University of Zambia
School of Medicine
Department of Pathology & Microbiology

Assessment of Hyperinsulinaemia, Serum Fatty Acid Synthase Levels and Hypertriglyceridaemia in Type 2 Diabetes Mellitus at UTH, Lusaka, Zambia

A Dissertation Submitted to the University of Zambia, in Partial Fulfilment of the Requirements for the Master of Sciences Degree in Pathology (Chemical Pathology)

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DECLARATION

This work/dissertation in substantially its present form has not been submitted or accepted previously for the award of a degree or diploma in this or any tertiary institution, and is not being submitted for a degree or diploma in any tertiary institution or for another degree or diploma at this institution. I declare that this Dissertation contains my own work except where specifically acknowledged. I further declare that I followed all the applicable ethical guidelines in the conduct of the research. This dissertation has been prepared in accordance with the Masters of Sciences in Pathology (Chemical Pathology), University of Zambia guidelines.

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CERTIFCATE OF APPROVAL

The University of Zambia approves this Dissertation on “Assessment of Hyperinsulinaemia, Serum Fatty Acid Synthase and Hypertriglyceridaemia in Type 2 Diabetes Mellitus in Lusaka”.

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ABSTRACT

Context— The transcriptional regulation of lipogenesis is a highly coordinated process that is controlled by insulin. Fatty acid synthase (FASN) plays a central role in de novo lipogenesis by converting acetyl-CoA and malonyl-CoA into the final end product, palmitate, which may subsequently be esterified into triacylglycerols and then stored in adipose tissue. Triacylglyceride and other lipids have been implicated in the development of atherocardiovascular complications in diabetes mellitus.

Aims— In this study we aimed to assess serum insulin and fatty acids synthase in patients with T2DM and their relationship with hypertriglyceridaemia.

Methods and Results— An analytical cross-sectional study was undertaken in black indigenous Zambians. Serum insulin, circulating FASN and triglycerides were assessed in T2DM and non-diabetic participants. Our results showed that participants with T2DM had significantly higher insulin and FASN levels ($p < 0.001$ and $p = 0.022$ respectively). Serum triglycerides were also significantly higher in diabetics ($p = 0.039$). Insulin correlated to FASN in non-diabetic participants with statistical significance ($r = 0.476$, $p = 0.034$). Serum insulin also correlated to circulating FASN in diabetic participants ($r = 0.333$), however the correlation was not statistically significant ($p = 0.139$). Multivariate linear regression showed that circulating FASN was the best predictor of triglycerides concentration (ß = 0.340, $p = 0.09$).

Conclusion— The results of this study showed that hyperinsulinaemia and high FASN are characteristic of T2DM. The existence of high FASN in the presence of hyperinsulinaemia could explain the hypertriglyceridaemia and the associated increase in atherocardiovascular complications observed in T2DM.

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LIST OF ABBREVIATIONS

- ACC – Acetyl CoA Carboxylase
- ACP – Acyl Carrier Protein
- ALB – Albumin
- ALT – Alanine Amino Transferase
- AMPK – AMP Activated Kinase
- AMPKK – AMP Activated Kinase Kinase
- AST – Aspartate Amino Transferase
- bHLH-LZ – Basic Helix-Loop-Helix – Leucine Zipper
- BMI – Body Mass Index
- UNZA BREC – University of Zambia Biomedical Research Ethics Committee
- CAT-1 – Carnitine Acyl Transferase I
- CoA – Coenzyme A
- CPT-1 – Carnitine Palmitoyl Transferase I
- DM – Diabetes Mellitus
- DRGS – Directorate of Research and Graduate Studies
- ELISA – Enzyme Linked Immunosorbent Assay
- ERK – Extracellular Signal-Regulator Kinase (MAPK)
- FASN – Fatty Acid Synthase
- FET – Fisher's Exact Test
- FFA – Free Fatty Acid
- FoxO1 – Forkhead Box 01
- G6Pase – Glucose 6-Phosphatase
- HDL – High Density Lipoprotein
- IDF – International Diabetes Federation
- IHCL – Intra Hepatic Lipid
- IMCL – Intra myocellular lipid
- IMGU – Insulin Mediated Glucose Uptake
- IL – Interleukin
- IRS – Insulin Receptor Substrate
- LCACoA – Long Chain Fatty Acyl-CoA

- LDL – Low Density Lipoprotein
- LIRKO – Liver-Specific Insulin Receptor Knockout
- LLD – Lower Limit of Detection
- MAPK – Mitogen Activated Protein Kinase
- MAPKK – Mitogen Activated Protein Kinase Kinase
- MAPKKK – Mitogen Activated Protein Kinase Kinase Kinase
- MCD – Malonyl – CoA Decarboxylase
- MCP1 – Monocyte Chemoattractant Protein 1
- MEK – Mitogen Activated Protein Kinase / Extracellular (MAPKK)
- mmol/L – millimoles per Litre
- mRNA – Messenger Ribonucleic Acid
- NEFA – Non-esterified Fatty Acid
- ng – Nano grams
- O.D. – Optical Density
- PEPCK – Phosphoenolpyruvate Carboxykinase
- PI-3K – Phosphatidylinositol 3 – Kinase
- PKC – Protein Kinase C
- Raf – Virus-induced Rapidly Accelerated Fibrosarcoma Protein (MAPKKK)
- Ras – Rat Sarcoma Protein
- SREBP – Sterol Regulatory Element Binding Protein
- SD – Standard Deviation
- T1DM – Type One (1) Diabetes Mellitus
- T2DM – Type Two (2) Diabetes Mellitus
- TG – Triglycerides
- TNF-α – Tumour Necrosis Factor Alpha
- UCL – University College of London
- U/L – Units per Litre
- UNZA – University of Zambia
- UTH – University Teaching Hospital
- VLDL – Very Low Density Lipoprotein
- WHO – World Health Organization

1.0 INTRODUCTION

1.1. BACKGROUND

Almost a century ago, the noted physician Sir William Osler defined diabetes mellitus as “a syndrome due to disturbance in carbohydrate metabolism from various causes, in which sugar appears in the urine, associated with thirst, polyuria, wasting and imperfect oxidation of fats”. With the advent of insulin and other therapeutic agents however these extreme features are unusual in properly managed patients with diabetes. Long term consequences persist, however. Hence, diabetes mellitus (DM) in the modern setting has been redefined as “a state of premature cardiovascular death that is associated with chronic hyperglycaemia and may also be associated with blindness and renal failure.” (Rubin, 2012).

The chronic hyperglycaemia observed in DM is related to insulin production and/ or utilisation. Two major forms of diabetes mellitus are recognized, distinguished by their underlying pathophysiology. The underlying pathology of Type 1 diabetes mellitus (T1DM) is that of deficient insulin production whereas in Type 2 diabetes mellitus (T2DM) there is ineffective utilization of insulin. Individuals with T2DM account for about 90% of diabetics around the world (WHO, 2012).

T2DM individuals are usually obese, and they manifest with insulin resistance, hyperinsulinaemia, and hyperglycaemia (Joven et al, 2011). Chronic hyperinsulinaemia is a major contributor to glucose and lipid metabolism abnormalities that culminate in hallmark features of acute and chronic pathophysiologic changes with ravaging consequences throughout the body (McGarry, 1992).

Insulin is the major fed state hormone produced by the pancreatic β-cell. The effect of insulin on cellular metabolism is vast, though the most emphasized aspect of insulin effect is the hormone’s ability to facilitate glucose uptake by skeletal muscles and fat tissue. Therefore, insulin does not only lower blood glucose concentration, but also supplies the required fuel necessary for normal function of tissues that are dependent on insulin for glucose uptake. However, the less emphasized point is that insulin is the major lipogenic hormone (Fernandez-Real et al, 2010; Badger et al, 1997) and it is an important effector of free fatty acid production in the liver via

regulation of the enzyme fatty acid synthase (FASN). FASN is a cytosolic multi-enzyme complex that functions normally in the liver and is minimally expressed in other tissues (Dorn et al, 2010). FASN is a key enzyme in *de novo* lipogenesis (Dorn et al, 2010, Menendez and Lupu, 2007), involved in the formation of free fatty acids that are esterified to glycerol, to yield triacylglycerols. It is biochemically predictable that hyperinsulinaemia may lead to up-regulation of fatty acid synthase expression in the liver which may lead to an increase in the triacylglycerol content of the liver which culminate in increased very low density lipoprotein (VLDL) and free fatty acids secretion into the systemic circulation, contributing to the development of hypertriglyceridemia, increased plasma non-esterified fatty acids (NEFAs) and high serum LDL- cholesterol that are usually observed in T2DM.

Dyslipidaemia in DM mostly affect the blood vessels, heart and liver. Derangements in lipid metabolism are the major factor contributing to the pathogenesis of chronic complication of DM (Eckel, et al, 2010). Chronic complications of diabetes mellitus include macrovascular complications and microvascular complications.

Macrovascular complications include cardiovascular disease including atherosclerotic heart disease and ischemic stroke, which account for more than half of all deaths among adults with diabetes (Rubin, 2012). Cardiovascular complications are by far the major cause of mortality among individuals with T2DM. Atherosclerotic peripheral vascular disease usually of the lower extremities is another macrovascular complication of diabetes and is associated with poor blood supply to the peripheral tissue and also contributes to poor wound healing.

Hyperinsulinaemia or serum levels of FASN in T2DM have not been described in Zambia. Neither has the association between Insulin and FASN, FASN and serum triglycerides concentration been described. This study had aimed to investigate hyperinsulinaemia and serum FASN in T2DM, and also asses the relationship between serum FASN levels with insulin and serum triacylglyceride levels in T2DM with the aim to provide sufficient knowledge and understanding of the pathophysiology of T2DM with respect to development of dyslipidaemia, thus outcome atherocardiovascular complications.

1.2. RATIONALE OF THE STUDY

A significant number of the T2DM patients that report at the UTH present with chronic complications of diabetes mellitus such as strokes, heart diseases, peripheral vascular disease with amputations and among others. Chronic complications of T2DM continue to be the major indication for admission and referral to UTH; however the management of T2DM is mostly based on glycaemic control.

The T2DM individual almost invariably manifests a serious breakdown in lipid dynamics, often reflected by elevated levels of circulating free fatty acids (FFAs) and triglycerides (TG), together with excessive deposition of fat in various tissues, which may compound on the existing obesity and insulin resistant state of such individuals.

More specifically, a compelling argument is made that an abnormal accumulation of free fatty acid and triacylglycerols may be due to over expression of the FASN enzyme due to insulin mediated enhanced FASN gene expression. Thus, it may be hypothesised that patients with T2DM may have increased serum FASN levels.

To investigate hyperinsulinaemia and its association with serum FASN concentrations among T2DM individuals at UTH, an analytical cross-sectional study was conducted with a view that the findings may enhance the management of diabetic patients in order to anticipate and appropriately manage diabetic vascular diseases attributed to increase in plasma triglycerides such as atherosclerosis, thus ultimately improve the diabetic patient's general well-being. This study is in line with a paradigm shift that, advocates that lipids control are as important as hyperglycaemic control in the pathogenesis of T2DM and thus treatment for diabetic dyslipidaemias should be part of any T2DM treatment and management regime.

1.3. SIGNIFICANCE OF THE STUDY

Effective treatment and management of diabetes mellitus requires a deep insight into the disease pathogenesis and pathophysiology itself. In Zambia, most of the knowledge that has come to the surface through research on diabetes mellitus has been largely epidemiological or centered on glucose homeostasis. Despite all that we have come to know over many decades, it's generally accepted that our knowledge of diabetic disease process remains diminutive and elusive.

For these reasons, the management of hyperglycaemia to the non-diabetic range has traditionally taken center stage and has a substantial impact on some diabetes-related acute and microvascular complications. However, research has failed to show a significant effect of intensive glucose control on the risk of major macrovascular events in individuals with long standing (< 8 years) T2DM. Thus macrovascular and thrombotic complications have continued to be the major cause of morbidity and mortality among individuals with T2DM. It's possible that one of the reasons for this impasse might relate to the fact that traditional views have considered T2DM to be a disorder primarily associated with abnormal glucose metabolism. However, this research proposed a more lipid centred approach to the problem, with the possibility that traditional concepts in this area might have carried the wrong emphasis and that the early hallmark abnormality in T2DM maybe that of lipid metabolism.

This study was aimed at providing evidence-based information that hyperinsulinaemia, FASN and hypertriglyceridaemia are early events in the pathogenesis of T2DM. Thus with proof-of-concept, suggest that, it may be appropriate to commence therapies against major macrovascular complication early in the disease process. The study had gone further as to describe the probable relationship between hyperinsulinaemia and serum FASN, and their correlation with serum triacylglyceride level in T2DM individuals in Zambia. FASN is of considerable clinical relevance because it is pathophysiologically linked to several serious medical problems including type T2DM, hypertension, atherogenic dyslipidemia, abnormalities of blood coagulation and fibrinolysis and non-alcoholic fatty liver disease; which are all important independent risk factors for cardiovascular disease.

1.4. LITERATURE REVIEW

1.4.1. Diabetes Mellitus

Diabetes Mellitus (DM) has become a growing epidemic disease that results from changes in lifestyle, obesity, sedentarism and the phenomenon of urbanization, as well as the longer life expectation of the population. It is one of the causes for the growing mortality due to cardiovascular diseases, especially in the developing countries (Torres and Maurice, 2011).

An estimate of 371 million people had diabetes in 2012, this figure accounts for approximately 5.3% of the global population. The number of people with T2DM is increasing in every country and its has been estimated that by 2030 this will have risen to 552 million**.** In addition, many potential sufferers are not included in the count because according to an estimate about 50% of cases remain undiagnosed for up to 10 years (Ahmad, 2012). The greatest number of people with T2DM are between 40 to 59 years aged. However most of these individuals do not know that they have diabetes (IDF diabetes atlas, 2012)

In Zambia, the comparative prevalence of diabetes mellitus according to the World Health Organisation (WHO) was 5.13% in the year 2012 and this figure is expected to almost double in the next 20 years. It is estimated in sub-Saharan Africa that about 81% of individuals with diabetes remain undiagnosed and this region has the highest global mortality rate due to diabetes (IDF diabetes atlas, 2012).

1.4.2. Diabetes and Obesity

T2DM is closely associated with obesity and its now generally accepted that the products of excess adipose tissue precede the perturbations of glucose metabolism (McGarry, 1992). The link between diabetes mellitus and obesity is supported in part by the work of Hu *et al* (2001), who showed that 61% of the acquired cases of T2DM could be attributed to overweight and obesity. Hu, F. *et al* also showed that a mild increase in BMI increases the risk of T2DM: e.g. women with a Body Mass Index (BMI) between 23 and 25 kg/m^2 have an almost three-fold increased risk of

developing diabetes compared with women with a BMI below 23 kg/m^2. This relative risk increases to 20 for women with BMIs ≥35 kg/m^2 (Hu *et al*, 2001). Another broadly accepted prediction about diabetes is that, although undoubtedly polygenic and heterogeneous in its roots, the condition has two hallmark features: (1) insulin resistance, defined here as an impaired ability of the hormone to suppress hepatic glucose output and to promote peripheral glucose uptake and (*2*) compromised function of the pancreatic β-cell such that insulin secretion is insufficient to match the degree of insulin resistance (McGarry, 2001). To counter insulin resistance and prevent hyperglycaemia, the pancreatic β-cells hyper-secrete insulin, thus leading to hyperinsulinaemia (Boden and Laakso, 2004). For this reason, overt diabetes mellitus develops only in individuals whose β-cells are unable to meet the increased and sustained demand for insulin secretion (McGarry, 1992).

1.4.3. Insulin Resistance, Hyperinsulinaemia and Dyslipidaemias

From an initial perception that a disorder of glucose metabolism was the primary event in the pathogenesis of T2DM, there is now a growing appreciation that chronic elevation of FFA levels is an early event that contributes to the development of this disease. This line of thought is supported by the fact that there is close association between insulin resistance and dyslipidemia. The latter is characterized by raised small dense LDL levels, elevated levels of triacylglycerides, and low levels of HDL (Notarnicola, *et al*, 2012). Boden (1991) demonstrated that if plasma FFA levels are elevated for more than a few hours, they will cause insulin resistance. Later findings by Jeppesen (1997) also showed that elevation of plasma free fatty acids (FFAs) plays a pivotal role in the development of T2DM by causing insulin resistance. Consistent with this notation, Lam *et al* (2002) suggested that circulating levels of FFAs, which are often elevated in obese and diabetic individuals, have an important causative link in the association of obesity with insulin resistance and T2DM. The mechanism by which FFA can cause insulin resistance, although not completely known, include generation of lipid metabolites (diacylglycerol), proinflammatory cytokines (TNF-α, IL1β, IL6, MCP-1) and cellular stress including oxidative and endoplasmic reticulum stress (Boden, 2011 and McGrady 2004).

Insulin resistance increases with excessive deposition of fat in various tissues including the muscle bed (Reaven, 1995; Schalch and Kipnis, 2010). Of important note are findings by Krssark *et al* 1999; Perseghin *et al.* 1998 and Perseghin *et al.* 2002, which showed a negative correlation between intramyocellular lipid (IMCL) content and whole - body insulin sensitivity (insulin resistance).

It also follows that FFAs induced insulin resistance increases as plasma FFA levels increase, and this can be a beneficial adaptive response during starvation and pregnancy. However, insulin resistance can become counterproductive when there is an excess of energy intake associated with physical inactivity. Under these conditions, glucose levels remain normal only as long as the hyperinsulinaemia is sufficient to compensate for the insulin resistance (Boden and Laakso, 2004).

1.4.4. Role of Insulin on Hepatic Lipid and Carbohydrate Metabolism

The metabolism of lipids in vertebrate cells is regulated by a family of transcription factors called sterol regulatory element binding proteins (SREBP's). SREBP's directly activate the expression of over 30 genes involved in the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids (Lin *et al*, 2005). Three different SREBP isoforms designated SREBP-1a, SREBP-1c, and SREBP-2 are encoded by two different genes and belong to the basic helix-loop-helix-leucine zipper (bHLH-LZ) family of transcription factors (Osborne, 2000; Brown and Goldstein, 1997). SREBP was originally identified as a transcription factor that binds to sterol regulatory element (SRE) for cholesterol regulation (Lin *et al*, 2005). Of the three members of the SREBP family, SREBP-1a, SREBP-1c and SREBP-2, SREBP-1c is highly expressed in lipogenic tissue, and is itself induced by feeding and/ or insulin (Horton *et al.* 2002).

The effect of insulin in a state of liver insulin resistance on hepatic lipogenesis was in part explained by Shimomura *et al* (2000) whose finding suggested that the compensatory elevation of insulin levels upregulates SREBP-1c expression which in turn activates hepatic lipogenic enzymes. Insulin mediated activation of atypical protein kinase C gamma (PKCλ), PKCζ and Akt via the phosphotidylinostol 3-kinase (PI-3K) pathway and/or Ras/Raf/MEK1/MEK2/ERK1/ERK2 mitogen activated protein

kinase (MAPK) signal transduction cascades, stimulate SREBP-1c and ultimately stimulate insulin FASN-dependent lipogenesis. (Van Obberghen *et al*, 2001 and Menendez *et al*, 2009).

SREBP-1c–responsive genes include those for citrate lyase and acetyl-CoA carboxylase (ACC) and FASN, which are enzymes, involved in *de novo* biosynthesis of palmitate (C 16:0). Other SREBP-1c target genes encode a rate-limiting enzyme of the fatty acid elongase complex, which converts palmitate to stearate (C18:0) (Moon *et al*, 2001); stearoyl-CoA desaturase, which converts stearate to oleate (C18:1); and mitochondrial glycerol-3-phosphate acyltransferase, the first committed enzyme in triacylglycerols and phospholipid synthesis (Edwards *et al*, 2000). Other supporting evidence indicates that the fatty liver of insulin resistance is caused by SREBP-1c, which is elevated in response to the high insulin levels (Shimomura *et al*, 1999a; Shimomura *et al*, 1999b).

So how does the liver respond to insulin despite the presence of insulin resistance in peripheral tissues? This mixed picture of insulin sensitivity and resistance cannot be easily explained. It has been proposed that insulin controls glucose and lipid metabolism through different pathways, with insulin receptor substrate 2 (Irs2) acting on glucose production via forkhead box O1 (FoxO1) and Irs1 acting on lipid metabolism via sterol regulatory element–binding protein-1 (SREBP-1) (Shimomura *et al*, 2000; Matsumoto *et al*, 2002) and FoxA2. To answer the question, Biddinger *et al*, (2008) had conducted a study to examine glucose and lipid metabolism in liver-specific insulin receptor knockout (LIRKO) mice. Biddinger *et al*. (2008) explored the consequences of hepatic insulin receptor deficiency for hypertriglyceridaemia. Herein lays the paradox. In LIRKO mice, the correlation with T2DM breaks down. Despite hyperglycaemia and hyperinsulinaemia, LIRKO mice manifest low plasma triglycerides and no elevation of hepatic triglycerides. The explanation for the paradox is that; first, insulin stimulates the phosphorylation of FoxO1, a transcription factor that activates gluconeogenesis (Matsumoto *et al*. 2006). Phosphorylation of FoxO1 downregulates genes required for gluconeogenesis, most prominently phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase), which leads to a decrease in hepatic glucose output, which helps to keep

blood glucose low. The second action of insulin is to activate the transcription factor SREBP-1c, which enhances transcription of genes required for fatty acid and triglyceride biosynthesis (Brown and Goldstein, 1997; Hurton *et al*, 2002). These studies assisted in the description of selective insulin resistance (Michael *et al*. 2000); despite extremely high insulin levels, the mRNAs for PEPCK and G6Pase remain high, and gluconeogenesis continues due to insulin resistance in the FoxO1 pathway. Despite insulin resistance in the FoxO1 pathway, insulin sensitivity is maintained in the SREBP-1c pathway (Shimomura *et al*. 2000). Thus, nuclear SREBP-1c levels are extremely high, which enhances fatty acid synthesis, and accelerates triacylglycerol accumulation (Shimomura *et al*. 2000; Shimomura *et al*. 1999b). Consistent with this notion, the excessive hepatic glucose output leads to further stress on the pancreatic β-cells to hyper-secrete insulin. And this could later lead to insulin insufficiency. This could explain why T2DM patients may no longer respond to oral hypoglycaemic medication to control the blood glucose and may have to be given insulin to control the blood glucose and prevent the complication of diabetes keto-acidosis.

1.4.5. Role of Insulin on Muscle Lipid and Carbohydrate Metabolism in T2DM

The concept that a lower than normal capacity to burn fat in extrahepatic tissue in a setting of hyperinsulinaemia has received support from both human and animal studies. Unlike the situation in liver, where malonyl-CoA can be actively converted into fatty acids, FASN is minimally expressed in skeletal and cardiac muscle (Ruderman *et al.* 1999). Accordingly, in muscle cells, malonyl-CoA act as a fuel sensors whose primary role is to regulate the rate of fatty acid oxidation in the mitochondria by inhibiting Carnitine-Acyl Transferase (CAT-I) also known as carnitine palmitoyltransferase I (CPT-1) – the enzyme that transports long chain fatty acids into mitochondria for oxidation (McGarry, 1995).

Furthermore, cellular concentrations of malony-CoA are regulated by acetyl-CoA carboxylase 2 (ACC2) (the muscle isoenzyme) and malonyl-CoA decarboxylase (MCD), both of which are regulated by AMP-activated kinase (AMPK) (Hardie *et al.* 1998; Hardie, 2004). AMPK itself is regulated by the upstream AMP-activated protein kinase kinase (AMPKK) (Hardie *et al.* 1998; Minchenko *et al.* 2008; Minchenko *et al.*

2011). A well-established role of AMPK is the phosphorylation and inactivation of ACC and also phosphorylation and activation of MCD (Winder *et al.* 1996; Saha *et al.* 2000). The net result would be a rapid fall in the malonyl-CoA level and an enhanced capacity of the cell to oxidize fatty acids (Chen *et al.* 2000). Therefore, ACC2 may be instrumental in the genesis of early insulin resistance in individuals prone to develop obesity and T2DM.

The most potent inhibitor of AMPK is a specific protein phosphatase that acts down stream the insulin-signalling pathway. Hyperinsulinemia may hence favour dephosphorylation of AMPK and/or ACC2 and MCD by upregulation of the relevant phosphatases and cause an increase in the activity of ACC2, leading to higher than normal levels of malonyl-CoA. The result would be a suppressed capacity of muscle to oxidize fatty acids, expansion of the LCACoA (and TG) pool, increased IMCL content (thus insulin resistance) and diminished insulin mediated glucose uptake (IMGU) (Saha *et al.* 2000; McGarry and Brown, 1997)

This concept received support from a number of human and animal studies, which showed an increase in muscle malonyl-CoA levels in insulin-resistant animal models (Saha *et al.* 2000) and T2DM humans (Bandyopadhyay *et al.* 2006; Choi *et al.* 2007; Olson *et al.* 2010).

1.4.6. Fatty Acid Synthase

FASN catalyses the synthesis of palmitate (C16:0) from acetyl-CoA as a primer and seven molecules of malonyl-CoA (acetate donor) derived from acetyl-CoA by ACC. FASN is active as a homodimer enzyme complex with seven different catalytic activities that include acetyl-CoA–Acyl Carrier Protein (ACP) transacetylase, malonyl-CoA–ACP transferase, β-ketoacyl-ACP synthase, β-ketoacyl-ACP reductase, β-hydroxyacyl-ACP dehydratase, enoyl-ACP reductase, and ACP thioesterase (Lomakin, Xiong and Steitz, 2007; Leibundgut *et al.* 2008). FASN catalyses all enzymatic steps essential for fatty acid biosynthesis (Leibundgut *et al.* 2008) in the liver for export to metabolically active tissues or storage in adipose tissue. Despite being an intracellular protein, FASN may be released into the extracellular space and may be a biomarker of metabolically demanding human diseases (Oliveras-Ferraros *et al.* 2009). This new paradigm proposes that insulin-resistant conditions such as obesity, T2DM, and cancer arise from a common FASN-driven “lipogenic state”. An

important question then is whether the development or the progression of insulin-related metabolic disorders can be prevented or reversed by the modulation of FASN status (Menendez *et al.* 2009).

According to the research literature, increased expression of FASN has emerged as a phenotype common to most human carcinomas. Increased FASN levels have been detected in serum of patients with different clinical stages of various cancers including breast cancer, colorectal cancer and maybe associated with tumour stage (Walter *et al.* 2009; Vazquez-Martin *et al.* 2009). In another research Richardson *et al* (2008) demonstrated that most human carcinomas, including those of the breast and prostate, overexpress FASN. In the vast majority of tumour cases, FASN has been described as a requirement for tumour cell survival and it also seems to play a role in conferring chemoresistance (Menendez *et al.* 2009). Recently, high serum levels of FASN have been detected in patients with chronic viral hepatitis infections and circulating FASN concentration correlated with the degree of liver steatosis (Joven *et al*. 2011). As many as 40% of T2DM develop evidence of hepatic steatosis (non-alcoholic fatty liver); a condition that leads to hepatic fibrosis and cirrhosis (Van Steenbergen and Lanckmans, 1995).

Menendez *et al* (2009) envisioned that, because energy metabolism, and especially dysfunction of glucose/lipid metabolism, is an early and nearly universal hallmark in most human malignancies, increased concentrations of extracellular/circulating FASN might also occur in other metabolic disorders in which insulin resistance is prominent, such as obesity, T2DM, or altered glucose tolerance. Intriguingly, Menendez *et al* (2009) observed a strong relationship between higher concentrations of circulating FASN and the most pronounced insulin resistance in the absence of any evidence of concurrent neosplasia (Fernandez-Real *et al.* 2010).

This literature review shows that hyperinsulinaemia increases fatty acid and TG synthesis in liver. The fatty acids are secreted into systemic circulation as triacylglycerols and VLDL particles, which are taken up, by muscle and adipose tissue. As triacylglycerols accumulate in muscle and in adipose tissue, fatty acids derived from these triglycerides worsen the insulin-resistant state in muscle and adipose tissue leading to a compromise in insulin-stimulated glucose uptake and

metabolism at these sites. The progress of these events leads from simple insulin resistance to glucose intolerance with elevated glucose levels causing even greater compensatory postprandial hyperinsulinaemia, which sets up a vicious cycle. The net result is the classic type 2 diabetic triad – hyperglycaemia, hyperinsulinaemia, and hypertriglyceridaemia (Michael *et al.* 2000).

In individuals with a genetic predisposition for diabetes, however, the pancreas cannot compensate for the increased secretory demands placed on it, resulting in pancreatic β-cell failure and decline in insulin levels, thus the development of Insulin – Requiring T2DM with its chronic complications (Boden and Laakso, 2004). Elevated plasma FFA levels do not only account for a large part of insulin resistance in obese patients with T2DM, but also account as an important contributor to β-cell demise. Islet lipotoxicity that leads to decreased insulin secretion in T2DM is largely due to FFAs accumulation in the pancreas leading to β-cell apoptosis (McGarry, 2001).

To determine the association between hyperinsulinaemia and FASN. Serum insulin concentration can be determined using a specific ELISA test kit for human insulin (Tasnim *et al*. 2012). Reference ranges for plasma insulin concentration are largely dependant on the testing method (McSorley, 2002). A global assessment on the ultimate role of FASN expression and/or activity in humans is largely precluded because immunohistochemical and/or mRNA studies should be performed in tissue biopsies from individuals. In this regard, the quantitative determination of FASN molecules in blood might be considered a non-invasive and objective method to easily and rapidly identify FASN-related metabolic altered states of insulin sensitivity in human subjects. Several studies have demonstrated that FASN, a cytoplasmic protein, can also occur in the extracellular space (Oliveras-Ferraros *et al.* 2009; Walter *et al.* 2009; Vazquez-Martin *et al.* 2009), hence can be determined using a specific ELISA test kit for human FASN (Vāvere and Lewis, 2008).

1.5. RESEARCH QUESTION

Does hyperinsulinaemia result in an increased plasma fatty acid synthase concentration, which may be associated with the observed hypertriglyceridaemia?

2.0. OBJECTIVES

2.1. GENERAL OBJECTIVE

To study serum FASN in patients with T2DM and establish its probable relationship with hyperinsulinaemia and hypertriglyceridaemia.

2.2. SPECIFIC OBJECTIVES

- To determine the mean difference in plasma insulin concentration and FASN concentration between T2DM and non-diabetic individuals.
- To provide proof-of-concept that hyperinsulinaemia is associated with up-regulation of FASN expression in T2DM.
- To evaluate the relationship between FASN and hypertriglyceridaemia as a biomarker of atherogenic dyslipidaemias in T2DM.

3.0. METHODOLOGY

3.1. STUDY DESIGN AND SITE

An analytical cross-sectional study was conducted, involving adult patients with T2DM and non-diabetic participants. The study was conducted at the UTH outpatient department (clinic 5), Lusaka, Zambia. Clinic 5 is a specialist clinic consisting of diabetic and cardiovascular patients that have been referred to UTH for further management.

3.2. TARGET POPULATION

All adults aged between 18 - 75 years old with T2DM that reported to clinic 5 at UTH.

3.3. STUDY POPULATION

All individuals that satisfied the inclusion criteria were enrolled into the study. A study non-diabetic group of individuals that did not have clinically or laboratory diagnosed DM, cancer, acute or chronic inflammatory condition, non-pregnant, were recruited from health individuals within the UTH and at Barclays Sports Complex. The two study groups were matched for age categories and sex in order to minimise bias.

3.4. SAMPLE SIZE

A total sample size of **44** participants **(22 patients and 22 non-diabetics)** had been calculated using the formula for determination of sample size for comparative research studies between two groups as given below;

$$N = \frac{4\sigma^2(z_{crit} + z_{pwr})^2}{D^2},$$

Where; *N* is the total sample size (the sum of the sizes of both comparison groups), σ is 8; the assumed SD of each group (assumed to be equal for both groups), the z_{crit} value is 1.960 as given in tables for Standard Normal Deviate (z_{crit}) corresponding to the desired significance criterion of 0.05 or 95% confidence interval (CI), the z_{pwr} value is 0.842 as given in Standard Normal Deviate (z_{pwr}) tables corresponding to 80% statistical power, and *D* is the minimum expected difference between the two means which has been estimated at 7. Both z_{crit} and z_{pwr} are cut-off points along the x-axis of a standard normal probability distribution that demarcate probabilities matching the specified significance criterion and statistical power, respectively. The two groups that make up *N* are assumed to be equal in number, also that the outcome variable of a comparative study is a continuous value for which means are compared, and it is assumed that two-tailed statistical analysis will be used (Eng, 2003).

3.5. SAMPLING METHODS

Systematic sampling in which consecutive individuals with T2DM that had reported to clinic 5 and met the inclusion criteria (described elsewhere) were included into the study sample. At least 4mls of blood sample were collected from each participant in 4mls plain vacutainers; the serum was separated from the blood cellular components and stored at a temperature of -80^0C for analysis on a later date. A non-diabetic group was selected by means of frequency matching of the same proportional characteristics (age broad categories and sex) as the study sample. The age categories were defined as follows: 1 (18 – 24yrs), 2 (25 – 64yrs), 3 (65 – 75yrs).

3.6. CASE DEFINITION

- **Type 1 diabetic individuals will be considered to be:**

(a) Those that were and had entirely been on insulin treatment from the onset of disease.

- **Type 2 diabetic individuals will be considered to be:**

(a) Those whose onset of disease was in adulthood (above 18 years old).

(b) Those that were and had been taking oral hypoglycaemic drugs

c) Those that were usually controlling their glycaemia through diet control.

NOTE: individuals that could not be classified under T2DM according to the above stated case definition had been excluded for the study.

3.6.1. Case Definition Limitations:

(a) Individuals with T1DM who initially had been misdiagnosed as having T2DM, thus initiated on oral hypoglycaemic drugs by this definition may have been considered as having T2DM.
(b) Individuals who had T1DM of adult onset may also been miscategorised as having T2DM.
(c) Individuals with T2DM who had been initiated on insulin treatment at the time of diagnosis and/or individuals with T2DM of childhood onset maybe miscategorised as having T1DM with respect to the above case definition. However such individuals had not been included in the study for being on insulin treatment.

3.6.2. Inclusion Criteria:

- Individuals with T2DM that had been diagnosed within the last 10 years and not on exogenous insulin treatment.
- Individuals aged between 18 and 75 years old.

- Those who had read (or read to) and understood the rationale of participating in the research, thereafter given written personal consent without undue duress.

3.6.3. Exclusion Criteria

- Individuals with T1DM or T2DM that were on insulin treatment.
- Patients that had malignant diseases or any acute or chronic inflammatory disease, as by determined clinical signs of infection*.
- Individuals who had undergone any major surgery at least a month before*.
- Non-Negroid Zambians.
- Pregnant women.
- Non-consenting individuals

➢ NOTE: Non – diabetic participants: Apart from not being diabetic, the inclusion criteria and exclusion criteria for this group were the same as that given for T2DM group.

* This information was obtained from the patient files, clinical signs of infection and from the interviews administered using a questionnaire.

3.7. DATA COLLECTION

3.7.1. Clinical Data and Demographic Data Collection

Participants were recruited at clinic 5 during normal clinic hours from 07:00 AM to 12:00 PM, from Monday to Friday. As researchers met the participants they were informed and explained to about the study. The researchers also provided the diabetic patients with the study information sheet. If patients autonomously agreed to participate in the study, they were required to sign the consent form and assigned a serial number.

Thereafter information on the participant's demographic data, medical history, and on-spot fasting blood glucose concentration were collected and compiled using a questionnaire. The demographic data included the participants' age, sex, occupation, marital status, exercise and residence. The medical history data included the specific

year in which the participant was diagnosed with diabetes mellitus, past and current medication list and dosage, and the presence of medical condition that may confound the research finding; included here were any major surgeries and cancer. The participant's routine on-spot fasting blood sugar concentration as measured by the researchers using a glucometer was also transcribed on to the questionnaire. The patients' files were also reviewed for any further relevant data to the research and also to confirm the accuracy of information provided by the participant and recorded in the questionnaire.

3.7.2. Specimen Collection

Study staff confirmed that participants had fasted for at least 8 hours before blood collection. Blood samples were collected from the conscious participants via venepuncture from the antecubital vein. 4ml of blood were collected in a 5ml syringe using 21G bore size needles. The collected blood specimen were then transferred into a 4ml blood volume plain (non-anticoagulated) vacutainers that were numbered with the unique participants' assigned serial number as recorded on the questionnaire. The blood specimens were transported to the UTH's Endocrinology Laboratory within 1 hour of collection for processing and analysis.

3.7.3. Specimen Preparation and Storage

In the laboratory, each specimen serial number was recorded on to a compilation summary sheet. Thereafter the blood specimens were centrifuged at 3000 revolutions per minute (3000 rpm) into order to separate the serum from the blood cellular component. Only serum were meticulously collected from the vacutainers using pasture pipettes and transferred to 2ml plastic cryovial containers with sealable screw caps, which were stored in a freezer at -80^{o}C until the specimens were required for analysis.

3.8. QUALITY CONTROL

To ensure reliable results, quality control was performed on all the analytical instruments and analysers used for any purpose during specimen analysis according to the UTH quality control guidelines. Quality control included equipment calibrations and analytical control runs on every analyser before each test analysis.

3.9. SPECIMEN ANALYSIS

3.9.1. Insulin ELISA Test Protocol

Serum insulin concentration was determined using the NeoBioLab® Human INS ELISA Kit; a quantitative competitive immunoassay for measurement of Human Insulin in cell culture fluid, body fluid, tissue homogenate, serum or plasma according to the manufacturer's protocol given below. This assay employed an antibody specific for Human Insulin coated on a 96-well plate.

3.9.1.1. Reagent Preparation

All kit components and samples were brought to room temperature before use. The microtiter plate was brought to room temperature before opening. The WASH SOLUTION concentrate (100×) was diluted with 990 mL of distilled water.

3.9.1.2. Assay Procedure

100 µL of SAMPLE or STANDARD (Insulin) (A to F) were added in duplicate to the appropriate wells in the supplied microtiter plate. Then 50 µL of CONJUGATE (Horseradish peroxidase) were added to each well and mixed well. The plate was then covered and Incubated for 1 hour at 37°C in a humid chamber. After incubation each well was washed 5 times with 300-400 µL 1X WASH SOLUTION (Buffered water) per well. After the last wash, the plate was inverted and blotted dry by tapping on absorbent paper to completely remove the liquid at each step. 50 µL of SUBSTRATE A (Peroxide) was added to each well followed by addition of 50 µL SUBSTRATE B (3, 3', 5, 5' Tetramethylbenzidine). The plate was here after covered and incubated for 10-15 minutes at room temperature away from direct sunlight. After incubation, 50 µL of STOP SOLUTION (Acid solution) were added to each well and the contents mixed well. The optical density (O.D.) was immediately read at 450 nm.

3.9.1.3. Data Processing

The O.D. of other non-zero standards were divided by that of the zero standard, then multiplied by 100 (used as X variables). Then, the base 10 logarithm of other standard concentration was calculated (taken as Y variables). A standard curve was generated from these variables in Microsoft Excel 2011 for Mac.

FIGURE 1: Insulin Calibration Curve

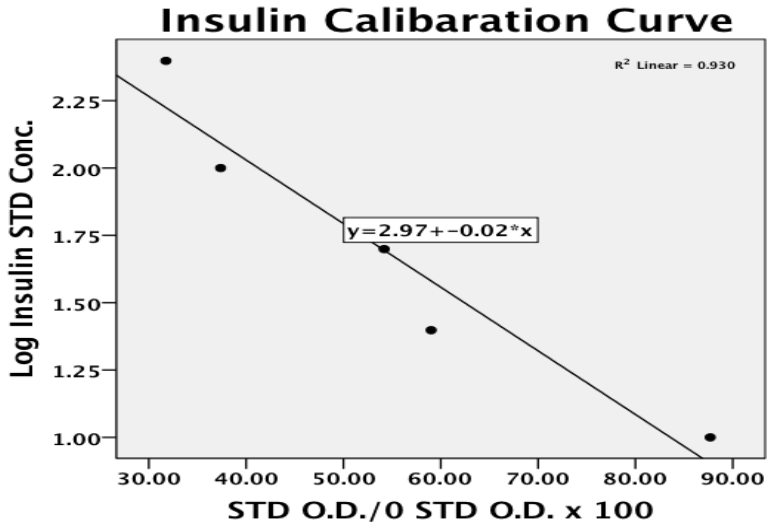

Fig. 1: Insulin calibration curve plotted from standard absorbances (O.Ds) againist concentrations. The regression equation was used to calculate sample concentration from their respective O.Ds.

To calculate results: the sample O.D. was processed as follows: O.D. of sample divided by that of standard 0, then multiplied by 100, to get Y values using the formulation y = -0.023x + 2.9743. To get the concentration of samples: 10 was powered to Y (10^{Y}).

3.9.1.4. Sensitivity And Specificity

The sensitivity obtained from this ELISA test kit is approximately 1.0 uIU/mL. The assay has high sensitivity and specificity for detection of insulin. No cross-reactivity or interference between insulin and any homologous proteins has been observed. Species cross reactivity has not been specifically determined.

3.9.2. Fatty Acid Synthase ELISA Test Protocol

Circulating FASN concentrations was measured in serum without additives by NeoBioLab® sandwich enzyme immunoassay (FAS ELISA) for the quantitative measurement of samples in serum, plasma, cell culture supernatants and urine.

3.9.2.1. Reagent Preparation

All kit components and samples were brought to room temperature before use. The microtiter plate was brought to room temperature before opening. The WASH SOLUTION concentrate (100×) was diluted with 990 mL of distilled water.

3.9.2.2. Assay Procedure

50 µL of SAMPLE or STANDARD was added in duplicate to the appropriate wells in the supplied microtiter plate. The wells had been pre-blocked and no additional blocking steps were required. The plate was then incubated for 1 hour at room temperature. After incubation, the wells were washed 3-5 times with 300-400 µL 1X WASH SOLUTION per well. Final wash was emptied and 100 µL of CONJUGATE added per well and mixed well. Then the plate was covered and incubated for 1 hour at 37°C in a humid chamber. After incubation, each well was washed 5 times with 1X WASH SOLUTION. After the last wash, the plate was inverted and blotted dry by tapping on absorbent paper to completely remove the liquid. 50 µL of SUBSTRATE A was added to each well followed by addition of 50 µL SUBSTRATE B. The plate was Covered and incubated for 10-15 minutes at room temperature away from direct sunlight. 50 µL of STOP SOLUTION was added to each well and mixed well. The optical density (O.D.) was read immediately at 450 nm. The mean blank value was subtracted from each SAMPLE or STANDARD value and the mean was calculated for duplicate wells. Standard curve was constructed in Microsoft Excel 2011 for Mac as given below. The concentration of all the samples was calculated using the equation y = 97.286x – 3.2617.

FIGURE 2: FASN Calibration Curve

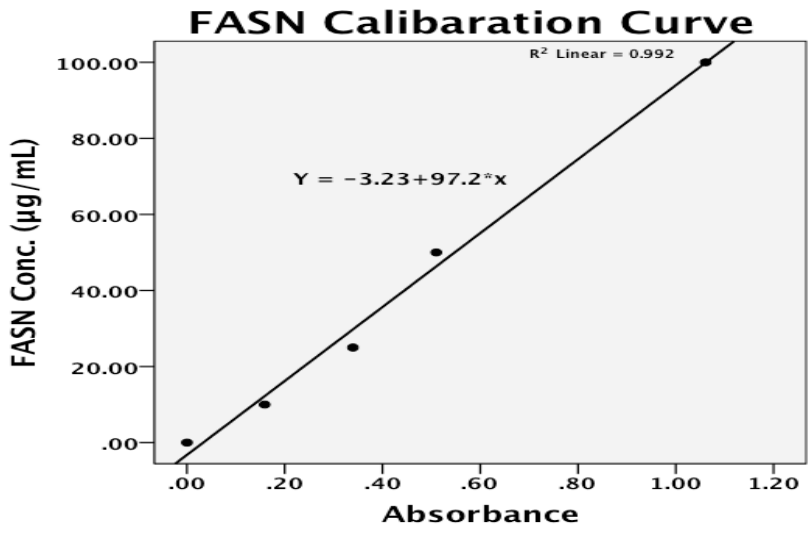

Fig. 2: FASN calibration curve plotted from standard concentratioins againist standard absorbances (O.Ds). The regression equation was used to calculate sample concentration from the respective O.Ds.

3.9.2.3. Sensitivity and Specificity

This assay has high sensitivity and excellent specificity for detection of FASN. No significant cross-reactivity or interference between FASN and any homologous protein assay has been observed. Species cross-reactivity has not been specifically determined. Note: Limited by current skills and knowledge, it is impossible to complete the cross-reactivity detection between FASN and all the analogues, therefore, cross-reaction may still exist.

The sensitivity of this assay or Lower Limit of Detection (LLD) is defined as the lowest protein concentration that could be differentiated from zero. The minimum detection limit is < 1ng/mL for this particular assay.

Both ELISA tests (FASN and Insulin) were performed in the UNZA – UCL Endocrinology Research Laboratory in the UTH. ELISA plates for serum FASN and insulin were read on the microwell reader available in the UNZA – UCL Endocrinology Research Laboratory.

3.9.3. Triglyceride, Cholesterol, LDL, HDL, AST, ALT and Albumin Test Protocol

These were determined using the available test kits on the Pentra 400 Chemistry Analyser available in UTH Clinical Chemistry Laboratory according to the manufacturer's recommendation and assay procedures for the automated analyser. All test protocols were calibrated and controls ran before samples could be assayed.

Note: A duplicate copy of all patients test results were sent to the patients' file for which the patients were notified of their results by the clinician. All non – diabetic participants were notified of their results by the researcher and they collected the results from the researcher or the Medical File Manager in Clinic 5 of the UTH.

3.10. ETHICAL CONSIDERATIONS AND PERMISSIONS

Patient information and results were kept confidential and access to this information was restricted to the researcher, supervisor, clinicians and the file managers in Clinic 5 only. The questionnaire had captured the participants' file number, which was assigned a serial number hence specimen containers were identified by serial numbers. The file number was obtained for the purpose of returning the results. All research participants were notified of their results, and results explained to them by the researcher or by the clinician.

The study participants were provided with an information sheet and given a thorough explanation of intent and rationale of the research after which the participants had given written informed consent without duress, thus insure a true meeting of minds between the researcher and the participants. All the above mentioned was done in private on a one to one basis to avoid undue influence that may have affected or substituted the patient's will for that of any other persons.

The research proposal for this study was submitted and approved by the University of Zambia Biomedical Research Ethics Committee (UNZA-BREC) for ethical approval.

Permission to conduct the study was be obtained from the: UTH medical superintendent, the consultants in the Department of Medicine, at LMGH through the Clinical Care Director's office and the Directorate of Research and Graduate Studies (DRGS) through the Assistant Dean, Postgraduate.

Permission to use equipment and facilities in the clinical chemistry laboratory in the UTH was obtained from the Head of the Department of Pathology and Microbiology at the University Teaching Hospital, and permission to use the ELISA equipment and laboratory facilities in the Endocrinology Research Laboratory was obtained from the Endocrinology Research Laboratory Head of Department.

4.0. DATA PROCESSING AND STATISTICAL ANALYSIS

Data were expressed as mean ± SEM for normally distributed continuous variables or median (interquartile range) for non-normally distributed variables. Normality was assessed using the Shapiro and Wilk statistic and the normality plots. Skewed variables were log – transformed prior to analysis.

The independent sample student's *t*-test was used to compare mean values of plasma insulin concentration to FASN concentration, mean serum FASN concentration to mean triglycerides concentration and other biochemical test (Cholesterol, LDL, HDL, ALT, AST, and Albumin) between the two groups (T2DM and non-diabetic group). The data was cleaned and then after showed no violation of normality as assessed by use of the Shapiro and Wilk statistic, and also showed homoscedasticity as assessed by use of the Levene test for equality of variance.

Bivariate linear regression and correlation coefficients were used to assess correlation between insulin and FASN, FASN and triglyceride in T2DM. Bivariate linear regression data of insulin vs. FASN and FASN vs. triglycerides were plotted and presented on scattergraphs. Step-wise multivariate linear regression analysis was used to examine the extent to which the different variables accounted for the variability of insulin, FASN and triglycerides. However multivariate linear regression was not utilised for rigorous hypothesis testing. When insulin was the dependent variable, the independent variables were BMI, glucose FASN, ALT, AST, albumin, cholesterol, LDL, HDL and triglycerides. When triglycerides was the dependent variable, the independent variables were Insulin, FASN, glucose, BMI, HDL, LDL, AST, ALT, cholesterol and albumin.

Data were analysed in IBM SPSS Statistics version 22 for Mac and Microsoft Excel 2011 for Mac. Results were summarised on to tables and graphs as given elsewhere. All statistical tests were performed at 5% significance level or 95% confidence interval and differences were considered significant if 2-tailed $p<0.05$.

5.0. RESULTS

5.1. Insulin, FASN and TG Conc. Mean Difference

The study found that T2DM participants had statistically significant higher insulin concentration (35.9 ± 3.6 µIU/mL) compared to non-diabetic participants (10.8 ± 1.4 µIU/mL), $t(40) = 6.518$, $p < 0.001$ (Fig. 3A). FASN concentration and Triglyceride concentration were also higher in T2DM participants (FASN; 18.2 ± 2.9 ng/mL and TG; 1.46 ± 0.17 mmol/L) than in non-diabetic participants (FASN; 11.8 ± 1.4 ng/mL and 1.03 ± 0.1 mmol/L) with statistical significance, $t(41) = 2.283$, $p = 0.022$ and $t(41) = 2.14$, $p = 0.039$ respectively (Fig. 3B and Fig. 3B, respectively).

FIGURE 3: Insulin, FASN and TG Mean Difference.

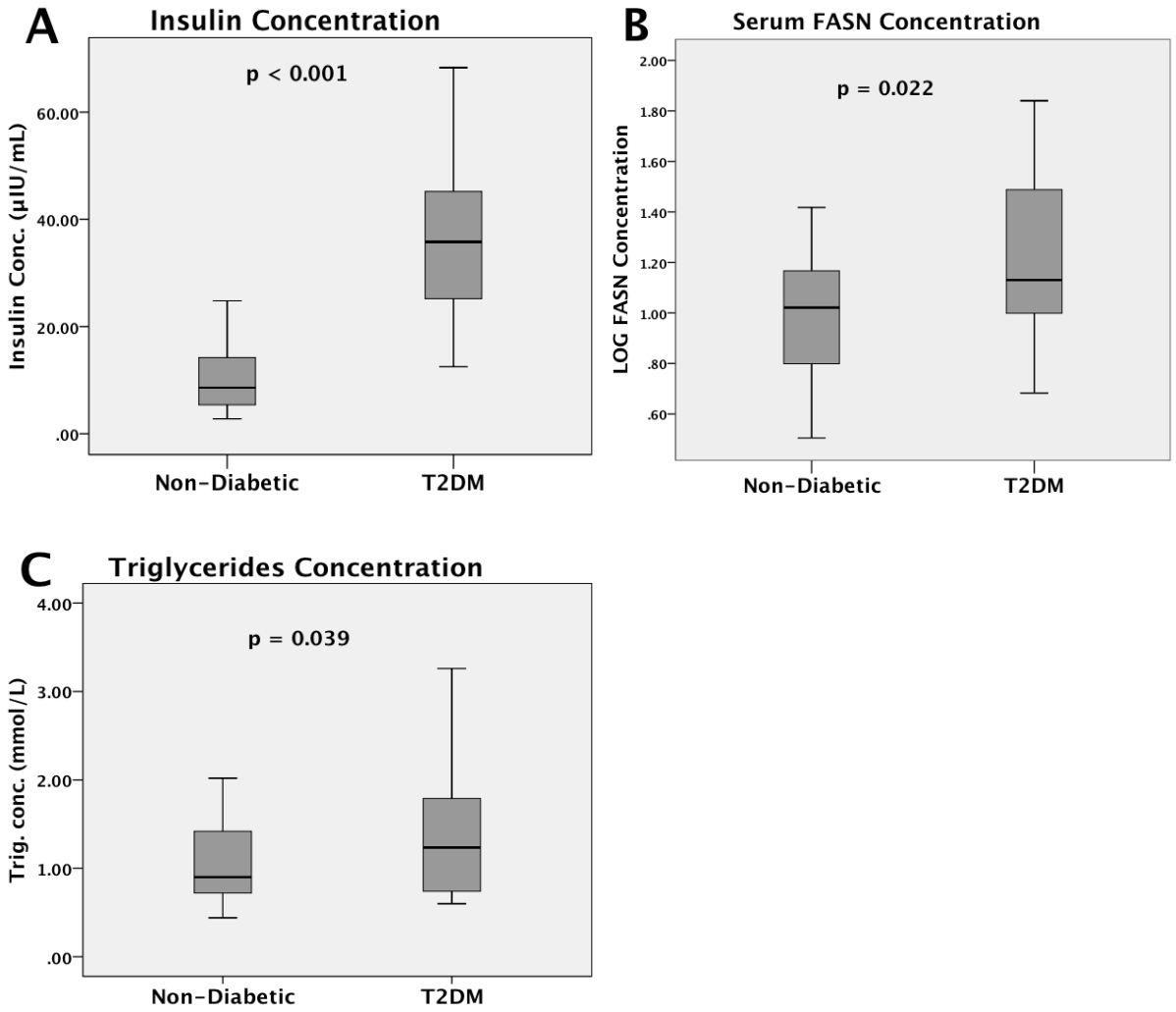

FIG. 3A: Insulin mean concentration difference between T2DM group and non-Diabetic Controls. **Fig. 3B**: Log FASN mean concentration difference between T2DM and non-Diabetic Controls. **Fig. 3C**: Triglyceride mean concentration difference between T2DM and non-Diabetic Controls. Difference assessed using Independent Sample *t*-Test with *p*-value significance at 95% confidence level.

5.2. Linear Regression of Insulin Vs. FASN and FASN Vs. TG

Bivariate linear regression analysis of FASN Vs. insulin showed moderate positive correlation in T2DM participants though not statistically significant (r=0.333, *p* = 0.163) (Fig. 4A). FASN Vs. insulin were moderately correlated in Healthy Non-diabetic participants with statistical significance (r = 0.476, *p* = 0.034) (Fig. 4A). FASN and triglycerides showed a weak correlation in both diabetic participants and Healthy Non-diabetic without statistical significance (r = 0.288, *p* = 0.193 and r = 0.121, *p* = 0.612 respectively) (Fig. 4C and Fig. 4D, respectively).

FIGURE 4. FASN:Insulin and TG: FASN Linear Correlation

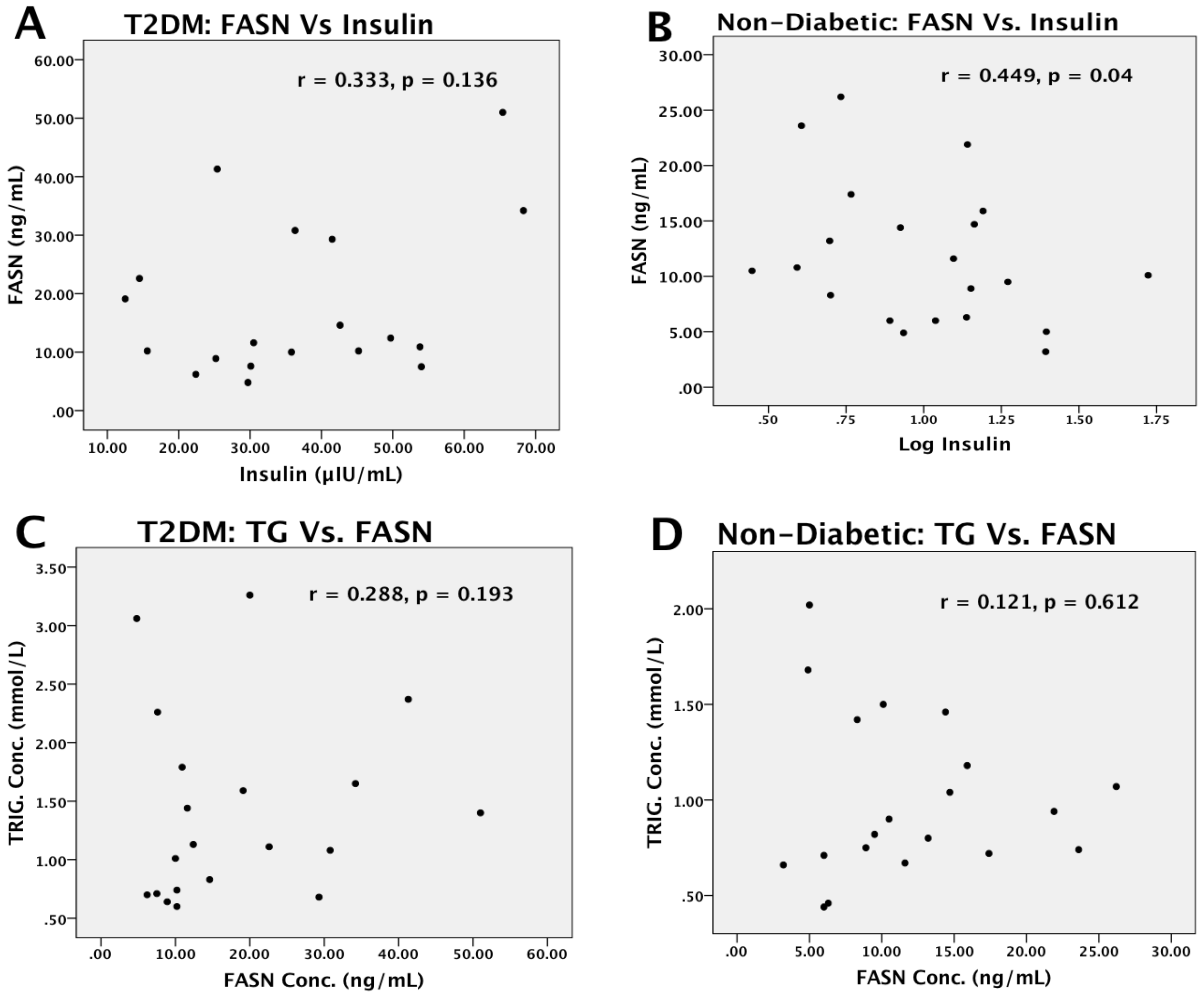

Fig. 4A: Linear association between insulin and FASN in T2DM group. **Fig. 4B**: Linear association between insulin and FASN in health non-diabetic control group. **Fig. 4C**: Correlation between FASN and TG in T2DM group. **Fig. 4D**: Correlation between FASN and TG in health non-diabetic control group. r = the correlation coefficient and p = p-value. Statistical significance determined at 95% confidence level.

5.3. Anthropometrics and Biochemicals Mean Difference

There was no age difference between T2DM participants (41.3 ± 2.2 years) and Healthy Non-diabetic participants (37.1 ± 1.8 years), as analysed using Fisher's exact test (FET) (1, N = 44) p = 1.00. Diabetic participants had similar BMI (27.6 ± 1.5 kg/m^2) to Healthy Controls (27.8 ± 1.3 kg/m^2), $t(42) = 0.071$; $p = 0.944$ (Table 1.0). Fasting blood glucose levels were higher in T2DM subjects (9.6 ± 0.9 mmol/L), than Healthy Control (5.1 ± 0.1 mmol/L) with statistical significance, $t(36) = 4.89$, $p < 0.001$ (Table 1.0). Total cholesterol, HDL-cholesterol, and LDL-cholesterol were similar in T2DM participants (5.28 ± 0.33 mmol/L, 1.31 ± 0.06 mmol/L, and 1.14 ± 0.08 mmol/L respectively) to the Control Group (4.75 ± 0.23 mmol/L, 1.39 ± 0.12 mmol/L, and 1.31 ± 0.13 mmol/L), $t(42) = 1.314$; $p = 0.196$, $t(39) = 1.118$; $p = 0.270$, and $t(42) = 0.678$; p = 501 respectively (Table 1.0). ALT, AST and albumin levels had shown no statistically significant difference, $t(42) = -.887$; $p = 0.38$, $t(42) = -0.722$; $p = 0.475$ and $t(39) = 0.602$; $p = 0.551$ respectively between diabetic participants (ALT; 22.4 ± 4.0 U/L, AST 27.2 ± 2.3 U/L and ALB 25.4 ± 0.37 mmol/L), and Non-diabetic participants (ALT; 18.1 ± 2.8 U/L, AST; 25.1 ± 1.8 U/L, and ALB; 25.7 ± 0.35 U/L (Table 1.0).

Table 1.0. Anthropometric and Metabolic characteristics of the study groups[1]

	Non-Diabetic (n=22)	T2DM (n=22)	*p*
Age (yr)	37.1 ± 1.8	41.3 ± 2.2	1.00*
BMI (kg/m^2)	27.8 ± 1.3	27.6 ± 1.5	0.944
Fasting Glucose (mmol/l)	5.1 ± 0.1	9.6 ± 0.9	< 0.001
Total Cholesterol	4.75 ± 0.23	5.28 ± 0.33	0.196
LDL – cholesterol (mmol/L)	1.31 ± 0.13	1.14 ± 0.08	0.551
HDL – Cholesterol (mmol/L)	1.39 ± 0.12	1.31 ± 0.06	0.270
ALT (U/L)	18.1 ± 2.8	22.4 ± 4.0	0.38
AST (U/L)	25.1 ± 1.8	27.2 ± 2.3	0.475
Albumin (mg/L)	25.7 ± 0.35	25.4 ± 0.37	0.551

[1] Health non-diabetic individuals and individuals with T2DM. [2]*P* represents overall significant differences across groups. *P*-values were derived from independent sample student's *t*-test. Age and BMI were included in the model as covariates. AST, ALT and ALB were used to assess the association between hepatic function with serum insulin, FASN and triglycerides concentration. *Obtained using Fisher's exact test (FET) of association.

5.4. Multivariate Linear Regression Analysis

Multivariate Linear regression analysis to assess factors that may have affected insulin (dependent variable) concentration, utilised AST, ALT (hepatocellular damage markers) and Glucose as independent variables, showed that only glucose met the 0.05 significance level for entry into the model, r = 0.494; *f* (3, 38) = 4.089, *p* = 0.013 (Table 2.0). Stepwise multivariate linear regression analysis was conducted to predict serum triglycerides concentration, with triglycerides as dependent variable and glucose, insulin, ALT, ALB, AST and FASN as independent variables. Results showed that serum FASN was the best predictor of triglycerides concentration but was not statistically significant (ß = 0.340, *p* = 0.09) (Table 3.0).

Table 2. Correlation between Insulin with ALT, AST and Glucose

Coefficients						
Model		Unstandardized Coefficients		Standardized	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	6.966	8.904		0.782	0.439
	Glucose	1.693	0.507	0.477	3.342	0.002
	ALT	0.113	0.217	0.105	0.521	0.605
	AST	0.024	0.366	0.013	0.065	0.949
a. Dependent Variable: Insulin						

Table 2: reveals a significant model for the predictor variables with a multiple correlation of 0.494, [F(3,38) = 4.089, *p* = 0.013, R^2 = 0.244]. Only glucose concentration met the significant criterion (*p* = 0.002). t = *t* – test statistics, *p* = (p – value) significance level.

Table 3. Correlation between TG with FASN and Other Variables

Coefficients						
Model		Unstandardized Coefficients		Standardized	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-1.424	1.733		-0.822	0.417
	Glucose	0.041	0.028	0.266	1.483	0.147
	FASN	0.014	0.008	0.340	1.742	0.090
	Insulin	-0.005	0.008	-0.125	-0.668	0.508
	ALT	0.006	0.010	0.118	0.530	0.600
	AST	0.005	0.018	0.066	0.297	0.768
	ALB	0.079	0.063	0.253	1.247	0.221
a. Dependent Variable: Triglycerides						

Table 3: Shows a model for the predictor variables with a multiple correlation of 0.236, [F(2,39) = 1.152, *p* = 0.327, R^2 = 0.056]. t = *t* – test statistics, *p* = (p – value) significance level.

6.0. DISCUSSION

The results of this study are in support of the researchers hypothesis that individuals with T2DM have significantly higher fasting serum insulin concentration and higher mean serum FASN concentration than non-diabetic individuals *(p* < 0.001; Fig. 3A and *p* = 0.022; Fig. 3B respectively). Increased serum FASN affirms the insulin-FASN axis, which describes insulin-stimulated up-regulation of FASN gene expression (Menendez *et al*. 2009; Postic and Girard, 2008; Dávalos *et al*. 2011). Evidence of the insulin-FASN axis was in part explained in this study by the moderate correlation between insulin and circulating FASN concentration in non-diabetic participants with statistical significance (r = 0.476, *p* = 0034; Fig. 4B). However, the correlation between insulin and circulating FASN in individuals with T2DM was not statistically significant (r = 0.115, *p* = 0.619; Fig. 4A). The lack of statistically significant correlation in the T2DM participant group may have been due to diabetic pathophysiologic changes and/or drug treatment effect (Algire *et al*. 2010; Nathan *et al.* 2009). These may have altered the metabolic interplay between insulin and FASN in diabetic individuals. However, any definitive conclusion on this discrepancy should await more studies and research that are statistically powered to access the above-mentioned effects. Nevertheless, the T2DM group showed statistically significant higher FASN concentration (*p* = 0.022) than the non-diabetic group. But then, what are the implications of increased FASN expression? Up-regulation of FASN expression in the liver may lead to an increase in the triglycerides content of the liver which culminate to increased VLDL and free fatty acids secretion into the systemic circulation (Shimomura *et al*. 2000; Van Obberghen *et al.* 2001; Menendez *et al*. 2009), contributing to the development of hypertriglyceridemia, as observed to be higher in participants with T2DM compared to normal controls (*p* = 0.039, Fig 3C). This metabolic link between insulin and FASN describes that, under hyperinsulinaemic conditions, the liver is turned into lipid 'biosynthesis factory' with all of its negative downstream effects, including the genesis of hypertriglyceridemia (Kahn et al. 2006; Berndt *et al*. 2007; Brown and Goldstein, 2008)

Therefore, hyperinsulinaemia may accounts for the dyslipidaemias that are observed in T2DM. However this study did not find a statistically significant positive correlation for FASN and TG (Fig. 4C and Fig. 4D) and for Insulin and TG (Appendix 3.1: Fig 5A

and Fig 5B) in both research groups. Notwithstanding, findings divergent to ours have been reported in various Caucasian studies that have demonstrated a positive correlation, e.g. Bansal *et al* (2007) demonstrating that fasting triglyceride levels in women correlated with traditional cardiac risk factors and markers of insulin resistance. Sierra-Johnson, *et al* (2006) and McLaughlin *et al* (2003) also demonstrated similar finding. However, finding of this study are consist with those of Sumner *et al* (2005), who showed that fasting triglycerides and the triglyceride–HDL cholesterol ratio are not markers of insulin resistance in African Americans, thus not predictive markers of hyperinsulinaemia. In addition, more compelling findings include those of Twang *et al*, (2007) and McGarry *et al* (2004), that demonstrated that Intrahepatic Triglycerides (IHTG) and IMCL content but not serum triglycerides in themselves are strong predictors of hyperinsulinaemia (insulin resistance).

Furthermore, since hyperinsulinaemia is an early event (Leahy, 2005; DeFronzo, 2009; DeFronzo, Mandarino, and Ferrannini, 2004) in the disease pathology and correlated to FASN, we may postulate that; hyper – expression of hepatic FASN (and other lipogenic enzymes) and the outcome dyslipidaemias in T2DM must also be early events of T2DM pathogenesis. With respect to the above observations, further conclusions are made that; the molecular lesions that culminate in chronic complications of T2DM such as macrovascular disorders may also be early event in the pathogenesis of T2DM. In addition, the findings of this research maybe interpreted with other finding [that overproduction of VLDL and FFA by the liver takes place early in the development of overt T2DM (McGarry *et al*. 2004; Adiels *et al*. 2006; Adiels *et al*. 2008; Rains and Jain, 2011)] suggesting that pancreatic β – cell failure as a results of islets lipotoxicity is a progressive mechanism that begins from the onset of T2DM (Lim *et al*. 2011; Poitout *et al*. 2011; Newsholme *et al*. 2007). This leads us to a reasonable conclusion that β – cell demise maybe an end point of untreated and/or poorly managed diabetic dyslipidaemias, which in themselves stem from increased FASN activity.

Here then, if vascular complications that lead to cardiovascular disease in T2DM are early events that stem from FASN-driven lipogenesis and progress over many years. We may postulate that, diabetic cardiovascular complications, which are by far the major cause of mortality among diabetics, may not be averted by rigorous glycaemic

control. This view is supported by various evaluations of insulin-regulated metabolic pathways (McGarry *et al*. 2004) and the molecular pathogenesis of T2DM. Totally in keeping with this view, the United Kingdom Prospective Diabetes Study (UKPDS), the American Diabetes Association and the European Association for the Study of Diabetes and other large scale longitudinal studies, have shown that dietary intervention and/or glycaemic control to the non-diabetic range have major benefits for diabetic related microvascular and neuropathic complications, but no benefits for cardiovascular complications and major macrovascular events (Van Dieren *et al*. 2011; Polak *et al*. 2011; Inzucchi *et al*. 2012). Also, the findings of this research show that fasting glucose did not correlate to serum triglycerides, cholesterol, LDL or HDL with statistical significance (Appendix 3.2: Fig. 6A, Fig. 6B, Fig. 6C and Fig. 6D, respectively), giving further impedance to the notation that glycaemic control in itself may have no effect on major macrovascular complications such as cardiovascular disease and coronary heart disease. We hereby present the argument that in T2DM, lipids are as important as carbohydrate and also that derangement in lipid metabolism maybe culpable for the majority of complications probably including hyperglycaemia itself (because insulin resistance which drives hyperglycaemia itself is largely due to increased IMCL and IHCL contents)(Twang *et al*. 2007; Petersen, 2006; Samuel, Petersen and Shulman, 2010; Muoio, 2012).

Consistent with prior hypothesis', findings of this study leave us with two important questions that; 1) can the development or progression of T2DM be prevented or reversed by modulation of FASN status or hyperinsulinaemic state? 2) And can early commencement of therapies targeted at diabetic dyslipidaemias prevent the development of atherocardiovascular complication in T2DM? The reason for the stated hypothesis' impasse maybe that FASN-driven endogenous FA biosynthesis is consistently not mentioned or overlooked in literature as a feasible molecular biomarker, which is associated with both dyslipidaemia and T2DM disease process. However, results generated from this study have offered persuasive evidence, which has exploited to deliver a proof-of-concept that establishes a casual role of FASN overexpression in T2DM. Thus, findings of this research emphasize an important point that is often overlooked when insulin resistance and T2DM is applied to whole body lipid and carbohydrate metabolism.

Various research findings involving insulin-signalling pathways have recognised the important link between insulin and dyslipidaemia. Furthermore, various new and emerging drug therapies are now aimed at addressing such important defect, which, have remained silent over many decades. Of important mention are the forthcoming FASN inhibitors (Fernandez *et al.* 2010; Huang *et al*. 2013; Yu *et al*. 2013), which further give support to clinical relevance of disrupting the insulin – FASN axis in the prevention and/or treatment of T2DM and present a very promising prospect. Modulation of FASN activity may therefore directly address the two core problems of T2DM – insulin resistance and diabetic dyslipidaemias.

However, despite all that has been discussed here, it should be noted that some the of the questions posed should not be answered with theory; but that any conclusions on these, should await more elaborate experiments and longitudinal studies that would assess the causative role of insulin and FASN to development of diabetic macrovascular complications.

6.1. CONCLUSION

The study showed that hyperinsulinaemia is characteristic of individuals with T2DM and is associated with an increase in circulating FASN. Insulin stimulated FASN-driven endogenous FAs biogenesis is itself associated with increased synthesis of fatty acids in the liver. Excessive production of FFAs may lead to diabetic dyslipidaemias, which are major risk factor for atherocardiovascular complications. Findings of this study recognise FASN as an important molecular biomarker that is over-expressed in hyperinsulinaemic state of T2DM.

6.2. IMPLICATIONS AND RECOMMENDATIONS

Treatment of T2DM is mostly focused on lowering blood glucose levels using anti-hyperglycaemic drugs to the non-diabetic range. With such a treatment regime, we should ask the question that, if blood glucose levels are being lowered with a drug

when postprandial insulin levels remain high, where does the excess glucose go? The answer is: most often being converted to lipids by FASN (Griffin and Sul, 2004), thus exuberating the existing triglyceridaemia and increase IMCL content, leading to greater insulin resistance and higher risk of cardiovascular disease. At best, such treatment regimen is a temporary solution with a narrow scope of benefit - and does not address the true source of the problem (insulin resistance and hyperlipidaemias- themselves causing the insulin resistance). In order to develop new, more efficient, preventive and therapeutic strategies for T2DM, there is an urgent need for increased understanding of the complexity of insulin signalling pathways. Furthermore, findings of this study suggest that circulating FASN and insulin are biomarkers of DM that could provide diagnostic and prognostic advantages by providing information on the metabolic state of diabetic individuals.

6.3. LIMITATIONS / WEAKNESSES AND ASSUMPTIONS

- The study was limited to diabetics who are not on insulin treatment thus presumed as T2DM individuals with 'normal' pancreatic β-cells function.
- The study did not provide any data pertaining to insulin resistance states of the research participants.
- Previous surgery, cancer and any chronic and acute inflammatory conditions were not determined but assessed by means of interviewer-administered questionnaire, clinical signs of infection, and checking participants hospital file records. However, the most important inflammatory condition that may have confounded study findings was hepatitis – assess by AST and ALT.
- SREBP-1c mRNA analysis would have been an important insulin – to – FASN link confirmer, however this study provides enough proof-of-concept that the perturbations of T2DM dyslipidaemias may lie in the Insulin-FASN axis.

- The association between FASN with IHLC, VLDL and/or VLDL specific apoprotein would have provided more valuable information concerning the relationship between FASN and diabetic dyslipidaemias.
- A third group of individuals with non-functional ß-cells (terminal [insulin – requiring T2DM] diabetes) or T1DM individuals would have been included in the study to check for dissociation of the insulin-FASN axis, and probably low serum lipids. However, the researcher did not have necessary tools for determining the extent of ß-cell failure or mere ß-cell failure in itself.

6.4. FUTURE DIRECTION

With respect to the above considerations, more supportive and difinitive investigation are required. Analysis of cellular FASN mRNA and/or SREBP-1c would provide even stronger molecular evidence that hyperinsulinaemic (insulin – resistant) states in T2DM arise from a common FASN driven lipogenic state.

Since some of our conclusions are merely theoritical, the insulin – FASN axis cause and effect should further be investigated. thereofere, it may be necessary to conduct animal model studies in order to conclusively elucidate whether the development and/or the progression of T2DM can be prevented or reversed by the modulation of FASN status.

To establish cause and effect, it maybe necessary to show that in healthy, insulin-sensitive subjects or animal models, manipulations that cause an increase FASN and/or Hyperinsulinaemia will cause reciprocal changes in insulin sensitivity, blood glucose concentration and lipid profile similar to those observed in T2DM.

7.0. REFERENCES

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8.0. APPENDICES.

Appendix 1: INCLUSION CRITERIA EXPLAINED

- Individual with T2DM who are currently on insulin treatment were excluded from the study for two reasons;
  1. The administrated exogenous insulin may also be detected by the Insulin ELISA test kit and hence may give high false positive values
  2. Individuals with T2DM on exogenous insulin therapy are more likely have pancreatic β-cells failure, hence past the hyperinsulinaemic phase of T2DM which precedes pancreatic β-cell failure
- Individuals with malignant disease were excluded from this study because high serum levels of FASN have been demonstrated in various malignant conditions.
- Individuals who underwent any major surgery one month prior to their evaluation for consideration into the study were excluded because major surgery has been describe to alter normal physiology with concomitant change in various cellular macromolecules and appearance or increase of many other substance in blood. Acute and chronic inflammation may also alter normal physiology, which may affect many plasma analytes; hence individuals with any inflammatory conditions that may alter any plasma analytes levels were not recruited into the study.
- Non – diabetic participants: Apart from not being diabetic, the inclusion criteria for non – diabetic participants is as that of individual with T2DM.

Appendix 2.1: Table 4: Non-Diabetic Participants' Raw Data

Non – Diabetic Participants												
#	AGE	FASN (µmol/l)	Insulin (µmol/l)	TG (mmol/l)	FBS (mmol/l)	ALT	AST (U/L)	Chol (mmol/L)	LDL (mmol/l)	HDL (mmol/l)	Alb (mmol/l)	BMI (Kg/m^2)
1	41	10.1	52.9	1.5	5.8	15	23	5.92	1.51	0.87	25.8	37.0
2	39	11.6	12.50	0.67	5.2	8	21	4.35	2.22	1.4	25.3	27.6
3	39	23.6	24.80	0.74	5.2	13	19	3.35	0.75	1.19	22.7	24.6
4	32	17.4	13.80	0.72	4.6	12	18	5.37	0.44	1.48	27.3	26.1
5	34	81.3	7.50	1.71	5.2	22	35	7.55	1.4	1.75	22	28.2
6	57	26.2	14.60	1.07	6.9	27	24	6.08	1.54	0.74	25.9	46.6
7	37	8.9	18.70	0.75	4.8	17	20	3.32	0.81	1.4	23.2	28.4
8	37	14.7	5.40	1.04	4.9	14	27	4.18	1.04	1.63	26.6	34.8
9	28	3.2	2.80	0.66	4.9	19	18	4.81	2.15	1.83	25.7	20.2
10	49	10.8	5.00	4.25	5.2	43	49	4.22	1.52	1.33	27.2	29.6
11	35	9.5	5.80	0.82	5.1	15	34	3.79	0.46	1.07	26.3	28.7
12	38	4.9	7.80	1.68	4.8	15	23	4.46	0.19	3.34	25.6	33.6
13	54	15.9	24.70	1.18	5.2	21	37	3.94	1.12	1	26.3	23.1
14	36	10.5	8.60	0.9	5.2	14	21	4.95	0.76	0.62	27.1	27.9
15	32	8.3	5.00	1.42	4.8	65	30	5.87	0.58	1.18	25	24.3
16	47	5.0	15.50	2.02	3.7	18	21	4.06	1.47	1.54	24.5	18.6
17	38	21.9	13.70	0.94	5.2	9	23	6.14	1.77	1.09	23.1	24.4
18	34	13.2	4.00	0.8	3.4	21	26	5.34	1.83	1.86	26.5	29.8
19	24	6.0	3.90	0.44	6.4	7	33	4.79	0.51	1.39	27.7	20.4
20	25	6.0	10.90	0.71	4.9	5	17	4.92	2.25	1.5	27	26.6
21	32	14.4	8.40	1.46	5.4	8	14	3.88	1.16	1.18	26.7	27.4
22	28	6.3	14.20	0.46	5.8	10	19	3.14	1.73	1.38	27.1	23.1

Appendix 2.2: Table 5: T2DM Participants' Raw Data

T2DM Participants												
#	AGE	FASN (µmol/l)	Insulin (µmol/mL)	TG (mmol/l)	FBS (mmol/l)	ALT	AST (U/L)	Chol (mmol/l)	LDL (mmol/l)	HDL (mmol/l)	Alb (mmol/l)	BMI (Kg/m^2)
1	49	30.8	36.3	1.08	6.7	11	22	5.1	1.69	1.15	18	36.8
2	60	10.9	53.8	1.79	14.4	21	31	7.36	1.16	1.39	27.1	28.3
3	40	20.0	209.9	3.26	12.4	19	27	3.79	0.56	1.66	26.6	29.9
4	62	10.0	35.8	1.01	6.8	14	38	2.92	1.47	1.52	26.6	30.1
5	58	7.5	54.0	0.71	5.8	7	16	5.85	2.31	1.23	23.4	28.9
6	35	34.2	68.3	1.65	24	11	14	6.38	1.69	1.6	19.7	24.0
7	54	6.2	22.4	0.7	7.4	16	25	3.91	0.41	1.8	25.6	28.3
8	40	8.9	25.2	0.64	6.5	11	21	4.37	1.12	1.22	24.9	28.0
10	44	19.1	12.5	1.59	9	13	15	7.71	0.99	1.77	26.2	29.6
11	55	69.0	13.5	2.7	19.1	16	23	6.66	0.87	1.19	24	22.3
12	33	11.6	30.5	1.44	23.9	15	14	2.68	1.11	0.93	26.8	19.7
9	52	10.2	15.6	0.74	15.6	32	43	3.8	1.02	1.07	21.5	29.5
13	41	51.0	65.4	1.4	11.8	13	20	5.15	0.89	1.13	22.3	21.1
14	40	29.3	41.5	0.68	4.4	21	32	3.75	1.33	1.14	24.5	23.8
15	58	22.6	14.5	1.11	8	19	26	5.37	1.37	0.8	24.8	22.5
16	58	14.6	42.6	0.83	5.4	31	25	5.89	1.1	1.23	26.7	20.0
17	63	7.6	30.1	2.26	13	19	18	6.37	1.78	1.27	26.4	35.6
18	50	69.1	42.3	1.34	8	25	35	7.57	1.48	1.9	17.2	20.4
19	52	10.2	45.2	0.6	12.4	43	42	3.09	1.13	1.17	25	23.8
20	29	12.4	49.7	1.13	9.7	97	57	6.78	1.17	1.57	26.7	26.3
21	61	4.8	29.7	3.06	6.7	17	24	5.34	0.99	0.89	26.7	50.8
22	48	41.3	25.4	2.37	9.1	22	30	6.28	0.68	1.16	26	28.2

Appendix 3.1: Figure 5: Linear Correlation for TG against Insulin

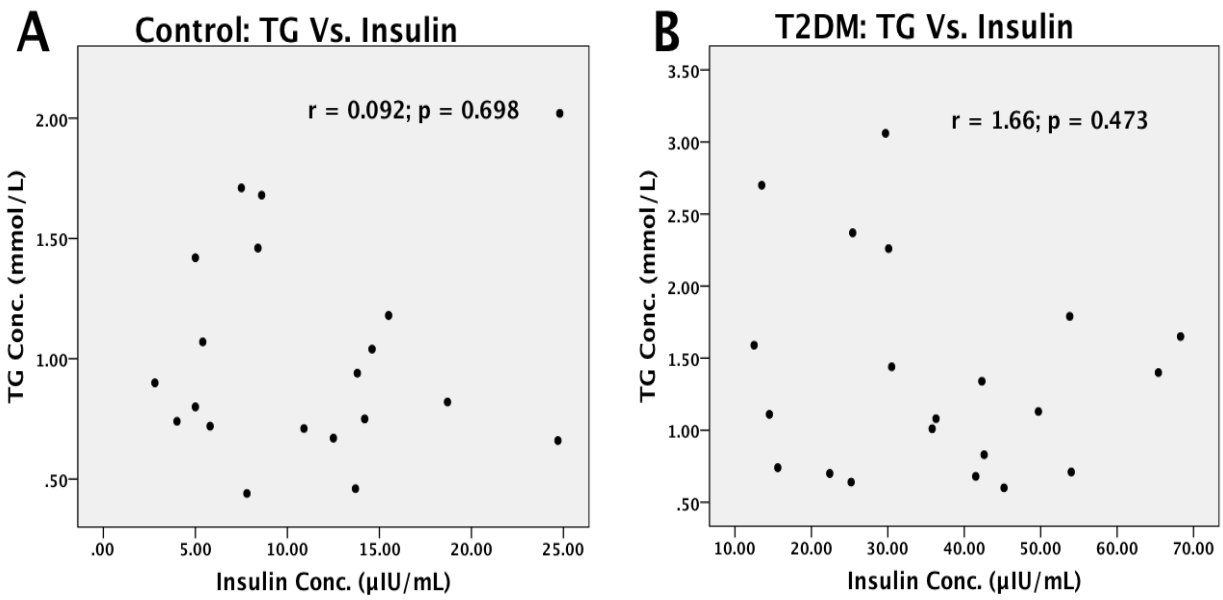

Fig. 5A: Linear association between insulin and TG in health non-diabetic control group **Fig. 4B**: Linear association between insulin and FASN in T2DM group. r = the correlation coefficient and *p* = p-value. Statistical significance determined at 95% confidence level.

Appendix 3.2: Figure 6: Linear Correlation of Glucose and Lipids

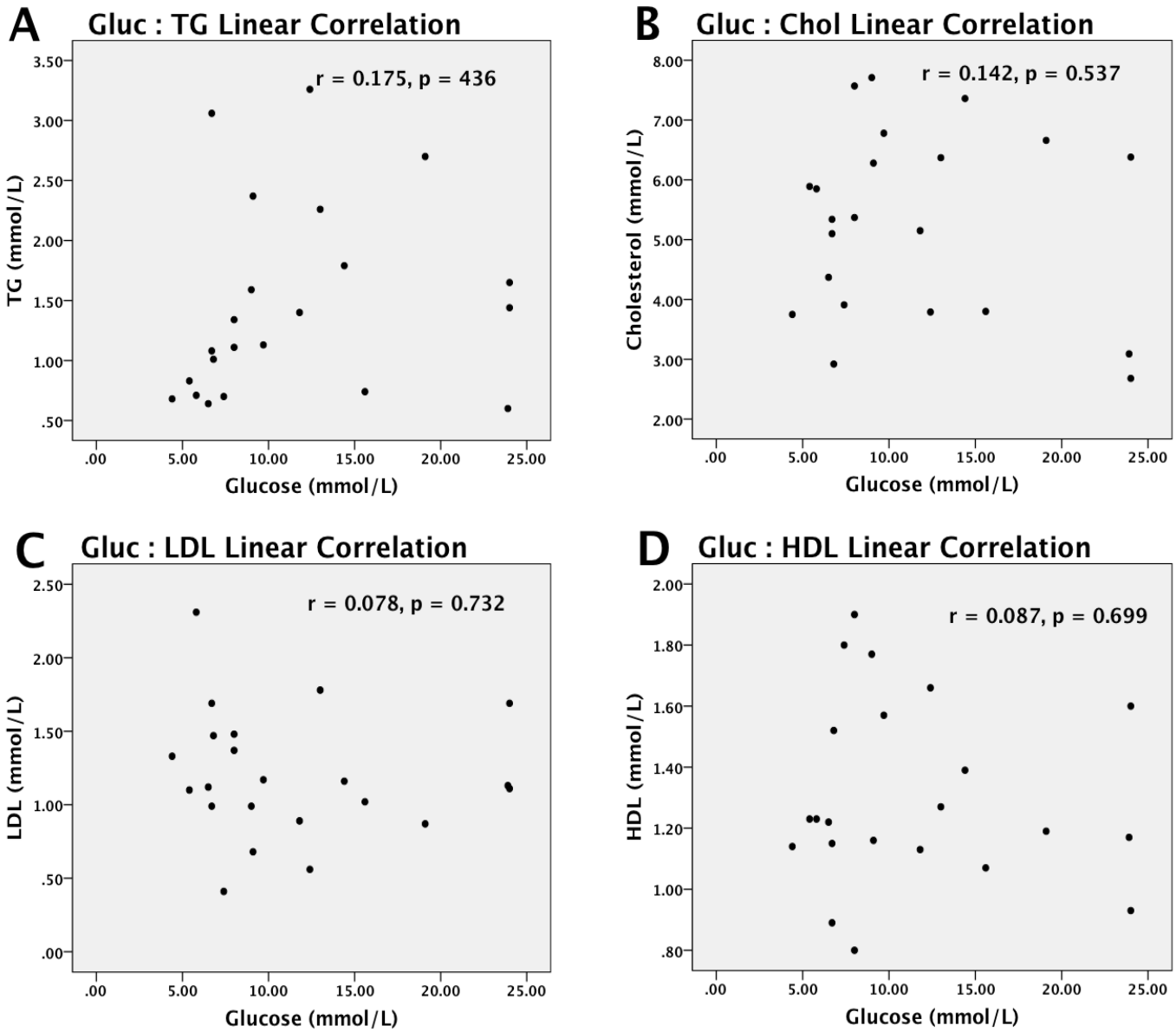

Fig. 6A: Linear association of glucose with TG in T2DM group. **Fig. 6B**: Linear association of glucose and Cholesterol in T2DM group. **Fig. 6C**: Correlation of glucose with LDL in T2DM group. **Fig. 6D**: Correlation of glucose with T2DM group. r = the correlation coefficient and p = p-value. Statistical significance determined at 95% confidence level.

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07th October, 2013

Mr. Musalula Sinkala
Department of Pathology & Microbiology
School of Medicine
UNZA
LUSAKA

Dear Mr. Sinkala,

RE: GRADUATE PROPOSAL PRESENTATION FORUM

Having assessed your dissertation entitled " **Assessment of Hyperinsulinaemia, Plasma Fatty Acid Synthase Levels and Hypertriglyceridaemia in Type II Diabetes Mellitus in the Outpatients Medical Clinic at the University Teaching Hospital, Lusaka".** We are satisfied that all the corrections to your research proposal have been done. The proposal meets the standard as laid down by the Board of Graduate Studies.

You can proceed and present to the Research Ethics.

Yours faithfully,

Dr. S.H. Nzala
ASSISTANT DEAN, POSTGRADUATE

CC: HOD, Pathology & Microbiology

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4th February, 2014.

Our Ref 002-11-13.

Mr. Masalula Sinkala,
University of Zambia,
School of Medicine,
Department of Biomedical Sciences,
P.O Box 50110,
Lusaka.

Dear Mr. Sinkala.

RE: RESUBMITTED RESEARCH PROPOSAL **"ASSESSMENT OF HYPERINSULINAEMIA, SERUM FATTY ACID SYNTHASE AND HYPERTRIGLYCERIDAEMIA IN TYPE 2 DIABETES MELLITUS IN THE OUTPATIENTS MEDICAL CLINIC AT THE UNIVERSITY TEACHING HOSPITAL, LUSAKA" (REF. No. 002-11-13)**

The above-mentioned research proposal was presented to the Biomedical Research Ethics Committee on 13th January, 2014. The proposal is approved.

CONDITIONS:

- This approval is based strictly on your submitted proposal. Should there be need for you to modify or change the study design or methodology, you will need to seek clearance from the Research Ethics Committee.
- If you have need for further clarification please consult this office. Please note that it is mandatory that you submit a detailed progress report of your study to this Committee every six months and a final copy of your report at the end of the study.
- Any serious adverse events must be reported at once to this Committee.
- Please note that when your approval expires you may need to request for renewal. The request should be accompanied by a Progress Report (Progress Report Forms can be obtained from the Secretariat).
- **Ensure that a final copy of the results is submitted to this Committee.**

Yours sincerely,

Dr. J.C. Mauthali
CHAIRPERSON

Date of approval: 4th February, 2014 **Date of expiry:** 3rd February, 2015.

Musalula Sinkala
Contact Number: +260966560148
Staff Development Fellow
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2013-10-22

The Medical Superintendent
University Teaching Hospital
Nationalist Road, P/Bag RW 1X
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Lusaka
Zambia

RE: PERMISSION TO CONDUCT RESEARCH

Dear Sir/Madam,

My name is Musalula Sinkala, a Master's of Science student in Pathology (Chemical Pathology) and Staff Development Fellow with the University of Zambia in the School of Medicine - Department of Biomedical Sciences.

I would like to conduct a research in fulfilment for the MSc. in Pathology requirement in your Outpatients Medical Clinic at the University Teaching Hospital. The proposed research study is entitled: **"Assessment of Hyperinsulinaemia, Plasma Fatty Acid Synthase Levels and Hypertriglyceridaemia in Type 2 Diabetes Mellitus in the Outpatients Medical Clinic at the University Teaching Hospital"**. The research has already been accepted and approved by the Graduate Proposal Forum in the University of Zambia.

I hereby request for permission through your office to conduct the study in the University Teaching Hospital. I also seek permission to utilize the laboratory facilities in the Clinical Chemistry Laboratory at UTH. Enclosed here-in is the complete full research proposal protocol.

I look forward to your timely response.

Yours faithfully

Musaluta Sinkala

ENCLOSURE

All correspondence should be addressed to the
Permanent Secretary
Telephone
Fax +260 211 253063

In reply please quote

No

REPUBLIC OF ZAMBIA
MINISTRY OF HEALTH

NDEKE HOUSE
P.O. BOX 30205
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16th April, 2014

Musalula Sinkala
Staff Development Fellow
University of Zambia
School of Medicine
Department of Biomedical Sciences
P.O. Box 50110
LUSAKA

RE: **PERMISSION TO CONDUCT RESEARCH – YOURSELF**

Refer to the above.

I write to acknowledge receipt of your letter dated 22nd October, 2013 in which you requested to carry out a research entitled **"Assessment of Hyperinsulinaemia, Plasma Fatty Acid Synthase Levels and Hypertriglyceridaemia in Type 2 Diabetes Mellitus in the Outpatients Medical Clinic at the University Teaching Hospital"**

Management has no objection to you doing your research at this institution.

You are further informed that this is so on condition that the institution bears no financial obligation to you during the period of this research and we are availed results prior to their dissemination.

Furthermore, you have to uphold professionalism as you interact with our clients.

Yours faithfully,

Dr. C. Chiluba
Head Clinical Care
For/ Medical Superintendent
LEVY MWANAWASA GENERAL HOSPITAL

From: Gibson Sijumbila <gsijumbila@yahoo.co.uk>
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Date: 22 July 2014 19:54:27 GMT+2
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On Tuesday, 22 July 2014, 16:47, editor jmst <editorjmst@hotmail.com> wrote:

Dear Author

Reviewers have now commented on your article **Plasma insulin and fatty acid synthase levels in patients with type 2 diabetes mellitus**.

It has been recommended for publication in our journal. But they are suggesting few minor corrections in your article. Do the revision and resubmit your article with in one week.

Reviewer comments compilation has been attached with this email for your reference.

Thank You

Regards
Admin JMST

From: Gibson Sijumbila <gsijumbila@yahoo.co.uk>
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On Monday, 4 August 2014, 18:52, editor jmst <editorjmst@hotmail.com> wrote:

Manuscript Reference ID: JMST-03/201422
Manuscript Category: Original article
Manuscript Title: Plasma insulin and fatty acid synthase levels in patients with type 2 diabetes mellitus.

Dear Author

I am pleased to inform you that your manuscript details mentioned above is accepted for publication in our Journal upcoming issue. Thank you for choosing Journal of Medical Science and Technology to publish your work. Here I would like to remind you that you could send your future manuscripts to our Journal. We highly appreciate you interest in our journal.

Best regards
Editor in Chief
Dr NM RAO

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Original Article Open Access

Plasma insulin and fatty acid synthase levels in patients with type 2 diabetes mellitus.

Musalula Sinkala[1], Trevor Kaile[1], Sandra Chileya[2], Clemence Marimo[1], Lydia Korolova[1], Geoffrey Kwenda[3], Gibson Sijumbila[1*]

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Abstract

Fatty acid metabolism is generally under regulation of hormones and nutritional status. Insulin being an anabolic hormone promotes synthesis of fatty acids and triglycerides, whereas insulin deficiency tends to increase mobilisation of fatty acids from triacylglycerols and increase fatty acid oxidation . Fatty acids are synthesized by fatty acid synthase (FASN) complex in mammalian cells. We carried out an analytical cross sectional study to find out the link between serum insulin and serum FASN levels. A total of 44 participants, 22 with type 2diabetes mellitus (T2DM) and 22 normal non-diabetic controls were recruited. Serum insulin and circulating FASN were assessed in T2DM patients and non-diabetic control subjects. Our results showed that diabetics have significantly higher insulin and FASN levels ($p< 0.0001$ and $p = 0.018$ respectively) than non-diabetics. Insulin levels were found to be significantly correlated to FASN in controls($r = 0.476$, $p = 0.034$). A non-significant correlation between serum insulin and circulating FASN was observed in diabetic participants ($r = 0.333$) ($p = 0.139$. In conclusion our results suggest that hyperinsulinaemia is a feature of T2DM and insulin increases serum concentration of FASN; hence the increased levels of serum FASN in diabetics with hyperinsulinaemia. Increased levels of FASN in turn stimulate lipogenesis which is responsible for some of the complications of diabetes mellitus. Therefore increased serum FASN may be a useful marker of glucose intolerance due to insulin resistance.

Key words: Diabetes mellitus, Insulin, Fatty acid synthase, Triglycerides

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Received: June 18, 2014 Accepted: August 04, 2014. Published: September 20, 2014. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Introduction

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both[1]. Blood glucose levels are controlled in part by insulin, a hormone in the body that helps move glucose from the blood to muscles and other tissues. The two types of diabetes are referred to as type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). T1DM results from inadequate insulin secretion by the pancreas and T2DM results from either deficiency or lack of responsiveness to insulin.

An estimate of 371 million people had diabetes in 2012, which accounts for approximately 5.3% of the global population. The number of people with T2DM is increasing in every country and its has been estimated that by 2030 this will have risen to 552 million. There has been substantial migration of people from rural to urban areas in Zambia. This movement of people from rural to urban areas in part accounts for the increasing cases of T2DM as other studies suggested[2, 3]. Obesity due to inactivity or unhealthy diet is one of the factors why rural-urban migration has seen increased prevalence of T2DM.

Uncontrolled diabetes mellitus leads to serious long-term complications which include cardiovascular diseases (doubled risk), chronic renal failure, retinal damage (which can lead to blindness), nerve damage (of several kinds), and microvascular damage, which may cause impotence and poor wound healing. T2DM individuals are usually obese, and they manifest with insulin resistance,

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hyperinsulinaemia, and hyperglycemia[4]. Chronic insulin resistance leading to hyperinsulinaemia, is a major contributor to glucose and lipid metabolism abnormalities that culminate in pathophysiologic changes with ravaging consequences throughout the body [5].

Insulin is the major fed state hormone produced by the pancreatic β cells. The effect of insulin on cellular metabolism is vast, though the most emphasized aspect of insulin effect is the hormone's ability to facilitate glucose uptake by skeletal muscles and fat tissue. Therefore, insulin does not only lower blood glucose concentration, but also supplies the required fuel necessary for normal function of tissues that are dependent on insulin for glucose uptake. However, the less emphasized point is that insulin is the major lipogenic hormone and it is an important effector of fatty acid production in the liver via activation of FASN [6]. FASN is a cytosolic multi-enzyme complex that functions normally in the liver and is minimally expressed in other tissues [7]. FASN a key enzyme in *de novo* lipogenesis [7, 8], is involved in the formation of fatty acids that are esterified to glycerol to yield triacylglycerols. It is biochemically predictable that hyperinsulinaemia may lead to up-regulation of FASN expression in the liver which could lead to an increase in the triacylglycerol content of the liver resulting in increased very low density lipoprotein (VLDL) and free fatty acids secretion into the systemic circulation. In circulation there would therefore be of hypertriglyceridaemia, increased plasma non-esterified fatty acids (NEFAs) and high serum LDL-cholesterol that are usually observed in T2DM. These derangements in lipid metabolism contribute significantly to the pathogenesis of chronic complication of T2DM[9].

Our study therefore aimed to investigate the relationship between serum insulin and serum FASN in T2DM. Our hypothesis was that hyperinsulinaemia is associated with increased concentration of FASN, which gives rise to observed dyslipidemia.

Materials and methods

To investigate hyperinsulinaemia and its association with serum FASN concentrations among T2DM individuals at UTH, an analytical cross sectional study was proposed. This involved adult patients with T2DM (cases) and without diabetes mellitus (controls).The study was conducted at the University Teaching Hospital (UTH) Outpatient Department (Clinic 5), Lusaka, Zambia. Adults aged between 18- 75 years old with T2DM who reported to clinic 5 at UTH and met the inclusion criteria were enrolled into the study. A study control group consisted of individuals without clinically or laboratory diagnosed diabetes mellitus who met the inclusion criteria. The study sample and the control group were categorically matched for age and sex in order to minimize bias.

A total sample size of 44 participants (22 patients and 22 controls) had been calculated using the formula for determination of sample size for comparative research studies between two groups as given below;

$$N = \frac{4\sigma^2(z_{crit} + z_{pwr})^2}{D^2},$$

Where, N is the total sample size (the sum of the sizes of both comparison groups); σ is 8, the assumed SD of each group (assumed to be equal for both groups); the z_{crit} value is 1.960 as given in tables for Standard Normal Deviate (z_{crit}) corresponding to the desired significance criterion of 0.05 or 95% confidence interval (CI); the z_{pwr} value is 1.282 as given in Standard Normal Deviate (z_{pwr}) tables corresponding to 90% statistical power; and D is the minimum expected difference between the two means which has been estimated at 7.

Systematic sampling in which consecutive individuals with T2DM that had reported to clinic 5 and met the inclusion criteria (given below) were included into the study sample. The control group was selected by means of frequency matching of the same proportional characteristics (age and sex) as the study sample.

Those individuals with T2DM that had been diagnosed within the last 10 years and not on exogenous insulin treatment, aged between 18 and 75 years old and gave a written consent without undue duress were included in the study. Individuals who were on insulin, pregnant, non-negroid Zambian, had chronic inflammatory conditions or declined to give consent were excluded. Participants were thoroughly examined by the Medical Officer and their clinical and demographic data recorded in confidential files. At least 4mls of blood was collected from the antecubital vein from each participant in plain vacutainers. The blood specimens were centrifuged at 3000 revolutions per minute (3000 rpm) in order to separate the serum (supernatant) from the blood cellular component (sediment). Only serum was then meticulously collected from the vacutainers using pipettes and transferred to 2ml plastic cryovial containers with sealable screw caps, which were stored in a freezer at -80°C until the specimens were required for analysis. Serum insulin concentration

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was determined with the NeoBioLab™Human INS ELISA Kit using a quantitative competitive Elisa for measurement of Human Insulin in cell culture fluid, body fluid, tissue homogenate, serum or plasma according the manufacturer's instructions.

Circulating FASN concentrations was measured in serum without additives by NeoBioLab sandwich enzyme immunoassay (FAS ELISA) for the quantitative measurement of samples in serum, plasma, cell culture supernatants and urine as described elsewhere[10]. Briefly the the standard curve was constructed using Microsoft Excel 2011 and from the standard curve concentrations **of** samples were determined. Optical density of the samples and controls were read at 450 nm. Data were expressed as mean ± SEM for normally distributed continuous variables or median (interquartile range) for non-normally distributed variables. Normality was assessed using the Shapiro and Wilk statistic and the normality plots. Skewed variables were log-transformed prior to analysis. The independent student's *t*-test was used to compare mean values of plasma insulin concentration to FASN concentration, between the two groups (T2DM and control groups). The data was cleaned and thereafter showed no violation of normality as assessed using the Shapiro and Wilk statistic, and also showed homoscedasticity as assessed using Levene test for equality of variance. Bivariate linear regression and correlation coefficients were used to assess correlation between insulin and FASN. The bivariate linear regression data on insulin vs. FASN were plotted and presented on scatter graphs. Data analysis was done using IBM SPSS Statistics version 22 for Mac and Microsoft Excel 2011 for Mac. Results were summarised on to tables and graphs as given below. All statistical tests were performed at 5% significance level or 95% confidence interval and differences were considered significant if 2-tailed p<0.05. The study was approved by the University of Zambia Biomedical Research Committee (UNZABREC).

Results and discussion

Insulin, FASN mean difference: The study found that T2DM participants had statistically significant higher insulin concentration (35.9 ± 3.6 µIU/mL) compared to non-diabetic participants (10.8 ± 1.4 µIU/mL), $t(40) = 6.518$, $p<0.0001$ (Fig. 1a). FASN concentration was higher in T2DM participants (FASN; 22.8 ± 4.1 ng/mL) than in control participant (FASN; 11.8 ± 1.4 ng/mL) with statistical significance, $t(41) = 2.471$, $p = 0.018$ (Fig. 1b).

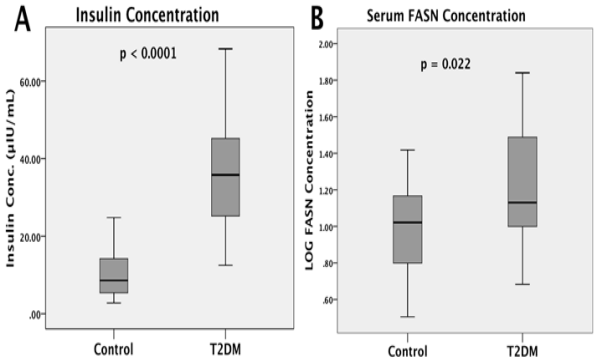
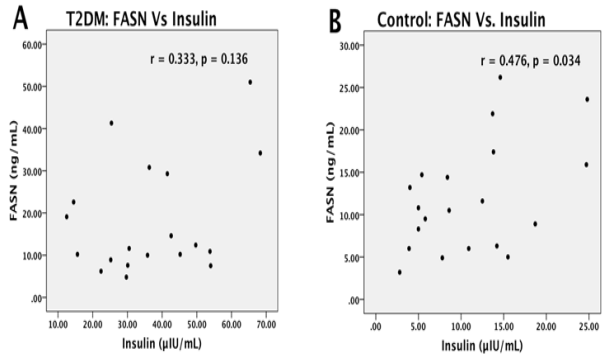


Figure 1: Insulin, FASN mean difference

(a) Insulin mean concentration difference between T2DM group and non-diabetic controls. (b) FASN mean concentration difference between T2DM and non-diabetic controls. Difference assessed using independent sample *t*-Test with *p*-value significance at 95% confidence level.

Linear regression analysis of insulin Vs. FASN: Bivariate linear regression analysis of FASN vs. insulin showed moderate positive correlation in T2DM participants though not statistically significant (r=0.333, $p = 0.163$) (Fig. 2a). FASN Vs. insulin were moderately correlated in healthy controls participants with statistical significance(r = 0.476, $p = 0.034$) (Fig. 2b).



a): Linear association between insulin and FASN in T2DM group. b): Linear association between insulin and FASN in health non-diabetic control group. r = the correlation coefficient and p = p-value. Statistical significance determined at 95% confidence level.

Anthropometrics and Biochemicals Mean Difference: The T2DM participants were older (41.3 ± 2.2 years) than healthy control participants (37.1 ± 1.8 years) with statistical significance, $t(42) = 4.314$; $p< 0.0001$. Diabetic participants had similar BMI (27.6 ± 1.5 kg/m^2) to healthy controls (27.8 ± 1.3 kg/m^2), $t(42) = 0.071$; $p = 0.944$) (Table 1). Fasting blood glucose levels were higher in T2DM subjects (9.6 ± 0.9 mmol/L), than healthy control (5.1 ± 0.1 mmol/L) with statistical significance, $t(36) = 4.89$, $p< 0.0001$ (Table 1). Alanine amino transferase (ALT),

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aspartate amino transferase (AST) and albumin levels had shown no statistically significant difference, $t(42) = -.887$; $p = 0.38$, $t(42) = -0.722$; $p = 0.475$ and $t(39) = 0.602$; $p = 0.551$ respectively between diabetic participants (ALT; 22.4 ± 4.0 U/L, AST 27.2 ± 2.3 U/L and ALB 25.4 ± 0.37 mmol/L), and control participants (ALT; 18.1 ± 2.8 U/L, AST; 25.1 ± 1.8 U/L, and ALB; 25.7 ± 0.35 U/L (Table 1).

	[1]Controls (n=22)	[2]T2DM (n=22)	*p*
Age (yr)	37.1 ± 1.8	41.3 ± 2.2	< 0.0001
BMI (kg/m^2)	27.8 ± 1.3	27.6 ± 1.5	0.944
Fasting Glucose (mmol/l)	5.1 ± 0.1	9.6 ± 0.9	< 0.0001
ALT (U/L)	18.1 ± 2.8	22.4 ± 4.0	0.38
AST (U/L)	25.1 ± 1.8	27.2 ± 2.3	0.475
Albumin (mmol/l)	25.7 ± 0.35	25.4 ± 0.37	0.551

Table 1: Anthropometric and metabolic characteristics of the study groups[1]

[1] Health control individuals and individuals with T2DM.[2]*P* represents overall significance of differences across groups. *P*-values were derived from independent sample student's *t*-test. Age and BMI were included in the model as covariates. AST, ALT and ALB were used to assess the association between hepatic function with serum insulin, FASN and triglycerides concentration.

Our results show that T2DM patients had statistically higher levels of insulin concentration than normal controls. This finding has also been observed in many studies and is generally one of the hallmarks of the condition. It has been postulated that the hyperinsulinaemia that is observed in this condition is brought about by insulin resistance on target tissues; and the body tries to offset this by producing more insulin. Increase in insulin resistance in a way is positively related to the magnitude of hyperinsulinaemia and this has been observed in the absence of conditions likely to affect insulin responsiveness like excess secretion of growth hormone[11]. The increase in insulin resistance also tends to worsen the glucose tolerance. It has also been observed that in the later stages of the condition impairment of insulin secretion may set in due to toxic damage by radical species on pancreatic β cells or exhaustion pancreatic β-cells giving rise to a full blown picture of diabetes mellitus[12].

Insulin facilitates glucose uptake in skeletal muscles by translocating glucose transporters from intracellular location to the plasma membrane where they pick up glucose and internalize it in cells. For translocation to take place there is an intracellular signalling pathway that is activated when insulin binds to its receptors on cell membranes. Insulin action involves a series of signaling cascades initiated by insulin binding to its receptor, eliciting receptor autophosphorylation and activation of the receptor tyrosine kinase, resulting in tyrosine phosphorylation of insulin receptor substrates (IRSs). Phosphorylation of IRSs leads to activation of phosphatidyl inositol 3-kinase (PI3K) and, subsequently, to activation of Akt and its downstream mediator AS160, all of which are important steps for stimulating glucose transport induced by insulin. Human studies have shown that insulin resistance is caused by dysregulation of signalling by IRS1 and IRS2 proteins as a common underlying mechanism[13, 14]. Fatty acids appear to cause this defect in glucose transport by inhibiting insulin-stimulated tyrosine phosphorylation of IRS and IRS-1 associated phosphatidylinositol 3-kinase activity[15]. Abnormalities in lipid metabolism lead to deposition and accumulation of lipids in muscles, liver and pancreatic β cells. Accumulation of these lipids in the muscle and liver lead to insulin resistance whereas in the pancreatic β-cells the result is impaired function.

This brings us to our second important funding of significantly higher FASN concentrations in T2DM than in controls. Mammalian FASN consists of 2 identical 270-kD polypeptide chains, each comprising all 7 enzyme activities. Our findings were in agreement with other studies, which showed that individuals with T2DM have significantly higher fasting serum insulin concentration and mean serum FASN concentration than non-T2DM individuals ($p < 0.0001$; and $p = 0.018$). Increased serum FASN affirms the insulin-FASN axis, which describes insulin-stimulated up-regulation of FASN gene expression[16-18]. Evidence of the insulin-FASN axis was in part explained in this study by the moderate correlation between insulin and plasma FASN concentration in control participants with statistical significance ($r = 0.476$, $p = 0034$). However, the correlation between insulin and circulating FASN in individuals with T2DM was not statistically significant ($r = 0.115$, $p = 0.619$). The lack of statistically significant correlation in the T2DM participant group may have been due to diabetic pathophysiologic changes and/or drug treatment effect[19, 20]. These may have altered the metabolic interplay between insulin and FASN in diabetic individuals. However, any definitive conclusion on this discrepancy should await more studies and research that are statistically powered to access the above-mentioned effects. Nevertheless, the T2DM group showed statistically significant higher FASN concentration ($p = 0.018$) than the non-

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diabetic group. But then, what are the implications of increased FASN expression? Up-regulation of FASN expression in the liver may lead to an increase in the triglycerides content of the liver which culminates in increased VLDL and free fatty acids secretion into the systemic circulation[16, 21, 22], contributing to the development of hypertriglyceridaemia. This metabolic link between insulin and FASN implies that, under hyperinsulinaemic conditions, the liver is turned into lipid 'biosynthesis factory' with all of its negative downstream effects, including the genesis of hypertriglyceridaemia [23, 24]. It is this derangement in lipid metabolism that is believed to be responsible for insulin resistance.

Furthermore, since hyperinsulinaemia is an early event[13, 25] in the disease pathology and correlated to FASN, we may postulate that over expression of hepatic FASN (and other lipogenic enzymes) and the outcome dyslipidaemias in T2DM must also be early events of T2DM pathogenesis. With respect to the above observations, further conclusions are made that the molecular lesions that culminate in chronic complications of T2DM such as macrovascular disordersmay also be early events in the progression of T2DM. Furthermore, the findings of this research may corroborate other finding that overproduction of VLDL and FFA by the liver takes place early in the development of overt T2DM [26-28]suggesting that pancreatic β-cell failure as a results of islets lipotoxicity is a progressive mechanism that begins from the onset of T2DM [29, 30]. This takes us to a reasonable conclusion that ß-cell demise maybe the end result of untreated and/or poorly managed diabetic dyslipidaemias, which in themselves stem from increased FASN activity. It must also be stated that the regulatory step in fatty acid synthesis is catalysed by acetyl-CoA carboxylase[31]. This enzyme carboxylates acetyl-CoA to form malonyl CoA; this malonyl CoA is the substrate for FASN. Acetyl-CoA carboxylase is inactive when it is phosphorylated and active when it is dephosphorylated. Insulin activates acetyl-CoA carboxylase by stimulating a pathway that dephosphorylates it [32]. Therefore apart from increasing the level of FASN, insulin also promotes dyslipidaemia by activating the enzyme which gives FASN the substrate.

In addition, if vascular complications that lead to cardiovascular disease in T2DM are early events that stem from FASN-driven lipogenesis and progress over many years then we may postulate that, diabetic cardiovascular complications, which are by far the major cause of mortality among diabetics, may not be averted by rigorous glycaemic control alone. This view is supported by various evaluations of insulin-regulated metabolic pathways and the molecular pathogenesis of T2DM [33]. In keeping with this view, the United Kingdom Prospective Diabetes Study (UKPDS), the American Diabetes Association and the European Association for the Study of Diabetes and others have shown that dietary intervention and/or glycaemic control to the non-diabetic range have major benefits for diabetic related microvascular and neuropathic complications, but no benefits on cardiovascular complications and major macrovascular events[34, 35].

Here we propose that manipulation of FASN through inhibitors may be one important mode for the control of dyslipidaemias associated with hyperinsulinaemia because FASN inhibitors may help to reduce insulin resistance and diabetic dyslipidaemias.

The fact that data on lipid profiles of both T2DM and controls was not collected and there was no follow up the participants were limiting factors. In addition it would have been appropriate to determine the phosphorylation status or activity of acetyl-CoA carboxylase since insulin is known to modulate its activity.

Conclusions

In conclusion therefore our study showed that T2DM patients in Lusaka, Zambia have significantly higher levels of insulin and FASN than non-diabetic controls. T2DM patients also have significantly higher fasting blood sugar level. The high insulin levels may be a result of insulin resistance in peripheral tissues; and the associated high FASN levels may be the main cause of dyslipidaemias responsible for some of the long term complications of T2DM.

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