2002) who stated that an increase in body mass index and blood pressure decreases glycaemic control. Some of the diabetics in this study were hypertensive (Appendix E) and this could be the reason why the blood pressure was high. A significant higher waist circumference was observed in diabetic than in non-diabetics (Table 4.1). This observation supports work by the InterAct Consortium, (2012), stating that the possibility of developing type 2 diabetes increases with increase in adiposity levels. Thus, a high waist circumference (WC) indicating high levels of adiposity upsurges the possibility of developing type 2 diabetes in adults.

The mean age of the diabetic subjects negatively correlated with kisspeptin levels. This result was consistent with earlier works (Pinto *et al.*, 2012; Leonor *et al.*, 2012; Irfan *et al.*, 2014) which

indicated that kisspeptin had a direct effect on the gonads causing a decrease in gonadal function as age advances. Also negative association was found among BMI and kisspeptin levels in the diabetic subjects. This result confirmed findings by researchers (Clement *et al.*, 1998; Kapoor *et al.*, 2005). The association of kisspeptin levels with blood pressure (SBP and DBP) and waist circumference in the diabetics was not significant (Table 4.5). This result confirms work done by (Donato *et al.*, 2011) that kisspeptin on no account influence blood pressure.

The diabetic subjects in this study were grouped according to duration of the disorder (Figure 4.1). It was observed that majority of the diabetic subjects in our study have been diabetic for five years and this number decreased as the diabetic subject's duration of diabetes increased. This could be as a result of poor management after first 5 years which might have led to diabetic complications and subsequently death in these males with T2DM.

The study also showed a significantly higher fasting blood glucose (FBG) and glycated haemoglobin (HbA1c) in diabetic compared with non-diabetic subjects (Table 4.2). This was because impaired glucose control tends to increase the concentration of glucose in the body. As the glucose concentration increases, the exposure of glucose to certain proteins to form glycated derivatives also increases (American Diabetes Association, 2016). As such, glycated haemoglobin in the diabetics in this study was greater than in the non-diabetics (Table 4.2) and this agreed with work done by Selvin *et al.*, (2010). Thus both FBG and HbA1c are risk factors for the development of type 2 diabetes complications (Nicholas *et al.*, 2013). The study also showed no association between kisspeptin and fasting blood glucose in diabetic subjects (Table 4.6). This means that blood glucose had no significant effect on kisspeptin levels. There was no association between glycated haemoglobin (HbA1c) and kisspeptin levels in the diabetic subjects. This was in agreement with work done by Rhoden *et al.*, (2005).

This study also showed significantly high levels of total cholesterol, triglycerides, low density lipoproteins, very low density lipoprotein, and cardiovascular risk (T. Chol/HDL) in diabetics than

non-diabetics. However, a low level of high density lipoproteins was found in diabetics than the non-diabetic controls (Table 4.2). This result was consistent with earlier works (Holt, 2004; Garg *et al.*, 2014; Ali *et al.*, 2015). According to Ali *et al.*, (2015), non-availability of glucose to cells hinders the uptake of lipids and lipoproteins in the body which can result in high lipid in plasma of diabetics and increasing atherosclerosis development. People living with T2DM often have low levels of HDL and high triglycerides which was also found in this study. Thus the high T. Chol/HDL ratio, an indicator of coronary functions is as a result of high cholesterol and low HDL in the diabetic males compared with the non-diabetics (Garg *et al.* 2014). The study further showed a positive correlation of kisspeptin levels with HDL; T. Chol/HDL whiles the other parameters (T. Chol, VLDL, LDL, and TG) showed a negative correlation with kisspeptin levels (Table 4.6). This result was not consistent with work by Overgaard *et al.*, (2015) who found a strong correlation between kisspeptin and triglycerides. Kisspeptin has been reported to take part in the maturation and metabolism of adipocytes, thus a direct effect on lipolysis hence less lipids (Pruszyriska-Oszmalek *et al.*, 2017), hence kisspeptin increases lipolysis. A significant association between kisspeptin and HDL was found however, there was a negative correlation between kisspeptin and T. Chol/HDL in the diabetic subjects (Table 4.6) which agreed with Overgaard *et al.*, (2015).

Dhillon *et al.*, (2005) initially reported that the administration of exogenous kisspeptin stimulated the Hypothalamic-Pituitary Gonadal axis in human males causing a rise in luteinizing hormone, follicle stimulating hormone and total testosterone. In this study low kisspeptin levels was described as kisspeptin levels below the reference range. Incidence of the level of kisspeptin was about three (3) times lower in diabetic subjects relative to non-diabetic subjects (figure 4.3). It is worth noting that among the diabetic males in our study, kisspeptin levels decreased with increasing duration of diabetes (Table 4.4). These results were consistent with earlier works (Castellano *et al.*, 2005; Farooqi & O'Rahilly *et al.*, 2009; Bruning *et al.*, 2000; Costanzo *et al.*, 2014; Wahab *et al.*, 2015). The gonadotropin hormones (LH and FSH) in diabetic subjects were

considerably higher than controls (Table 4.3), however, testosterone levels were lower in the diabetics than the non-diabetics (Table 4.3).

Since kisspeptin levels in the diabetic subjects were low, it was expected that LH and FSH will be lower than the non-diabetics but that was not so. This was because the Leydig cells in the testis in the diabetic subjects had been damaged by high glycaemia and therefore was not responding well to the gonadotropin hormones (Messanger *et al.*, 2005) leading to low testosterone in the diabetic subjects (Table 4.3). The low testosterone led to lack of negative feed-back inhibition on LH and FSH secretion, a case of primary hypogonadism. These findings were consistent with earlier studies (Smith *et al.*, 2006; Dahl *et al.*, 2009; Asare-Anane *et al.*, 2013; Daka *et al.*, 2015). The low testosterone level could also be as a result of insulin resistance causing an increase in the conversion of testosterone to estradiol (E₂). Also, the incidence of low testosterone with high LH and FSH further confirms the possibility of testicular dysfunction in these men (Asare-Anane *et al.*, 2013). This could be the reason why although a significant association existed between kisspeptin and LH and FSH in type 2 diabetic males (Table 4.7), these hormones could not be used as significant indicators for low kisspeptin levels in the diabetic subjects (Table 4.8). In addition, low kisspeptin with high gonadotropins recorded in this study among diabetic subjects showed that kisspeptin secretion could not only be from the hypothalamus region alone but other tissues (testis, kidney, pancreas, ovary, and placenta) contributed to kisspeptin levels in humans (Katagiri *et al.*, 2007).

A highly significant association was found in this study between kisspeptin and testosterone in type 2 diabetic males (Table 4.7). A nominal regression analysis done between testosterone and low kisspeptin levels in diabetic subjects showed a significant odd ratio (OR) value of 1.5 times of developing low kisspeptin (Table 4.8) making low testosterone strong predictive variable to low kisspeptin in males with type 2 diabetes mellitus. These results were in agreement with previous works done (Dhillon *et al.*, 2005; Smith *et al.*, 2006; Pinto *et al.*, 2012; Irfan *et al.*, 2014).

5.1. Conclusion

Plasma kisspeptin levels were considerably low in type 2 diabetic males in Ghana relative to the non-diabetic males. The hypothesis is, however, rejected for kisspeptin levels which were decreased significantly in type 2 diabetic males compared with controls. The study showed no significant association between SBP, DBP, waist circumference, FBG, HbA1c, T. Chol., TGs, LDLs, and VLDLs with kisspeptin levels in type diabetic males in Ghana.

Although the association between glycated haemoglobin and kisspeptin levels was not significant, a higher percentage of glycated haemoglobin was seen in the diabetics than the control group.

Negative significant association of age, body mass index (BMI), and cardiovascular risk (T. Chol/HDL) with kisspeptin levels in type 2 diabetic males in Ghana was observed in this investigation. While a positive significant association was found between high density lipoproteins (HDL), testosterone, luteinizing hormone, and follicle stimulating hormone with kisspeptin levels in diabetic males in Ghana.

This study also demonstrated that advancement in age and low testosterone are negative influences for the progress of low kisspeptin in type 2 diabetic males in Ghana. This research showed that low testosterone can lead to low kisspeptin levels in T2DM males.

5.2. Recommendations

Further investigation should be made on larger sample size, different age category, on females with shorter duration of diabetes.

Further work should consider effect of exogenous administration of kisspeptin on GnRH production and release of gonadotropins and insulin secretion in the plasma.

5.3. Limitations

The study could not investigate into the diagnostic parameters such as C-peptide and estradiol that may help our understanding between kisspeptin and diabetes type 2 diabetes males. This limitation was due to logistic challenge, unavailability of reagents and machines.

Another limitation encountered in this study was lack of reference range of kisspeptin levels for males and females apparently healthy Ghanaians. This limitation may be the result of little work being done about the hypothalamic hormone kisspeptin in Ghana.



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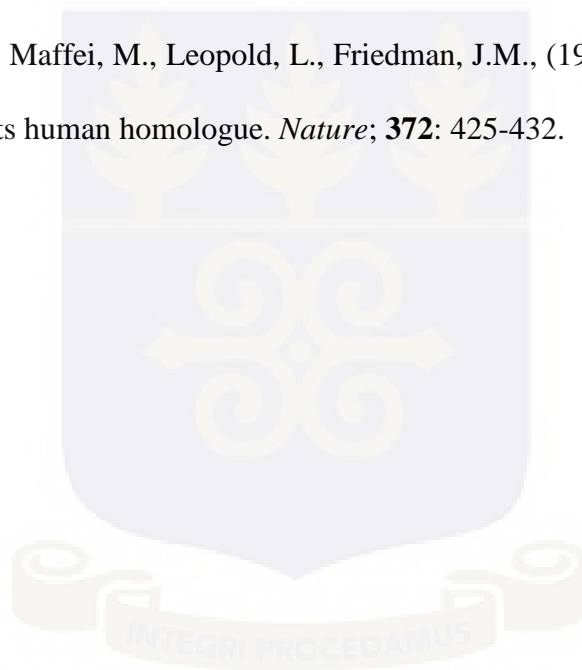
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APPENDIX

APPENDIX A: ETHICAL APPROVAL



UNIVERSITY OF GHANA COLLEGE OF HEALTH SCIENCES

ETHICAL AND PROTOCOL REVIEW COMMITTEE

Ref. No.:

22nd September, 2016.

Genevieve Kwao-Zigah
Department of Chemical Pathology
School of Biomedical and Allied Health Sciences
University of Ghana
Korle-Bu, Accra

ETHICAL CLEARANCE

Protocol Identification Number: **CHS-Et/M.1 – P 4.2/2016-2017**

The Ethical and Protocol Review Committee of the College of Health Sciences on the 22nd of September, 2016 unanimously approved your research proposal.

TITLE OF PROTOCOL: **“Plasma Kisspeptin Levels in Type 2 Diabetic Males at the Korle-Bu Teaching Hospital”**

PRINCIPAL INVESTIGATOR: **Genevieve Kwao-Zigah**

This approval requires that you submit six-monthly review reports of the protocol to the Committee and a final full review to the Ethical and Protocol Review Committee at the completion of the study. The Committee may observe, or cause to be observed, procedures and records of the study during and after implementation.

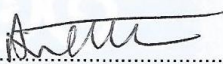
Please note that any significant modification of this project must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this study to the Ethical and Protocol Review Committee within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this study. You will therefore be required to furnish the Committee with any manuscript for publication.

This ethical clearance is valid till 31st August, 2017.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

Signed: 

PROFESSOR ANDREW A. ADJEI
CHAIRPERSON, ETHICAL AND PROTOCOL REVIEW COMMITTEE

cc: Provost, CHS
Dean, SMD
Head of Department

APPENDIX B: INFORMED CONSENT FORM

I, Genevieve Kwao-Zigah of Chemical Pathology Department, University of Ghana School of Biological and Allied Health Sciences (SBAHS) wish to embark on a study entitled, Plasma Kisspeptin Levels in Ghanaian males with Type 2 Diabetes Mellitus (T2DM). The purpose of this study is to determine the levels of plasma kisspeptin hormone in Type 2 Diabetes mellitus males in the Ghanaian population. The development of Type 2 Diabetes mellitus has being found to be due to low levels of kisspeptin in plasma and consequently leads to low levels of testosterone in these men.

You will be asked few routine questions about your personal details, demographic background, your lifestyle etc. The advantages of the study are to inform and educate you on hormone Kisspeptin levels in the plasma and its susceptibility to the development of T2DM in males. It will be appreciated if you volunteer, though participation is entirely and strictly confidential. You may choose to withdraw from the study whenever you wish. Participants of the study will undergo an overnight fast after which 10mls of blood will be withdrawn. Both procedures may involve slight discomfort. The amount of blood to be taken by phlebotomists and used for the research study will not exceed 10mls.

You will be assured of the strictest confidentiality of your personal information. This study will adhere to all applicable protocols and will maintain quality assurance in accordance with good laboratory practice. The blood samples collected will bear an identification code to ensure anonymity, confidentiality, and ease of identification.

There is the possibility that you might not benefit directly from participation. However, the information obtained and conclusions were drawn will be applied in the adoption of relevant health policies as well as the appropriate care and management.

You will incur no costs and you will also not be paid for participating in this study. However, you will be entitled to know the outcome of the laboratory results and this will be well explained to

you. All data will be entered onto a lock/word-protected Microsoft Excel spreadsheet. Study questionnaires will be kept in a locked cabinet in a locked office.

My contact is 0540894579. You may call me for any further clarifications. Thank you for the cooperation and anticipated compliance to the study requirements.

Signature of Participant:

Date:

Signature of Researcher:

Date:

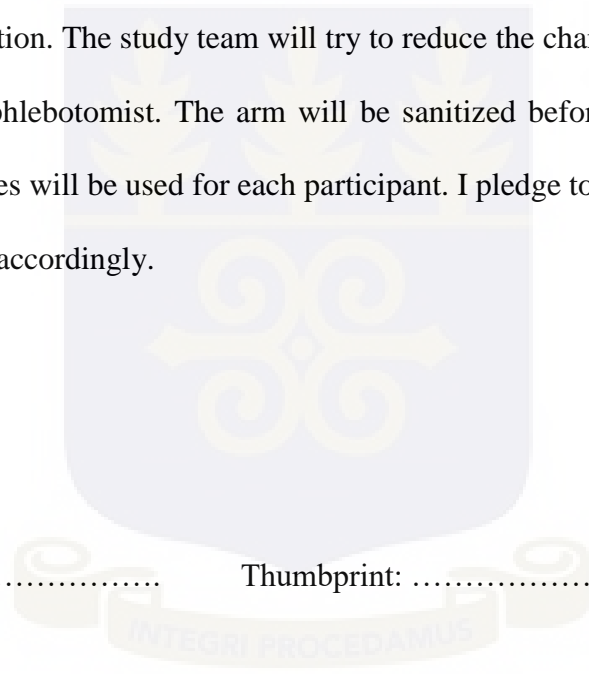


APPENDIX C: PARTICIPANT CONSENT RESPONSE

Research title: Plasma Kisspeptin levels in Type 2 Diabetes Mellitus males in the Ghanaian population.

I, have been invited to participate in this study research. I have been told of the purpose of this study and procedures have been explained to me which am to answer questions raised about plasma kisspeptin levels in Type 2 Diabetes Mellitus males. I have agreed to give information about my personal information such as my educational background, health history etc. I understand I will not be reimbursed monetarily for participating in this study. I am aware also of the dangers, risk, and discomforts that might be associated with the pain of blood collection. The study team will try to reduce the chances of those risks happening by employing trained phlebotomist. The arm will be sanitized before blood collection, and new sterile needles and gloves will be used for each participant. I pledge to adhere to the requirement of the study and I consent accordingly.

Signature: Thumbprint:



APPENDIX D: STUDY QUESTIONNAIRE-DATA SHEET

Participants ID:

Date:

A. SOCIO- DEMOGRAPHIC DATA

1. Age years 30-39 [] 40- 49 [] 50-59 [] 60 -69 []
2. Weight (kg): 3. Height (m): 4. BMI: (kg²/m)
5. BP 6. Pulse 7. Waist circumference

B. HEALTH HISTORY

8. Are you a diabetic? Yes [] No []
- 8b. if yes, how long have you been diabetic?
- 8c. Please indicate the type: i. Type 1 [] ii. Type 2 [] iii. Not certain []
9. Are you currently taking any insulin or pill to control diabetes? Yes [] No []
- 9b.If yes, how often are you supposed to take insulin/ pill? i. I don't take Insulin/pill [] ii. Occasionally as needed [] iii. Once a day [] iv. Twice a day [] v. Three or more times a day []
- 9c. Common diabetic drugs: i. Metformin oral [] ii. Actos oral [] iii. Glucophage oral [] iv. Januvia oral [] v. Others []
10. Do you have any medical condition? Yes [] No []
- 10b. if yes, please state the condition
11. Are you on any medication? Yes [] No []

11b. If yes, please mention.....

12. Do you take other medication or herbal treatment? Yes [] No []

12b. If yes, please state

C. LIFE-STYLE BEHAVIOURS

13. Do you smoke? Yes [] No []

13b. If yes, how often? i. Once a day [] ii. A couple a week [] iii. Monthly [] iv Once awhile []

14. Do you drink alcohol? Yes [] No []

14b. If yes, how often? i. Once a day [] ii. A couple a weekly [] iii. Monthly [] iv Once awhile []

14c. On average, how many servings of alcohol do you take daily? i. One

servings of alcohol: 120ml of wine (½ medium glass of dry wine) []

ii. 285ml of beer (1/2 large beer bottle, one full mini Guinness) []

iii. 30ml (1 tot of spirit, whiskey gin, akpeteshi and alcoholic bitters) []

iv. 60ml of (brandy, vermouth, aperitif) []

APPENDIX E: HYPERTENSIVE DRUGS TAKEN BY SUBJECTS

	Total	Total	Lisinopril	Nifedepine	Amlodipine	Losartan	Other
	subjects	number					drugs
	hypertension	treatment					
Diabetic	34	34	11	4	12	2	5
subjects							
(60)							
Control	10	5	2	4	3	0	1
group							
(60)							

