SERUM IMMUNOGLOBULIN LEVELS IN TYPE 2 DIABETIC PATIENTS IN KORLE-BU TEACHING HOSPITAL, GHANA

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THIS THESIS IS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES, UNIVERSITY OF GHANA, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MASTER OF PHILOSOPHY (M.PHIL) DEGREE IN CHEMICAL PATHOLOGY

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DECLARATION

I, Collins Paa Kwesi Botchey, do hereby declare that this thesis, which is being submitted in fulfillment of the requirements for an M.Phil. Degree in Chemical Pathology is the result of my own research conducted at the National Diabetic Research and Management Centre (Korle-Bu) and the Central Laboratory unit of the Korle-Bu Teaching Hospital (KBTH) under the supervision of Dr. Henry Asare - Anane and Dr. Kwadwo Asamoah Kusi. References to other people's work have been duly acknowledged. This thesis presents results of original research undertaken by me and neither all nor part of this thesis has been presented for another degree in this institution or elsewhere.

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DEDICATION

*To my dear parents **Symonds** and **Mary**.*

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ABSTRACT

The incidence of type 2 diabetes is on the increase and on-going research suggests that the increased concentrations of some circulating immunoglobulins among type 2 diabetic patients may be non-specific signs of the development of diabetic complications. Immunoglobulins, of which the most abundant are IgA, IgG and IgM, play an essential role in the body's immune system by assisting in destroying foreign substances when they invade the body. Elevated immunoglobulin levels have however been strongly linked to the development and progression of diabetic complications. The aim of this study was to investigate serum levels of immunoglobulins (IgA, IgG and IgM) in Ghanaian type 2 diabetics, and to identify factors that may be associated with the variation in immunoglobulin levels that may lead to diabetic complications. One hundred and ninety two (96 type 2 diabetic and 96 non-diabetic) subjects were recruited for the study. Diabetic subjects were contacted during outpatient clinic appointments at the National Diabetes Research and Management Centre, Korle-Bu Teaching Hospital. Matched non-diabetic controls were recruited from the catchment area. The immunoglobulins (IgA, IgG and IgM) and interleukin 6 (IL-6) concentrations were measured in subjects sera using commercially available ELISA assays performed according to manufacturer's instructions. Blood chemistry, blood pressure, body mass index and waist circumference of subjects were assessed. Serum IgA (g/L) was higher in the diabetic than in the non-diabetic subjects (0.89 vs 0.74, $p=0.043$). Serum IgG (g/L) was also significantly higher in the diabetic compared with the non-diabetic subjects (7.58 vs 7.29, $p<0.001$). The difference in serum IgM between diabetics and the non-diabetics was not significant. Serum IL-6 (pg/mL) was higher (1.70) in Type 2 diabetic

subjects than in the non-diabetics (0.99) ($p<0.001$). The results showed that waist circumference (WC) and body mass index (BMI) were significantly higher in the diabetics than in the non-diabetic subjects ($p<0.001$ in both cases). However, there were no relationships between the WC and the immunoglobulin levels; and BMI and the immunoglobulin levels. There was no significant correlation between BMI and IL-6 ($p=0.337$). Albumin, Low Density Lipoprotein (LDL), Very Low Density Lipoprotein, and cardiovascular risk (Total Cholesterol / HDL) did not show any significant association with the immunoglobulin levels. There was association between fasting blood glucose (FBG) and IgA ($p=0.001$). FBG was also associated with IL-6 ($p=0.025$). Serum IgG levels inversely correlated with glycated haemoglobin (HbA_{1c}) ($p=0.036$). Serum IL-6 showed significant correlation with both IgA ($p=0.001$) and IgM ($p=0.003$). It can be concluded that the serum immunoglobulin levels were elevated in the Type 2 diabetic subjects.

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LIST OF ABBREVIATIONS

ADA……..American Diabetes Association

ALB……..…….……....…Albumin

β-cell…….……beta cell

BCG……....Bromocresol Green

BMI…….……Body Mass Index

BP……..Blood Pressure

cm……..centimeter

CDC……Centre for Disease Control

DBP……Diastolic Blood Pressure

DHT…….……..…Dihydrotestosterone

DM……Diabetes Mellitus

EDTA…….Ethylene diamine tetra-acetic acid

FBG……..…Fasting Blood Glucose

FDA……..Food and Drug Administration

Fig……..Figure

GDM……..Gestational Diabetes Mellitus

g/L…… Gram per liter

GLU……..…….Glucose

GMP……..…….Guanosine monophosphate

HbA_{1c}……...Glycated haemoglobin

HDL……..High Density Lipoprotein

HRP……..Horseradish Peroxidase

IDA…….International Diabetes Association

IgA……Immunoglobulin A

IgG……Immunoglobulin G

IgM……Immunoglobulin M

IL-6……..……..Interleukin 6

Kg/m^2…….Kilogram per meter square

LDL……...…Low Density Lipoprotein

MetS…….Metabolic Syndrome

mL.....……...Milliliters

mmol/L……Millimole per liter

NDMRC……National Diabetes Management and Research Center

^oC……..…Degree Celsius

%.....Percentage

OD…….Optical Density

PBS……Phosphate Buffered Saline

SD…….Standard Deviation

SBP…….Systolic Blood Pressure

TBS……Tris-Buffered Saline

T.Chol……..……Total Cholesterol

TG……..……Triglyceride

TMB……..3, 3', 5, 5', - tetramethyl benzidine

T.Prot……..……Total Protein

UK……..….United Kingdom

VLDL-C……..Very Low Density Lipoprotein

Vs……...…….Versus

WC…….……Waist Circumference

WHO……..……World Health Organization

µL……....Microliters

>……..……..Greater Than

<..... Less Than

CHAPTER ONE

INTRODUCTION

1.1. Background

Diabetes mellitus is a complex metabolic disorder marked by persistent hyperglycaemia, resulting from defects in insulin secretion, insulin action or both (WHO, 1999). Majority of the cases of diabetes fall into two broad categories. Type 1 diabetes which is caused by an absolute deficiency of insulin secretion and type 2 diabetes mellitus (T2DM), which is much more prevalent, and is caused by a combination of resistance to insulin action and inadequate compensatory insulin response.

The changes in human activities and lifestyle over the past century have resulted in a dramatic rise in the incidence of diabetes throughout the world. According to Dobson (2000), the incidence of type 2 diabetes has doubled globally over the past three decades. In the 1950s and 1960s, diabetes prevalence in Ghana was estimated at between 0.2 and 0.4 percent in adults (Amoah, 2002). However, analysis of admissions to the Korle-Bu Teaching Hospital reveals that diabetes accounts for 6.8 percent of all adult admissions (Amoah, 2002). With increasing numbers of patients suffering from type 2 diabetes, the consequent prevalence of complications resulting from chronic inflammation is also rising. Heart disease and stroke are the leading causes of death in these patients. Patients with type 2 diabetes are also at risk of nerve damage (neuropathy) and abnormalities in blood vessels (vascular injuries). Though the causes of type 2 diabetes are unknown, risk factors such as family history of diabetes, adults over 45 years, being overweight, history

of gestational diabetes, hypertension, stroke and metabolic syndrome are believed to predispose to the disease (Heather, 2008).

Serum immunoglobulins play a significant role in the body's defense against pathogens and their levels are determined routinely in clinical practice to provide key information on the humoral immune status of patients. There are five classes of immunoglobulins: immunoglobulins A (IgA), G (IgG), M (IgM), D (IgD) and E (IgE). The major immunoglobulins based on their abundance and sizes are the IgA, IgG and IgM while the minor ones are IgD and IgE. IgA protects mucosal surfaces because of its presence in body secretions. It provides immunity to infant digestive tract because of its presence in the colostrums. IgG enhances phagocytosis, neutralizing toxins and viruses. It also protects foetus and new born babies because it crosses the placenta. IgM is the first antibody produced during an infection and it is effective against microbes and agglutinating antigens. Determining immunoglobulin levels of populations with specific disease conditions is important for assessing the extent to which these levels have changed. Generally, serum immunoglobulin concentrations tend to increase with age (Gonzalez-Quintela *et al.,* 2008) or exposure to pathogens (antigens) (Shetty, 1993). Earlier studies have also reported increase in serum immunoglobulin A levels in type 2 diabetics (Gonzalez-Quintela *et al.,* 2008; Ardawi *et al*., 1994) However, the variations and relationships between the major immunoglobulin (IgA, IgG and IgM) levels in type 2 diabetics, the most prevalent form of diabetes, are not completely understood.

Interleukin 6 (IL-6) is a cytokine produced by many different cell types such as immune cells and adipose tissue. It plays an important role in the mediation of inflammatory

responses (Orban *et al*., 1999). It is also involved in the development and acceleration of microvascular complications in patients with diabetes (Wegner *et al.*, 2013).

Many other pro-inflammatory cytokines such as IL-12 and TNF-α are linked with inflammatory reaction and were shown to increase the risk of T2DM (Hu *et al*., 2004; Schmidt *et al*., 1999). These pro-inflammatory cytokines can enhance insulin resistance directly in adipocytes, muscle and hepatic cells, leading to systemic disruption of insulin sensitivity and impaired glucose homeostasis (Pickup and Crook, 1998; Hu *et al*., 2004; Schmidt *et al*., 1999). Inflammation is considered a key regulator of pathogenesis of T2DM especially in the development of complications as a result of physiological and cellular activities, but what triggers this inflammation is still unknown (Pickup and Crook, 1998). An experiment performed using rats showed that IL-6 induced a dose-dependent inhibition of glucose–stimulated insulin release from rat pancreatic islets of Langerhans (Southern *et al*., 1990). If diabetes which is a disorder of insulin deficiency or impaired effectiveness of insulin action, and interleukin 6 is known to inhibit glucose stimulated insulin release, then interleukin 6 is an important cytokine that needs to be thoroughly investigated.

1.2. Problem Statement

The incidence of type 2 diabetes in Ghana is increasing at an alarming rate (Amoah, 2002). The high prevalence of type 2 diabetes among Ghanaian adults leads to high morbidity and low socio-economic activities. This has been a high health-burden to the nation in terms of health care management.

It is known that diabetes increases susceptibility to various infections (Heather, 2008). Therefore, people with type 2 diabetes are more susceptible to developing infections than non-diabetics since their immune system is likely to become weakened.

Changes in immunoglobulin levels may be implicated in diabetic complications and this further reduces productivity and may even increase mortality rates (Rodriguez-Segade *et al*., 1996).

1.3. Justification

Changes in immunoglobulin levels may be implicated in the susceptibility to infection and diabetic complications. However, changes in immunoglobulin and cytokine levels in type 2 diabetes mellitus are not fully understood. Studies in Spain have shown that increased concentrations of some circulating immunoglobulins may be nonspecific signs of the development of diabetic complications (Rodriguez-Segade *et al*., 1991; and Rodriguez-Segade *et al*., 1996). Elevated levels of some immunoglobulins which may serve as early signs in the aetiogenesis of diabetic complications have however not been demonstrated in Ghana. Considering the important role immunoglobulins play in the body's immune system, there is the need to elucidate the serum immunoglobulin levels in type 2 diabetics and their association with diabetic complications such as nephropathy and hypertension so as to improve the management of the disease. There is lack of data regarding changes in immunoglobulin concentrations in patients with diabetes mellitus in Ghana. This work therefore sought to determine the serum immunoglobulins (IgA, IgG and IgM) levels in type 2 diabetic patients and compare with non-diabetic individuals.

The economic and social costs of diabetes and its management will be lessened for health care services if these clinical complications can be identified early enough and prevented or managed.

1.4. Hypotheses

Type 2 diabetes mellitus does not influence levels of serum immunoglobulins IgA, IgG and IgM as well as Interleukin 6

1.5. Aim of the Study

The aim of this study was to investigate serum levels of immunoglobulins (IgA, IgG and IgM) and interleukin 6 in Ghanaians with type 2 diabetes.

1.6. Specific Objectives

1. To investigate the associations between immunoglobulins (IgA, IgG and IgM) and interleukin 6 levels and the general (demographic and clinical) characteristics of the study population.
2. To assess the relationship between biochemical indices (fasting blood glucose, fasting lipids, total proteins and albumin) and serum immunoglobulin levels in the study population.
3. To investigate the association between serum immunoglobulin levels and interleukin 6 in the study population.

CHAPTER TWO

LITERATURE REVIEW

2.1. Definition of Diabetes Mellitus

Diabetes mellitus is a complex metabolic disorder caused by defects in insulin secretion, or action or both (WHO, 1999). It is characterized by hyperglycemia which is often accompanied by excessive thirst, polyuria and polyphagia (Celik and Zimmet, 2001). A range of biochemical aberrations are present in diabetes mellitus, but the fundamental defects to which most of the abnormalities can be traced are reduced entry of glucose into various "peripherial" tissues (insulin resistance) and increased release of glucose into the general circulation from the liver (gluconeogenesis and glycogenolysis) (Knop *et al.*, 2007). These events culminate in high level of plasma glucose (hyperglycaemia) with the cells "starving" due to low level of intracellular glucose. In diabetes therefore, the body tissues have to metabolize protein and fat reserve for energy (Rizza, 2010).

Besides carbohydrate dysmetabolism, diabetes is also associated with aberration in protein and lipid metabolism. In diabetes, amino acids catabolism increases, with the liver converting amino acids into glucose under stimulation of glucagon (Knop *et al.*, 2007). The principal abnormalities of fat metabolism in diabetes are acceleration of lipid catabolism, with increased formation of ketone bodies, and decreased synthesis of fatty acids and triglycerides (Mazzone *et al.*, 2008).

2.2. Classification of Diabetes Mellitus

There are three main types of diabetes mellitus. These are type 1 (T1DM), type 2 (T2DM) and gestational diabetes mellitus (GDM). Prior to the late 1970s, there was no consensus on the diagnostic criteria for diabetes. This led to much confusion and precluded any meaningful comparison of the prevalence of diabetes within or between populations. As noted by West (West, 1975), diabetologists classified as normal more than half of the one and two-hour values of glucose tolerance test considered to be abnormal by other well-qualified diabetologists. The first expert report on the criteria for the diagnosis of diabetes presented by the WHO in 1965, were subsequently modified and simplified by the WHO and the National Diabetes Group in the United States in 1979, 1980 and 1985 (World Health Organisation, 1980, 1985). The 1980 WHO Expert Committee proposed two major classes of diabetes mellitus, referred to as insulin-dependent diabetes mellitus (IDDM) or type 1 and non-insulin-dependent diabetes mellitus (NIDDM) or type 2. In the 1985 Study Group Report, the terms type 1 and type 2 were omitted but the classes IDDM and NIDDM were retained, and a class, Malnutrition Related Diabetes Mellitus (MRDM) was introduced. In both the 1980 and 1985 reports, other classes of diabetes mellitus included “other types” and impaired glucose tolerance (IGT) as well as gestational diabetes mellitus (GDM) (Alberti and Zimmet, 1998; World Health Organization, 1980, 1985).

An amendment of classification of diabetes based on both clinical stages and aetiopathogenesis of diabetes mellitus and other categories of hyperglycaemia was proposed (Kuzuya and Matsuda, 1997). It was argued that the terms “insulin-dependent diabetes mellitus” and “non-insulin-dependent diabetes mellitus” and their acronyms

"IDDM" and "NIDDM", should no longer be used; these terms were confusing and often resulted in patients being classified based on treatment rather than on pathogenesis. Therefore, terms type 1 and type 2 were reintroduced. The diabetes class named type 1 encompasses those cases attributable to an autoimmune process, as well as those with beta-cell destruction and who are prone to ketoacidosis for which neither the aetiology nor pathogenesis is known (idiopathic). It does not include those forms of beta-cell destruction of failure to which specific causes can be assigned such as cystic fibrosis, mitochondrial defects, etc. The type named type 2 includes the common major form of diabetes mellitus which results from defect(s) in insulin secretion, almost always with a major contribution from insulin resistance (Kuzuya & Matsuda, 1997).

The evidence for, and characteristics of, diabetes mellitus seen in undernourished populations was reviewed in a subsequent international workshop (Hoet *et al*., 1997; Tripathy and Samal, 1997). Whilst it appeared that malnutrition may influence the expression of several types of diabetes, the evidence that diabetes mellitus can be caused by malnutrition or protein deficiency per se was not convincing. Therefore it was recommended that the class, "malnutrition-related diabetes mellitus" (MRDM) be removed. The former subtype of MRDM, protein-deficient pancreatic diabetes, may be considered as malnutrition modulated or modified form of diabetes mellitus for which more studies were needed. The other former subtype of MRDM, fibrocalculous pancreatic diabetes, is now classified as a disease of the exocrine pancrease, fibrocalculous pancreatopathy, which may lead to diabetes mellitus (The Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes, 2010). The class "impaired glucose tolerance (IGT)" is now classified as a stage of impaired glucose

regulation, since it can be observed in any hyperglycaemic disorder and is itself not diabetes. A clinical stage of impaired fasting glycaemia (IFG) was introduced to classify individuals whose fasting glucose values were above the normal range but below those diagnostic of diabetes mellitus.

GDM was retained but now encompasses the group formerly classified as gestational impaired glucose tolerance and gestational diabetes mellitus. Thus the new classification system identifies four major types of diabetes mellitus; type 1, type 2, "other specific types" and GDM (Alberti & Zimmet, 1998).

GDM is carbohydrate intolerance resulting in hyperglycaemia of variable severity with onset or first recognition during pregnancy. It does not exclude the possibility that the glucose intolerance may occur before pregnancy, but has been previously unrecognized. The definition applies irrespective of whether or not insulin is used for treatment or the condition persists after pregnancy (Reece *et al*., 2010). Individuals at high risk for gestational diabetes include older women, those with previous history of glucose intolerance, those with history of large for gestational age babies, women from certain high risk ethnic group, and any pregnant woman who has elevated fasting or casual blood glucose levels (Xiang *et al.,* 2010). It may be appropriate to screen pregnant women belonging to high–risk populations during the first trimester of pregnancy in order to detect previously undiagnosed diabetes mellitus. Formal systematic testing for gestational diabetes is usually done between 24 and 28 weeks of gestation (Reece *et al*., 2010).

The aetiological classification of diabetes mellitus refers to physiological processes, defects or disorders which often result in chronic hyperglycaemia. The main aetiological

classes of diabetes mellitus are type 1 idiopathic diabetes mellitus, type 2 diabetes mellitus (T2DM) and "other types" diabetes mellitus. However the major classes of diabetes mellitus are the type 1 and type 2. Type 2 diabetes comprises approximately 90-95% of all cases of diagnosed diabetes mellitus, whilst type 1 comprises about 5-10% (Alberti and Zimmet, 1998; American Diabetes Association, 2012). In both sub-classes of type 1 diabetes mellitus there is absolute deficiency of insulin. The onset of symptoms is abrupt. Insulin is often required for survival to prevent the development of ketoacidosis, coma and death (The Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes, 2010).

Autoimmune TIDM results from an inflammatory autoimmune and T-cell-mediated destruction of the insulin-producing beta cells of the pancreas, usually leading to absolute insulin deficiency. Insulin resistance does not play a major role in its pathogenesis (American Diabetes Association, 2012). Majority of these individuals are lean, young and with autoimmune markers associated with diabetes mellitus and most of them have susceptibility. The human leukocyte antigen (HLA) haplotypes with linkage to the Major Histocompatibility Complex (MHC) Class II genes DQA, DQB and DRB (Pociot *et al*., 2010) are responsible for regulation of immune system in humans. Individuals with this subclass of diabetes mellitus often become dependent on insulin for survival eventually and are at risk for ketoacidosis (Polychronakos and Li, 2011). At this stage of the disease there is little or no insulin secretion as manifested by low or undetectable levels of plasma C-peptide. The rate of destruction of beta-cells is quite variable, being rapid in some individuals and slow in others (Alberti and Zimmet, 1998). The rapidly progressive form is commonly observed in children, but also may occur in adults (American Diabetes

Association, 2012). The slowly progressive form generally occurs in adults and is sometimes referred to as latent autoimmune diabetes in adults (LADA). A number of patients, particularly children and adolescents, may present with unprovoked ketoacidosis as the first manifestation of the disease (The Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes, 2010). Others have modest fasting hyperglycaemia that can rapidly change to severe hyperglycaemia and/or ketoacidosis in the presence of infection or other stress. Still others, particularly adults, may retain residual beta-call function, sufficient to prevent ketoacidosis, for many years (Alberti & Zimmet, 1998).

Idiopathic TIDM is a subclass of TIDM with an unknown aetiology, but it is likely related to insulin resistance and transient B-cell dysfunction, perhaps because of glucose desensitization (Giugliano *et al*., 2008). Patients with idiopathic T1DM have permanent insulinopenia, and they are prone to ketoacidosis, but have no evidence of autoimmunity and it is not HLA associated (Polychronakos and Li, 2011). Idiopathic T1DM has been described mostly in African-Americans and Asians as well as other ethnic groups (Pociot *et al.*, 2010). In most patients with idiopathic T1DM, insulin therapy is better in terms of glycaemic control than either oral hypoglycaemic agents or diet therapy alone and that long-term glycaemic control is better maintained with insulin treatment (American Diabetes Association, 2012).

T2DM refers to a condition of chronic hyperglycaemia as a result of insulin resistance with a background of gradual beta-cell destruction. Insulin levels may be normal, decreased or increased. There are two identifiable defects in T2DM. There is predominantly insulin resistance, which is decreased ability of insulin to act on peripheral tissues (McFarlane *et al*., 2001). Thus initially, and usually throughout their lifetime,

these individuals may not need insulin treatment for survival. There may also be predominantly impaired insulin secretion due to B-cell dysfunction. However insulin resistance is the primary defect, preceding the derangement in insulin secretion and clinical diabetes by about 20 years (Giugliano *et al*., 2008). The specific aetiologies of this class of diabetes mellitus are unknown, however autoimmune destruction B-cells of the pancreas does not occur and patients do not have other known specific causes of diabetes mellitus listed under "other specific types" diabetes mellitus.

2.2.1. Type 1 Diabetes Mellitus

Type 1 diabetes is caused by an absolute deficiency of insulin and develops when the body's immune system destroys the insulin-producing pancreatic beta cells. In Africa, the presence of atypical forms of diabetes makes it difficult to classify patients, based on usual clinical criteria, as having T1DM and T2DM (Perret *et al*., 1996; Perret and Nguemby-Mbina, 1991). This typical form of diabetes has been suggested by the 'accelerator hypothesis' to be overlay, rather than overlap, between TIDM and T2DM; implying that T1DM and T2DM may share some common aetiologies (Wilkin, 2001). The difficulty in classifying and diagnosing some forms of diabetes in Africa may explain why approximately half (42-64%) of Africa patients initially treated with insulin do not have classical T1DM and may enter prolonged remission (Li *et al*., 2004; McFarlane *et al*., 2001). It is widely believed that classical T1DM is less common in Africa than in Europe or North America, and this is certainly common clinical experience. However, early out-of-hospital mortality may confound this impression, and the question remains open (Gill *et al*., 2009).

Epidemiology studies of T1DM in sub-Saharan Africa are difficult to conduct because of problems in finding cases as well as enumeration of the background population; few reports are available in literature (Motala *et al*., 2008). In published studies, prevalence of T1DM is low: 0.33 per 1000 in Nigerian and 0.95 per 1000 in Sudanese school children (Majaliwa *et al*., 2008). Incidence of T1DM has been reported to be as low as 1.5 per 100,000 per year in Tanzania and high as 10.1 per 100,000 per year Sudan (Motala, 2002). This large difference could be attributable to methodological discrepancies between studies, or true ethnic dissimilarities, because Tanzanian people are predominantly of Africa origin, whereas Sudanese populations are of mixed Arab and African heritage. However, the prevalence of T1DM is lower than that of western countries (Mbanya *et al*., 2010).

Finding of clinical studies from South Africa, Tanzania and Ethiopia suggest that the characteristics of T1DM in people from sub-Saharan Africa differ from typical European populations (Mbanya *et. al*., 2010). Unsurprisingly, insulinopenia is a prominent feature (Motala *et al*., 2008). However, the age at onset in African communities (age 22-29 years) is about 10 years later than in populations from Europe, with peak age at onset of 15-19 years in Tanzania, 22-23 years or 21-30 years in South Africa, and 20-25 years in Ethiopia (Motala *et al*., 2008). A female preponderance was reported in affected people from South Africa, Ehiopia, Sudan, Nigeria, and Libya (Majaliwa *et al*., 2008).

2.2.2. Type 2 Diabetes Mellitus

Insulin resistance is a primary pathological feature of type 2 diabetes and predates the onset of this diabetes. Insulin resistance and/or impaired glucose tolerance are also part of the metabolic syndrome which comprises of abnormal serum lipid profile, central obesity and hypertension (WHO, 1999). The authors indicated that type 2 diabetes is a heterogeneous disorder that results from an interaction between a genetic predisposition and environmental factors and accounts for around 90% of all cases of diabetes. Most patients with this form of diabetes are obese and in most cases the disease is diagnosed in those over 40 years old (Jonsson, 2002). Obesity itself causes some degree of insulin resistance. However, the demographics of this disease are changing, and it is now becoming increasingly common in children and young adults (American Diabetes Association, 2000). Type 2 diabetes has a gradual and insidious onset, with nearly a third of the cases being identified as an incidental finding or in the coronary care unit (Melmberg *et al.*, 1995). This form of diabetes frequently goes undiagnosed for many years because the hyperglycemia develops gradually and at earlier stages it is often not severe enough for the patient to notice any of the classic symptoms of diabetes (American Diabetes Association, 2011). The incidence of type 2 diabetes increases with age, with most cases diagnosed after the age of 40 years (Centers for Disease Control and Prevention, 2011). The prevalence of diabetes is increasing rapidly. The World Health Organization (2003) has predicted that by 2030 the number of adults with diabetes will have almost doubted worldwide, from 177 million in 2000 to 370 million. It was also projected that 221 million people will be diabetic in 2010 and 324 million by 2025 (Zimmet *et al.*, 2001). According to Pratley and Weyer (2001), abnormalities in beta cell function are found early in the natural history of type 2 diabetes and in first degree

relatives of people with type 2 diabetes, suggesting that they are an integral component of pathogenesis of type 2 diabetes.

Most of the studies done between 1960s and early 1980s reported the prevalence of T2DM, using urine analysis in localized settings in countries such as Ethiopia, Ghana, Lesotho, Uganda and Malawi, to be lower than 1%; except the prevalence in South Africa (0.6 – 3.6%) and Cote d'Ivoire (5.7%) (McLarty *et al*.,1990). However, T2DM is presently the most common form of diabetes in sub-Saharan Africa, similar to other regions of the world. Most of the available, albeit still scarce, data for diabetes prevalence in sub-Saharan Africa are based on standardized 1985 WHO criteria (World Health Organization, 1985), with several reports from west, east and northeast Africa, and South Africa (Mbanya *et al*., 2010; McLarty *et al.,* 1990). These published data show that, contrary to previous notion, diabetes is not rare in sub-Saharan Africa. Although rates of less than 3% have been recorded in rural and urban communities in west and east Africa, frequencies of 3-10% are noted in urban and peri-urban populations in South Africa and in Sudanese communities of Africa origin, comparable with rates in developed countries (Motala, 2002; Motala *et al*., 2008).

Few epidemiological studies in sub-Saharan Africa have been published in which 1998/2003 American Diabetes Association (American Diabetes Association, 2012) and 1998 WHO (Alberti and Zimmet, 1998) criteria were used to determine the prevalence of diabetes. The prevalence of diabetes ranges from 3.9% in rural South Africa to 6.7% in urban Guinea (Mbanya *et al*., 2010). Although low diabetes prevalence has been noted in some rural and urban populations. The high prevalence reported in people from urban

areas of Kenya (Christensen *et al*., 2009) could be attributable to non-probability sampling in that study, so these results should be interpreted with caution.

The WHO Stepwise chronic disease risk factor surveillance programme (STEPS) undertaken in many Africa countries aims to clarify the burden of diabetes in sub-Saharan Africa. Reported prevalence varies widely (Benin 3%; Mauritania 6%; Cameroon 6.1%; Congo 7.1%; Zimbabwe 10.2%; Democratic Republic of Congo 14.5%) (Motala *et al*., 2008). In most of these studies, diagnosis is based on fasting blood glucose concentration, measured in either capillary whole blood or venous plasma. The high rates noted in some studies are inconsistent with other reports in indigenous African people. Whether this inconsistency is an indication of a sudden upsurge of diabetes in sub-Saharan Africa or is related to study methods (sampling strategy, true fasting blood samples) needs further assessment (Mbanya *et al*., 2010).

2.2.3. Gestational Diabetes Mellitus

Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance with onset or first recognition during pregnancy (Ross, 2006). Risk factors for developing GDM are: a previous diagnosis of gestational or prediabetes, impaired fasting glycaemia, a family history revealing a first degree relative with Type 2 diabetes, maternal age, ethnic background, being overweight and a history of previous pregnancy which resulted in a child with a high birth weight >4kg (Ross, 2006). Maternal obesity increases the risk of a number of other pregnancy complications, including preeclampsia and Caesarean delivery (Leddy *et al*., 2008).

2.3. Prevalence of Diabetes

According to estimates of diabetes mellitus in Africa, the 2010 diabetes mellitus national prevalence for Ghana was 3.6 % (International Diabetes Federation, 2010). However, in an independent community-based diabetes prevalence study in Greater Accra-Ghana, Amoah *et al*. (2002) determined the crude prevalence of diabetes to be 6.3 %. Their findings indicated that diabetes was more common in males (7.7 %) than females (5.5%) and increased with age. In the United States, 11.3 % of people aged 20 years and above have diabetes (Center for Disease Control and Prevention, 2011). The incidence of type 1 diabetes as well as the prevalence of type 2 diabetes is increasing in the developed and developing world. In adults, type 1 diabetes accounts for approximately 5 % of all diagnosed cases of diabetes according to Center for Disease Control and Prevention (2011). Gestational diabetes mellitus occurs in about 2 - 5 % of all pregnancies and may improve or disappear after delivery.

2.4. Risk Factors of Type 1 Diabetes

The etiology of type 1 diabetes remains poorly understood, but it is likely that an environmental factor triggers an autoimmune process in a predisposed individual. Thus, the risk factors for type 1 diabetes may include genetic susceptibility, autoimmunity or environmental factors. The genetic risk factors are passed on from generation to generation (inherited). With autoimmune mechanism as a risk factor, the T lymphocytes ($CD4^+$ and $CD8^+$) infiltrate the islets of Langerhans in the pancreas and destroy the insulin-producing beta cell population (Akerblom *et al*., 2002) and hence are implicated in the pathogenesis and etiology of the insulin-dependent diabetes mellitus (Nerup and

Lernmark, 1981). Although genetic susceptibility to type 1 diabetes is inherited, only 12-15 % of type 1 diabetes occurs in families (Akerblom *et al*., 2002).

2.5. Risk Factors of Type 2 Diabetes

The risk factors in type 2 diabetes are; genetic predisposition, age, obesity, alcohol intake, history of gestational diabetes and life style. The life style which may pre-dispose people to type 2 diabetes include; intake of high carbohydrate and fatty foods, alcohol intake, and lack of physical exercise. Researchers have demonstrated a strong link between having a large waist circumference (greater than 88 cm for women and greater than 102 cm for men) and an increased risk of developing Type 2 diabetes, independent of a person's body mass index (World Health Organization, 2011; The InterAct Consortium, 2012). They found out that waist circumference is a simple measure that can be used to diagnose abdominal obesity and identify individuals at increased risk of Type 2 Diabetes Mellitus (The InterAct Consortium, 2012). Interaction between genetic predisposition and environmental factors is believed to account for 90.95 % of diabetes cases (Center for Disease Control and Prevention, 2011).

2.6. Complications of Diabetes

The formation of advanced glycation end products (AGEs) and inflammation are important biochemical abnormalities that accompany diabetes mellitus. Studies by Basta *et al.*(2004) indicate that the effects of AGEs on vessel wall homeostasis may account for the rapidly progressive atherosclerosis and other complications associated with diabetes

mellitus. Severe long term abnormalities such as eye complications, heart disease, kidney and foot problems can result if blood glucose levels are poorly controlled in diabetics (Brophy *et al*., 2007). With the introduction of treatments that allow patients with diabetes to live through the acute metabolic consequences of the disease, it has become evident that diabetes is associated with a number of chronic complications. These complications are usually of two kinds: microvascular complications - that include retinopathy, nephropathy, neuropathy and peripheral vascular disorders; and macrovascular complications that include cardiovascular, cerebrovascular, renal and nervous disorders (Polidori, 2001; Daneman, 2006). According to Ardawi *et al.* (1994), changes in IgA and other immunoglobulins may be implicated in the pathogenesis of diabetic complications, such as susceptibility to infection. High levels of serum IgA may affect kidney function. Immunoglobulin IgA nephropathy is the most common glomerulonephritis and is characterized by deposition of the IgA antibody in glomerulus (D'Amico, 1987).

2.6.1. Microvascular Complications

Microvascular complications are mostly present ay the diagnosis of patients with type 2 diabetes. Retinopathy and cataracts affect around 15% of individuals with type 2 diabetes and are frequently present at diagnosis (UK Prospective Diabetes Study Group, 1988; American Diabetes Association, 2001). The risk of developing nephropathy is lower in type 2 diabetes than in type 1 diabetes because of the generally later onset of the former disorder. The prevalence of microalbuminuria, a higher than normal albumin excretion that cannot be detected by standard urine reagent strips and a sign of early

diabetic nephropathy is around 25-30%. Approximately, 5-13% of patients with type 2 diabetes have frank proteinuria (American Diabetes Association, 2001). Although, the individual risk of developing end-stage renal failure is lower in type 2 diabetes, patients with type 2 diabetes requiring renal replacement therapy outnumber patients with type 1 diabetes because of the much greater prevalence of type 2 diabetes. Neuropathy affects 20-50% of patients with type 2 diabetes, and its sequelae, such as foot ulceration and amputation, cause consideration morbidity and mortality (Boulton and Malik, 1998).

2.6.2. Macrovascular Disease

Patients with diabetes have a two-fold to four-fold increased risk of having myocardial infarction and stroke in men and up to a ten-fold increased risk in pre-menopausal women (American Diabetes Association, 2001) and 60 – 75% of all people with diabetes die from cardiovascular disease (Neaton *et al*., 1993). It is thought that increased prevalence of metabolic syndrome features in people with diabetes largely explains the excess cardiovascular disease in these patients (Stratton *et al*., 2000). In the Prospective Cardiovascular Munster Study, for example, 49% of individuals with diabetes had hypertension, 24% had a low level of high-density lipoprotein cholesterol and 37% had hypertrigylceridaemia, compared with values of 31%, 16% and 21% respectively, in people without diabetes (Assmann and Schulte, 1988).

2.7. Immunoglobulins

Immunoglobulins are glycoprotein molecules that are produced by plasma cells and plasmablasts in response to immunogens and play essential role in the body's immune

system. They function by attaching themselves to foreign substances, such as bacteria, and destroy them. There are five classes of immunoglobulins: immunoglobulins A, G, M, D and E known as IgA, IgG, IgM, IgD and IgE respectively. However, for the purpose of this study, IgA, IgG and IgM will be considered in the reviewed literature.

The U.S. National Library of Medicine (2012) gives the U.S. ranges of IgA, IgG and IgM to be 0.76 to 3.90 g/L, 6.50 to 15.00 g/L and 0.40 to 3.45 g/L respectively. However, a study conducted in Northern Nigeria to determine the levels of circulating immune complexes, immunoglobulins A, G and M among apparently healthy individuals had ranges of IgA, IgG and IgM as 0 to 1.91 g/L, 6.58 to 11.63 g/L and 0.18 to 2.37 g/L respectively (Tanyigna *et al.*, 2004). According to Gonzalez-Quintela *et al.* (2008), serum IgA levels are usually higher in males than in females. However, serum IgG and serum IgM levels are higher in females than in males and Serum IgA and IgG levels tended to increase with age, whilst serum IgM showed no significant variation with age.

Deficiencies in the levels of immunoglobulins result in reduction in the humoral defense system in the body. Immunoglobulin deficiencies are the result of congenital defects affecting the development and function of B lymphocytes (B-cells). This can happen when B-cells fail to develop into antibody-producing cells. Since antibodies are essential in fighting infectious diseases, people with immunoglobulin deficiency syndromes become sick more often as they are prone to invading microbes such as Streptococci, Meningococci, *Haemophilus influenzae*, enterovirus and the hepatitis B virus.

Deficiencies of IgA are found in hypogammaglobulinemia (Young *et al*., 2008), X-linked agammaglobulinemia and Selective IgA deficiency syndrome (Morimoto, 2008). Levels

of IgG are decreased in hypogammaglobulinemia (Young *et al*., 2008), X-linked agammaglobulinemia (Morimoto, 2008) and HIV.

Deficiencies of IgA are found in Selective IgM deficiency syndrome (Belgemen *et al*., 2009) and X-linked agammaglobulinemia.

2.7.1. Immunoglobulin A

Immunoglobulin A (IgA), according to Shetty (1993) is actively secreted by mucosal associated lymphoid tissue (MALT). IgA appears selectively in sero-mucus secretions such as saliva, tears, nasal fluids, colostrums, blood, and in secretions of the lung, genito-urinary and gastro-intestinal tracts. It is also synthesized locally by plasma cells.

The immunoglobulin IgA is most abundant in body and performs the role of defending the exposed external surfaces of the body against attack by micro organisms. It functions by inhibiting the adherence of coated microorganisms to the surface of the mucosal cells, thereby preventing entry into the body tissues. It contributes to the protection of the newborn babies by being in abundance in colostrums.

2.7.2. Immunoglobulin G

Immunoglobulin G (IgG) constitutes 75 percent of the total serum immunoglobulins in humans (Shetty, 1993). Throughout the secondary immune response, IgG is the major immunoglobulin to be synthesized. It plays a vital role in the defense against infection. IgG is responsible for the protection of the embryo during the first few months of life since it is the only immunoglobulin that crosses the placenta. IgG enhances phagocytosis by attracting macrophages as well as polymorphonuclear phagocytic cells.

2.7.3. Immunoglobulin M

Immunoglobulin M (IgM) is the largest immunoglobulin in size. The IgM antibodies appear early in response to infection and because of their size, are largely confined to the blood stream. They are an important defense mechanism against bacteria. The size and valency of IgM makes it a very effective agglutinating and cytolytic agent as well as the most efficient complement fixing immunoglobulin. According to Shetty (1993), IgM predominates in certain antibody responses such as response to the typhoid 'O' antigen. Immunoglobulin M response is short lived, hence its presence may be helpful in establishing an acute infection.

2.8. Immunoglobulins Associated with Diabetes Mellitus

Studies showed that serum IgA levels increased in diabetic patients (Singh and Kulig, 1992; Gonzalez-Quintela *et al*., 2008). Handwerger *et al*. (1980) reported mildly elevated levels of IgA and mildly decreased IgG levels in insulin-dependent diabetics. Significant increases in serum IgA and IgG concentrations were observed whereas the concentration of IgM was significantly decreased in diabetic patients compared with non-diabetic subjects (Ardawi *et al.*, 1994). They found out that serum IgA concentrations of diabetic patients were influenced by the degree of glycaemic control. Also Rodriguez-Segade *et al.* (1996) confirmed increased concentration of circulating IgA among type 1 and type 2 diabetic patients, with the levels higher in males than in females. They concluded that serum IgA levels were a non-specific sign of the development of diabetic complications such as retinopathy, neuropathy, nephropathy, macrovascular disease and hypertension.

2.9. Interleukin 6 and Diabetes Mellitus

Interleukins are cytokines (secreted proteins and signaling molecules) which largely affect the function of the immune system. Yudkin *et al*. (2000) posed this question: *Inflammation, obesity, stress and coronary heart disease: is interleukin the link?* Interleukin 6 (IL-6) plays a central role in inflammation and tissue injury (Ridker *et al*., 2000) and acts as both a pro-inflammatory and anti-inflammatory cytokine. It has been observed that *in vitro* IL-6 induced a dose-dependent inhibition of the glucose-stimulated insulin release from rat pancreatic islets of Langerhans (Southern *et al*., 1990). Sandler *et al*. (1990) also found out that IL-6 affects insulin secretion and glucose metabolism of rats' pancreatic islets *in vitro*. Mediators of inflammation such as the IL-6 family of cytokines have been proposed to be involved in the events causing both type 1 and type 2 diabetes (Kristiansen and Mandrup-Poulsen, 2005). According to Kristiansen and Mandrup-Poulsen (2005), individuals who develop type 2 diabetes display features of low-grade inflammation years in advance of disease onset. IL-6 has in addition to its immunoregulatory actions been proposed to affect glucose homeostasis and metabolism directly and indirectly by action on skeletal muscle cells, adipocytes, hepatocytes, pancreatic β-cells, and neuroendocrine cells (Kristiansen and Mandrup-Poulsen, 2005).

According to Harris *et al*. (1999) stated that elevated IL-6 levels are associated with mortality in the elderly.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study Area and Population

The study site was the National Diabetes Management and Research Centre (NDMRC), Korle-Bu Teaching Hospital. The Korle-Bu Teaching Hospital is a leading tertiary referral hospital in Accra with 1,600 bed capacity and 3,000 members of staff. The hospital serves the city of Accra (with a population of about three million) and 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, however, holds over 80,000 patient records, and each day, approximately 160 patients attend the clinic from in and around the catchment area (NDMRC Records). The clinic manages both type 1 and type 2 diabetic cases, nonetheless, majority of the patients seen are type 2 diabetics reporting for routine check-up.

3.2. Target Population

The study was carried out on patients diagnosed with type 2 diabetes according to WHO (1999) criteria undergoing management of their conditions in the out-patient's clinic at NDMRC between April to November 2013. A total of 192 subjects including controls were recruited (96 type 2 diabetic subjects and 96 controls). Subjects who served as controls were screened to make sure that they were non-diabetic. The selection criteria for the subjects was based on response to the questionnaire, which were intended to

obtain information on subject's age, duration of the type 2 diabetes disease and drug usage.

3.3. Study Design

This was a hospital-based cross-sectional study with matched controls. The study was intended primarily to investigate immunological alterations associated with type 2 diabetes.

3.4. Sample Size Determination

The minimum sample size was determined by the formula;

$N = Z^2 (p * q) / e^2$ (Spiegl & Stephens, 2008)

$N = (1.96)^2 (0.063) (1-0.063)/(0.05)^2$

$N = 91$

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

P, 0.063 is the sample proportion of the 6.3% prevalence of diabetics in Ghana (International Diabetes Federation, 2010);

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

Based on the formula above, a sample size of 192 was estimated for the study, comprising 96 subjects with type 2 diabetes and 96 subjects without diabetes allowing for drop outs.

3.5. Inclusion and Exclusion Criteria

3.5.1. Inclusion Criteria

The subjects recruited for the study were between the ages 35 to 60 years. They included Diagnosed type 2 diabetic patients on management at the NDMRC. The controls consisted of non-diabetics within the same age brackets.

3.5.2. Exclusion Criteria

Subjects above 60 and below 35 years as well as smokers and heavy drinkers were excluded from the study. Patients who were immunosuppressed such as those with immunoglobulin deficiency syndrome, HIV and Hepatitis B were excluded from the study. Also those who tested positive for the urine nitrite test or had bacterial and parasitic infections were excluded from the study. The above have been proven to affect immunoglobulin levels in subjects (Shetty, 1993; van den Berg *et al.*, 2009; Schmiemann *et al*., 2010).

3.6. Ethical Issues

Approval was obtained from Research and Ethical Review Committee of the University of Ghana Medical School, College of Health Sciences.

3.7. Sampling and Sample Collection

3.7.1. Specimen Collection, Transport and Storage

Fasting venous blood samples (10 mL) were obtained from the subjects between 07:00 and 09:00 hours each day, according to Helsinki protocol declaration (2008). Two milliliters (2 mL) of whole blood was transferred into sodium fluoride containing tube and the plasma separated for the estimation of glucose. Three milliliters (3 mL) of whole blood was transferred into ethylene diamine tetra-acetic acid (EDTA) containing tubes for the estimation of glycated haemoglobin (HbA_{1c}). The remaining five milliliters (5 mL) of whole blood was then placed into serum separator tubes for processing. Serum samples were then aliquoted in 1 mL portions into 5 sterile eppendorf tubes and stored at -20^{o}C until they were required for use.

3.7.2. Anthropometric Measurements

Weight and height of participants were measured using a standardized weighing scale and a standardized stadiometer respectively after subjects had taken off their footwear. Body mass index (BMI) was then calculated from body weight (in kilograms) divided by square of the height (in meters). Waist circumference of subjects were measured at the navel region, parallel to the hip bone according to WHO (2003) protocol for waist circumference measurements.

3.7.3. Blood Pressure

Blood pressure (diastolic and systolic) measurements was 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

Collected blood samples from each recruited subject according to standardized venipuncture procedure was transferred into serum separator, EDTA and fluoride tubes respectively. The blood collected in serum separator tubes were allowed to clot and serum separated by centrifugation. Enzyme-Linked Immunosorbent assay (ELISA) technique for the immunoglobulins (IgA, IgG and IgM) and Interleukin 6 using specific and high affinity antibodies were used to determine their serum levels in the subjects. Fasting blood glucose, lipid profile, total protein and albumin of the subjects were analysed using VITROS system auto analyser (version 950). Glycated hemoglobin (HbA_{1c}) was measured using Randox Daytona auto analyzer. The determination of HbA_{1c} was based on a latex agglutination inhibition assay.

3.8.1. Measurement of Immunoglobulin A

Principle of the Procedure

The test uses a sandwich ELISA technique and the ELISA kit. The wells contain a pre-coated human IgA monoclonal antibody and the detecting antibody is polyclonal

antibody with biotin a label. Samples and biotin labeled antibody were added into ELISA plate wells and washed out with (phosphate buffered saline) PBS or (tris-buffered saline) TBS. Then Avidin-peroxidase conjugates are added to the ELISA wells. For coloring, 3, 3', 5, 5', - tetramethyl benzidine (TMB) substrate is used after reactant is thoroughly washed out wit PBS. TMB turns into blue in catalytic peroxidase and finally turns into yellow under the action of acid. The intensity of colour formed was measured at 450 nm and the concentration estimated.

Experimental Procedure

Serum samples stored in eppendorf tubes at -20^{o}C were taken out of the refrigerator to thaw after which each sample was vortexed for 10 seconds. ELISA Kit (MyBiosource – USA) which was previously stored at 4 ^{o}C in the refrigerator was brought to room temperature. Hundred microliters (100 µL) of serum test samples and controls, as well as different concentrations of human IgA pre-prepared standard samples were added to corresponding wells . A blank well was filled with standard diluent provided. The reaction wells were sealed with adhesive tapes and incubated in an incubator at 37^{o}C for 90 minutes. The ELISA plates were taken out of the incubator and washed three times in wash buffer. A volume of 100 µL of Biotinylated human IgA antibody liquid prepared 30 minutes before use, was add to each well. The reaction wells were again sealed with adhesive tapes and incubated at 37^{o}C for 60 minutes. The ELISA plates were aspirated three times.

Hundred microliters (100 µL) of enzyme-conjugate liquid prepared 30 minutes before being used was added to each well except the blank well. The reaction wells were sealed

with adhesive tapes and incubated at 37^{o}C for 30 minutes. The ELISA plate was then aspirated five times with wash buffer. A volume of 100 µL Colour Reagent liquid was then added to the individual wells (including the blank well) and incubated in the dark at 37^{0}C. Incubation was done for 30 minutes by which colour for high concentration of standard curve wells had become darker and colour gradient appeared. Hundred microliters (100 µL) of stop solution was added to all wells to stop the reaction and then mixed well. The Optical Density (OD) was measured with MULTISKAN MS Microplate reader vesion 1.5 (USA) at 450nm after 10 minutes.

With the aid of standard curve Serum IgA concentrations of samples were determined employing Curve Expert 1.3 to analyze and compute the OD results.

3.8.2. Measurement of Immunoglobulin G

Principle of the Procedure

The IgG present in the sample reacts with the anti-IgG antibodies which have been adsorbed to the surface of polystyrene microtitre wells. After the removal of unbound proteins by washing in wash buffer, anti-IgG antibodies conjugated with horseradish peroxidase (HRP), are added. These enzyme-labelled antibodies form complexes with the previously bound IgG. Following another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3, 3', 5, 5' – tetramethylbenzidine (TMB). The quantity of bound enzyme varies directly with the concentration of IgG in the sample tested; thus, the absorbance, at 450 nm, is a measure

of the concentration of IgG in the test sample and can be interpolated from the standard curve constructed from the standards, and corrected for sample dilution.

Experimental Procedure

Serum samples stored at -20^{o}C to be used for IgG determinations were brought to room temperature and vortexed for 10 seconds. All ELISA reagents to be used were also brought to room temperature before use. Standard samples of concentrations: 15.6 ng/mL, 31.25 ng/mL, 62.5 ng/mL, 125 ng/mL, 250 ng/mL and 500 ng/mL were prepared in duplicates according to protocol provided. A 1/80,000 dilution of each test sample and control sample were prepared according to protocol provided. Hundred microliters (100 µL) of the standards and diluted samples were pipetted into predesignated wells of micro titre ELISA plates and then incubated at room temperature for sixty minutes. After incubation the contents were then aspirated.

The wells were then washed in diluted wash solution and aspirated. This was repeated three times, for a total of four washes. Residual buffer was removed by the use of the aspirator. A volume of 100 µL of diluted Enzyme-Antibody Conjugate was pipetted and added to each well. This was followed by incubation at room temperature in the dark for twenty minutes. The plate was washed four times and 100 µL of TMB substrate solution was then added to each well. The plate was incubated in the dark at room temperature for ten 10 minutes. Stop solution (100 µL) was then added to each well.

The absorbance at 450 nm of the contents of each well was determined with MULTISKAN MS Microplate reader version 1.5 (USA). Serum IgG concentrations of the samples were determined employing Curve Expert 1.3 to analyze and compute the

OD results. Concentrations obtained were multiplied by the dilution factor of 80,000 to obtain true serum IgG levels in samples.

3.8.3. Measurement of Immunoglobulin M

Principle of the Procedure

This test follows the same procedure used for measuring IgA. The test employs a sandwich Elisa technique and the ELISA kit. The wells contain a pre-coated human IgM monoclonal antibody and the detecting antibody is polyclonal antibody with biotin labeled. Samples and biotin labeling antibody are added into ELISA plate wells and washed in PBS. Then Avidin-peroxidase conjugates are added to ELISA wells in order; TMB substrate is used for coloring after reactant is thoroughly washed in PBS. TMB turns into blue in the presence of peroxidase catalytic and finally turns yellow in the presence of acid. The color depths in samples at 450 nm are positively correlated to the concentrations of IgM.

Experimental Procedure

Serum samples stored at -20^{o}C were brought to room temperature and vortexed for 10 seconds. The ELISA Kit was also brought to room temperature. Hundred microliters (100 µL) each of serum test samples and controls, as well as different concentrations of human IgA pre-prepared standard samples were added to corresponding wells . A blank well was filled with standard diluent provided. The reaction wells were sealed with adhesive tapes and then incubated at 37^{o}C for 90 minutes. The Elisa plates were then taken out of the incubator and washed three times in wash buffer. A volume of 100 µL of Biotinylated

human IgM antibody liquid prepared 30 minutes before use, was add to each well. The reaction wells were again sealed with adhesive tapes and incubated in the incubator at 37^{o}C for 60 minutes. The Elisa plates were washed three times.

Hundred microliters (100 µL) of enzyme-conjugate liquid prepared 30 minutes before being used was added to each well except the blank well. The reaction wells were sealed with adhesive tapes and incubated in the incubator at 37^{o}C for 30 minutes. The Elisa plate was then washed five times with wash buffer. A volume of 100 µL Colour Reagent liquid was then added to the individual wells (including the blank well) and incubated in a dark incubator at 37^{o}C. Incubation was done for 30minutes by which colour for high concentration of standard curve wells had become darker and colour gradient appeared. Hundred microliters (100 µL) stop solution was then added to individual wells (including the blank well) and then mixed well. The Optical Density (OD) was measured with MULTISKAN MS Microplate reader vesion 1.5 at 450 nm after 10 minutes.

With the aid of standard curve Serum IgM concentrations of diabetic samples and controls were determined employing Curve Expert 1.3 to analyze and compute the OD results.

3.8.4. Measurement of Interleukin 6

Principle of the Procedure

The human IL-6 ELISA test is based on standard sandwich enzyme-linked immune-sorbent assay technology. Human IL-6 specific –specific monoclonal antibodies are precoated onto the well plates. The human specific detection polyclonal antibodies are

biotinylated. The test samples and biotinylated detection antibodies are added to the wells subsequently and followed by washing with prepared wash buffer. Avidin-Biotin-Peroxidase Complex is added and unbound conjugates are washed away with prepared wash buffer. HRP substrate TMB is used to visualize HRP enzymatic reaction. TMB is catalyzed by HRP to produce a blue color product that changes into yellow after adding acidic stop solution. The intensity of yellow colour is proportional to the human IL-6 amount present in test sample captured in plate.

Experimental Procedure

The serum samples stored in eppendorf tubes at -20^{o}C to be used for IL-6 determinations were taken out of the refrigerator to thaw after which each sample was vortexed for 10 seconds. All reagents to be used were also brought to room temperature before use. Standard samples of concentrations: 300 pg/mL, 150 pg/mL, 75 pg/mL, 37.5 pg/mL, 18.75 pg/mL, 9.38 pg/mL and 4.69 pg/mL were prepared in duplicates according to protocol provided. A 1/10 dilution of each test sample and control sample was prepared according to protocol provided. Hundred microliters (100 µL) of the 300 pg/mL, 150 pg/mL, 75 pg/mL, 37.5 pg/mL, 18.75 pg/mL, 9.38 pg/mL, 4.69 pg/mL human IL-6 standard calibrator solutions prepared were pipetted into the precoated 96-well plates in dublicates. Hundred microliters (100 µL) of the sample diluent buffer was placed into a control well (zero well). A volume of 100 µL of each diluted test samples were placed into empty wells in duplicates. The plates were sealed with adhesive cover and incubated at 37^{o}C for 90minutes.

After incubation, the plate contents were discarded and the plate aspirated.

Hundred microliters (100 µL) of biotintylated anti-human IL-6 antibody working solution was then placed into each well and the plate incubated at 37^{o}C for 60 minutes. The plate was washed three times with prepared wash buffer in an aspirator.

Prepared working solution (100 µL) was then added into each well and the plate incubated at 37^{o}C for 30 minutes. After incubation, the plate was washed five times with prepared wash buffer in an aspirator. Ninety microliters (90 µl) of prepared TMB

Colour developing reagent was added into each well and the plate incubated at 37^{o}C for 30 minutes. Hundred microliters (100 µL) of prepared TMB stop solution was then added into each well the O.D absorbance read at 450 nm in the MULTISKAN MS Microplate reader vesion 1.5 within 30 minutes after adding the TMB stop solution. Concentrations obtained were multiplied by dilution factor of 10 to obtain true serum IL-6 levels in the samples.

3.8.5. Measurement of Glucose

Principles of the Procedure

The VITROS Glucose (GLU) slide method was performed using the VITROS GLU slides and Calibrator kit on VITROS Chemistry System (Johnson & Johnson, New Jersey, USA). Six microliters (6µ1) of sample was deposited on the slide and was eventually distributed by the spreading layer to the underlying layers. The oxidation of glucose is catalyzed by glucose oxidase to form hydrogen peroxide and gluconate. The reaction is followed by an oxidative coupling catalyzed peroxidase in the presence of dye precursors to produce a dye. The intensity of the dye is measured by reflected light at a

wavelength of 540 nm. The Chemistry of glucose slides used in this study is as described by Curme *et al.*, (1978). The incubation time and reaction condition for the entire reaction were 5 minutes and 37^{o}C respectively.

Reaction Sequence

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{D – gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4 – aminoantipyrine} + \text{1, 7 – dihydroxynaphthalene} \xrightarrow{\text{peroxidase}} \text{red dye}$$

3.8.6. Measurement of Total Cholestral (T. CHOL)

Principles of the Procedure

The VITROS Cholesterol (CHOL) slide method was performed using the VITROS CHOL slides and Calibrator kit on VITROS Chemistry System (Johnson & Johnson, New Jersey, USA). The method used in this study is based on an enzymatic method proposed by Allain *et al.*, (1974). Six microliter (6 µL) sample is deposited on the slide and is eventually distributed by the spreading layer to the underlying layers. The Triton X-100 (TX 100) surfactant in the spreading layer aids in dissociating the cholesterol and cholesterol esters from lipoprotein complexes present in the sample. Hydrolysis of the cholesterol is catalyzed by cholesterol ester hydrolase. Free cholesterol is then oxidized in the presence of cholesterol oxidase to form cholestenone and hydrogen peroside. Finally, hydrogen peroxide oxidizes a leuco dye in the presence of peroxides to generate a colored dye. The density of the dye formed is proportional to the cholesterol concentration present in the sample and is measured by reflectance spectrophotometry at

a wavelength of 540 nm. The incubation time for the entire reaction was 5 minutes at 37^{o}C.

Reaction Sequence

lipoprotein $\xrightarrow{\text{TX-100}}$ cholesterol + cholesterol ester + proteins

cholesterol ester + H_20 $\xrightarrow{\text{cholesterol ester hydrolase}}$ cholesterol + fatty acids

cholesterol + O_2 $\xrightarrow{\text{cholesterol oxidase}}$ cholest-4-en-3-one + H_2O_2

H_2O_2 + leuco dye $\xrightarrow{\text{peroxidase}}$ dye + $2H_2O$

3.8.7. Measurement of Triglyceride (TRIG)

Principles of the Procedure

The VITROS Triglyceride (TRIG) slide method was performed using the VITROS TRIG slides and Calibrator kit on VITROS Chemistry System (Johnson & Johnson, New Jersey, USA). The method used in this study is based on the enzymatic method as described by Spayd *et al*., (1978). Six microliters (6 µL) of patient sample is deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. The Triton X-100 surfactant in the spreading layer aids in dissociating the triglycerides from lipoprotein complexes present in the sample. The triglyceride molecules are then hydrolyzed by lipase to yield glycerol and fatty acids. Glycerol diffuses to the reagent layer, where it is phosphorylated by glycerol kinase in the presence of adenosine triphosphate (ATP). In the presence of oxygen, L-α-glycerophosphate is oxidized to dihydroxyacetone phosphate and hydrogen peroxide. The final reaction involves the

oxidation of a leuco dye by hydrogen peroxide, catalyzed by peroxidase, to produce a dye. The density of the dye formed is proportional to the triglyceride concentration present in the sample and is measured at a wavelength of 540nm by reflectance spectrophotometry. The incubation time for the entire reaction was 5 minutes at 37^{o}C.

Reaction Sequence

lipoprotein $\xrightarrow{\text{surfactant}}$ triglycerides + proteins

triglycerides + H_2O $\xrightarrow{\text{lipase}}$ glycerol + fatty acids

glycerol + ATP $\xrightarrow{\text{glycerol kinase}}$ L-α-glycerophosphate + ADP

L-α-glycerophosphate + O_2 $\xrightarrow{\text{oxidase}}$ dihydroxyacetone phosphate + H_2O_2

H_2O_2 + leuco dye $\xrightarrow{\text{peroxidase}}$ dye + H_2O

3.8.8. Measurement of High Density Lipoprotein (HDL)

Principles of the Procedure

The VITROS HDL cholesterol slide method was performed using the VITROS HDL cholesterol slides and Calibrator kit on VITROS Chemistry System (Johnson & Johnson, New Jersey, USA). The method is based on a non-HDL cholesterol precipitation method similar to one described by Burstein *et al*., (1970). This is followed by an enzymatic detection similar to that proposed by Allain *et al*., (1974). Ten microliters (10 µL) of patient sample is deposited on the slide and is eventually distributed by the spreading layer to the underlying layers. HDL cholesterol is separated by the precipitation of non-high density lipoproteins (non-HDL) using phosphotungstic acid (PTA) and magnesium chloride ($MgCl_2$) in the spreading layer. The Emulgen B-66 surfactant in the spreading

layer aids in the selective dissociation of the cholesterol and cholesterol esters from the HDL cholesterol complexes present in the sample. Hydrolyses of the HDL-derived cholesterol ester to cholesterol is catalyzed by a selective cholesterol ester hydrolase. Free cholesterol is then oxidized in the presence of cholesterol oxidase to form cholestenone and hydrogen peroxide. Finally, hydrogen peroxide oxidizes a leuco dye in the presence of peroxidase to generate a colored dye. The density of dye formed is proportional to the HDL cholesterol concentration present in the sample and is measured at a wavelength of 670 nm by reflectance spectrophotometry. The incubation time for the entire reaction was 5 minutes at 37^{o}C.

Reaction Sequence

HDL-C + non-HDL-C $\xrightarrow{PTA/MgCl_2}$ high density lipoproteins + non-HDL

High density lipoproteins $\xrightarrow{Emulgen\ B\text{-}66}$ cholesterol + HDL Chol. Esters + proteins

HDL-C esters + H_2O $\xrightarrow{chol.\ Ester\ hydrolase}$ cholesterol + fatty acids

Cholesterol + O_2 $\xrightarrow{cholesterol\ oxidase}$ cholest-4-en-3-one + H_2O_2

H_2O_2 + leuco dye $\xrightarrow{peroxidase}$ dye + $2H_2O$

3.8.9. Measurement of Serum Albumin (ALB)

Principles of the Procedure

The VITROS Albumin (ALB) slide method was performed using the VITROS ALB slides and Calibrator kit on VITROS Chemistry System (Johnson & Johnson, New Jersey, USA). Six microliter (6µ1) is deposited on the slide and is evenly distributed by

the spreading layer to the underlying layers. When the fluid penetrates the reagent layer, the bromcresol green (BCG) dye diffuses to the spreading layer and binds to albumin from the sample. This binding results in a shift in wavelength of the reflectance maximum of the free dye. The color complex that forms is measured by reflectance spectrophotometry at a wavelength of 630nm. The amount of albumin bound dye is proportional to the concentration of albumin in the sample. Incubation time for the entire reaction was 3 minutes at a temperature of 37^{o}C.

Reaction Sequence

albumin + bromecresol green (BCG) ⟶ BCG-albumin complex

3.8.10. Measurement of Total Protein (TP)

Principles of the Procedure

The VITROS Total Protein (TP) slide method was performed using the VITROS TP slides and Calibrator kit on VITROS Chemistry System (Johnson & Johnson, New Jersey, USA). The principle of the test used in this study is based on the biuret reaction, which produces a violet complex when protein reacts with cupric ion (Cu^{2+}) in an alkaline medium (Kingsley, 1942). Ten microliter (10 µL) sample is deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. When the fluid penetrates the reagent layer, the reagent diffuses up to the spreading layer and reacts with protein. The reaction between protein and copper tartarte takes place largely in the spreading layer where the protein is confined because of its high molecular weight. The amount of colored complex formed is proportional to the amount of total protein in the

sample and is measured at a wavelength of 540 nm by reflectance spectrophotometry. The incubation time for the entire reaction was 5 minutes at 37^{o}C.

Reaction Sequence

Protein + copper tartarte $\xrightarrow{\text{LiOH}}$ colored complex

3.8.11. Measurement of Glycated Haemoglobin (HbA_{1c})

Principles of the Procedure

The measuremnt of glycated haemoglobin (HbA_{1c}) involved the following steps.

Sample Pre-treatment

The first step of the procedure involved the pre-treatment of the whole blood sample. This was done by adding 10 µL of whole blood to 400 µL haemoglobin denaturant reagent (1:41 dilution). This lysed red blood cells and caused hydrolysis of the haemoglobin by the action of a protease enzymes in the haemoglobin denaturant reagent.

3.8.12. Determination of Total Haemoglobin

The total haemoglobin reagent was used to determine the concentration of total haemoglobin. The method invovled the conversion of all the haemoglobin derivatives into haematin in an alkaline solution of a non-oinic detergent as described by Wolf *et al*., (1984). The reaction was initiated by the addition of the pre-treated sample to the total haemoglobin reagent, resulting in a green solution. The conversion of different

haemoglobin species into alkaline haematin with one defined absorption spectrum allows the endpoint measurement of total haemoglobin at 600 nm.

3.7.12.1. Determination of HbA_{1c}

The determination of HbA_{1c} was based on a latex agglutination inhibition assay. The agglutinator, which consists of a synthetic polymer containing multiple copies of the immunoreactive portion of HbA_{1c}, causes agglutination of latex coated with HbA_{1c} specific mouse monoclonal antibodies. In the absence of HbA_{1c} in the sample, the agglutinator in the HbA_{1c} R2 Reagent and the antibody coated micro particles in the HbA_{1c} R1 Reagent will agglutinate, resulting in an increase in absorbance. The presence of HbA_{1c} in the sample will slow the rate of agglutination as it competes with the HbA_{1c} agglutinator for antibody binding sites on the latex. Hence, the increase in absorbance is inversely proportional to the concentration of HbA_{1c} in the sample. An increase in absorbance due to agglutination was measured at a wavelenght of 700nm and the extent of agglutination was used to calculate the concentration of HbA_{1c} from a calibration curve. The percentage (%) of HbA_{1c} was then calculated using the g/dl HbA_{1c} and total haemoglobin values.

3.8. Data Handling

All data were entered into Microsoft excel spreadsheet for storage and subsequent analysis. Data was handled confidentially. Unique identifiers were used for computer based data entry. The supervisors and Investigator ensured that all study forms together with all identification code lists were kept safe and confidential.

3.8. Statistical Analysis

Data gathered was analyzed in accordance with the requirements of the research objectives. The Statistical Package for the Social Sciences (SPSS) version 20.0 was used. The Mann-Whitney U-test for median comparison was used to test the differences in immunoglobulin and IL-6 levels between diabetic and non-diabetic subjects. The median, minimum and maximum values were given as measures of dispersion. For comparison of demographic, clinical and biochemical parameters, quantitative data was presented as mean ± SD. The statistical significance between means was estimated by student's t–test when appropriate. Spearman's correlation coefficient (r) was used to measure the strength of the association between the variables. Differences were considered statistically significant at $p<0.05$.

CHAPTER FOUR

RESULTS

4.1. General Characteristics and Clinical Parameters of the Study Population

The number of volunteers who participated in the study, demographic and clinical indices have been presented in Table 1. The subjects comprised ninety-six (96) type 2 diabetics who were matched for age with ninety-six (96) non-diabetic subjects. Each test group comprised of 38 males and 58 females. The mean age for the diabetic study group and non-diabetic control group were 50.6 ± 7.4 and 48.1 ± 8.4 years respectively.

The difference between the means of the ages of the diabetics and non-diabetics was not significant ($p=0.052$) (Table 1). Though the means of the systolic and diastolic blood pressures were slightly higher for the diabetic than the non-diabetic subjects, the observed difference was not significant ($p=0.08$ and $p=0.111$ respectively). The mean waist circumference and the mean body mass index were significantly higher in the diabetic than in the non-diabetic subjects ($p<0.001$in both cases) (Table 1).

The frequency distribution of the type 2 diabetic subjects at various duration of the diabetes is presented in Figure 1.

Table 1: Demographic information and clinical parameters of the study population

Parameters	Type 2 Diabetics (N=96)	Non-Diabetics (N=96)	p-value
Age(yrs)	50.6 ± 7.4	48.1 ± 8.4	0.052
Males (%)	38 (37.5)	38 (37.5%)	
SBP (mmHg)	137.8 ± 17.2	132 ± 19.9	0.08
DBP (mmHg)	82.7 ± 10.1	78.8 ± 19.3	0.111
BMI (Kg/m^2)	29.7 ± 5.8	25.1 ± 4.1	<0.001**
Waist Circumference (cm)	99.7 ± 15.2	88.5 ± 11.4	<0.001**
Period of diabetes (yrs)	8.6 ± 6.4		

Data for the demographic and clinical parameters (Age, Gender, SBP, DBP, BMI, Waist circumference and Period of diabetes). Values for Age, SBP, DBP, BMI, Waist circumference and Period of diabetes are given as mean ± standard deviation while values for gender was given as frequency (percentage). *mean difference is significant ($p<0.05$). **mean difference is highly significant ($p<0.01$).

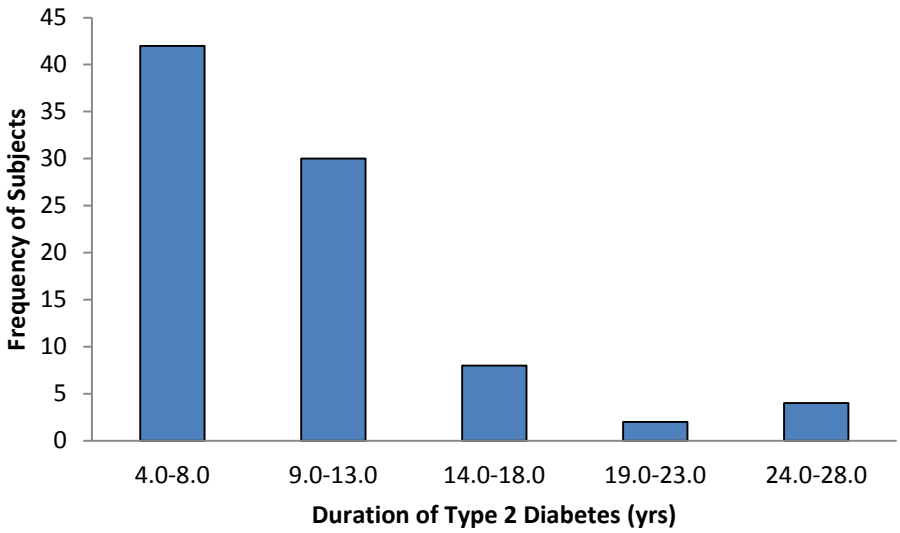

Fig. 1: Frequency distribution of type 2 diabetic subjects at various duration of diabetes

The figure (Fig. 1) showed a decline in the number of Type 2 diabetic subjects with increase in duration of diabetes in years.

4.2. Biochemical Parameters of the Study Population

The means of the measured biochemical parameters of the study subjects are shown in Table 2. Fasting blood glucose (FBG) was significantly higher in the diabetic than the non-diabetic subjects ($p<0.001$). The mean glycated haemoglobin (HbA1c) was found to be significantly higher in the diabetic than the non-diabetic subjects ($p=0.023$). The total protein however, was significantly higher in the non-diabetic than in the diabetic subjects ($p=0.007$). No significant difference was observed in any of the other biochemical parameters between type 2 diabetics and non-diabetics (Table 2).

Table 2: Biochemical parameters of the study population

Parameters	Type 2 Diabetics (N=96)	Non-Diabetics (N=96)	p-value
FBG (mmol/L)	7.7 ± 3.7	4.3 ± 0.7	<0.001**
HbA1C (%)	6.1 ± 1.3	5.7 ± 0.9	0.023*
T. Chol (mmol/L)	4.9 ± 1.4	5.2 ± 1.1	0.194
TG (mmol/L)	1.2 ± 0.6	1.2± 0.8	0.983
HDL (mmol/L)	1.3 ± 0.5	1.3 ± 0.3	0.467
LDL (mmol/L)	3.1 ± 1.3	3.3 ± 0.9	0.157
VLDL (mmol/L)	0.5 ± 0.3	0.5 ± 0.3	0.998
LDL/HDL-ratio	2.4 ± 1.1	2.7 ± 1.0	0.076
T.Chol/HDL-ratio	3.8 ± 1.2	4.2 ± 1.4	0.079
T. Protein (g/L)	76.2 ± 8.1	79.1 ± 4.8	0.007**
Albumin (g/L)	44.0 ± 3.9	50.5 ± 4.4	0.188

Data shows the biochemical parameters of the study population. Values are given as mean ± standard deviation. *mean difference is significant ($p<0.05$). **mean difference is highly significant ($p<0.01$). FGB is fasting blood glucose, HbA1c is glycated hemoglobin, T.Chol is total cholesterol, TG is triglyceride, HDL is high density lipoprotein, VLDL is very low density lipoprotein and T.Protein is total protein.

4.3. Distribution of Serum Immunoglobulin Levels in Type 2 Diabetic Subjects

The levels of all the three immunoglobulins IgA, IgG and IgM did not follow normal distribution, (Fig. 2a, 2b & 2c). Majority of the Type 2 diabetic subjects (65%) presented IgA levels ranging from 0.5 - 1.4 g/L. The subjects who showed IgA ranging from 1.5 - 3.4 g/L constituted 21%, while 1% of the subjects showed IgA concentration of 4.5 – 4.9 g/L. (Table 3). The 25th percentile and 75th percentile of IgA level in the diabetic subjects were found to be 0.63 and 1.33 respectively (Fig 2a).

The values of IgG were higher than IgA and IgM, ranging from 6.4 – 8.4 g/L (Table 3). Majority of the Type 2 diabetics fell into the range between 7.5-7.9 g/L (66%). The 25th percentile and 75th percentile of IgG were 7.44 and 7.77 respectively (Fig 2b).

Majority of the diabetic subjects showed IgM levels ranging between 0.08-8.35 g/L (Table 3). The median, 25th and 75th percentile were 0.73, 0.33 and 2.12 respectively (Fig 2c).

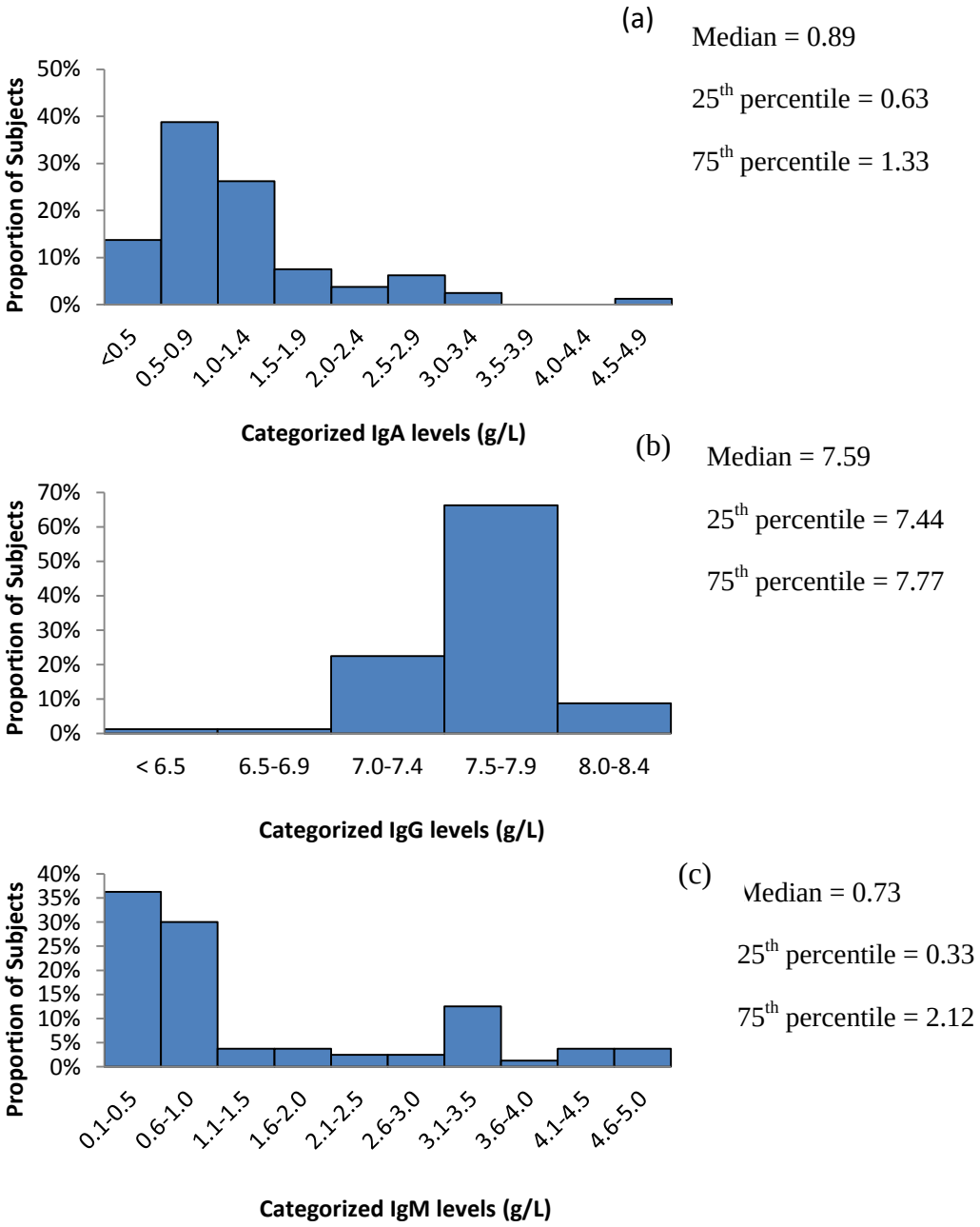

Fig. 2: Proportion of type 2 diabetic subjects at various categorized serum immunoglobulin levels. (a) IgA (b) IgG (c) IgM

4.4. Immunoglobulin and Interleukin 6 Levels in Diabetic and Non- diabetic Subjects.

The median IgA concentration level (0.89 g/L) in Type 2 diabetic subjects was found to be significantly higher than the corresponding IgA level (0.74 g/L) in the non-diabetic subjects (p=0.043) (Table 3). Similarly, median IgG level (7.58 g/L) was found to be higher in the type 2 diabetics than in the non-diabetic subjects (7.29) (p<0.001). On the other hand, the median value of IgM observed in the type 2 diabetic subjects was not significantly different from the non-diabetics (p=0.270). The median IL-6 concentration level (1.70 pg/mL) in the diabetic subjects was significantly higher than the corresponding IL-6 level (0.99 pg/mL) in the non-diabetic subjects.

Table 3: Immunoglobulin and IL-6 levels in type 2 diabetic subjects compared with non-diabetic Controls

Parameters	Type 2 Diabetics (N=96)	Non-Diabetics (N=96)	p-value
IgA (g/L)	0.89 (0.36-4.81)	0.74 (0.34-3.51)	0.043*
IgG (g/L)	7.58 (6.43-8.35)	7.29 (4.68-11.65)	<0.001**
IgM (g/L)	0.73 (0.08-4.99)	0.57 (0.01-4.06)	0.270
IL-6(pg/mL)	1.70 (0.39-14.73)	0.99 (0.01-4.52)	<0.001**

Data presented as medians and minimum-maximum ranges (within parenthesis) * significant (p<0.05). ** highly significant (p<0.01). IgA: immunoglobulin A, IgG: immunoglobulin G, IgM: immunoglobulin M.

4.5. Association of Immunoglobulin and Interleukin 6 Levels with General Characteristics and Clinical Parameters of Type 2 Diabetic Subjects

The results obtained showed that there was significant association between age and immunoglobulin A in diabetic subjects. However there was no association between age on one hand, and IgG, IgM and IL-6 (Table 4).

There was no significant relationship between waist circumference of the diabetic subjects and their immunoglobulin as well as interleukin 6 levels (Table 4).

The results as presented in Table 4 did not show any significant association between systolic and diastolic blood pressure of the diabetic subjects and their immunoglobulin and interleukin 6 levels.

However, Body Mass Index (BMI) was found to correlate positively with IgG ($p<0.05$). No significant association was observed between BMI and IgA, IgM and IL-6 levels in the diabetic subjects (Table 4).

Table 4: Correlation of immunoglobulins and interleukin-6 levels with general characteristics and clinical parameters of diabetic subjects

		IgA	IgG	IgM	IL-6
Age	**R**	0.159*	-0.036	0.047	0.114
	P	0.047	0.065	0.558	0.154
BMI	**R**	0.025	0.161*	0.069	0.077
	P	0.751	0.043	0.388	0.337
Waist circumference	**R**	-0.013	0.049	0.073	0.025
	P	0.872	0.541	0.364	0.759
SBP	**R**	0.018	-0.042	0.023	0.054
	P	0.824	0.600	0.775	0.502
DBP	**R**	0.016	0.071	0.023	0.016
	P	0.838	0.374	0.779	0.841

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

4.6. Association of Immunoglobulin and Interleukin 6 Levels with Biochemical Indices of the Diabetic Subjects

The results of the various associations of immunoglobulin and IL-6 levels with biochemical indices of the diabetic study population are presented in Table 5.

Low Density Lipoprotein (LDL), Very Low Density Lipoprotein, the ratio: LDL/High Density Lipoprotein (HDL) as well as the ratio: Total Cholesterol (T.Chol)/HDL did not show any significant association with the immunoglobulin and IL-6 of the study population. Albumin correlates significantly and inversely with IL-6 ($p<0.05$). Similarly, HDL correlates positively with IL-6 ($p<0.05$). Total protein showed positive correlation with IgA (Table 5).

Fasting blood glucose (FBG) correlated significantly and positively with IgA ($p=0.033$) IgG ($p=0.001$) and IL-6 ($p<0.001$) (Table 5). As the fasting blood glucose level increased, IgA, IgG and IL-6 levels also increased in the diabetic study subjects. Glycated haemoglobin (HbA1c) correlated significantly, positively with IL-6 ($p<0.05$) but inversely with IgG ($p<0.05$) (Table 5).

Serum IgA and IgM (but not IgG) tended to increase significantly with IL-6 levels. ($p=0.001$, 0.031 and 0.500 respectively) (Table 6).

Table 5: Correlation of immunoglobulins and interleukin-6 levels with biochemical parameters of diabetic subjects

		IgA	IgG	IgM	IL-6
FBG	**R**	0.170*	0.252**	0.050	0.296**
	P	0.033	0.001	0.530	<0.001
HbA1C	**R**	0.035	-0.196*	-0.089	0.185*
	P	0.662	0.013	0.265	<0.020
T. Protein	**R**	0.174*	0.013	0.003	-0.079
	P	0.029	0.873	0.971	0.326
Albumin	**R**	0.035	0.021	-0.007	-0.159*
	P	0.662	0.791	0.927	0.046
HDL	**R**	-0.053	0.050	0.028	0.159*
	P	0.506	0.529	0.723	0.047
LDL	**R**	-0.090	-0.026	-0.108	0.049
	P	0.260	0.750	0.176	0.541
VLDL	**R**	0.110	0.069	-0.042	0.032
	P	0.168	0.389	0.604	0.689
LDL/HDL	**R**	-0.012	-0.027	-0.124	-0.058
	P	0.879	0.736	0.121	0.473
CHOL/HDL	**R**	0.036	-0.017	-0.107	-0.062
	P	0.657	0.830	0.181	0.440

Data presented as Spearman's correlation coefficient, r. *Correlation is significant at p<0.05; **Correlation is highly significant at p<0.01

FBG: fasting blood glucose; HbA1c: glycated haemoglobin; HDL: high density lipoprotein; LDL: low density lipoprotein, VLDL: very low density lipoprotein.

Table 6: Correlation between interleukin-6 and immunoglobulin levels of diabetic subjects

		IgA	IgG	IgM
IL-6	**R**	0.326**	0.054	0.172*
	P	0.001	0.500	0.031

Data presented as Spearman's correlation coefficient, r. *Correlation is significant at $p<0.05$; **Correlation is highly significant at $p<0.01$
IL-6: interleukin-6, IgA: immunoglobulin A, IgG: immunoglobulin G, IgM: immunoglobulin M.

CHAPTER FIVE

DISCUSSION

Results of the study on 96 Type 2 diabetic and 96 non-diabetic individuals showed a higher waist circumference (WC) in the diabetic than the non-diabetic subjects (Table 1). This observation supports the findings of The InterAct Consortium (2012) which indicated that high WC is an indicator of high adiposity which has the potential for diabetes development. The risk of type 2 diabetes in adults increases continuously with increasing obesity, and decreases with weight loss (World Health Organization, 2011; The InterAct Consortium, 2012). Increased abdominal fat surrounding the liver and other abdominal organs releases metabolically active fatty acids, inflammatory agents and hormones that ultimately lead to higher LDL cholesterol, triglycerides, blood glucose and blood pressure. A careful analysis of the relationship between obesity and adult-onset diabetes, according to World Health Organization (2011), confirms that abdominal obesity is an important risk factor, even after controlling for age, smoking and family history. However, the usefulness of waist circumference measurement as a first-step diagnostic tool when assessing an individual's risk of diabetes is still unclear. This may be because most published studies are cross - sectional, so the interpretation of results is likely to be confounded by other concurrent conditions such as hypertension and dyslipidaemia (World Health Organization, 2011). Indeed the subjects in this study with significantly high WC had already developed Type 2 diabetes. Early identification of increasing WC, and managed before the onset of type 2 diabetes could have prevented the condition. Therefore WC measurements should be carried out routinely on

particularly obese individuals to identify high risk individuals for preventive action or for early lifestyle intervention.

In the study, body mass index (BMI) was also found to be significantly higher in the diabetic subjects than the non- diabetics. This result confirmed findings by researchers (Wang *et al*., 2005; Knowler *et al*., 2002) indicating that BMI is high in diabetic patients. Actually, BMI was identified as a pre-disposing factor in diabetic development (The InterAct Consortium, 2012). Risk is particularly high in individuals with large amount of abdominal (visceral) fat (Kannel *et al.*, 1991), which is a source of bioactive mediators that directly contributes to insulin resistance (Xu *et al*., 2003). These findings also provide solid genetic support indicating that a higher BMI causes a raised risk of type 2 diabetes and high blood pressure.

Systolic and diastolic blood pressures were not significantly different in diabetic and non-diabetic subjects. According to Amoah *et al*. (2002), worsening glycaemic status tended to be associated with increase in body mass index as well as systolic and diastolic blood pressures. It was therefore expected that the diabetics in the study would present higher diastolic and systolic pressures compared to the non-diabetics. The diabetics in this study were on medication. Those who had developed hypertension were on anti-hypertensive drugs (Appendix E). These management practices could be responsible for the systolic and diastolic pressures which were not significantly different from the non-diabetics.

In this study, the distribution of the three immunoglobulins did not follow normal distribution (Fig 2). They were skewed because they are non-parametric measures . The results did not agree with the findings of Rodriguez-Segade *et al*., (1996) and Gonzalez-Quintela *et al*. (2008) who indicated that the immunoglobulin levels follow a normal

distribution but slightly skewed. The observed distribution of this study informed the choice of statistical analysis of the results for inferences to be made.

Analysis of the results revealed that the concentration of IgG was greater than the other immunoglobulins in both the Type 2 diabetics and non-diabetic subjects (Table 3). This result confirms the existing information that IgG is the most abundant of the total serum immunoglobulins in humans (Shetty, 1993). The immunoglobulin ranges of the non-diabetics measured in the study were consistent with established ranges from the U.S. National Library of Medicine (2012) for healthy individuals.

The concentrations of IgA and IgG were significantly higher in the diabetics than in the non-diabetic controls. It is known from literature that IgA concentration are elevated in the serum of diabetics. The significantly high IgA level observed in the diabetic subjects compared with the non-diabetic controls may reflect the possible accumulation of inflammatory conditions with the diabetes (Gonzalez-Quintela *et al*., 2008). According to Rodriguez-Segade *et al*. (1996), IgA is a non-specific sign of the development of diabetic complications. This is possible considering the fact that most of the subjects had lived with the diabetic condition for between 4 and 28 years. Also, IgA correlated highly with the serum concentration of interleukin 6, which according to Gonzalez-Quintela *et al*. (2008) is a marker of inflammation and a co-factor for immunoglobulin synthesis. It was also observed by Rodriguez-Segade *et al*. (1996) that people with micro- or macrovascular diabetic complication had higher serum IgA concentrations than the diabetics without complications. Therefore monitoring IgA may provide early warning of possible presence of complications. The observed significantly higher IgG in the diabetic subjects than the non-diabetic subjects could be associated with probable infection in the

diabetic patients.. In this study, serum IgM showed no significant difference in both type 2 diabetes and nondiabetics This finding agrees with those of Gonzalez-Quintela et al. (2008). Glycation of immunoglobulin occurs in patients with diabetes in proportion with the increase in HbA1c, and this may have harmed the biological function of the IgA and IgG antibodies (Peleg *et al.*, 2007), hence, their elevation in the diabetics.

The serum IL-6 level in Type 2 diabetic subjects was significantly higher in the diabetics than in the non-diabetic controls. This finding is in agreement with the observation made by Kado *et al*. (1999) and Marques-Vidal *et al*. (2013). However, this observation contradicts the findings of Al-Shukaili *et al*. (2013) who stated that the mean value of IL-6 was significantly lower in Type 2 diabetes mellitus cases, compared to control subjects. It has been reported that normal individuals with elevated levels of IL-6 had an independently increased risk to develop Type 2 diabetes. High levels of IL-6 and other inflammatory cytokines appear in early stages of Type 2 diabetes and are capable of promoting Type 2 diabetes through diminishing insulin sensitivity (Schmidt *et al*., 1999; Festa *et al*., 2002). Based on this information, occasionally, the inflammatory cytokine levels should be checked in the general population to screen for early signs of diabetic development.

There was no observed association between BMI and IL-6. This cytokine is known to be associated with obesity. It was expected that people with diabetes would have a higher BMI and WC than the non-diabetics. Glycated haemoglobin (HbA1c) in the study subjects was found to be significantly higher in the Type 2 diabetics than in the non-diabetic subjects. It was also positively correlated with IL-6. This observation agrees with previous findings (Predhan *et al*., 2001; Spranger *et al*., 2003).

5.1. Conclusion

The findings of the study showed that serum IgA and IgG were significantly higher in type 2 diabetics than in the non-diabetics but there was no significant difference in IgM between the two groups. The work revealed association between age and IgA levels. Body mass index is associated with IgG. Fasting blood glucose (FBG), glycated haemoglobin (HbA1c) and IL-6 are associated with alterations in immunoglobulin levels which are responsible for humoral immune response in Type 2 diabetic subjects. Body mass index correlated positively with IL-6.

Humoral immune responses were markedly altered with poorly controlled FBG and HbA1c which is likely to be one of the reasons why Type 2 diabetics have increased susceptibility to infections. The study supports the importance of controlling glycated haemoglobin levels, general sugar levels as well as BMI in Type 2 diabetics.

Type 2 diabetes is not associated with serum IgM but associated with serum IgA and IgG as well as interleukin-6. Thus the hypothesis that Type 2 diabetes mellitus does not influence levels of serum immunoglobulins IgA, IgG, IgM and interleukin 6 is accepted for IgM because the levels of IgM observed in the type 2 diabetic subjects were not significantly different from the non-diabetics. The hypothesis is, however, rejected for IgA, IgG and interleukin 6 whose levels were significantly higher in the diabetics compared to their levels in the non-diabetics.

5.2. Recommendations

Further research should be done to relate immunoglobulin levels to diabetic complications. Future work should consider the association between immunoglobulin levels and pathogenic infections in type 2 diabetic patients. Further studies should be done on larger sample size and different age categories with narrow range of duration of the diabetes.

Further investigations based on well-designed prospective studies with incident type 2 diabetes as the outcome would be needed to make recommendations on the usefulness of the waist circumference measurement as a first - step diagnostic tool when assessing an individual's risk of diabetes.

Further work should also be done to be able to establish whether elevated immunoglobulin leads to higher levels of interleukin 6 levels or vice versa.

5.3. Limitations

One difficulty encountered in the study was the lack reference range for immunoglobulin levels of apparently normal Ghanaians. The range of duration of diabetes of the subjects was large (4 to 28 years). The highly variable duration of the disease of the study subjects could have played an essential role in varied immunoglobulin and cytokine production among these two study groups.

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APPENDICES

APPENDIX A: ETHICAL APPROVAL

UNIVERSITY OF GHANA MEDICAL SCHOOL
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Your Ref. No.

P O Box 4236
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15th November, 2013

Collins Paa Kwesi Botchey
Dept. of Chemical Pathology
UGMS, Korle-Bu

ETHICAL CLEARANCE

Protocol Identification Number: MS-Et/M.2 – P 4.9/2013-2014

The Ethical and Protocol Review Committee of the University of Ghana Medical School on 14th November, 2013 unanimously approved your research proposal.

TITLE OF PROTOCOL: **"Serum Immunoglobulin Levels in Type 2 Diabetic Patients in Korle-Bu Teaching Hospital, Ghana"**

PRINCIPAL INVESTIGATOR: Collins Paa Kwesi Botchey

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 August, 2014.

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

Signed:.....
PROFESSOR JENNIFER WELBECK
(CHAIRPERSON, ETHICAL AND PROTOCOL REVIEW COMMITTEE)

cc: Ag. Dean
Head of Department
Research Office

APPENDIX B: INFORMED CONSENT FORM

TITLE OF STUDY: 'Serum Immunoglobulin Levels in Type 2 Diabetic Patients in Korle-Bu Teaching Hospital, Ghana

Serum immunoglobulin levels are determined routinely in clinical practice because they provide key information on the humoral immune status. Determining the distribution of immunoglobulin levels in general populations is important for interpreting reference values.

The major contributor to the increasing number of diabetic patients is type 2 diabetes. Diabetes is believed to be associated with changes in serum immunoglobulin level causing these levels to rise or fall. These immunoglobulins play important roles in the body's immune system. Studies have shown that increased concentrations of some circulating immunoglobulins may be non-specific signs of the development of diabetic complications. Furthermore, immunoglobulin levels which may serve as signs in the aetiogenesis of diabetic complications have not been studied in Ghana. It is the researcher's belief that the elevated immunoglobulin levels in diabetes may be an early warning sign for the development of diabetic complications and by taking part in this research; you will help address some of these issues.

I……have been asked to volunteer as a participant in the research study "Immunoglobulin levels in diabetic patients in Ghana".

I have been informed of the purpose of the study and how it will help in the management of the disease to reduce morbidity and mortality.

I have also been told that the procedure of the study is that:

I will have my name, age and sex taken and where I come from will be noted and everything recorded on a questionnaire. However, though the field collection will bear my name I will be referred to only by a unique identification number. High confidentiality will be maintained at all time on my information and personality.

I will be asked to provide blood sample. A trained technician will take venous blood from my arm using a syringe and needle. This may cause minor pain, discomfort, and bruising at the site of needle insertion.

The risk and discomfort are:

By participating in this research, I am likely to have some discomfort. This includes the discomfort of questioning and the pain of collection of blood. This team will try and reduce the chances of those risks happening, but in case it happens, they will provide me with free medical care in hospital.

Benefit:

There is no personal benefit that I will derive by participating in this study except my results could be released to my doctor upon my request.

Confidentiality:

The information that is collected for this research project will be kept confidential.

Information about me that will be collected for the study will be stored in a file which will not have my name on it, but a number assigned to it. The number assigned will not be disclosed to anyone except the principal investigator. The findings of this study may be reported at meetings or in medical journals, but my name will not be used in the report.

My right to refuse or withdraw:

I have the right to take part in this research or not without losing any benefits that I would normally have. I may stop participating in this research anytime I wish.

The alternatives to participating:

I have been told that this study does not involve the administration of investigational drugs or use of new curative procedures.

Contact information:

If I have any questions, I may ask them now or later, I may contact:

COLLINS P. K. BOTCHEY, Department of Chemical Pathology, UGMS Korle-Bu

(Principal Investigator)

Mobile: 0540980640

OR

DR. H. ASARE-ANANE, Department of Chemical Pathology, UGMS Korle-Bu

(Principal Supervisor)

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Consent

I have read (or have had someone interpret to me) the entire explanation about this study and have been given the opportunity to discuss any questions. I understand the nature, risks and benefits of the study and that I may withdraw at any time. I hereby consent to take part in this study.

……. Date……...

Signature of participant

…….. Date……...

Signature of Witness

……. Date……...

Signature of Investigator

APPENDIX C: STUDY QUESTIONNAIRE – DATA SHEET

Name (Surname in blocks): ……

Date: …… Serial Number: ……..

Contact Tel. Number: ……

Email: …….

Address: ……

Demographics and Anthropometric Characteristics

1. Age: ……. (35-60 years)
2. Height (m): …….. 3. Weight (kg): ……..... 4. BMI: ……

5. BP: ……6. Pulse: …… 7. Waist Circumference

(Pleases tick [√] the appropriate box where applicable)

7. Sex: [ ] Male

   [ ] Female

8. Marital status: [ ] Single

   [ ] Married

   [ ] Divorced

   [ ] Widowed

9. Number of children: [ ] None

   [ ] 1 – 2

   [ ] 3 – 4

   [ ] 5 or more

10. Level of education: [ ] None

[ ] Primary

[ ] Secondary

[ ] Tertiary

11. Occupation: [ ] Unemployed

[ ] Government worker

[ ] Trader

[ ] Others

Lifestyle

12. What type of food do you mostly eat?

[ ] More carbohydrates

[ ] Less carbohydrates

[ ] More meat or fish

[ ] Less meat or fish

[ ] More fruits

[ ] Less fruits

[ ] More vegetables

[ ] Less vegetables

13. How many times do you eat in a day?

[ ] Two

[ ] Three

[ ] Four

[ ] Five

14. Do you Exercise? [ ] Yes

[ ] No

15. If yes to question 19, how many times a week do you exercise?

[ ] Once a week

[ ] Twice a week

[ ] 3-5 times a week

[ ] Everyday of the week

Medical History /Assessment of diabetic complications:

16. Are you diabetic? [ ] Yes If yes, how long have you had it?.....

[ ] No

17. Are you hypertensive? [ ] Yes If yes, how long have you had it?.....

[ ] No

17. Have you had any infections such as malaria, typhoid or hepatitis B in the past week?

[ ] Yes If yes, how long have you had it?.....

[ ] No

Any other information/Remarks:

……..……

……

APPENDIX D: MANN-WHITNEY U-TEST FOR MEDIAN COMPARISON

	Cases	Control	P-value
IgA	0.8875	0.735	0.063
IgG	7.581	7.285	0.000
IgM	0.7346	0.5736	0.270
IL-6	1.701	0.991	0.000

CASES

	IgA (g/L)	IgG (g/L)	IgM (g/L)	IL-6 (pg/mL)
Number of values	96	96	96	96
Minimum	0.355	6.43	0.07919	0.3916
25% Percentile	0.625	7.435	0.3289	1.519
Median	0.8875	7.581	0.7346	1.701
75% Percentile	1.325	7.77	2.124	1.918
Maximum	4.81	8.35	4.99	14.73

CONTROL

	IgA (g/L)	IgG (g/L)	IgM (g/L)	IL-6 (pg/mL)
Number of values	96	96	96	96
Minimum	0.34	4.675	0.000364	0.008583
25% Percentile	0.5563	7.011	0.3085	0.4271
Median	0.735	7.285	0.5736	0.991
75% Percentile	0.9988	7.646	1.607	1.747
Maximum	3.505	11.65	4.056	4.522

APPENDIX E: DRUGS TAKEN BY SUBJECTS WHO ARE HYPERTENSIVE

	Hypertensive	Subjects on anti-hypertensive or anti-cholesterol medication						
	Subjects	Number on treatment	Amlodipine	Nifedipine	Losartan	Statin	Lisinopril	Other drugs
Diabetic Subjects (N=96)	35	35	13	5	2	8	18	13
Non-diabetic Subjects (N=96)	9	3	2	0	0	1	0	1